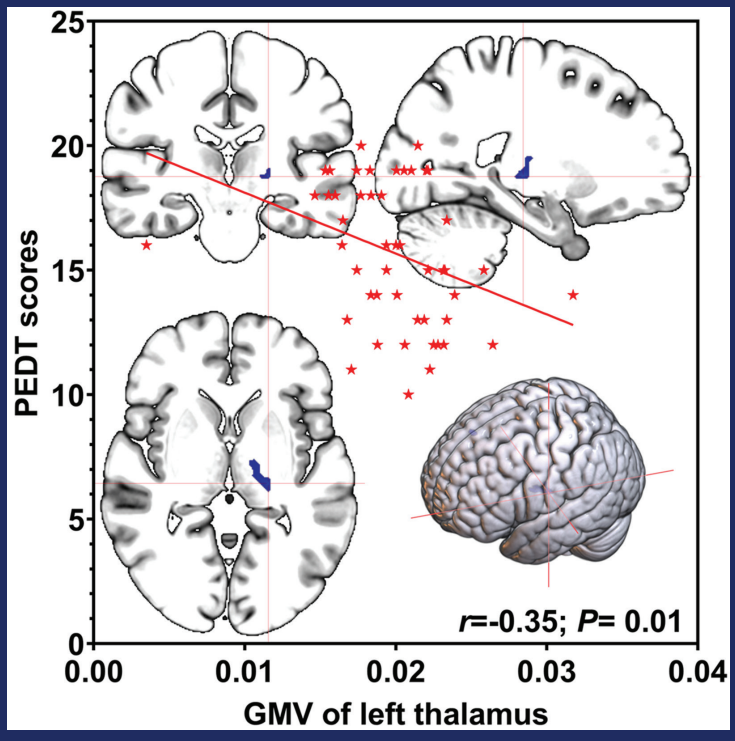
Online ISSN: 2047-2927 | Volume 12, Issue 4, May 2024

ANDROLOGY

www.wileyonlinelibrary.com/journal/andrology

Editor-in-Chief:
Aleksander Giwercman
Deputy Editor:
Wei Yan



Cover Illustration: Relationships between Grey Matter Volume (GMV) of impaired brain region and Premature Ejaculation Diagnostic Tool (PEDT) scores. From: Gao et al. Decreased grey matter volume in dorsolateral prefrontal cortex and thalamus accompanied by compensatory increases in middle cingulate gyrus of premature ejaculation patients. In this issue: pp: 841-849.

The official journal of the American Society of Andrology and the European Academy of Andrology

WILEY

American Society of Andrology

EUROPEAN ACADEMY OF ANDROLOGY

Andrology

EDITOR IN CHIEF

Aleksander Giwercman, Department of Translational Medicine, Lund University, CRC-91-10-058, Jan Waldenströms gata 35, SE 214 28 Malmö, Sweden and Reproductive Medicine Centre, Skane University Hospital, Östra Varvsgatan 11F, SE 211 73 Malmö, Sweden

DEPUTY EDITOR

Wei Yan, MD, PhD, The Lundquist Institute for Biomedical Innovation at Harbor-UCLA Medical Center, 1124 West Carson Street, Torrance, CA 90502, USA and Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA.

ASSOCIATE EDITORS

Kristian Almstrup, Copenhagen, Denmark
John Amory, Seattle, WA, USA
Lars Björndahl, Stockholm, Sweden
Paolo Capogrosso, Milan, Italy
Patricia Cuasnicú, Buenos Aires, Argentina
Michael Eisenberg, Palo Alto, CA, USA
Sandro Esteves, Campinas, Brazil
Alberto Ferlin, Brescia, Italy
Kathleen Hwang, Pittsburgh, PA, USA
Emmanuele A. Jannini, Rome, Italy
Niels Jørgensen, Copenhagen, Denmark
Zdravko Kamenov, Sofia, Bulgaria
Jackson Kirkman-Brown, Birmingham, UK
Maciej Kurpisz, Poznan, Poland
Maris Laan, Tartu Estonia
Francesco Lotti, Florence, Italy
Andreas Meinhardt, Giessen, Germany
Monica Muratori, Florence, Italy
Cristian O'Flaherty, Montreal, Canada
Giulia Rastrelli, Florence, Italy
Andrea Salonia, Milan, Italy
Daniele Santi, Modena, Italy
Jan-Bernd Stukenborg, Stockholm, Sweden
Katja Teerds, Wageningen, The Netherlands
Pablo Visconti, Amherst, MA, USA

EDITORIAL BOARD

Kenneth Aston, Salt Lake City, UT, USA
Maria Agustina Battistone, Boston, USA
Nelson Bennet, Chicago, USA
Nicolas Brukman, Haifa, Israel
Livio Casarini, Modena, Italy
Frederic Chalmel, Rennes, France
Tony Chen, Palo Alto, USA
Eric Chung, Brisbane, Australia
Angelo Cignarelli, Bari, Italy
Giovanni Corona, Bologna, Italy
Carlos Da Ros, Porto Alegre, Brazil
Alan Diekman, Little Rock, AR, USA
Joel Drevet, Clermont-Ferrand, France
Erma Drobnis, Columbia, MO, USA
Waltraud Eggert-Kruse, Heidelberg, Germany
Ahmed El-Sakka, Ismailia, Egypt
Thomas Garcia, Houston, TX, USA
Vito Giagulli, Conversano, Italy
Soledad Natalia Gonzalez, Buenos Aires, Argentina
Dimitrios G. Goulis, Thessaloniki, Greece
Jingtao Guo, Beijing, China
Xuejiang Guo, Nanjing, China
Geert Hamer, Amsterdam, the Netherlands
William J. Huang, Taipei, Taiwan
Ates Kadioglu, Istanbul, Turkey
Dario Krapf, Rosario, Argentina
Csilla Krausz, Florence, Italy
Sandra Laurentino, Münster, Germany
Guillermina Luque, Buenos Aires, Argentina
Sara Marchiani, Florence, Italy
Alexander Pastuszak, Salt Lake City, UT, USA
Edoardo Pozzi, Milan, Italy
Rodolfo Rey, Buenos Aires, Argentina
Vincenzo Rochira, Modena, Italy
Swati Sharma, Münster, Germany
Gunapala Shetty, Houston, TX, USA
Arthi Thirumalai, Seattle, USA
D.N. Rao Veeramachaneni, Fort Collins, CO, USA
Margarita Vigodner, New York, NY, USA
Tom Walsh, Seattle, WA, USA
Joachim Wistuba, Münster, Germany
Alexsander Yatsenko, Pittsburgh, PA, USA

EDITORIAL ASSISTANT

Shubhangi Chuttar
(E-mail: andrology.office@wiley.com)

PRODUCTION EDITOR

Aswathi KP
(E-mail: akp@wiley.com)

Publisher: Andrology is published by Blackwell Publishing Ltd, 9600 Garsington Road, Oxford OX4 2DQ, UK. Tel: +44 (0) 1865 776868; Fax: +44 (0) 1865 714591. Blackwell Publishing Ltd is now part of John Wiley & Sons.

Disclaimer: The Publisher, The American Society of Andrology and Editors cannot be held responsible for any errors in or any consequences arising from the use of information contained in this journal. The views and opinions expressed do not necessarily reflect those of the Publisher, The American Society of Andrology or Editors, neither does the publication of advertisements constitute any endorsement by the Publisher, The American Society of Andrology, Editors or Authors of the products advertised.

Journal Homepage

For submission instructions, subscription and all other information visit: www.wileyonlinelibrary.com/journal/Andrology

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13452 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

ANDROLOGY
Contents
Volume 12, Issue 4, May 2024

ANDROLOGY AND HUMANITIES

703 **Simone Cilio, Alessia d'Arma, Francesco Montorsi and Andrea Salonia**
Parenting desire among men who have sex with men in a heteronormative context

REVIEW ARTICLES

705 **MariaYu Konoshenko, Pavel Laktionov and Olga Bryzgunova**
Prostate cancer therapy outcome prediction: are miRNAs a suitable guide for therapeutic decisions?

719 **Wen Yang, Rong Hua, Yunxia Cao and Xiaojin He**
A metabolomic perspective on the mechanisms by which environmental pollutants and lifestyle lead to male infertility

740 **Ishith Seth, Damien Gibson, Gabriella Bulloch, Konrad Joseph, Jevan Cevik, Kirby R. Qin, Shekib Shahbaz and Warren M. Rozen**
Vasovasostomy: A systematic review and meta-analysis comparing macroscopic, microsurgical, and robot-assisted microsurgical techniques

768 **Daniele Santi, Angelo Cignarelli, Matteo Baldi, Andrea Sansone, Giorgia Spaggiari, Manuela Simoni and Giovanni Corona**
The chronic alcohol consumption influences the gonadal axis in men: Results from a meta-analysis

SOCIETY NEWS

781 **Alberto de la Iglesia, Dorte L. Egeberg, Daniel Marcu, Guillaume Richer, Brendan J. Houston, Omar Ammar, Gülizar Saritas, Emily Delgouffe, Davor Jezek, Csilla Krausz, Ewa Rajpert-De Meyts and Hermann M. Behre**
Leading at the vanguard of andrology: The Network for Young Researchers in Andrology joins forces with the European Academy of Andrology

ORIGINAL ARTICLES

785 **Yan Wang, Bin Zhao, Haiyan Yang and Zheng Wan**
A real-world pharmacovigilance study of FDA adverse event reporting system events for sildenafil

793 **Fuxun Zhang, Yang Xiong, Yangchang Zhang, Kan Wu and Bo Zhang**
Genetically proxied intestinal microbiota and risk of erectile dysfunction

801 **Kathleen Herkommer, Valentin H. Meissner, Andreas Dinkel, Matthias Jahnen, Stefan Schiele, Martina Kron, Donna P. Ankerst and Jürgen E. Gschwend**
Prevalence, lifestyle, and risk factors of erectile dysfunction, premature ejaculation, and low libido in middle-aged men: first results of the Bavarian Men's Health-Study

809 **Amanda de Deus, Gabriele Gonçalves, Jenilson da Silva, Luís Cláudio de Jesus, Ana Paula S. Azevedo-Santos, Hivana Dall Agnol and Silma Regina Pereira**
Microbiome reveals inflammatory-related bacteria and putative functional pathways involved in human papillomavirus-associated penile squamous cell carcinoma

821 **Lukas Schäfer, Jann F. Cremers, Bahne Witschel, Victor Schüttfort, Timo O. Nieder, Frederik König, Malte W. Vetterlein, Philipp Gild, Roland Dahlem, Margit Fisch, Sabine Kliesch and Armin Soave**
What do patients with Peyronie's disease expect from therapy? A prospective multi-center study

830 **Douglas Schneider, Mitchell O'Leary, Eliad Amini, Jake Miller, Nick Hassas, Jeanie Nguyen, Muhammed A. Moukhtar Hammad, David Barham and Faysal A. Yafi**
Peyronie's disease response to intralesional collagenase clostridium histolyticum therapy is independent of baseline testosterone

835 **Alberto Costa Silva, Vasco Rodrigues, Carlos Martins Silva and Afonso Morgado**
Complications after surgical correction of penile fractures—Is there a clinical impact?

841 **Songzhan Gao, Jianhuai Chen, Jia Liu, Yichun Guan, Rusheng Liu, Jie Yang and Xianfeng Yang**
Decreased grey matter volume in dorsolateral prefrontal cortex and thalamus accompanied by compensatory increases in middle cingulate gyrus of premature ejaculation patients

WILEY

Discover this journal online at
Wiley Online Library
wileyonlinelibrary.com/journal/andrology

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13452 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

850 **Changsheng Huan, Mian Wang, Yu Song, Zexin Jia, Dandan Wei, Lulu Wang, Qingqing Xu, Juan Wang, Mengzhen Zhao, Jintian Geng, Jiayu Shi, Cuicui Ma, Zhenxing Mao, Chongjian Wang and Wenqian Huo**
Inflammatory markers and androstenedione modify the effect of serum testosterone on obesity among men: Findings from a Chinese population

862 **Sara Dabizzi, Costanza Calamai, Selene Degl'Innocenti, Luca Boni, Linda Vignozzi, Mario Maggi, Elisabetta Baldi and Monica Muratori**
Tip-microVapour Fast Freezing: A novel easy method for cryopreserving severe oligozoospermic samples

870 **Ana Tímermans, Fátima Otero, Manuel Garrido, Jaime Gosálvez, Stephen Johnston and José Luis Fernández**
The relationship between sperm nuclear DNA fragmentation, mitochondrial DNA fragmentation, and copy number in normal and abnormal human ejaculates

881 **Riddhi Kirit Pandya, Ameya Jijo, Aswathi Cheredath, Shubhashree Uppangala, Sujith Raj Salian, Vani R Lakshmi, Pratap Kumar, Guruprasad Kalthur, Sanjay Gupta and Satish Kumar Adiga**
Differential sperm histone retention in normozoospermic ejaculates of infertile men negatively affects sperm functional competence and embryo quality

891 **David J. Handelsman, Amanda Idan, Sue Sleiman, Fey Bacha, Georgina V. Long, Alexander M. Menzies, Tejnei Vaishnav, Noosha Litkouhi, Xanthie Volckmar, William Ledger and Antoinette Anazodo**
Testicular function after non-cytotoxic and immunotherapy drug treatment

899 **Amy Tran-Guzman, Amina Khan and Martine Culty**
Differential roles of cyclooxygenase enzymes in the regulation of murine juvenile undifferentiated spermatogonia

918 **Raul A. Gonzalez-Castro, Luke A. Whitcomb, Emma C. Pinsinski and Elaine M. Carnevale**
Cryopreservation of equine spermatozoa reduces plasma membrane integrity and phospholipase C zeta 1 content as associated with oocyte activation

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13452 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 26 June 2023 | Revised: 30 July 2023 | Accepted: 5 September 2023

DOI: 10.1111/andr.13529

ANDROLOGY AND HUMANITIES

ANDROLOGY WILEY

Parenting desire among men who have sex with men in a heteronormative context

Simone Cilio[1,2] | Alessia d'Arma[1] | Francesco Montorsi[1,3] | Andrea Salonia[1,3]

[1]Division of Experimental Oncology, Unit of Urology, Urological Research Institute, IRCCS Ospedale San Raffaele, Milan, Italy

[2]Department of Neurosciences, Reproductive Sciences and Odontostomatology, Urology Unit, University of Naples "Federico II", Naples, Italy

[3]Vita-Salute San Raffaele University, Milan, Italy

Correspondence
Andrea Salonia, FECSM; University Vita-Salute San Raffaele, Division of Experimental Oncology/Unit of Urology, Urological Research Institute, IRCCS Ospedale San Raffaele, Via Olgettina 60, Milan 20132, Italy.
Email: salonia.andrea@hsr.it

Abstract

Introduction: The desire for parenthood in the LGBT+ community is still a matter of critical debate worldwide.

Objectives: We aimed to (i) analyse the prevalence of desire for fatherhood in a cohort of white-European sexually active men who have sex with men and (ii) explore the characteristics of men who have sex with men with respect to those without the desire for fatherhood.

Materials and methods: An anonymous self-compiled survey with 23 closed socio-demographic general questions, one section aimed to assess medical history and recreational habits and two sections devoted to investigate genital and sexual health, was provided to each participant. Likewise, all participants were invited to complete a number of questionnaires, including the Male Sexual Health Questionnaire and the Beck Inventory for Depression.

Results: Of 191 men who have sex with men white-European participants who eventually completed the survey, 112 (58.6%) clearly expressed their desire for fatherhood. Of all, the wish to become a parent was strong in 99 (51.8%) individuals and higher in younger participants (35 [28–46] vs. 43 [32–50] years, $p = 0.01$). No other significant differences in terms of educational level and relational status were identified between the two groups. Of all, the wish to become a parent was strong in 99 (51.8%) individuals and higher in younger participants (35 [28–46] vs. 43 [32–50] years, $p = 0.01$).

Conclusion: Current preliminary findings highlight that more than one in two men who have sex with men has a desire for fatherhood, with almost 81% of all having a high desire for fathering, which is clearly more intense in younger men.

KEYWORDS
fatherhood, LGBT+, parenting desire

Decades have gone by since the first review dealing with growth and development of children in same-sex couples was published. Overall, much more significant than the sexual orientation of parents, the quality of family relationships was identified as a key point in terms of children's development.[1–4] Indeed, children raised by sexual minority couples grew up as successfully as children raised by heterosexual parents.[5] Therefore, it is surprising to say the least that nowadays the legitimacy of parenting desires in the sexual minority community is still a matter of critical debate in some countries, especially in the most heteronormative ones.

© 2023 American Society of Andrology and European Academy of Andrology.

Members of our research team have developed a pilot survey of closed questions exploring sociodemographic, genital and sexual health characteristics of men who have sex with men (MSM) in a heteronormative context. The primary aim was to attempt and assess the proportion of parenting desire among cisgender MSM. Preliminary results within a homogenous group of 191 white-European participants (median [interquartile range] age 39 [29–49] years) showed that even 112 (58.6%) clearly expressed their desire for fatherhood. Of all, the wish to become a parent was strong in 99 (51.8%) individuals and higher in younger participants (35 [28–46] vs. 43 [32–50] years, $p = 0.01$). Of relevance, the wish for parenting was greater in those individuals who had experienced a traumatic process of acceptance of their own sexual orientation (40 [70.2%] vs. 62 [52.5%], $p = 0.04$). Conversely, the desire of fathering did not differ according to different relationship status or educational levels.

In 1999, Denmark was the first country to promulgate a national law allowing adoptions to men and women regardless of their sexual orientation. Since then, several countries have discussed and enacted laws to rule non-binary couples to foster or adopt children or stepchildren worldwide. With the legalisation of same-sex marriage and adoption in Cuba in September 2022, the world count of countries where non-heterosexual couples can adopt children has increased to 53. Conversely, in other countries, on the one hand, no rule has been provided to allow same rights—thus including adoption—to either single people or couples belonging to the sexual minority community; on the other hand, the issue has not been clearly legislated by national laws.

Here, we provide further real-life world data corroborating the concept that the desire for parenting does exist and is strong in more than one in two MSM individuals. Notwithstanding the historical view of homosexuality as a possible threat to the heteronormative dominant way of life,[6] being translational researchers heavily involved in the field of sexual and reproductive health, we believe that good and bad feelings, as well as the underlying mechanisms of science—both the one that embraces biology in the most traditional form but also psychology and behaviours—do not belong only to one or the other sexual orientation. However, the contemporary political debate is still largely ignoring or even opposing this desire at least in some countries, despite the scientifically proven absence of negative repercussions on children's growth in families formed by MSM.[7] Consequently, we would strongly support the idea that care and attention, a harmonious growth, the necessary affection and the loving care received, acculturation, opportunities and even the happiness deriving from important things as well as from the daily life of having a person close to you and who loves you are fundamental for each child, regardless of who the parent is, that is, heterosexual, religious, married, single, sexual minority—for example, MSM, LGBT+, LGBQT+ and even longer acronyms. At least we scientists believe so.

AUTHOR CONTRIBUTIONS

Conceptualisation: Simone Cilio, Andrea Salonia and Francesco Montorsi. *Data curation*: Simone Cilio and Alessia d'Arma. *Methodology*: Simone Cilio and Andrea Salonia. *Writing—original draft*: Simone Cilio and Alessia d'Arma. *Writing—review and editing*: Andrea Salonia and Francesco Montorsi.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

ORCID

Andrea Salonia https://orcid.org/0000-0002-0595-7165

REFERENCES

1. Patterson CJ. Children of lesbian and gay parents. *Child Dev*. 1992;63:1025-1042.
2. Zhang Y, Huang H, Wang M, et al. Family outcome disparities between sexual minority and heterosexual families: a systematic review and meta-analysis. *BMJ Glob Health*. 2023;8:e010556.
3. Wainright JL, Patterson CJ. Delinquency, victimization, and substance use among adolescents with female same-sex parents. *J Fam Psychol*. 2006;20:526-530.
4. Ioverno S, Carone N, Lingiardi V, et al. Assessing prejudice toward two-father parenting and two-mother parenting: the beliefs on same-sex parenting scale. *J Sex Res*. 2018;55:654-665.
5. Patterson C. Parents' sexual orientation and children's development. *Child Dev Perspect*. 2016;11:45-49. doi:10.1111/cdep.12207
6. Warriner K, Nagoshi CT, Nagoshi JL. Correlates of homophobia, transphobia, and internalized homophobia in gay or lesbian and heterosexual samples. *J Homosex*. 2013;60:1297-1314.
7. Carone N, Lingiardi V, Chirumbolo A, Baiocco R. Italian gay father families formed by surrogacy: parenting, stigmatization, and children's psychological adjustment. *Dev Psychol*. 2018;54:1904-1916.

How to cite this article: Cilio S, d'Arma A, Montorsi F, Salonia A. Parenting desire among men who have sex with men in a heteronormative context. *Andrology*. 2024;12:703–704. https://doi.org/10.1111/andr.13529

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13529 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 11 July 2023 | Revised: 4 September 2023 | Accepted: 9 September 2023

DOI: 10.1111/andr.13535

ANDROLOGY WILEY

REVIEW ARTICLE

Prostate cancer therapy outcome prediction: are miRNAs a suitable guide for therapeutic decisions?

MariaYu Konoshenko | Pavel Laktionov | Olga Bryzgunova

Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia

Correspondence
Maria Yu Konoshenko, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, CBFM SB RAS, 8 Lavrentiev Avenue, Novosibirsk 630090, Russia.
Email: msol@ngs.ru

Funding information
Russian Science Foundation, Grant/Award Number: 23-25-10026; Government of the Novosibirsk Region, Grant/Award Number: 000000540699598235120582/No. p—45

Abstract

Background: Radical prostatectomy, radiotherapy, chemotherapy, and androgen-deprivation therapy are among the most common treatment options for different forms of prostate cancer (PCa). However, making therapeutic decisions is difficult due to the lack of reliable prediction markers indicating therapy outcomes in clinical practice. The involvement of miRNAs in all mechanisms of the PCa development and their easy detection characterize them as attractive PCa biomarkers. Although there are extensive data on the role of miRNAs in PCa therapy resistance and sensitivity development, the issues of whether they could be used as a guide for therapy choice and, if so, how we can progress toward this goal, remain unclear. Thus, generalizable reviews and studies which summarize, compare, and analyze data on miRNA involvement in responses to different types of PCa therapies are required.

Objectives: Data on the involvement of miRNAs in therapy responses, on the role of cross-miRNA expression in different therapies, and on miRNA targets were analyzed in order to determine the miRNA-related factors which can lend perspective to the future development of personalized predictors of PCa sensitivity/resistance to therapies.

Materials and methods: The data available on the miRNAs associated with different PCa therapies (resistance and sensitivity therapies) are summarized and analyzed in this study, including analyses using bioinformatics resources. Special attention was dedicated to the mechanisms of the development of therapy resistance.

Results and discussion: A comprehensive combined analysis of the current data revealed a panel of miRNAs that were shown to be most closely associated with the PCa therapy response and were found to regulate the genes involved in PCa development via cell proliferation regulation, epithelial-mesenchymal transition (EMT), apoptosis, cell-cycle progression, angiogenesis, metastasis and invasion regulation, androgen-independent development, and colony formation.

Conclusion: The selected miRNA-based panel has the potential to be a guide for therapeutic decision making in the effective treatment of PCa.

KEYWORDS
chemotherapy, miRNA, prostate cancer, prostatectomy, radiotherapy, therapy resistance

© 2023 American Society of Andrology and European Academy of Andrology.

1 | INTRODUCTION

Prostate cancer (PCa) is a widespread, malignant neoplasm, one of the cancer types with leading positions in morbidity and fifth in mortality worldwide as well as in developed countiese.[1,2] Twenty percent of newly diagnosed cases are advanced or metastatic with low survival rates.[3] Localized PCa often runs an indolent course without any threat to mortality with nearly 100% 5-year survival rates.[4] The most frequent treatment options of localized forms of PCa (stages I, IIA, IIB) include the following: radical prostatectomy (RP), radiotherapy (RT), and androgen-deprivation therapy (ADT), brachytherapy. The treatments for locally advanced PCa (T3-T4, NxN0, M0) include the following: RT, RP, and ADT.[5–7] These therapies are often chosen for symptomatic patients with extensive stage T3–T4 tumors, with prostate specific antigen (PSA) levels > 25—50 ng/mL and PSA doubling time of less than 1 year. Treatments for patients with regional metastasis (cN+, M0) include the following: RP, RT, and ADT (as a component of the multimodal therapy after RP or RT, or as a therapy for patients with contraindication to the local therapy).[8] Thus, each patient has several therapy options available to their condition; however, it remains unclear which of them will be the most effective for certain patients because of the lack of reliable prediction markers of therapeutic outcomes in clinics. The therapy types all have their own advantages and disadvantages; however, all of them have serious side effects and greatly impact patients' quality of life.[9] Thus, inefficient therapies are not only a waste of time but cause harm to patient health. Moreover, and even more seriously, the timely choice of the correct type of therapy is a key in saving a patients' life, especially those patients with aggressive forms of PCa.[10]

The mechanisms of tumor resistance to therapy are variable and partly overlap between different therapies. They include the following: mechanisms underlying cell-cycle regulation, apoptosis, and autophagy; mechanisms supporting cancer stem cells (CSCs), DNA repair, angiogenesis, and androgen-independent development. These mechanisms include a number of pathways and are controlled by numerous regulators, as follows: cellular receptors, transcription factors, and gene expression regulators, such as histone modifiers, DNA methylation mutation regulators, and miRNA-driven supramolecular silencing regulators.

miRNAs are 19—25 nucleotide-long noncoding RNAs that post-transcriptionally regulate gene expression. Typically, miRNA suppress translation or induce degradation of mRNA via binding the target RNA. One miRNA could target several mRNAs, involved in regulation of different processes and signaling pathways as well as one mRNA may be regulated by several miRNAs. miRNAs have been proven to be essential for normal execution of cell growth, tissue differentiation, cell proliferation, embryonic development, and apoptosis ect.[11] Dysregulated miRNA and, as a consequence the impaired balance of related biological processes, plays critical roles in the development and progression of cancer, cardiovascular, neurodegenerative and other diseases.[12–14] Numerous studies have indicated the driving role of miRNAs in cancer progression.[15–18] Simultaneously, the stability of miRNAs in tissues and biological fluids, as well as their ease of detection, made them attractive biomarkers for the diagnosis and prognosis of various cancers.[19]

There are several key functions for the application of miRNAs in PCa monitoring: in the staging and diagnosis of PCa forms; in prognosis; in therapy decision making; in the assessment of therapy efficacy; as a part of co-therapy (as a therapy sensitizing agent); for patient surveillance to detect cancer relapse (Figure 1). The known data indicate that miRNAs may be used for determining tumor progression and sensitivity or resistance to different therapy types, as well as biomarkers for patient outcomes.[20–23] As prognostic markers, miRNAs may be used when measured before therapy and when measured in post-operative tissues and biofluids after or during therapy. Continuous monitoring of miRNA levels in biological fluids enables clinicians to predict and evaluate the efficacy of chosen therapies and to monitor a given patient's long-term cancer-related processes (highlighting therapy switch-points). Through this monitoring, clinicians are also able to identify and introduce new biomarkers for different types of therapies. There remains great demand for this kind of progress, because it enables the survival rates of PCa patients to improve (Figure 1). The discovery of biomarkers which can predict responses to anticancer therapies is crucial for enhancing the efficacy of treatment and to improve the survival rates of PC, which currently remain poor.

Previously, we reviewed the involvement of miRNAs in the development of biochemical recurrence (BCR) of PCa after prostatectomy,[20] involving mechanisms of radiosensitivity, radioresistance,[21] chemosensitivity, chemoresistance,[22] and ADT sensitivity and resistance.[23] However, to the best of our knowledge, there has been no combined, comprehensive analysis of the impact of miRNAs on the development of resistance to different types of PCa therapies which accounts for miRNA intersections between therapies or the specificity of miRNA-regulated biological processes and pathways.

The present review summarizes the information presented in our previous reviews on miRNAs under different types of the PCa therapy[20–23] alongside recently published data, and provides a comprehensive analysis of the importance of miRNAs in the development of sensitivity and resistance to different types of PCa therapies:

- miRNAs and radical prostatectomy
- miRNAs and radiotherapy
- miRNAs and chemotherapy
- miRNAs and androgen-deprivation therapy (ADT)

Then, miRNA panels to select the effective therapy type are selected based on analyzed data. Challenges and perspectives of miRNA-based therapy effectiveness markers are further discussed.

Data on the involvement of miRNAs in therapy responses, on the role of cross-miRNA expression in different therapies, and on miRNA targets were analyzed in order to determine the miRNA-related factors which can lend perspective to the future development of personalized predictors of PCa sensitivity/resistance to therapies. These miRNAs

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

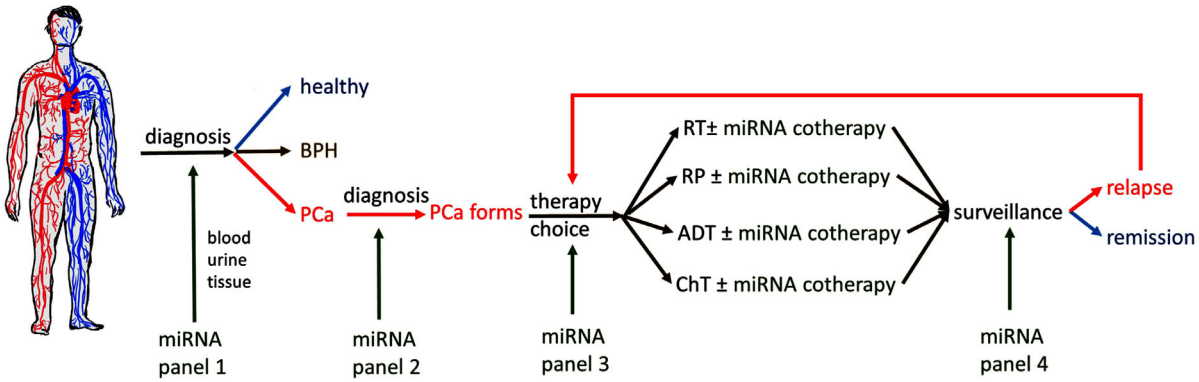


FIGURE 1 Key points of miRNA application in PCa diagnosis and therapy. Abbreviations: BPH, benign prostate hyperplasia; PCa; prostate cancer, RP, radical prostatectomy; RT, radiotherapy; ADT, androgen deprivation therapy; ChT, chemotherapy.

may represent the promise of the future development of drugs for miRNA-based PCa co-therapies.

1.1 | miRNAs and radical prostatectomy

RP is commonly used first-line treatment for patients with clinically localized PCa and is characterized by an average life expectancy of at least 10 years.[24] Like any surgical intervention, RP affects patient health and standard of living, through blood loss and high transfusion rates, additional procedures such as pelvic lymph node dissection, long hospital stays, and postoperative complications. Postoperative complications may adversely affect both the physical and emotional states of patients, as they include a substantial risk of experiencing long-term urinary incontinence or retention, erectile dysfunction, rectal injury, hematoma, lymphocele/lymphorrhea, deep venous thrombosis, pulmonary embolism, wound complications, neurapraxia, reoperation, and mortality.[25]

Almost 30% of PCa patients experience BCR after a successful RP.[26,27] BCR is detectable through rising PSA measurements and is considered a failure of primary treatment. Distant metastasis is a frequent consequence of BCR. The transition from BCR to the metastasis development can occur quickly or slowly, even extending over several years.[28,29] Moreover, it remains disputable whether BCR is indicative of tumor relapse or metastasis. Clinicopathological parameters (tumor volume, pathological grade, the status of lymph node metastasis, preoperative PSA, and the Gleason score) are used for routine prognosis. Unfortunately, current prognostic markers fail to determine the outcome with high reliability and cannot precisely prognosticate whether a tumor will develop or remain indolent.[30] An alternative is the use of molecular markers, including genomic, transcriptomic, proteomic, and metabolomic markers: for example, KLK2, PCA3, prostate-specific membrane antigen, prostate stem cell antigen, altered expressions of Bcl-2 and Bax,[31] p53, PTEN, OHdG, and 8-Iso-PGF2α in the urine and others,[32–35] including miRNAs.[36] Most studies in the literature that aim to analyze the involvement of miRNAs in PCa response to RP can be divided into the following categories:

- Studies reporting an alteration of miRNA expression in response to RP;
- Studies indicating the association between miRNAs expression and shorter BCR/survival after RP.

The data from such studies were previously comprehensively analyzed[20] and are presented in Figure 2. The present study includes the analysis of studies including those which were published after June 2020.[37–39]

The Venn diagram allows us to identify those miRNAs which do not match in different studies, and to group miRNAs according to their contribution to therapy resistance/sensitivity and predictive value. Among potential reasons of the observed discrepancies, patient heterogeneity (PCa stage, presence or absence of PCa relapse, etc.), technical variability (from different RP techniques to various sample types and miRNA isolation procedures), different observation period should be mentioned.

According to the current data (Figure 2), numerous miRNAs are involved in the response to RP and BCR development; however, some of the findings are contradictory. The same miRNAs were shown to be up- and downregulated after RP (miR-141), associated with BCR presence and absence, or induced by RP (miR-21, miR-141, miR-133b, miR-145-5p). Obviously, these miRNAs are the first candidates in the exclusion list, and the common reasons for the discordance in the data will be discussed in the last subchapter of this paper. miRNAs change in expression after RP for many different reasons, including changes in the oncological status of the patient, surgery peculiarities, and the postoperative complications mentioned earlier. That is why they cannot be considered as markers of the effective/noneffective therapy. Over- and underexpressed miRNAs associated with shorter BCR/survival after RP are more regularly proven as decision-making tools in the choice of RP as a therapy. However, there is greater scholarly attention on those miRNAs which are associated with BCR and simultaneously alter their expression after RP. The monitoring of such miRNAs may enable the prediction of BCR and possible relapse. Additional validated cohort studies are necessary to shed light on whether such miRNA-based panels are better for relapse prediction than a rise in PSA.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

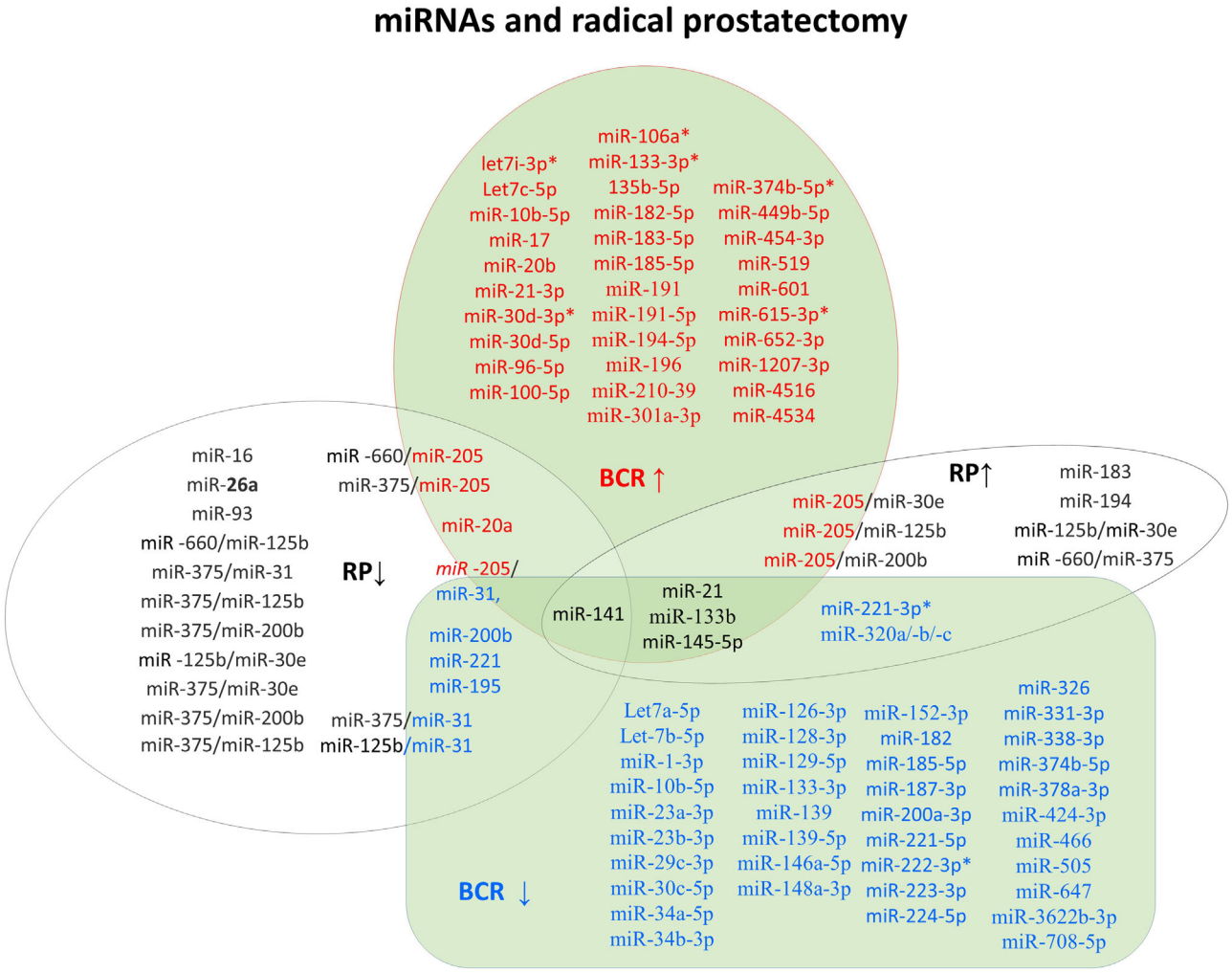


FIGURE 2 The association between miRNAs expression and the response to radical prostatectomy: miRNAs upregulated by RP (RP↑), miRNAs downregulated by RP (RP↓), overexpressed miRNAs associated with shorter BCR/survival after RP (BCR ↑, in red color), downexpressed miRNAs associated with shorter BCR/survival after RP (BHR ↓, in blue color). In the case of relative miRNA expression (e.g., miR-660/miR-205), the RP cased alteration of relative expression (miR-660/miR-205), whereas the expression of miR marked by color only (miR-205) was enhanced in BCR samples.

1.2 | miRNAs and radiotherapy

RT is a typical treatment for nonmetastatic PCa. Approximately, 60% of PCa patients undergo RT as a monotherapy or as a part of complex co-therapy with RP and/or chemotherapy. RT damages malignant cells; however, it also has a significant influence on adjacent tissues and proliferating cells. The hematopoietic, endothelial, vascular, and immune systems are among the primary vital systems which suffer from radiation.[40–42] Even though RT protocols and techniques have advanced greatly, posttreatment biopsies show a 15%–20% residual disease rate.[43–45] Radioresistance results in more aggressive phenotypes, increased proliferation, metastases, increased morbidity, and reduced patient survival.

Most studies in the literature that aim to analyze the involvement of miRNAs in PCa responses to RT can be divided into the following categories:

- Studies reporting an alteration of miRNA expression in response to RT;
- Studies reporting an influence of miRNA expression on radioresistance/sensitivity.

The data from such studies were previously comprehensively analyzed.[21] These data, together with data published later[46–48] and not included in our previous review, are presented in Figure 3.

Ionizing radiation damages cells by producing intermediate ions and free radicals that cause DNA single-strand breaks or double-strand breaks. Response to RT namely radiosensetivity or radioresistance depends on multiple processes caused by ionizing radiation energy or indirectly by reactive oxygen species generated via water dissociation. These include DNA damage response, autophagy, apoptosis, cell-cycle checkpoint activation, survival pathway engagement, oncogene or tumor-suppressor mutations, microenvironmental hypoxia and

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

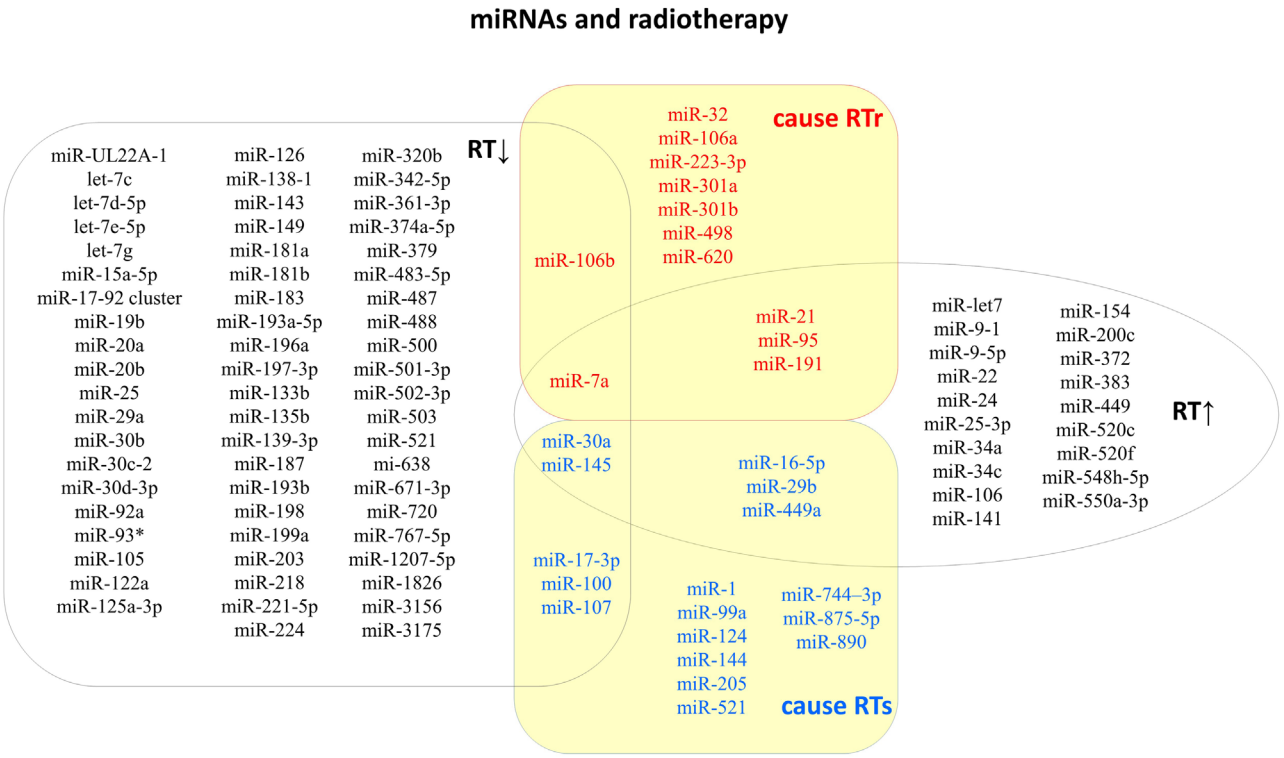


FIGURE 3 miRNAs associated with the development of radioresistance and radiosensitivity: miRNAs upregulated by radiotherapy (RT↑), miRNAs downregulated by radiotherapy (RT↓), upregulated miRNAs provoking radiotherapy resistance (cause RTr, in Red), upregulated miRNAs provoking radiotherapy sensitivity (cause RTs, in Blue).

CSCs development. The miRNAs presented in Figure 3 are involved in the regulation these processes which greatly affect responses to RT.[21] The multifactorial nature of radioresistance as well as involvement of single miRNA in several pathways contributes to challenges of miRNA role in PCa radioresponce.

Unlike the miRNAs presented in Figure 2 and the miRNAs associated with BCR, there is no evidence of an intersection between miRNAs which provoke radioresistance and those which provoke radiosensitivity. However, in some studies, miRNA-7a, -30a, and -145 were up- and downregulated after RT. Nevertheless, these results may be not contradictory if the studied patients were characterized by different percentages of relapse. Other miRNAs with an association with the development of radioresistance and radiosensitivity shown by two different types of experiments (miR-17-3p, 21, 95, 100, 106b, 107, 191 и 1605p, 29B и 449a) are apparently reflect the type of response to RT (i.e., to determine the phenotype of the tumor associated with the response to RT).

1.3 | miRNAs and chemotherapy

Chemotherapeutic agents are usually not recommended for localized PCa treatment. However, if PCa is diagnosed at a late stage, when metastasis can occur, then chemotherapy is often demanded. Both neoadjuvant and adjuvant chemotherapies using chemotherapeutic drugs such as docetaxel, paclitaxel, mitoxantrone, cisplatin, azacytidine, cyclopamine, estramustine, trichostatin A, and thapsigargin were shown to treat metastatic PCa effectively.[49,50] Among these drugs, taxanes are usually used as a front-line therapy. Taxanes stabilize microtubules by inhibition of their depolymerization, promoting cell-cycle arrest at the G2M phase, attenuating the effects of bcl-2 and bcl-xL gene expression, and further initiating apoptosis and cytotoxicity.[51,52] Taxanes may affect androgen receptor (AR) signaling and inhibit ligand-induced AR nuclear translocation, which leads to the cytoplasmic accumulation of ARs[53] and the inhibition of AR target genes, such as prostate-specific antigens.[54] Docetaxel is the most frequently used first-line chemotherapy. Cabazitaxel and abiraterone are usually used as second-line chemotherapies. Cabazitaxel is a semisynthetic taxane which is active in taxane-resistant tumors.[55] Abiraterone acetate is a CYP17 inhibitor that blocks androgen biosynthesis and is prescribed in combination with prednisolone. The typical side effects of PCa chemotherapy include neutropenia, an increased risk of infections, easy bruising or bleeding, hair loss, mouth sores, loss of appetite, nausea, vomiting, diarrhea. The main problem posed during chemotherapy is the frequent development of multidrug resistance (MDR), which decreases the efficacy of chemotherapeutic sources/drugs[56] and is responsible for over 90% of deaths in patients receiving both traditional chemotherapeutic drugs and targeted drugs.[57]

Most studies in the literature that aim to analyze the involvement of miRNAs in chemotherapy resistance can be divided into three categories:

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

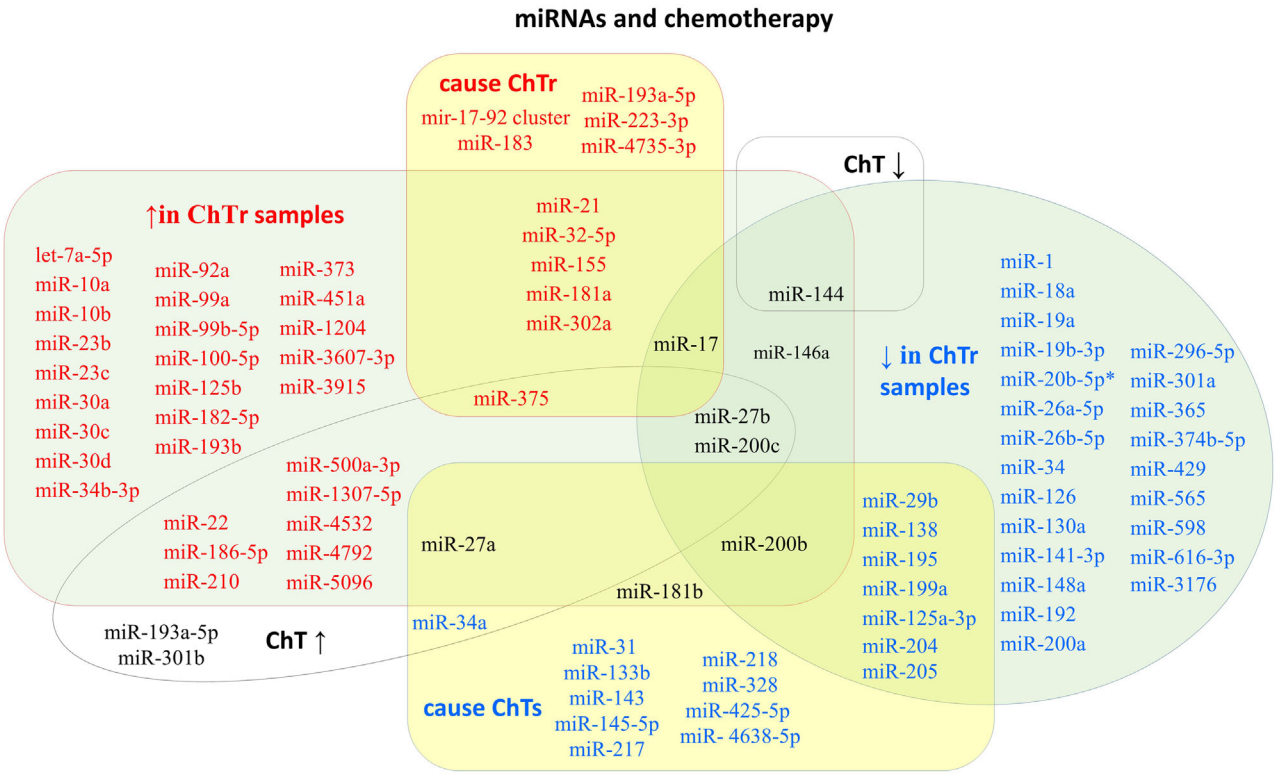


FIGURE 4 miRNAs associated with chemoterapy: miRNAs upregulated by ChT (ChT↑), miRNAs downregulated by ChT (ChT↓), miRNAs overexpressed in chemotherapy-resistant samples (↑ in ChTr samples), miRNAs downregulated in chemotherapy-resistant samples (↓ in ChTr samples), upregulated miRNAs provoking chemotherapy resistance (cause ChTr), upregulated miRNAs provoking chemotherapy sensitivity (cause ChTs). The red color indicates miRNAs, which were shown to be associated with chemotherapy resistance, the blue color indicates miRNAs, which were shown to be associated with chemotherapy sensitivity.

- Studies reporting an alteration of miRNA expression in the response to chemotherapy in PCa;
- Studies reporting an influence of miRNA expression on chemoresistance/sensitivity;
- Studies aiming to compare miRNA expression in chemoresistant and chemosensitive samples (tissues, serum of PCa patients, cell lines, and xenograft models).

In our previous study,[22] we analyzed studies which predominantly investigated the role of miRNA in the development of resistance to docetaxel, cisplatin, cabazitaxel, paclitaxel, and doxorubicin. The miRNAs shown to be involved in the development of chemotherapy resistance, as shown by different methods, are presented in Figure 4.

According to the known literature data, miRNAs (Figure 4) are involved in all known chemotherapy resistance mechanisms, including the following: EMT, hypoxia, autophagy, androgen resistance, drug efflux pump, increased DNA repair capacity, alterations in microtubule structure and/or function, apoptotic defects, and highly chemoresistant-tumor-initiating CSCs.[22] As soon as each miRNA regulates multiple genes and processes, many of the miRNAs presented in Figure 3 are simultaneously involved in the regulation of different chemotherapy resistance mechanisms.[22]

For example, ChT-upregulated miRNAs could be divided into a few groups, as follows: miRNA-34a are the inducers of ChT sensitivity; nine miRNAs are involved in the development of Ch resistance (one of them is in the list of ChTr inducers); two miRNAs (301b and 193a-5p) are upregulated (these have not been confirmed to be involved in PCa by other studies); two have been observed to be up- or downregulated in chemoresistant samples in accordance with the data of different authors, and thus may not yet be considered. To predict ChT efficacy before ChT, the most promising miRNAs are those which are up- and downregulated in ChTr samples (excluding those at the intersection of the ChTs area), whereas the efficacy could be confirmed by miRNAs at the intersection of ChT-induced upregulated miRNAs and ChTr, and by those which can cause ChTr/s miRNAs: miR-22, −186-5p, −210, −500a-3p, −1307-5p, −4532, −4792, −5096, −21, −32-5p, −155, 181a, −302a, and especially miR-375, as well as miR-29b, −125a-3p, −138, −195, −199a, −204, and −205.

1.4 | miRNAs and androgen-deprivation therapy

ADT is usually used to treat PCa patients with advanced metastatic, recurrent, and castration-resistant prostate cancer (CRPC). In clinical practice, there are several types of ADTs, which have different mechanisms of action: orchiectomy, antiandrogen monotherapy, LHRH agonist, LHRH antagonists, complete androgen blockade, estrogens, and second-generation hormonal therapies (in combination with ADT).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Side effects strongly depend on the type of ADT; however, the most frequent side effects include the following: decreased libido, erectile dysfunction, decreased energy, depressed mood and cognition, emotional lability, reduced quality of life, vasomotor symptoms, fatigue, weight gain, alterations in muscle mass/body fat distribution, gynecomastia, reduction in penile/testicular size, anemia, insulin resistance, diabetes mellitus, possible cardiovascular disease, development of osteoporosis, increased fractures, anxiety, impaired sleep quality, and irritability, among others.[58–61]

Moreover, PCa can often escape therapeutic pressure by entering a dormant state and subsequently transforming into an incurable, castration-resistant disease.[62] CRPC is a heterogeneous, highly aggressive disease, and is determined by rising PSA with castration testosterone levels.

One of the main problems associated with ADT lays in predicting the effectiveness of an ADT and the time from the beginning of therapy to the progression of PCa. Many clinical and laboratory parameters have been suggested as predictors of the clinical response to ADT, as follows: PSA, Gleason score, extent of bone metastases, serum alkaline phosphatases, performance status, hemoglobin, testosterone levels,[63] dyhydrotestosterone, androstenediol, dehydroepiandrosterone levels, and markers of the AR activity. Markers of the AR activity include mRNA expression, protein expression, protein localization, gene amplifications, gene polymorphisms (including CAG repeat polymorphisms,[64,65] gene mutation, splice variants, and other genetic polymorphisms—hormones (LHRH, LH), receptors (LHRHR, LHR), enzymes (androgen biosynthesis and metabolism, estrogen biosynthesis), chromosomal alterations (TPMRSS2:EGR fusion gene), gene expression profiling, proteomics, and noncoding RNA, etc.[66]). However, these predictors correlate poorly with clinical outcomes. Thus, an urgent need to develop novel biomarkers to predict responses to ADT remains, and candidate biomarkers have been proposed.

Most studies in the literature that aim to analyze the involvement of miRNAs in chemotherapy resistance can be divided into four categories:

- Comparison of miRNA expression in androgen-resistant and androgen-sensitive cell lines in CRPC and androgen-sensitive specimens;
- Influence of ADT on miRNA expression;
- Resistance or sensitivity to ADT of PCa cell lines and xenografts in response to induction/inhibition of miRNA expression;
- miRNAs associated with shorter CRPC progression of PCa after ADT.

The data from such studies were previously comprehensively analyzed[9] and are presented in Figure 5. The present study includes an analysis of all the manuscripts listed in our previous review[9] and those studies which were carried out after it was published.[67,68]

The selected miRNAs are involved in the ADT response and CRPC development via multiple mechanisms, including the following: within the hypothalamic–pituitary–gonadal axis, AR regulation, hypoxia, the immune system, reactive oxygen species, apoptotic processes, cell-cycle regulation, and responses to stress. Many of the miRNAs presented in Figure 5 regulate the expression of genes involved in signaling pathways that are crucial for the PCa development, such as NKX3.1, GSTP1, SPINK1, PTEN, FOXP3, C-Myc, ERHB2, PIM1, and EZH2.[9] Representation of data in the form of a Venn diagram, as in the previous therapy cases, enables the identification of miRNAs which possess the minor value (data from different studies do not match in relation to the following miRNAs: miRNA-15a, 16, 99a, 100, 125b, 143, 148a, 221, 220a, 222, 203, 616, and 663), sample-set-specific miRNAs, and miRNAs with synergistic growth (located on the intersection of similar functions in medical samples: miR-1, 7c, 21, 23b, 27b, 92a, 141, 145, 146a,149,181c-5p, 188-5p, 196b-5p, 200b, 204, 210, and 214-3p for ADT sensitiveness; miRNA-32, 126, 136, 181c, 196b-3p, 218, 300b, 302a, 302b, 302c, 345, 346, 361, 367, 375, 381, 424, 551b, and 644a). Obviously, the potential of miRNA as an ADT-resistant or -sensitive marker rises with the increase in the number of types of studies that indicate its involvement in the development of resistance/sensitivity. Detailed analysis of the miRNAs involved in the ADT response could be carried out as has been completed for previous therapy types, using the Venn diagram presented.

1.5 | miRNA panels to select effective therapies

To select an miRNA panel with the potential for use in the selection of effective and personalized therapies, miRNAs involvement in sensitivity to different types of the therapy was analyzed. The data were combined and only those miRNAs with significance in therapy responses, shown by two or more different types of studies, were selected as the most reliable options. For such miRNAs, we have indicated whether their up- or downregulation is associated with therapy sensitivity (Figure 6, marked by "+"). miRNAs located at the intersections of sample sets and up- and downregulated miRNAs are labeled in black (Figures 2–5). Those involved in the regulation of non-synergistic groups were excluded from the selected miRNA sets.

In cases where only one type of study indicated a given miRNA's involvement in therapy sensitivity, such miRNAs were also excluded from further analysis. If one of the miRNAs which was selected according to the above-mentioned criteria (Figure 6, marked by "+") also indicated a resistance to another therapy type, but only by one type of study, they were additionally marked, indicating that the second therapy type is not recommended (marked by "−").

The selected miRNAs are presented in Figure 6. Those miRNAs with altered expression shown to be associated with therapy resistance are marked by the abbreviated therapy name and "−" because such therapies are not recommended for patients with such miRNA expression. Those miRNAs with altered expression shown to be associated with therapy sensitivity are marked by the abbreviated therapy name and "+" because such therapies are recommended for patients with such miRNA expression.

Thanks to the inclusion/exclusion criteria, sample sets with opposite miRNA expression do not have intersections, but these do have intersections with other sample sets (no intersections between ChT+ and

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

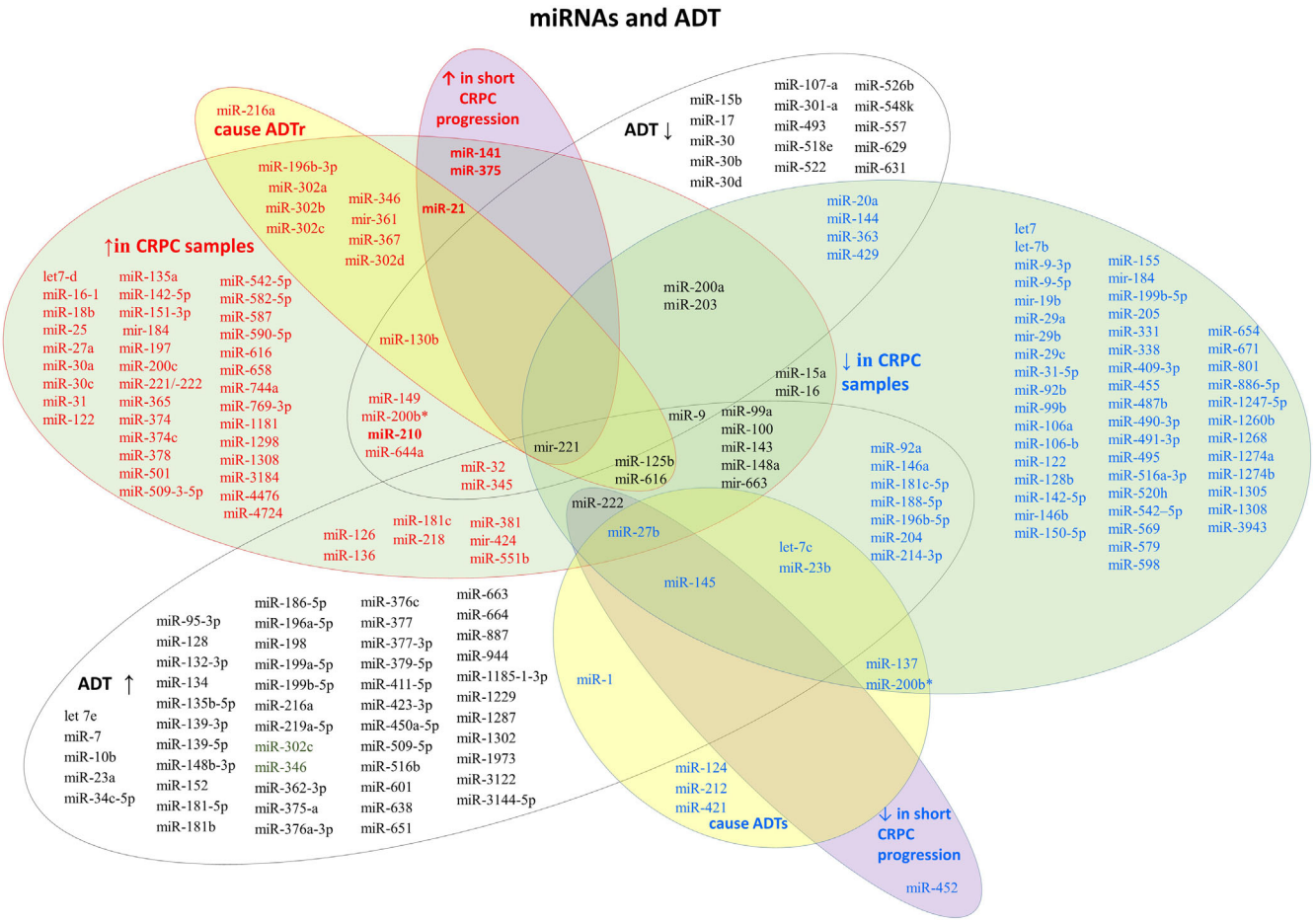


FIGURE 5 miRNAs and ADT response: miRNAs upregulated by ADT (ADT↑), miRNAs dowregulated by ADT (ADT↓), miRNAs upregulated in CRPC samples (↑ in CRPC samples), miRNAs downregulated in CRPC (↓ in CRPC samples), upregulated miRNAs provoking ADT resistance (cause ADTr), upregulated miRNAs provoking ADT sensitivity (cause ADTs), upregulated miRNAs in samples of patients with further short CRPC progression (↑ in short CRPC progression), downregulated miRNAs suppressed in samples of patients with further short CRPC progression (↓ in short CRPC progression). The red color indicates miRNAs, which were shown to be associated with ADT resistance, the blue color indicates miRNAs, which were shown to be associated with ADT sensitivity.

ChT−, RT+ and RT−, RP+ and RT−, ADT+ and ADT−). Nevertheless, the sample sets have multiple intersections with each other, which could interfere with the analysis of patient-specific miRNA expression panels. Moreover, these miRNAs, such as miR-21, miR-29b, and mir-30a, etc., could be used as a convenient universal normalizer to study paired miRNA expression in different groups. Another approach could be based on the inclusion of all miRNAs, with the use of those miRNAs demonstrating similar expression in different therapies as a confirmation of a choice for a given therapy (example.g., miR-204, −302a, and −375 can be indicators for ChT or ADT but not for RP or RT).

As a result, an miRNAs panel which has potential for use in the selection of an effective type of PCa therapy was selected ("therapy choice panel"). The future development of this panel involves precise identification of the thresholds of miRNA expression to differentiate patient groups, where the verification and validation of these thresholds, and the possible use of the selected panel, comprise an assessment of the expression. In fact, there may be patients possessing a miRNAs set which is simultaneously "recommend" for several types of therapy. Thus, thresholds for the best therapy choice should be additionally developed (for example, the effectiveness of therapy recommendations could be indicated by it having the greatest number of miRNAs; alternatively, the value of a given difference between miRNA expression and threshold may be informative for therapy selection). There may also be cases for which different therapies may have the same effectiveness; in such cases, the least severe therapy may be used.

The analysis of the known literature data revealed that the miRNAs from the "therapy choice panel" are deeply involved in the regulation of the biological processes which underlie carcinogenesis, such as proliferation, apoptosis, cell-cycle regulation, metastasis and invasion, androgen-independent growth, angiogenesis, epithelial–mesenchymal transition (Figure 7).

All the selected miRNAs are involved in the regulation of at least one of the analyzed processes, and most of these miRNAs regulate several of the analyzed processes.

The genes that are regulated by the miRNAs from the "therapy choice panel" were analyzed using databases to assess their

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

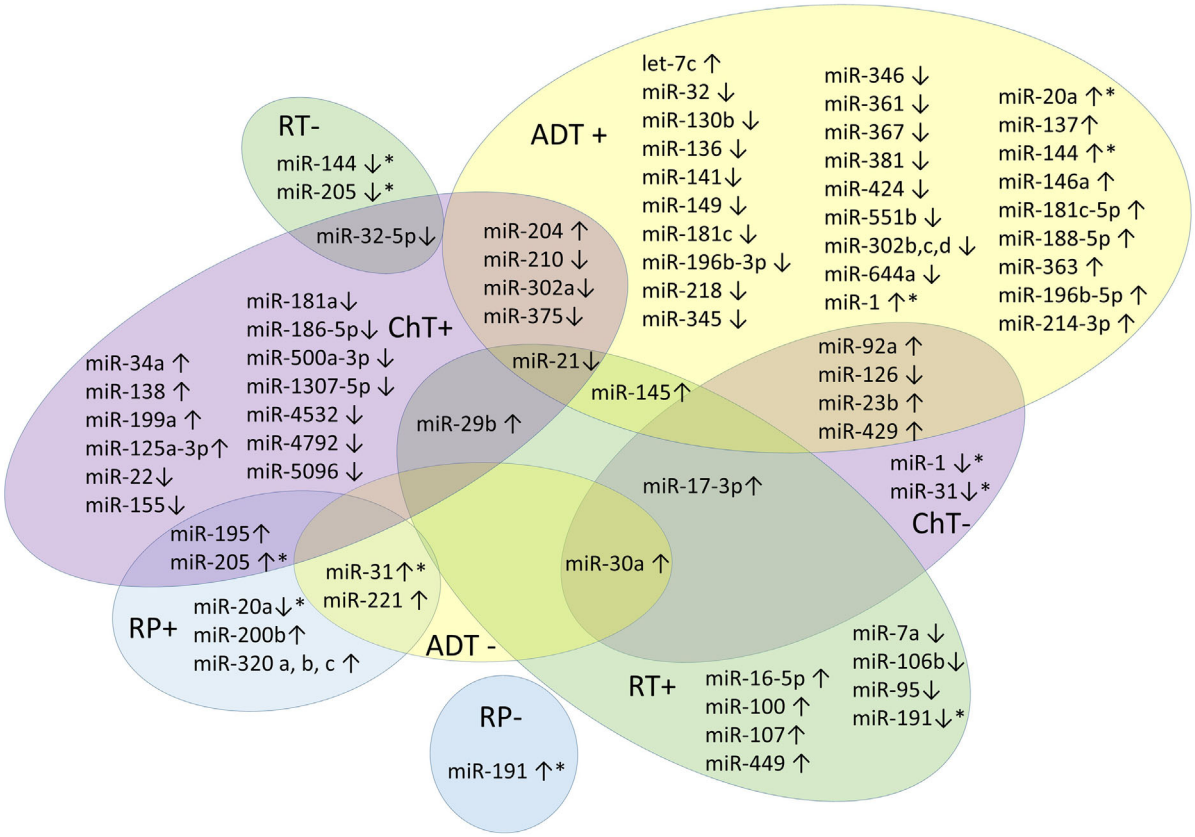


FIGURE 6 miRNAs panel for prostate cancer therapy choice ("therapy choice panel"). "+"—miRNAs, which expression associated with PCa therapy sensitiveness. "-"—miRNAs, which expression associated with PCa therapy resistance. "*"—duplicate miRNAs. RP, radical prostatectomy; RT, radiotherapy; ADT, androgen deprivation therapy; ChT, chemotherapy.

involvement in PCa and the development of PCa therapy resistance/sensitivity. Using the DIANA database, we uncovered the therapy-sensitive genes which are involved in the PCa development and regulated by a presented miRNA panel (Figure 8).

The DIANA database does not contain information on the work of miRNAs such as let-7c, miR-210, −4532, −5096, −644, −1, −7a, or −95, in the processes associated with the development of PCa; therefore, in further analysis, these microRNAs were not involved. The genes regulated by the selected miRNAs are mostly not individually interconnected with any given therapy type, and these are obviously responsible for general processes which are characteristic of PCa cells. To confirm the universality of the ongoing processes, we built an ontology of the genes involved in the sensitivity of all four study therapy types (using the String database) (Figure 9). The genes involved in the most important cellular processes are highlighted in color.

Thus, the presented database analysis confirms the involvement of an miRNA panel (Figure 6) in the regulation of those genes which are crucial for PCa development and therapy effectiveness. The gene ontology revealed the involvement of the analyzed genes in the high-importance pathway in the PCa development, as follows: the Pi3K-Akt signaling pathway, the mTOR signaling pathway, the FoxO signaling pathway, and the VEGF signaling pathway, etc. Thus, these genes can potentially be used as an addition to the "therapy choice panel" to increase its stability and sensitivity. However, the high interception of those genes which are regulated by miRNAs and involved in the regulation of resistance/sensitivity to different types of therapy evidences the importance of a careful investigation of the specificity of miRNAs from the "therapy choice panel" for each therapy type.

The genes which are involved in the regulation of sensitivity to only one type of the therapy have potential to make a great contribution to "the therapy choice panel." However, this is not the case because the genes which are "specific" for "RT+" (Figure 8) refer to the PIK3 family, which is known to be involved in responses to other therapy types (Figure 8). Concerning the genes which are involved in the regulation of ADT sensitivity, CASP 9 and BAD are in a list of the central regulators of apoptosis. ARAF is involved in the MAPK signaling pathway. IGFR is a strong tumor growth promoter in a wide variety of cancers. Moreover, according to STRING, five out of the six genes which are "specific" to "RT+" and "ChT+" are part of a joint gene web (data are not presented). This indicates that the studied genes are not specific for any single therapy type. However, only comprehensive research can state with certainty whether these genes may enhance the sensitivity/specificity of the "therapy choice panel" or not. Obviously, the selected panel must be validated by a series of cohort experiments, and the threshold of expression for each miRNA should be comprehensively determined.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

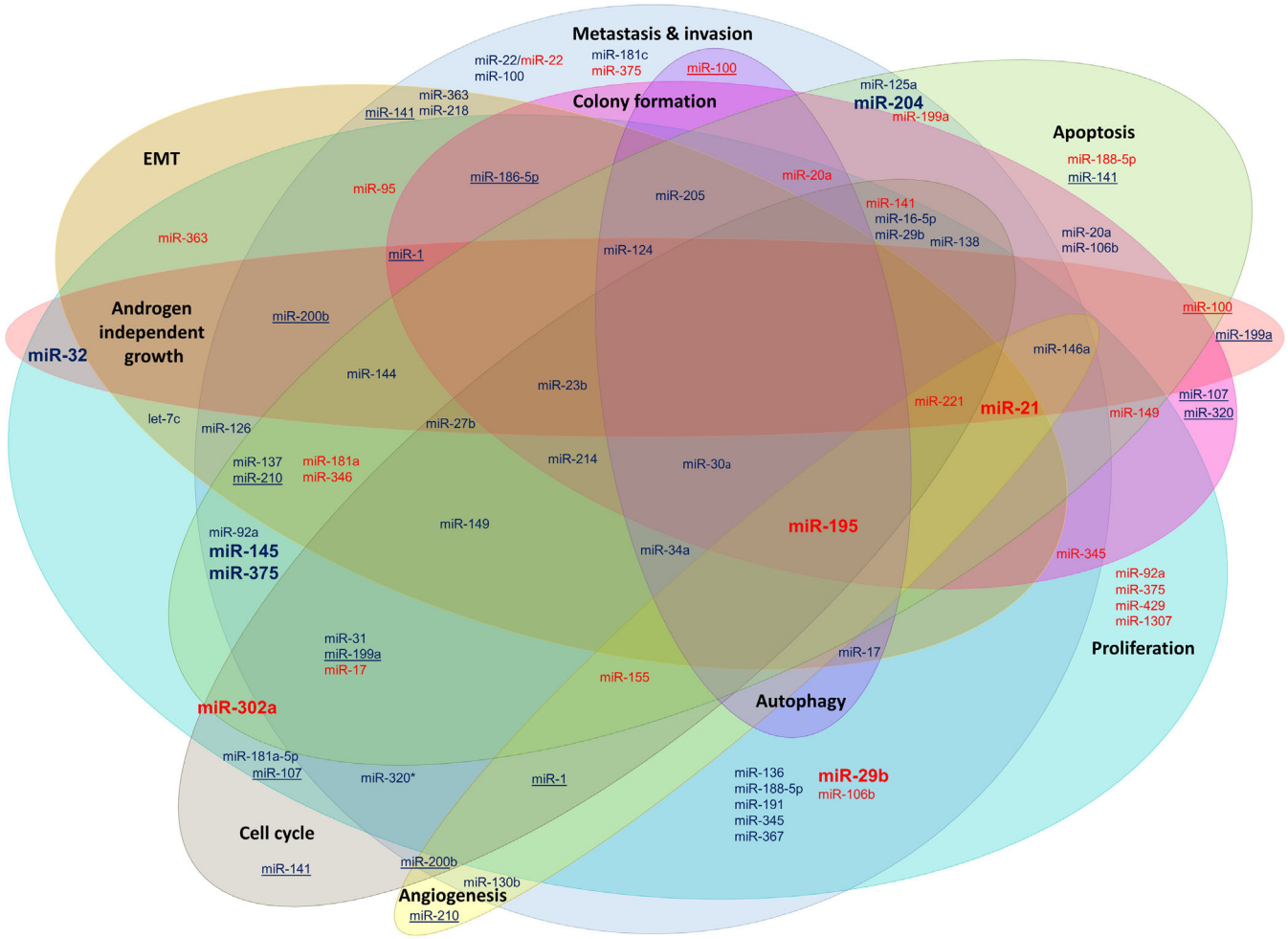


FIGURE 7 The involvement of miRNAs from "therapy choice panel" in the regulation of biological processes which underlie cancerogenesis and therapy resistance. Oncogenic miRNAs are marked by red color, tumor suppressors—by blue color. Duplicate miRNAs (example.g., miR-107, −141, −363 etc.) are underlined. miRNAs, which indicate the sensitivity to several therapy types are marked by large print.

1.6 | miRNA-based therapy effectiveness markers: challenges and perspectives

A great number of studies have demonstrated the high potential of miRNA-based biofluid biopsy for cancer diagnosis, prognosis, and therapy effectiveness assessment. Indeed, miRNAs are intrinsic in the regulation of all processes which are important for cancer therapy resistance and sensitivity. The expression of miRNAs can be assessed in different sources: tissues, biofluids, and extracellular vesicles. miRNA-based liquid biopsy presents an opportunity for noninvasive analysis of real-time patient status. This would allow the timely detection of the development of therapy resistance and indicate the need for dose/therapy type adjustments where needed.

However, a number of unsolved or insufficiently studied issues remain. First of all, despite the rise in studies aiming to analyze the potential of miRNAs as predictors of therapy effectiveness during recent years, this area remains poorly studied. Moreover, the pathobiology of miRNAs during different cancer therapies remains poorly understood. The main concerns are the low number of studied miRNAs (there are still a very limited number of studies using large-scale methods, e.g., sequencing, microchips) and low sample sizes. The latter is explicitly prevalent when long-term patient-state studies investigating dynamical miRNAs are executed. This is due to complications with the selection and formation of patient cohorts possessing similar clinical–pathological characteristics and therapy courses, as well as being attributable to the long-term follow-up with patients. The heterogeneity of PCa also contributes to differential miRNA expression and may obstruct the search for miRNA-based markers. For example, samples from the patients with androgen dependence/independence, different PCa stage, invasiveness, presence or absence of metastasis, etc., may be characterized by different miRNA expressions.[23,67,68] The diversity of PCa therapies, for example, different types of ADT, different dosages, and the duration of chemo- and radiotherapies, is another issue which should be taken into account while developing of miRNA-based markers. A separate and very complicated issue is the analysis of miRNAs during the combined therapy, for example, ADT+ RP +RT. The variability in sample collection and processing also

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

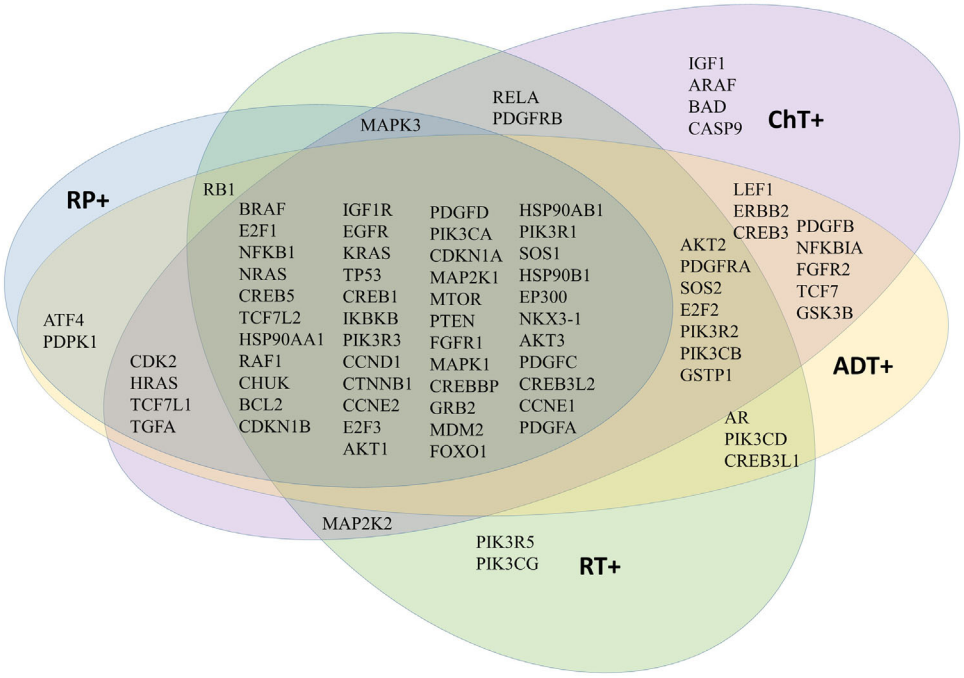
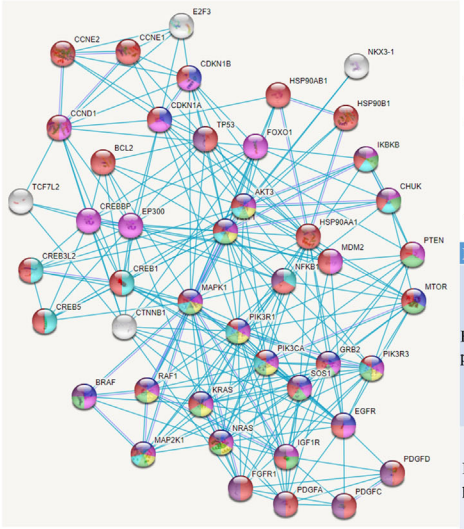


FIGURE 8 Genes, involved in PCa regulation and influenced by specific miRNAs sets from Figure 6 (miRNAs sets that indicate therapy sensitivity to specific therapy type); the data of DIANA database are presented.



Blue - ErbB signaling pathway
Raspberry - FoxO signaling pathway
Yellow - VEGF signaling pathway
Green - mTOR signaling pathway
Light blue - TNF signaling pathway
Red - PI3K-Akt signaling pathway
Violet - MAPK signaling pathway

Name	Description
ErbB signaling pathway	The ErbB family of receptor tyrosine kinases (RTKs) couples binding of extracellular growth factor ligands to intracellular signaling pathways regulating diverse biologic responses, including proliferation, differentiation, cell motility, and survival. The Shc- and/or Grb2-activated mitogen-activated protein kinase (MAPK) pathway is a common target downstream of all ErbB receptors. Similarly, the phosphatidylinositol-3-kinase (PI-3K) pathway is directly or indirectly activated by most ErbBs. Several cytoplasmic docking proteins appear to be recruited by specific ErbB receptors and less exploited by others. These include the adaptors Crk, Nck, the phospholipase C gamma (PLCgamma), the intracellular tyrosine kinase Src, or the Cbl E3 ubiquitin protein ligase.
FoxO signaling pathway	The forkhead box O (FOXO) family of transcription factors regulates the expression of genes in cellular physiological events including apoptosis, cell-cycle control, glucose metabolism, oxidative stress resistance, and longevity. A central regulatory mechanism of FOXO proteins is phosphorylation by the serine-threonine kinase Akt/protein kinase B (Akt/PKB), downstream of phosphatidylinositol 3-kinase (PI3K), in response to insulin or several growth factors.
VEGF signaling pathway	There is now much evidence that VEGFR-2 is the major mediator of VEGF-driven responses in endothelial cells and it is considered to be a crucial signal transducer in both physiologic and pathologic angiogenesis. The binding of VEGF to VEGFR-2 leads to a cascade of different signaling pathways, resulting in the up-regulation of genes involved in mediating the proliferation and migration of endothelial cells and promoting their survival and vascular permeability.
mTOR signaling pathway	The mammalian (mechanistic) target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase, which exists in two complexes termed mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 is activated by the presence of growth factors, amino acids, energy status, stress and oxygen levels to regulate several biological processes, including lipid metabolism, autophagy, protein synthesis and ribosome biogenesis. On the other hand, mTORC2 responds to growth factors and controls cytoskeletal organization, metabolism and survival.
TNF signaling pathway	Tumor necrosis factor (TNF), as a critical cytokine, can induce a wide range of intracellular signal pathways including apoptosis and cell survival as well as inflammation and immunity.
PI3K-Akt signaling pathway	The phosphatidylinositol 3' -kinase(PI3K)-Akt signaling pathway is activated by many types of cellular stimuli or toxic insults and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival.
MAPK signaling pathway	The mitogen-activated protein kinase (MAPK) cascade is a highly conserved module that is involved in various cellular functions, including cell proliferation, differentiation and migration.

FIGURE 9 STRING data: interaction network of genes, regulated by miRNAs, involved in sensitivity of all four studied therapy types. The data of STRING database are presented.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

contribute to the results obtained and discrepancies between different studies as well. miRNA expression is assessed in different biofluids (tissue, plasma, serum, plasma extracellular vesicles (EVs), cell-free urine, urine EVs, including different storage conditions and techniques for EVs isolation), using various protocols of miRNA isolation and real time polymerase chain reaction (RT-PCR). The gold standards are to be found. Moreover, not only the effectiveness of selected protocol should be taken into account but also the possibility to use them in clinical laboratory. For example, effective techniques like miRNA isolation using phenol–chloroform and EVs isolation using ultracentrifugation have limitations in clinical laboratory use.

One of the approaches to search for therapy-associated miRNA expression alterations is to analyze miRNA expression during or after the therapy. However, patients' oncological status after/during therapy is not the only possible cause of miRNA level variation. Each type of therapy causes the side effects and complications mentioned above, and these processes should be taken into account while analyzing miRNA expression alterations caused by therapies. That is why the variation of miRNAs expression after therapy is not sufficient to determine miRNAs' association with therapy effectiveness. Moreover, therapy sometimes induces therapy resistance, and the alteration of the expression of some miRNAs during therapy may reflect this process. Therefore, sample timing and its connection with signatures of healing should be carefully analyzed and introduced into the analysis of miRNA-based predictors of post-RP patient status. A possible way to exclude those alterations in miRNA expression which are not specific to therapy sensitivity is to analyze those miRNAs whose expression before therapy differed from the equivalent miRNAs' expression in healthy donors, and whose expression during or after therapy align with their expression in healthy donors if the therapy is efficient. Such miRNAs may have higher potentialities as diagnostic and prognostic markers.

On the other hand, miRNA studies based on PCa cell lines or xenografts also have limitations. PCa cell lines differ by their molecular signature, for example, through TP53 loss of heterozygosity, nucleotide mutations, gene expression level, neuroendocrine differentiation, cytogenetics, etc. Due to this heterogeneity, miRNA expressions induced by therapy can differ between cell lines due to reasons other than therapy sensitivity. Such miRNA expression changes may complicate the search for miRNA-based therapy markers. Moreover, cell line studies, unlike xenograft models and human studies, cannot reflect the complex phenomenon of therapy response including microenvironment-level mechanisms and adjusted tissues.

Another significant limitation of the studies discussed here is the high variability in their experimental design and different methodological complications. For example, there was an absence of proper standard normalizers for potential diagnostic miRNAs and different methodological procedures were used, starting from simple ones such as the isolation of miRNAs (enrichment by vesicle-associated miRNAs, efficacy of miRNA extraction, etc.). Both miRNA extraction efficacy and inter-sample and inter-experimental efficacy of RT-PCR may strongly interfere with miRNA expression data.

A further challenge in miRNA-based markers development is the specificity of miRNA markers to selected types of oncology and specific types of therapy. The markers predicting therapy effectiveness do not have to be specific toward a given cancer type, but it would be preferable if such markers could predict the effectiveness of different tumor therapies. At the same time, therapy-type specificity is important. Indeed, miRNAs regulate multiple processes involved in the cancer development, including therapy resistance and sensitivity mechanisms. Moreover, mechanisms of the development of resistance partly overlap for different therapy types. Therefore, miRNAs involved in the development of resistance to different therapy types overlap, and the intersections of the genes regulated by these miRNAs are even more pronounced (Figures 8). Therefore, finding a single miRNA marker for the prediction of cancer therapy effectiveness is unlikely. A solution to the issue may be provided by a combination of miRNA markers in a panel which can increase the specificity, robustness, and reproducibility of a given assessment of therapy outcome prediction. Another solution is a combination of miRNA-based markers with markers of a different nature: for example, PSA, gene methylation, or mRNA expression level. For example, a recent study by Tomeva et al.[68] showed that a discriminant function model combined with AR mutation, cell-free DNA methylation, and a panel of 12 different circulating miRNAs improved the performance of single-type markers and could classify several cancer types with 95.4% accuracy, 97.9% sensitivity, and 80% specificity.[68] The fact that each analyzed type in the study provided distinct information and increased the value of the model highlights the potential of such multi-nature-based test panels for diagnosis and, probably, prognosis of cancer.

2 | CONCLUSION

The vast amount of evidence and the literature and database analysis presented in the present work suggest that miRNAs are prominent players in the therapy-resistance development and that their expression can be a predictor of PCa therapy effectiveness and, thus, a useful tool in selecting effective personalized therapies. A meta-analysis of the current data revealed a panel of miRNAs that were shown to be most closely associated with PCa therapy responses and which were seen to regulate the genes involved in the PCa development. However, a very scrupulous experimental verification of the selected miRNA panel (with careful sample preparation and sample and data analysis) is needed. This is especially the case since the number of miRNAs involved in investigations of their roles in the development of PCa therapy resistance remains limited and insufficiently studied. Among main complication faced by miRNA researches are lack of standardized research, including variable doses and protocols, limited number of studied miRNA and patient sample, duration of surveillance. Due to above-mentioned standardized research, cohort validation studies and collaborative efforts to establish a robust miRNA-based prognostic panels are needed.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

AUTHOR CONTRIBUTIONS

Conceptualization: M.Y.K., O.E.B., and P.P.L. *Methodology*: M.Y.K., O.E.B. *Visualization*: M.Y.K. and O.E.B. *Writing—original draft*: M.Y.K. and O.E.B. *Writing—review and editing*: P.P.L. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

The research was carried out within the Russian Science Foundation project No. 23-25-10026, https://rscf.ru/project/23-25-10026/ within project supported by the Government of the Novosibirsk Region 00000054069959982351205 82/No. p—45.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

MariaYu Konoshenko https://orcid.org/0000-0003-2925-9350

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: gLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
2. Siegel RL, Miller KD, Wagle NS, et al. Cancer statistics. *CA Cancer J Clin*. 2023;73(1):17-48.
3. Li J, Siegel DA, King JB. Stage-specific incidence rates and trends of prostate cancer by age, race, and ethnicity, United States, 2004–2014. *Ann Epidemiol*. 2018;28:328-330.
4. Steele CB, Li J, Huang B, Weir HK. Prostate cancer survival in the United States by race and stage (2001–2009): findings from the CONCORD-2 study: US PCa Survival by Race and Stage. *Cancer*. 2017;123:5160-5177.
5. Lei JH, Liu LR, Wei Q, et al. Systematic review and meta-analysis of the survival outcomes of first-line treatment options in high-risk prostate cancer. *Sci Rep*. 2015;5:7713.
6. Greenberger BA, Zaorsky NG, Den RB. Comparison of radical prostatectomy versus radiation and androgen deprivation therapy strategies as primary treatment for high-risk localized prostate cancer: a systematic review and meta-analysis. *Eur Urol Focus*. 2020;6(2):404-418.
7. Ellinger J, Alajati A, Kubatka P, et al. Prostate cancer treatment costs increase more rapidly than for any other cancer-how to reverse the trend? *EPMA J*. 2022;13(1):1-7.
8. Shah H, Vaishampayan U. Therapy of advanced prostate cancer: targeting the androgen receptor axis in earlier lines of treatment. *Target Oncol*. 2018;13(6):679-689.
9. Shariat SF, Kattan MW, Vickers AJ, et al. Critical review of prostate cancer predictive tools. *Future Oncol*. 2009;5(10):1555-1584.
10. Pellegrino A, Cirulli GO, Mazzone E, et al. Focal therapy for prostate cancer: what is really needed to move from investigational to valid therapeutic alternative? A narrative review. *Ann Transl Med*. 2022;10(13):755.
11. Carthew RW. Gene regulation by microRNAs. *Curr Opin Genet Dev*. 2006;16(2):203-208.
12. Hata A. Functions of microRNAs in cardiovascular biology and disease. *Annu Rev Physiol*. 2013;75:69-93.
13. Sheinerman KS, Toledo JB, Tsivinsky VG, et al. Circulating brain-enriched microRNAs as novel biomarkers for detection and differentiation of neurodegenerative diseases. *Alzheimers Res Ther*. 2017;9(1):89.
14. He B, Zhao Z, Cai Q, et al. miRNA-based biomarkers, therapies, and resistance in cancer. *Int J Biol Sci*. 2020;16(14):2628-2647.
15. Cannistraci A, Di Pace AL, De Maria R, Bonci D. MicroRNA as new tools for prostate cancer risk assessment and therapeutic intervention: results from clinical data set and patients' samples. *BioMed Res Int*. 2014;2014:1-17.
16. Aveta A, Cilio S, Contieri R, et al. Urinary microRNAs as biomarkers of urological cancers: a systematic review. *Int J Mol Sci*. 2023;24(13):10846.
17. Bertoli G, Cava C, Castiglioni I. MicroRNAs: new biomarkers for diagnosis, prognosis, therapy prediction and therapeutic tools for breast cancer. *Theranostics*. 2015;5(10):1122-1143.
18. Mayr C, Beyreis M, Wagner A, et al. Deregulated microRNAs in biliary tract cancer: functional targets and potential biomarkers. *Biomed Res Int*. 2016;2016:4805270.
19. Condrat CE, Thompson DC, Barbu MG, et al. miRNAs as Biomarkers in disease: latest findings regarding their role in diagnosis and prognosis. *Cells*. 2020;9:276.
20. Konoshenko MY, Laktionov PP. MiRNAs and radical prostatectomy: current data, bioinformatic analysis and utility as predictors of tumour relapse. *Andrology*. 2021;9:1092-1107.
21. Konoshenko MY, Bryzgunova OE, Laktionov PP. miRNAs and radiotherapy response in prostate cancer. *Andrology*. 2021;9:529-545.
22. Konoshenko MY, Laktionov PP. The miRNAs involved in prostate cancer chemotherapy response as chemoresistance and chemosensitivity predictors. *Andrology*. 2022;10:51-71.
23. Konoshenko MY, Bryzgunova OE, Laktionov PP. miRNAs and androgen deprivation therapy for prostate cancer. *Biochim Biophys Acta Rev Cancer*. 2021;1876:188625.
24. Sun M, Sammon JD, Becker A, et al. Radical prostatectomy vs. radiotherapy vs. observation among older patients with clinically localized prostate cancer: a comparative effectiveness evaluation: comparative effectiveness of RP, RT and observation. *BJU Int*. 2014;113:200-208.
25. Novara G, Ficarra V, Mocellin S, et al. Systematic review and meta-analysis of studies reporting oncologic outcome after robot-assisted radical prostatectomy. *Eur Urol*. 2012;62:382-404.
26. Amling CL, Blute ML, Bergstralh EJ, et al. Long-term hazard of progression after radical prostatectomy for clinically localized prostate cancer: continued risk of biochemical failure after 5 years. *J Urol*. 2000;164:101-105.
27. Han M, Partin AW, Zahurak M, et al. Biochemical (Prostate Specific Antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer. *J Urol*. 2003;169:517-523.
28. Pound CR. Natural history of progression after PSA elevation following radical prostatectomy. *JAMA*. 1999;281:1591.
29. Mahdy A, Patil R, Parajuli S. Biochemical recurrence in prostate cancer and temporal association to bone metastasis. *Am J Case Rep*. 2019;20:1521-1525.
30. Albertsen PC. Treatment of localized prostate cancer: when is active surveillance appropriate? *Nat Rev Clin Oncol*. 2010;7:394-400.
31. Miyake H, Fujisawa M. Prognostic prediction following radical prostatectomy for prostate cancer using conventional as well as molecular biological approaches: prognosis after radical prostatectomy. *Int J Urol*. 2013;20:301-311.
32. Carneiro A, et al. The role of immunohistochemical analysis as a tool for the diagnosis, prognostic evaluation and treatment of prostate cancer: a systematic review of the literature. *Front Oncol*. 2018;8:377.
33. Yan W, Jamal M, Tan SH, et al. Molecular profiling of radical prostatectomy tissue from patients with no sign of progression identifies ERG as the strongest independent predictor of recurrence. *Oncotarget*. 2019;10:6466-6483.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

34. Lin X, Kapoor A, Gu Y, et al. Assessment of biochemical recurrence of prostate cancer (Review). *Int J Oncol*. 2019;55(6):1194-1212.
35. Di Minno A, Aveta A, Gelzo M, et al. 8-Hydroxy-2-Deoxyguanosine and 8-Iso-Prostaglandin F2α: putative biomarkers to assess oxidative stress damage following robot-assisted radical prostatectomy (RARP). *J Clin Med*. 2022;11(20):6102.
36. Konoshenko MY, Lekchnov EA, Bryzgunova OE, et al. The panel of 12 cell-free micrornas as potential biomarkers in prostate neoplasms. *Diagnostics*. 2020;10:38.
37. Strand SH, Schmidt L, Weiss S, et al. Validation of the four-miRNA biomarker panel MiCaP for prediction of long-term prostate cancer outcome. *Sci Rep*. 2020;10:10704.
38. Cheng B, He Q, Cheng Y, et al. A three-gene classifier associated with microrna-mediated regulation predicts prostate cancer recurrence after radical prostatectomy. *Front Genet*. 2020;10:1402.
39. Zhan B, Huang L, Chen Y, et al. miR-196a-mediated downregulation of p27 kip1 protein promotes prostate cancer proliferation and relates to biochemical recurrence after radical prostatectomy. *Prostate*. 2020;80:1024-1037.
40. Yang FE, Vaida F, Ignacio L, et al. Analysis of weekly complete blood counts in patients receiving standard fractionated partial body radiation therapy. *Int J Radiat Oncol Biol Phys*. 1995;33:617-617.
41. Verheij M, Dewit LG, Boomgaard MN, et al. Ionizing radiation enhances platelet adhesion to the extracellular matrix of human endothelial cells by an increase in the release of von Willebrand factor. *Radiat Res*. 1994;37:202-207.
42. Hallahan DE, Chen AY, Teng M, Cmelak AJ. Drug-radiation interactions in tumor blood vessels. *Oncol Williston Park*. 1999;13:71-77.
43. Tetreault-Laflamme A, Crook J. Options for salvage of radiation failures for prostate cancer. *Semin Radiat Oncol*. 2017;27:67-78.
44. Chang L, Graham PH, Hao J, et al. Emerging roles of radioresistance in prostate cancer metastasis and radiation therapy. *Cancer Metastasis Rev*. 2014;33:469-496.
45. Zelefsky MJ, Yamada Y, Fuks Z. Long-term results of conformal radiotherapy for prostate cancer: impact of dose escalation on biochemical tumor control and distant metastases-free survival outcomes. *Int J Radiat Oncol*. 2008;71:1028-1033.
46. Mao A, Tang J, Tang D, et al. MicroRNA-29b-3p enhances radiosensitivity through modulating WISP1-mediated mitochondrial apoptosis in prostate cancer cells. *J Cancer*. 2020;11:6356-6364.
47. Lo HC, Hsu JH, Lai LC, et al. MicroRNA-107 enhances radiosensitivity by suppressing granulin in PC-3 prostate cancer cells. *Sci Rep*. 2020;10:14584.
48. Iinuma K, Kawakami K, Mizutani K, et al. miRNA-93 in serum extracellular vesicles before and after low dose rate prostate brachytherapy. *Anticancer Res*. 2021;41:2411-2418.
49. Dagher R, Li N, Abraham S, et al. Approval summary: docetaxel in combination with prednisone for the treatment of androgen-independent hormone-refractory prostate cancer. *Clin Cancer Res*. 2004;10:8147-8151.
50. de Bono JS, Oudard S, Ozguroglu M, et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet*. 2010;376:1147-1154.
51. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci*. 1980;77:1561-1565.
52. Clarke SJ, Rivory LP. Clinical pharmacokinetics of docetaxel. *Clin Pharmacokinet*. 1999;36:99-114.
53. Ganju A, Yallapu MM, Khan S, et al. Nanoways to overcome docetaxel resistance in prostate cancer. *Drug Resist Updat*. 2014;17:13-23.
54. Darshan MS, Loftus MS, Thadani-Mulero M, et al. Taxane-induced blockade to nuclear accumulation of the androgen receptor predicts clinical responses in metastatic prostate cancer. *Cancer Res*. 2011;71:6019-6029.
55. Vrignaud P, Sémiond D, Lejeune P, et al. Preclinical antitumor activity of cabazitaxel, a semisynthetic taxane active in taxane-resistant tumors. *Clin Cancer Res*. 2013;19:2973-2983.
56. Sánchez C, Mendoza P, Contreras HR, et al. Expression of multidrug resistance proteins in prostate cancer is related with cell sensitivity to chemotherapeutic drugs: multidrug resistance in prostate cancer. *Prostate*. 2009;69:1448-1459.
57. Bukowski K, Kciuk M, Kontek R. Mechanisms of multidrug resistance in cancer chemotherapy. *Int J Mol Sci*. 2020;21:3233.
58. Trost LW, Serefoglu E, Gokce A, et al. Androgen deprivation therapy impact on quality of life and cardiovascular health, monitoring therapeutic replacement. *J Sex Med*. 2013;10:84-101.
59. Taylor LG, Canfield SE, Du XL. Review of major adverse effects of androgen-deprivation therapy in men with prostate cancer. *Cancer*. 2009;115:2388-2399.
60. Teleni L, Chan RJ, Chan A, et al. Exercise improves quality of life in androgen deprivation therapy-treated prostate cancer: systematic review of randomised controlled trials. *Endocr Relat Cancer*. 2016;23:101-112.
61. Grossmann M, Hamilton EJ, Gilfillan C, et al. Bone and metabolic health in patients with non-metastatic prostate cancer who are receiving androgen deprivation therapy. *Med J Aust*. 2011;194:301-306.
62. Nabavi N, Saidy NRN, Venalainen E, et al. miR-100-5p inhibition induces apoptosis in dormant prostate cancer cells and prevents the emergence of castration-resistant prostate cancer. *Sci Rep*. 2017;7:4079.
63. Varenhorst E, Klaff R, Berglund A, et al. Predictors of early androgen deprivation treatment failure in prostate cancer with bone metastases. *Cancer Med*. 2016;5:407-414.
64. Suzuki H, Akakura K, Komiya A, et al. CAG polymorphic repeat lengths in androgen receptor gene among Japanese prostate cancer patients: potential predictor of prognosis after endocrine therapy. *Prostate*. 2002;51:219-224.
65. Shimbo M, Suzuki H, Kamiya N, et al. CAG polymorphic repeat length in androgen receptor gene combined with pretreatment serum testosterone level as prognostic factor in patients with metastatic prostate cancer. *Eur Urol*. 2005;47:557-563.
66. Grivas PD, Robins DM, Hussain M. Predicting response to hormonal therapy and survival in men with hormone sensitive metastatic prostate cancer. *Crit Rev Oncol Hematol*. 2013;85:82-93.
67. Rönnau CGH, Fussek S, Smit FP, et al. Upregulation of miR-3195, miR-3687 and miR-4417 is associated with castration-resistant prostate cancer. *World J Urol*. 2021;39:3789-3797.
68. Tomeva E, Switzeny OJ, Heitzinger C, et al. Comprehensive approach to distinguish patients with solid tumors from healthy controls by combining androgen receptor mutation p.H875Y with cell-free DNA methylation and circulating miRNAs. *Cancers*. 2022;14:462.

How to cite this article: Konoshenko M, Laktionov P, Bryzgunova O. Prostate cancer therapy outcome prediction: are miRNAs a suitable guide for therapeutic decisions? *Andrology*. 2024;12:705–718. https://doi.org/10.1111/andr.13535

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13535 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 10 May 2023 | Revised: 17 August 2023 | Accepted: 3 September 2023

DOI: 10.1111/andr.13530

ANDROLOGY

WILEY

REVIEW ARTICLE

A metabolomic perspective on the mechanisms by which environmental pollutants and lifestyle lead to male infertility

Wen Yang[1,2,3] | Rong Hua[1,3] | Yunxia Cao[1,3] | Xiaojin He[2,3]

[1]Reproductive Medicine Center, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, Hefei, China

[2]Reproductive Medicine Center, Department of Obstetrics and Gynecology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

[3]NHC Key Laboratory of Study on Abnormal Gametes and Reproductive Tract, Anhui Medical University, Hefei, China

Correspondence
Xiaojin He, Reproductive Medicine Center, Department of Obstetrics and Gynecology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.
Email: hxj0117@126.com

Yunxia Cao, Reproductive Medicine Center, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, Hefei, China.
Email: caoyunxia5972@ahmu.edu.cn

Funding information
National Key R&D Program of China, Grant/Award Number: 2021YFC2700901; National Natural Science Foundation of China, Grant/Award Numbers: 81971441, 82101681; Outstanding Talents Support Program of Anhui Province, Grant/Award Number: gxbjZD2020061; Collegial Collaborative Innovation Project of Anhui Province, Grant/Award Number: GXXT-2021-071; Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences, Grant/Award Number: 2019PT310002

Abstract

The incidence of male infertility (MI) is rising annually. According to epidemiological studies, environmental pollution (e.g., organic, inorganic, and air pollutants), occupational exposure (e.g., high temperature, organic solvents, and pesticides), and poor lifestyle (e.g., diet, sleep, smoking, alcohol consumption, and exercise) are important non-genetic causative factors of MI. Due to multiple and complex causative factors, the dose–effect relationship, and the uncertainty of pathogenicity, the pathogenesis of MI is far from fully clarified. Recent data show that the pathogenesis of MI can be monitored by the metabolites in serum, seminal plasma, urine, testicular tissue, sperm, and other biological samples. It is considered that these metabolites are closely related to MI phenotypes and can directly reflect the individual pathological and physiological conditions. Therefore, qualitative and quantitative analysis of the metabolome, the related metabolic pathways, and the identification of biomarkers will help to explore the MI-related metabolic problems and provide valuable insights into its pathogenic mechanisms. Here, we summarized new findings in MI metabolomics biomarkers research and their abnormal metabolic pathways triggered by the presented non-genetic risk factors, providing a metabolic landscape of semen and seminal plasma in general MI patients. Then, we compared the similarities and differences in semen and seminal plasma biomarkers between MI patients exposed to environmental and poor lifestyle factors and MI patients in general, and summarized some common biomarkers. We provide a better understanding of the biological underpinnings of MI pathogenesis, which might offer novel diagnostic, prognostic, and precise treatment approaches to MI.

KEYWORDS
biomarkers, male infertility (MI), metabolic profiling, metabolomics, MI pathogenesis, non-genetic factors

Wen Yang and Rong Hua contributed equally to this work.

© 2023 American Society of Andrology and European Academy of Andrology.

1 | INTRODUCTION

1.1 | Non-genetic factors play an important role in male infertility but are difficult to treat

Infertility affects about 8%–12% of couples globally, and male factors are its major cause in approximately 50% of the cases.[1–3] The clinical manifestations of male infertility (MI) mainly include oligozoospermia, asthenospermia, teratozoospermia, and azoospermia, as well as their combinations.[4] It is estimated that there are many factors leading to MI, both genetic and non-genetic. Currently, high-throughput sequencing technology enables the screening of patients with MI caused by genetic factors for pathogenic mutations and abnormalities in their genetic material.[5,6] This provides excellent insights into its pathogenesis. However, the pathogenesis of MI caused by non-genetic factors has proved challenging. Environmental and occupational exposures, lifestyle factors (such as diet, sleep, smoking, alcohol, and exercise), obesity, aging, and varicocele are potential non-genetic risk factors for MI.[7–12] The pathogenicity of these non-genetic factors appears to be more complex than that of genetic factors, and due to their dose–effect, pathogenicity can vary depending on the individual.[13–15]

Current treatments for clinical MI due to non-genetic factors mainly include changing lifestyle, drug treatment (hormone, antioxidant, anti-infection therapy, etc.), food supplements, surgical treatment, and assisted reproductive technologies.[16–18] However, due to a lack of understanding of its pathogenesis, the success of symptomatic treatment is challenging.[16,19,20] The most commonly used semen analysis and other detection methods in clinical practice cannot clarify MI aetiology and potential pathogenesis.[21] Therefore, there is an urgent need for new technologies to find disease-related biomarkers and elucidate MI pathogenesis more efficiently. We consider that it can open a new path for the precise and targeted clinical treatment of MI.

1.2 | Disturbances in metabolic pathways may explain the pathogenesis of male infertility

Several hypotheses have been proposed for the pathogenesis of non-genetic MI, including oxidative stress and energy metabolism imbalance in the testis and seminal plasma, hormone metabolism disorder and inflammation response in peripheral blood, as well as epigenetic modifications that can occur in individuals after exposure to harmful factors,[6,22–27] and the combination of the above mechanisms. When these processes occur, they are reflected by changes in the levels of sperm transcriptome, proteome, gut microbiome, metabolome, and epigenome.[28–31] At present, the application of functional genomic ("-omic") technologies and high-throughput analyses, including gut microbiomics, transcriptomics, proteomics, and metabolomics, are commonly used to screen molecular biomarkers for many human pathologies.[30,32,33] Thus, they provide a new perspective on the underlying mechanisms of the disease's pathogenesis and progression. However, metabolomics has better sensitivity, and is both effective and comprehensive.[34,35] In contrast to the relatively simple chemical constitutions of the genome (four nucleotide bases) and proteome (20 proteogenic amino acids), the metabolome consists of thousands of different chemical classes and the number of metabolites is estimated to be around 1 million, while the number of genes and proteins are about 20,000 and 620,000, respectively.[34] These metabolites are closely correlated with genes in which a single deoxyribonucleic acid (DNA) base change in a given gene can result in a 10,000-fold change in the levels of endogenous metabolites.[34,36] In addition, the metabolome is believed to reflect the molecular profile closest to an individual's phenotype and subtle alterations in biological pathways (occurring in real time or over the time scale of seconds, minutes, hours, or even days) can be detected to provide insight into the mechanisms that underlie various physiological conditions and aberrant processes, including diseases.[37]

Metabolomics is usually defined as the study of the overall qualitative and quantitative composition of metabolites in biological systems.[38–44] These metabolites can be endogenous or can originate from the metabolism of drugs, environmental chemicals, and microbiota.[37,45] The two main analytical platforms used for metabolomics are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS); Table 1 summarizes their advantages and disadvantages. The data derived from these platforms provide qualitative and quantitative metabolomic analyses, allowing the identification of MI biomarkers and the analysis of associated metabolic pathways, thus describing MI-related metabolic issues and providing valuable insights into them. Currently, researchers have elucidated that the differential metabolites between MI and matched controls can be classified as amino acids, fatty acids, acylcarnitines, lipids, purines, organic acids, and sugars, which are involved in pathways associated with the metabolism of branched-chain amino acids (BCAAs), tryptophan, lipids, energy, and purines, as well as oxidative stress/redox homeostasis pathways.[42,46–50]

2 | SEARCH METHODS

2.1 | Search strategy

The PubMed database was searched for published articles, using the keywords: ("male infertility" OR "sperm quality" OR "sperm parameters") and ("metabolomics" OR "biomarkers" OR "metabolic pathways" OR "metabolic fingerprint" OR "metabolic profiling" OR "nuclear magnetic resonance spectroscopy" OR "mass spectrometry") and ("environmental pollution" OR "phthalate" OR "benzene, toluene and xylenes" OR "arsenic" OR "copper" OR "cadmium" OR "air pollutants" OR "$PM_{2.5}$" OR "nanoparticles" OR "SO_2" OR "O_3" OR "poor lifestyle" OR "high fat diet" OR "obesity" OR "circadian rhythm" OR "sleep deprivation" OR "smoking" OR "cigarettes". In addition, to provide a metabolic landscape of semen and seminal plasma in patients with general MI, additional searches were performed in PubMed using the keywords: ("male infertility" OR "oligozoospermia" OR "asthenozoospermia" OR "teratozoospermia" OR

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 The advantages and limitations of NMRS, LC–MS, and GC–MS as analytical tools for metabolomics research, adapted from ref.[51,52]

	NMRS	LC–MS	GC–MS
Sample	Liquid, solid, gas phase, and tissue	Nonvolatile, polar compounds	Volatile, nonpolar compounds
Sample preparation	Simple	Simple	More demanding
Experiment cost	Cheap	Expensive	Expensive
Experiment time	A few minutes to hours	Slow	Slow
Metabolite identification	Difficult, because of the convoluted spectra and peak overlap	Difficult, because of the lack of comprehensive spectral libraries	Accurate
Sensitivity	Low but can be improved with higher field magnets, cryo- and microprobes, and dynamic nuclear polarization.	Very sensitive	Sensitive
Reproducibility	Very high	Difficult	Moderate
Sample recovery	Nondestructive		
sample can be recovered and stored for a long time	Destructive to the sample	Destructive to the sample	
Target analysis	Not relevant for targeted analysis	Superior for targeted analysis	Superior for targeted analysis
Libraries	Libraries of limited use due to a complex matrix	Limited commercial libraries	Large commercial and public libraries
Tissue samples extraction	NO, using HRMAS-NMR	Require extraction	Require extraction

Abbreviations: GC–MS, gas chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; NMRS, nuclear magnetic resonance spectroscopy; HRMAS-NMR, high-resolution magic angle spinning-nuclear magnetic resonance.

"oligoasthenoteratozoospermia" OR "azoospermia" OR "unexplained male infertility" OR "andrology diseases" OR "varicocele" OR "erectile dysfunction" OR "Kallmann syndrome" OR "normosmic congenital hypogonadotropic hypogonadism" and ("metabolomics" OR "biomarkers" OR "metabolic pathways" OR "metabolic fingerprint" OR "metabolic profiling" OR "nuclear magnetic resonance spectroscopy" OR "mass spectrometry") and ("semen" OR "seminal plasma").

2.2 | Inclusion and exclusion criteria

This narrative review has no time frame or species restrictions. Eligible studies were selected according to the following criteria: (i) metabolomic changes in sperm, semen, seminal plasma, blood, urine, and other biological samples of MI patients and animal models after exposure to environmental and poor lifestyle factors; (ii) metabolomic changes in semen and seminal plasma of patients with general MI (not related to environmental pollution and poor lifestyle); (iii) a case group of men diagnosed with infertility or decreased sperm quality and a control group of men without infertility. Outcomes had to contain at least the identified biomarkers or differential metabolic pathways. The major exclusion criteria were as follows: (i) duplicate data; (ii) case series $n < 5$, letters to the editor, pilot studies, reviews, and comments; (iii) insufficient data reported. The selection was divided into three phases. First, studies were selected by title and duplications were removed. Second, abstract screening was performed. Third, the full texts of articles were screened for eligibility. Suitable articles were then included.

3 | METABOLOMIC INVESTIGATION OF THE ROLE OF ENVIRONMENTAL FACTORS IN MALE INFERTILITY

Recently, there have been increasing reports on the adverse effects of environmental toxicants (e.g., organic, inorganic, and air pollutants) on male reproductive health.[53,54] For example, in a large, retrospective, cohort study in southern China, ambient air pollutants (carbon monoxide [CO], sulfur dioxide [SO_2], ozone [O_3], particulate matter 2.5 [$PM_{2.5}$], and particulate matter 10 [PM_{10}]) were significantly associated with reduced sperm counts, especially for the period over 10—14 lag days.[55] In addition, the decreased sperm motility was significantly correlated with O_3.[55] Moreover, a cross-sectional study in a fertility center in Italy found that bisphenol A (BPA) and phthalates and their metabolites in the urine of male volunteers had significant negative effects on semen parameters (e.g., concentration, motility, and malformation rates).[56] In addition, various heavy metals, such as copper and cadmium, have been identified as pathogenic agents of MI due to their toxic and endocrine-disrupting properties.[57,58] However, there are still unresolved questions and gaps in how these environmental toxicants affect the male reproductive system.[59] The application of metabolite profiling in MI can help understand the underlying pathogenesis of the disease at a molecular level, which serves as a hub linking these pollutants to MI pathogenesis (details shown in Table 2). In the following sections, we will review primary metabolomics-based findings in the metabolomes of different sample matrices obtained from MI patients in relation to organic pollutants (phthalate esters [PAEs], benzene, toluene, and xylenes), inorganic pollutants (arsenic, copper,

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Metabolomics findings act as a hub linking environmental pollution to male infertility.

Categories	Study design	Subjects	Biological matrix	Technique	Discriminant metabolites or findings	Pathways	Reference
Exposure to phthalate	Wuhan, China case–control	660 adult men (high and low semen quality)	Seminal plasma (human)	Untargeted LC–HRMS	(−): oleic acid and L-acetylcarnitine	1. Unsaturated fatty acid biosynthesis 2. Acetylcarnitine metabolism	Wang et al.[60]
	Xiamen, China case–control	150 fertile males 139 infertile males	Urinary (human)	Untargeted HPLC–QTOF–MS	(+): carnitine C2, carnitine C3:1, carnitine C8, carnitine C10:1, carnitine C10:2, carnitine C12-DC; aspartate, hydroxyestrone and leucylproline (−): uridine and methylxanthine	1. Carnitine and/or acetylcarnitine metabolism 2. Amino acid 3. Oxidative stress 4. Energy production	Liu et al.[61]
Exposure to benzene, toluene and xylenes	Netherlands case–control	99 workers occupationally exposed to organic solvents 27 controls	Urinary (human)	TargetedHPLC	High levels of glycol ethers, hippuric acid, and methyl hippuric acid were significantly correlated with a lower risk of low semen quality	1. Energy production	Tielemans et al.[62]
	Ningbo, China case–control	56 workers occupationally exposed to organic solvents 37 controls	Semen and blood (human)	Targeted LC–MS	(+): fructose (−): γ-GT and LDH-C4 activities	1. Energy production	Xiao et al.[63]
Exposure to arsenic	Xiamen, China cross-sectional	67 adult men (high and low semen quality)	Seminal plasma (human)	Untargeted UPLC/MS/MS	(+): eicosatetraenoate (−): L-carnitine, pivaloylcarnitine, hydroxyhexanoycarnitine and DHA	1. Oxidative stress 2. Energy production 3. Carnitine metabolism 4. Unsaturated fatty acid biosynthesis	Huang et al.[22]
	Nanjing, China case–control	76 fertile males 179 infertile males	Urinary (human)	Untargeted HPLC–QTOF–MS	(+): acetyl-*N*-formyl-5-methoxykynurenamine, carnitine, estrone, 2-oxo-4-methylthiobutanoic acid, malonic acid, valine, and LysoPC (10:0)	1. One-carbon metabolism 2. Oxidation stress 3. Steroidogenesis dysfunction.	Wu et al.[64]
	Xiamen, China animal models	10 mice (1 mg/L sodium arsenite) 10 mice (5 mg/L sodium arsenite) 10 mice (25 mg/L sodium arsenite) 10 controls	Testis (mouse)	Untargeted UPLC–MS	(+): L-Leucine, trigonelline, L-Methionine, L-Phenylalanine, L-Tyrosine, 2-Keto-6-acetamidocaproate, L-Acetylcarnitine, and LysoPE(0:0/20:4(5Z,8Z,11Z,14Z)) (−): hypoxanthine, L-2,3-Dihydrodipicolinate, inosine, allopregnanolone, and cortolone	1. Amino acid biosynthesis and metabolism 2. Energy production 3. Lipid metabolism 4. Oxidation stress	Huang et al.[65]

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 (Continued)

Categories	Study design	Subjects	Biological matrix	Technique	Discriminant metabolites or findings	Pathways	Reference
Exposure to copper	Guangzhou, China animal models	6 germ cell samples of copper sulfate-treated 6 controls	Germ cell line (mouse)	Untargeted LC–MS	1. In ESI+ mode: (+): 39 metabolites (such as 13-hydroxykaur-16-en-18-oic acid, indole-3-acrylic acid, DL-tryptophan, ophthalmic acid and gamma glutamylleucine) 2. In ESI- mode: (+): 114 metabolites (such as 2-phosphinomethylmalic acid, hexose, uridine, ophthalmic acid and gamma glutamylleucine) (−): 13 metabolites (such as 22-oxodocosanoic acid, alpha-Naphthoflavone, and (2R)−2,3-dihydroxypropyl nonadecanoate)	1. Oxidation stress 2. Polyunsaturated fatty acids 3. Amino acid biosynthesis and metabolism	Lin et al.[23]
	Riyadh, India, Egypt animal models	mice exposed $CuSO_4$/copper oxychloride controls	Blood and testis (mouse)	ELISA Western blotting	(+): MDA and NO (−): GSH, SOD, and CAT; LH, FSH, and testosterone	1. Hormones biosynthesis and metabolism 2. Oxidation stress	Heba Nageh et al.[66]; Sarawi et al.[67]; Mandil et al.[68]
Exposure to cadmium	Egypt, China, Iran, Italy animal models	mice exposed $CdCl_2$ controls	Blood and testis (mouse)	ELISA Western blotting	(+): MDA and NO (−): GSH, SOD, and CAT; testosterone	1. Hormones biosynthesis and metabolism 2. Oxidation stress	Elmallah et al.[69]; Zeng et al.[70]; Tayebe et al.[71]; Venditti et al.[72]
Exposure to $PM_{2.5}$	Chongqing, China animal models	cells treated with different concentrations of PM2.5 suspensions (12.5, 25, 50, 100, 200 μg/mL) controls	GC-2spd cell line (mouse)	Targeted UHPLC–QE–HFX–MS/MS	(−): saccharopine, L-Histidine, S-Adenosylmethionine, L-Asparagine, L-Lysine, Citrulline, L-Methionine, L-Phenylalanine, L-Isoleucine, L-Valine; dGTP, guanosine, ADP, deoxyguanosine, deoxyinosine, adenosine and hypoxanthine	1. TCA cycle 2. oxidative metabolism 3. Amino acid biosynthesis and metabolism 4. Energy production	Shi et al.[73]
	China animal models	mice exposed concentrated ambient PM2.5 (CAP) controls	Serum and testis (mouse)	ELISA	(−): testosterone, FSH, and LH	1. Hormones biosynthesis and metabolism	Zhou et al.[74]; Yang et al.[75]; Qiu et al.[76]
Exposure to carbon black nanoparticles	Hebei, China animal models	12 mice exposed carbon black nanoparticles 6 controls	Serum (mouse)	Untargeted UHPLC–Q-TOF–MS/MS	(+): taurocholic acid, LysoPC (16:0), amino acid and nucleotide derivatives (−): hormones and lipids	1. Hormone metabolism 2. Lipid metabolism 3. Amino acid 4. Nucleotide metabolism.	Du et al.[77]

Abbreviations: ADP, adenosine diphosphate; CAT, catalase; DHA, docosahexaenoic acid; FSH, follicle stimulating hormone; GC–MS, gas chromatography–mass spectrometry; GSH, glutathione; HPLC–MS, high-performance liquid chromatography– mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; LDH-C4, Lactate dehydrogenase C4; LH, luteinizing hormone; MDA, malondialdehyde; NMRS, nuclear magnetic resonance spectroscopy; NO, nitric oxide; SOD, superoxide dismutase; UHPLC–Q-TOF/MS, ultra-high-performance liquid chromatography–quadrupole time-of-flight–mass spectrometry; UPLC–MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; γ-GT: gamma-glutamyl transpeptidase; dGTP, deoxyguanosine triphosphate.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

and cadmium), and air pollutants ($PM_{2.5}$, nanoparticles, SO_2, and O_3). Research of the literature shows that these pollutants adversely affect the reproductive health of men, and their pathogenic mechanisms have been increasingly investigated using metabolomics.

3.1 | Organic pollutants and male infertility

Although economic growth, as a result of industrialization, has improved the health-related quality of life indicators, it has also led to the augmentation of the release of chemical toxins into the environment.[53] These chemicals (PAEs, benzene, toluene, and xylenes) were not fully tested for toxicity before commercialization and they may thus eventually pose a threat to the environment and threaten human reproductive health.[78]

3.1.1 | Urinary phthalate ester metabolites are associated with the incidence of male infertility

PAEs are widely used in various industrial sectors as plasticizers, solvents, and additives in a number of consumer products, such as vinyl flooring, wall coverings, food containers, cosmetics, coatings of pharmaceutical pills, and nutritional supplements to enhance their flexibility, transparency, and durability.[78,79] As EDs, PAEs can enter the body through the skin, inhalation, or oral intake, and can interfere with the endocrine system of the body which, in turn, affects the male reproductive function.[80] A study of 660 Chinese adults by Wang et al. using non-targeted liquid chromatography–mass spectrometry (LC–MS) identified 25 potential biomarkers, mainly including docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), oleic acid, 9,10-dihydroxystearic acid, and carnitine or its derivatives (hexanoylcarnitine, propionylcarnitine, L-palmitoylcarnitine, L-acetylcarnitine, and linoelaidyl carnitine), that were associated with poor semen quality in human seminal plasma.[60] Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway metabolic pathway database (www.genome.jp/kegg), they also found that the increased primary and secondary PAE metabolites in the urine affected semen quality by inducing metabolic disorders associated with polyunsaturated fatty acids (PUFAs) and acylcarnitine in the seminal plasma.[60] In addition, a study by Liu et al. using high-performance liquid chromatography–quadrupole time-of-flight–mass spectrometry (HPLC–QTOF–MS) showed that both the increased levels of phthalate metabolites in the urine and increased MI risk were significantly associated with changes in metabolites such as uridine, methylxanthine, aspartate, and hydroxyestrone in urine. These metabolites are mainly involved in energy metabolism, oxidative stress, and hormone metabolism.[61]

From the accumulated data, we conclude that elevated levels of primary and secondary metabolites of PAEs in the urine can affect semen quality by altering the metabolites in the seminal plasma and urine. However, the design of these studies was cross-sectional, which makes it challenging to establish a causal relationship between the altered metabolites and MI.[60,61] Therefore, further prospective research is needed to verify these potential mechanisms underlying MI pathogenesis.

3.1.2 | The metabolites of benzene, toluene, and xylenes in urine may cause male infertility through impaired energy and amino acid metabolism in sperm

A multicenter case-referent study conducted in the UK showed that exposure to organic solvents such as benzene, toluene, and xylene was associated with MI.[81] After the exposure of workers to benzene, toluene, and xylene, Tielemans et al. performed a metabolic analysis of their urine using reversed-phase HPLC and gas chromatography (GC).[62] Interestingly, they confirmed an association between urinary glycol ethers, hippuric acid (a metabolite of toluene), and methyl hippuric acid (a metabolite of xylene) and abnormal semen parameters, with a stronger association in men with primary infertility.[62] In another study, Liu et al. collected urine from workers exposed to the same organic solvents and performed a metabolomic analysis using LC–TOF–MS. The findings revealed a total of 27 potential biomarkers that were closely related to combined exposure to benzene, toluene, and xylene. These biomarkers were significantly enriched in three pathways, namely, lysine metabolism, amino sugar metabolism, and nucleotide sugar metabolism.[82] Meanwhile, Xiao et al. found that after exposure to these solvents, the levels of fructose in the semen were significantly increased, while lactate dehydrogenase C4 (LDH-C4) levels and acrosin activity were significantly reduced. This suggested that both the sperm energy metabolism and fertilization capacity were severely affected.[63] On the other hand, in the studies discussed above,[63,82] the exposure groups were composed of 20 and 24 individuals, respectively. Disturbances in these metabolic pathways were not specific to organic solvent exposure. Thus, more workers need to be recruited to validate these potential mechanisms.

3.2 | Inorganic pollutants and male infertility

Besides the organic pollutants discussed above, some toxic inorganic pollutants, due to their non-biodegradable and persistent nature, can accumulate in the environment over long periods and can, therefore, interfere with different physiological processes in the body even at very low concentrations.[83,84] Exposure to toxic inorganic pollutants such as arsenic, copper, and cadmium can cause damage to the male reproductive system, resulting in lower semen quality parameters, testicular dysfunction, and hormonal imbalances.[85]

3.2.1 | Disturbances in urinary, testicular, and semen metabolites are associated with male infertility after arsenic exposure

Arsenic, a metalloid, mostly found in groundwater as a byproduct of soil/rock erosion and industrial and agricultural processes, has been

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

identified as a toxicant that affects virtually every organ or tissue of human body.[86,87] Food and water supplies are the main intake routes in daily life.[87] The reported mechanisms by which arsenic exposure contributes to the development of MI are alterations in the body's hormone levels, increased oxidative stress, DNA damage, and metabolic disturbances associated with spermatogenesis.[88] Huang et al. performed a UPLC/MS-based metabolomic analysis of 98 human seminal plasma. Data showed that the reduced L-carnitine, pivaloylcarnitine, hydroxyhexanoycarnitine, and DHA, as well as the elevated eicosatetraenoate, were associated with an increased risk of low-sperm-concentration for arsenic exposure.[22] In another large case–control study, eight biomarkers associated with MI were found in the urine of the arsenic exposure group. These metabolic biomarkers were AFMK, estrone, carnitine, valine, and fatty acid metabolites when compared to controls by the HPLC–QTOF–MS platform. The authors further constructed the meet-in-metabolite analysis (MIMA)-related metabolic network and indicated that arsenic methylation coupled one-carbon metabolism disruption with oxidation stress, leading to impaired fatty acid oxidation and steroidogenesis, thus ultimately inducing MI.[64]

In a study of arsenic exposure in rats, 40 Sprague–Dawley (SD) male rats that were randomly distributed into four groups, each group containing 10 rats.[65] Animals from all groups were administered with deionized water, 1 mg/L, 5 mg/L, and 25 mg/L of sodium arsenite (SA) via drinking water, respectively. After 6 months of rats' exposure, samples from their testicular tissues were collected and a metabolomic analysis based on the ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) analytical platform was performed.[65] The obtained results showed that, compared with controls, 8 metabolites, such as L-leucine, trigonelline, L-methionine and L-phenylalanine, were significantly increased, while another 5, such as cortolone, allopregnanolone, and inosine were significantly decreased. By employing the MetaboAnalyst 2.0 software (http://www.metaboanalyst.ca), the authors further found that these metabolites have mainly occurred in four metabolic pathways, which were characterized as important for the aminoacyl-tRNA biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, as well as for the ubiquinone and another terpenoid–quinone biosynthesis.[65]

Taken together, many biomarkers, such as amino acids, cholines, fatty acids, and cortisol have been identified in the organism after long-term arsenic exposure. These biomarkers may bring new insights to elucidate the reduction of sperm quality and quantity in men due to arsenic exposure.

3.2.2 | Copper and cadmium exposure result in lower semen quality parameters, testicular dysfunction, and hormonal imbalances

Copper is a heavy metal that is widespread in the environment and is known to be toxic to humans on exposure.[89] Lin et al. studied the damage produced by copper exposure on the reproductive system by analyzing mouse spermatogonia cell lines with LC–MS/MS.[23] The results showed significant effects on the biosynthesis and metabolism of amino acids, while the levels of two biomarkers of oxidative stress, namely, ophthalmic acid and γ-glutamyl leucine, were observed to be significantly increased.[23] These results suggested that oxidative stress and amino acid metabolism disorders play an important role in the reproductive toxicity induced by copper exposure. Furthermore, excessive levels of Cu^{2+} in the body can alter the conformation of the luteinizing hormone receptor (LHR), follicle stimulating hormone receptor (FSHR), and testosterone receptor, leading to dysfunction of the hypothalamic-pituitary-gonadal (HPG) axis and ultimately impairing normal spermatogenesis.[90]

Cadmium is another common heavy metal and is considered one of the major reproductive toxicants in males.[91] The biological half-life of cadmium in the human body is very long (about 20—40 years), and the excretion rate is very low, so that accumulation in the body for a long time can affect male reproductive function.[91,92] Cadmium-induced reproductive toxicity among males occurs mainly in two ways: (1) by interfering with the development and function of Leydig cells to induce disruption of hormone metabolism in the testis[93] and (2) by significantly increasing the level of reactive oxygen species (ROS) in the testis, leading to lipid peroxidation (LPO) of sperm membranes, reduced adenosine triphosphate levels, and decreasing sperm quality.[94–96] In addition, Zhu et al.[93] concluded that all antioxidants partially or completely antagonized the toxic effects mediated by cadmium, such as L-carnitine,[97] vitamin E,[98] and vitamin C.[99]

3.3 | Air pollutants and male infertility

Air pollutants are closely related to human health and usually exist in the form of solid particles, droplets or gases, mainly including particles ($PM_{2.5}$ and nanoparticles), sulfur dioxide (SO_2), and ozone (O_3).[100] Based on current metabolomics studies in both clinical populations diagnosed with MI and experimental models, researchers found that air pollutants affect male fertility mainly by inducing oxidative stress and hormone metabolism disorders.[101,102]

3.3.1 | Oxidative damage in testes and hormonal imbalances in serum caused by $PM_{2.5}$ and nanoparticles lead to male infertility

There are solid data showing that ambient PM with a diameter of fewer than 2.5 aerodynamic micrometers, designated as $PM_{2.5}$ and nanoparticles, is currently considered to be the most important component of air pollution.[103] Indisputable data show the accumulation of these particles in the reproductive organs via the blood–testis barrier and the placental barrier, ultimately lead to the development of MI.[104,105] In in vitro experiments, after exposure of the GC-2spd cell line to different concentrations of PM2.5 (12.5, 25, 50, 100, 200 μg/mL), researchers observed significant inhibition of proliferation and mitochondrial

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

damage and dysfunction in the cells.[73] To further investigate the possible pathogenic mechanism of PM2.5, metabolomics analysis was conducted to evaluate metabolic changes induced by PM2.5 exposure. The results showed that the levels of saccharopine, L-histidine, S-adenosylmethionine, L-asparagine, L-lysine, citrulline, L-methionine, L-phenylalanine, L-isoleucine, and L-valine were significantly decreased compared to the control group. In terms of purine metabolism, the concentrations of deoxyguanosine triphosphate (dGTP), guanosine, ADP, deoxyguanosine, deoxyinosine, adenosine, and hypoxanthine were significantly reduced by more than 50% compared to the controls. These possible biomarkers are mainly enriched in pathways associated with energy production, oxidative stress, and amino acid metabolism.[73] In addition, studies on rats exposed to carbon black nanoparticles utilized the UHPLCQ–TOF–MS/MS-based non-targeted metabolomic platform to analyze the metabolic changes of these animals' blood serum and found that the detected metabolites, associated with the biosynthesis of steroid hormones (e.g., estradiol, pregnenolone, cortexolone, and cortisol), were significantly affected and difficult to recover.[77] These hormones are essential for spermatogenesis, growth, development, and maturation. Based on mouse models, some toxicological experiments also found that exposure to $PM_{2.5}$ significantly reduced the antioxidant capacity of reproductive organs and markedly increased the concentration of ROS and the expression of the enzyme superoxide dismutase (SOD) in murine testes.[106–108] On the other hand, 8-hydroxy-2′-deoxyguanosine (8-Oh-dG) is a sensitive marker of DNA damage and is widely used as such of oxidative damage.[105,109] Zhang et al. observed that the content of 8-Oh-dG was increased in rats' testes after exposure to $PM_{2.5}$ (0, 1.8, 5.4, and 16.2 mg/kg.bw.) through intratracheal instillation for 30 days, compared with this of the control group. The result of it was that the sperm quantity and quality of these rats were decreased.[110] These results suggested that exposure to $PM_{2.5}$ could induce testicular oxidative stress, which then caused oxidative damage to sperm.

3.3.2 | Exposure to SO_2, and O_3 lead to oxidative stress during spermatogenesis

Studies on the damage mechanism of SO_2, O_3 and other gas pollutants to the male reproductive system mostly focused on oxidative stress.[111,112] Zhang et al. pointed out that SO_2 caused oxidative damage to human sperm in the early stage of the sperm development, while increased levels of malondialdehyde (MDA), a marker of oxidative damage, were detected in human seminal plasma.[111] In mouse experiments, LPO and changes in antioxidative status in testis were also observed after exposure to SO_2.[113,114] Extrapulmonary toxicity studies have shown that O_3 or O_3 products could penetrate the blood–gas barrier and were absorbed by circulating blood,[115] thus inducing oxidative stress that damages the function of the male reproductive system and the integrity of DNA within the sperm through this pathway.[116]

4 | METABOLOMICS STUDY OF THE EFFECT OF LIFESTYLE ON MALE INFERTILITY

4.1 | A high-fat diet or poor nutrition can lead to disruption of the metabolome in the reproductive system

Nowadays, people gradually realize the importance of "light-fat" and "low-carbon" diet. The food uptake and energy balance play an important role in the maintenance of normal male fertility.[117] ^{1}H-NMR metabolomic profiling of mice that were given a high-fat diet (HFD) found that the testicular metabolome was enriched in AMP, glutamine (Gln), and glutathione (GSH), whereas acetate content was strikingly decreased.[118] These biomarkers affect spermatogenesis through various metabolic pathways, which include cAMP-activated signaling pathways (AMPK), Gln accumulation that leads to energy metabolism impairment in the Sertoli cells (SCs), and elevation in glutathione levels misbalancing the redox responses.[118] In a study conducted by Palmer and colleagues, mice had been given an HFD for 10 weeks. The mice were further divided into four groups, each receiving one of the following interventions for 8 weeks: (1) continuation of HFD, (2) change to a low-fat diet (LFD), (3) continuation of HFD with exercise, (4) change to LFD with exercise.[119] The results showed that diet alone reduced adiposity (1.6-fold) and serum cholesterol levels (1.7-fold, $P < 0.05$), while exercise alone did not change them. Interestingly, the diet and/or exercise improved sperm quantity and quality, and reduced ROS and mitochondrial membrane potential, while the exercise combined with an LFD also improved glucose tolerance.[119]

When energy intake is inadequate, male fertility is also affected.[120] After the Wistar rats were fed for 28 days with 30% less of the standard chow diet than the control group, the researchers collected their plasma and testes.[121] The results, acquired by nuclear magnetic resonance spectroscopy (NMRS), showed that testicular tissue metabolism was severely affected. It was mainly manifested as: (1) the levels of glutamate (Glu), proline (Pro), alanine (Ala), aspartic acid (Asp), and BCAAs were significantly decreased, (2) the concentrations of carbonyl and NT-substituted proteins, markers of oxidative damage, were significantly reduced, followed by an increase in the transcript levels of NRF1 in testicular tissue, suggesting a decrease in oxidative damage in the testis. Moreover, in rat plasma, the hormonal profile was found to be out of balance, seen particularly in higher levels of ghrelin and glucagon-like peptide-1 (GLP-1), and lower levels of leptin. Disruptions in these metabolic pathways can affect spermatogenesis and reduce sperm quality, which in turn can affect male fertility.[121]

In another study, where mice (F_0) were fed with HFD, the sperm parameters were significantly affected in their offspring like sons (F_1) and/or grandsons (F_2), compared to these the descendants of the chow-fed mice. Meanwhile, the metabolomics analysis of testicular tissue via NMRS revealed increased levels of glycine, leucine and acetate and decreased levels of inosine and Gln in the F_1. The sperm

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

parameters and testicular metabolites were correlated by using the Pearson correlation method, and the correlation matrixes for each generation were obtained independently.[27] In addition, even when F_0 was given transient HFD, the results showed changes in the testicular metabolism and the sperm parameters in their offspring.[27] These data demonstrated that there were paternally-mediated intergenerational and transgenerational effects on the testicular metabolome and sperm quality caused by an HFD.

4.2 | Circadian rhythm disturbances and sleep deprivation lead to increased oxidation levels, suppressed sex hormone production, and abnormal testicular function and spermatogenesis

Sleep is also an important influencing factor in MI, including circadian rhythm disturbances and sleep deprivation (SD).[122,123] Shift workers are reportedly more likely to have diabetes mellitus, dyslipidemia, hypertension, gastrointestinal disturbances, and reproductive dysfunction according to published findings.[124] Ortiz et al. studied 20 rotating shift workers and found that elevated levels of 5-hydroxyindoleacetic acid (5-HIIA), a metabolite of serotonin, in the urine were associated with an increased risk of infertility in these workers.[125] Serotonin, a neurotransmitter and its receptors have been reported to be localized in the Leydig cells and to have an inhibitory effect on the synthesis and release of testosterone.[126] This suggests that the increased risk of infertility in shift workers may be mediated by neuroendocrine imbalances. Melatonin, as a neurohormone, is one of the final products of serotonin's catabolism and plays a regulatory role in the male's reproductive system. When circadian rhythms are disturbed, the synthesis and secretion of melatonin in humans are affected.[123] Melatonin is an antioxidant substance and influences the synthesis and release of hypothalamic GnRH and thus pituitary LH and FSH and eventually testicular function.[127] Therefore, low fertility in shift workers may also be due to disruption of melatonin metabolism.[123]

In a SD mouse model, researchers found significant changes in the hormone metabolic pattern of these mice. The obtained results showed significantly higher serum levels of cortisol, corticosterone, and significantly lower levels of testosterone.[128,129] The authors further suggested that the increased corticosteroids reduced the testosterone levels by causing inhibition of the HPG axis and activation of the hypothalamus–pituitary–adrenal (HPA) axis. Meanwhile, SD male rats showed higher serum and testicular tissue levels of ROS and MDA, while the serum total antioxidant capacity (TAC) and testicular tissue GSH levels were lower compared to the control group, assuming that the redox processes in the testis were imbalanced.[128] Moreover, SD altered the nitric oxide (NO) pathway in mice testes, leading to an increase in NO levels.[130] NO could inhibit DNA replication and induce LPO, ultimately resulting in apoptosis of Sertoli and germ cells in the testis as well as spermatogenesis.

4.3 | Smoking—a long-term concern

The impact of smoking on male reproductive health is a long-term concern.[131] Compared to nonsmokers, the sperm metabolomic profiling of regular smokers by LS–MS/MS revealed activation of nitric oxide synthase (a marker of oxidative stress), reduced mitochondrial uptake of fatty acids, leading to inadequate energy supply and disturbed metabolism of phenylalanine and tryptophan indicating altered biosynthesis of tetrahydrobiopterin.[132] Ascorbic acid is an important antioxidant that protects the body from oxidative damage. Dawson et al. have revealed that in male heavy-smokers there was a 20%–40% reduction in serum ascorbic acid levels, but when the ascorbic acid was given as a supplement then the authors observed improvement in the participants' sperm quality.[133] It is proposed that these disturbances may be mediated by mitochondrial dysfunction, which is mainly manifested as a decrease in the expression of Ca (2+)-ATPase and creatine kinase in sperm.[134,135]

Notably, the effects of smoking on steroid hormone levels remain controversial.[136] In some large retrospective case–control studies, researchers discovered a significant positive association between serum total testosterone (TT), free testosterone (FT), and smoking.[137,138] Conversely, in other studies, authors stated opposing views,[139] which were supported by the fact that the accumulated data showed no effect of smoking on the total levels of testosterone.[140]

5 | A METABOLIC LANDSCAPE OF SEMEN AND SEMINAL PLASMA IN MALE INFERTILITY PATIENTS

The semen and seminal plasma not only provide nutritional support for spermatozoa but also modulate the process of sperm maturation. Therefore, as a potential source of biomarkers, they have a more complex molecular composition than blood and urine. Table 3 summarizes the findings of metabolomic studies of the semen and seminal plasma of patients with general MI (not related to environmental pollution and poor lifestyle). We observed 31 common biomarkers in the semen and seminal plasma of patients with oligoasthenospermia, asthenozoospermia, teratozoospermia, unexplained MI, azoospermia, normosmic congenital hypogonadotropic hypogonadism, and varicocele. The metabolic roles of these biomarkers vary according to the disease, and detailed information on them is summarized in Table 4.

Then, we compared the similarities and differences in these semen and seminal plasma biomarkers between MI patients who had been exposed to environmental and poor lifestyles factors and those with general MI (Tables 2 and 5 vs. Table 3). The results showed that a number of biomarkers were common to the two groups, including elevated levels of Gln, glycine, and valine and decreased levels of carnitine and its metabolites (acylcarnitines, L-acetylcarnitine, pivaloylcarnitine, and hydroxyhexanoycarnitine), unsaturated fatty acids (DHA and oleic acid), fructose, citric acid, succinic acid, valine, glycine, serine, leucine, isoleucine, phenylalanine, alanine, threonine, proline, glutamic acid, lysine, tyrosine, tryptophan, spermidine, and spermine. These

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 Overview of metabolomic studies of the semen and seminal plasma in general male infertility.

Categories	Study design	Subjects	Biological matrix	Technique	Discriminant metabolites or findings	Pathways	Reference
Oligoasthenospermia (OA)	Cagliari, Italy case–control	18 OA 29 controls	Semen	Untargeted ^{1}H NMR spectroscopy	(+): choline and fructose (−): myo-inositol and aspartate	1. Energy production 2. Choline metabolism	Federica et al.[141]
	Angers, France case–control	20 severe oligoasthenospermia (SOA) 20 controls	Seminal plasma	Untargeted HPLC-MS	(−): 17 phosphatidylcholines and four sphingomyelins; acylcarnitines, with free L-carnitine being the most discriminating metabolite; polyunsaturated fatty acids; six amino acids (glutamate, aspartate, methionine, tryptophan, proline, and alanine); four biogenic amines (spermine, spermidine, serotonin, and alpha-aminoadipate)	1. Acylcarnitines metabolism 2. Phospholipids (choline) metabolism 3. PUFA biosynthesis and metabolism 4. Amino acids metabolism	Magalie et al.[142]
	Angers, France case–control	19 extreme oligospermia (EO) 20 controls	Semen	Targeted HPLC–MS	(−): amino acid level, polyamines (spermine and spermidine); polyunsaturated fatty acids (PUFA); free carnitine	1. Amino acids metabolism 2. Spermine biosynthesis and metabolism 3. Carnitine metabolism 4. Fatty acid metabolism	Orianne et al.[143]
Asthenozoospermia (AS)	Wenzhou, China case–control	87 AS 73 controls	Seminal plasma	Untargeted UPLC–MS/MS	(+): lactic acid (−): the tricarboxylic acid cycle-related products, such as pyruvic acid, succinic acid, malic acid, citric acid, hypoxanthine, tryptophan, inosine, phenylalanine, methionine, and valine	1. Energy production 2. Amino acid metabolism 3. Purine metabolism 4. Methionine cycle	Chen et al.[144]
	Shenzhen, China case–control	33 AS 30 controls	Seminal plasma	Untargeted ^{1}H NMR spectroscopy	(+): choline containing metabolites (choline, phosphocholine, and glycerophosphocholine); the Krebs cycle metabolites (citrate and α-ketoglutarate); cholesterol metabolism metabolites (cholesterol, 5α-cholesterol and 7-ketocholesterol); creatinine; glutamine, glutamate, cysteine, histidine and taurine; (−): lipids and cytidine; phenylalanine and tyrosine	1. Amino acids metabolism 2. Lipids metabolism, 3. Phospholipids (choline) metabolism 4. Cholesterol metabolism 3. Nucleoside metabolism 4. Energy production 5. Oxidative stress	Zhang et al.[145]
	Nanjing, China case–control	30 AS 30 controls	Seminal plasma	Targeted GC–MS	(+): both palmitic acid (saturated fatty acid); oleic acid (monounsaturated fatty acid); valine	1. Fatty acid metabolism 2. Amino acids metabolism	Tang et al.[146]
	Punjab, India case–control	13 AS 50 controls	Seminal plasma	Targeted LC–MS–MS	glucose, triglycerides, cholesterol, and microprotein; cholesterol, glutamic pyruvic transaminase, and lactate dehydrogenase	1. Steroid biosynthesis 2. Steroid hormone biosynthesis 3. Energy production	Ashutosh et al.[147]
	Manipal, India case–control	20 AS 20 controls	Seminal plasma	Untargeted ^{1}H NMR spectroscopy	(−): guanidoacetate and fructose	1. Energy production	Varshini et al.[148]

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 (Continued)

Categories	Study design	Subjects	Biological matrix	Technique	Discriminant metabolites or findings	Pathways	Reference
	Zhejiang, China case–control	20 AS 20 controls	Seminal plasma	Untargeted UHPLC–Q-TOF/MS	(+): creatinine, uric acid, N6 -methyladenosine (m6 A), uridine, and taurine (−): carnitine, nicotinamide, *N*-acetylputrescine and l-palmitoylcarnitine	1. Energy production 2. Amino acids metabolism 3. Oxidative stress 4. Acylcarnitines metabolism 5. Nucleoside metabolism	Li et al.[149]
Teratozoospermia	Tehran, Italy case–control	14 infertile patients with teratozoospermia 15 controls	Seminal plasma	Untargeted ^{1}H NMR spectroscopy	(+): citric acid, choline, D-glucose, L-tyrosine, L-alanine, L-proline, L-leucine, L-lysine, myo-inositol, L-lactic acid, L-threonine, pyruvate, glutamine, valine and isoleucine (−): L-glutamic acid, cholesterol and taurine	1. Amino acids metabolism 2. TCA cycle 3. Oxidative stress	Bahareh et al.[150]
	Zanjan, Iran case–control	15 infertile patients with teratozoospermia 12 controls	Seminal plasma	Targeted LC–MS–MS	(+): glutamine, asparagine, and glycylproline, whereas downregulated metabolites include cysteine, γ-aminobutyric acid, histidine, hydroxylysine, hydroxyproline, glycine, proline, methionine, ornithine, tryptophan, aspartic acid, argininosuccinic acid, α-aminoadipic acid, and β-aminoisobutyric acid; glutamine, asparagine (−): cysteine, tryptophan, glycine, and valine	1. Amino acids metabolism 2. Energy production	Elham et al.[151]
Idiopathic Oligoasthenoteratozoospermia (OAT)	Malatya, Turkey case–control	31 OAT 28 controls	Seminal plasma	Untargeted ^{1}H NMR spectroscopy	(+): tyrosine (−): lactate, citrate, lysine, arginine, valine, glutamine, creatinine, α-ketoglutaric acid, spermine, and putrescine	1. Amino acid metabolism 2. Lipid metabolism, 3. Phospholipid metabolism 3. Cholesterol metabolism 4. Energy production 5. Nucleotide metabolism	Mumcu et al.[152]
Azoospermia	Manipal, India case–control	20 azoospermia 20 controls	Seminal plasma	Untargeted ^{1}H NMR spectroscopy	(−): guanidoacetate and fructose	1. Energy production	Varshini et al.[148]
Non-obstructive azoospermia (NOA)	Tehran, Italy case–control	9 (NOA, TESE+) 11 (NOA, TESE-) 10 controls	Seminal plasma	Untargeted GC–MS	The tartaric acid, 2,2,4,4,6,6-hexamethyl-1,3,5-trithiane, and 2-Pyrrolidineacetic acid were special biomarkers for (TESE-) group	–	Kambiz et al.[153]
	Tehran, Italy case–control	10 (NOA, TESE+) 10 (NOA, TESE-)	Seminal plasma	Targeted Raman Spectrometer	The ROS level was 901 RLU for TESE (+) and 1968 RLU for TESE (−) patients.	Oxidative stress	Kambiz et al.[154]
Unexplained male infertility (UMI)	Tehran, Iran case–control	19 UMI 15 controls	Seminal plasma	Untargeted Raman spectrometer	The –CH functional group (2,800 3,000 cm^{-1}) was significantly increased whereas the –SH group (2,550 2,600 cm^{-1}) was missing in UMI men. The CH group is a biomarker of oxidative stress while the –SH group is a functional antioxidant.	Oxidative stress	Naser et al.[155]

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 (Continued)

Categories	Study design	Subjects	Biological matrix	Technique	Discriminant metabolites or findings	Pathways	Reference
	Manipal, India case–control	17 UMI 20 controls	Seminal plasma	Untargeted ^{1}H NMR spectroscopy	(–): valine, 2- hydroxyisovalerate, lysine, hippurate and fructose	1. Energy production 2. Amino acids metabolism 3. Oxidative stress	Varshini et al.[148]
	Nanjing, China	50 UMI 50 controls	Seminal plasma	Untargeted GC–MS	(+): urea and glutamine; (–): various amino acids, including tryptophan, phenylalanine, glycine, serine, threonine, isoleucine, proline, glutamic acid, and valine	1. Amino acids metabolism 2. Oxidative stress	Qiao et al.[156]
Varicocele (VC)	Brazil case–control	21 VF (fertile with VC) 35 VI (infertile with VC) 24 C (fertile without VC)	Seminal plasma	Untargeted ^{1}H NMR spectroscopy	(+): valine, 3-hydroxybutyrate, lactate, GABA, citrate, glycosides, and n-acetyltyrosine (–): caprate, isoleucine, uridine, glutamine, arginine, and tyrosine	1. Energy production 2. Amino acids metabolism 3. Oxidative stress 4. Fatty acid metabolism 5. Nucleoside metabolism	Filipe et al.[157]
Kallmann Syndrome (KS)	Beijing, China case–control	21 KS 22 controls	Seminal plasma	Targeted UPLC–QTOF–MS	(+): lipid species (triacylglycerol (TAG), sphingomyelin (SM), phosphatidylethanolamine (PE), lyso phosphatidylethanolamine (LPE), free fatty acid (FFA) and hexosylceramides (HexCer)); (–): TAG(53:2)_FA 17:0, TAG(56:5)_FA20:4, TAG(56:4)_FA18:1, TAG(54:4)_FA20:3, TAG(54:4)_FA16:0, PC (16:0/20:4), PC (16:0/16:1), PC (18:1/20:3), PC (14:0/18:1), and PC (16:0/14:0).	Lipid metabolism	Li et al.[158]
	Beijing, China case–control	17 KS 20 controls	Seminal plasma	Untargeted UPLC–QTOF–MS	(–): linoleic acid, 13-Hydroxyoctadecadienoic acid, LysoPC (18:1(9Z)), and Glycerylphosphorylcholine (GPC)	1. Linoleic acid metabolism 2. GPC metabolism	Guo et al.[159]
Normosmic congenital hypogonadotropic hypogonadism (ncHH)	Beijing, China case–control	25 ncHH 23 controls	Seminal plasma	Untargeted HPLC–qTOF–MS	(–): phenylalanine, tyrosine, and tryptophan biosynthesis, valine, leucine and isoleucine biosynthesis, arginine and proline metabolism, glycine, serine and threonine metabolism; triacylglycerol, phosphatidylethanolamine, sphingomyelin, phosphatidylcholines, lyso phosphatidylethanolamine, and hexosylceramides.	1. Amino acid metabolism 2. Lipid metabolism	Li et al.[160]
Kidney–Yang Deficiency syndrome (KYDS)	Shanghai, China case–control	18 KYDS 18 controls	Seminal plasma	Untargeted LC–MS	There are forty-one discriminating metabolites (18 increased and 23 decreased) were identified between the KYDS and controls.	1. Phenylalanine metabolism 2. Phenylalanine, tyrosine, and tryptophan biosynthesis 3. Glyoxylate and dicarboxylate metabolism 4. Tyrosine, and sphingolipid metabolism	Chen et al.[161]

Abbreviations: GC–MS, gas chromatography–mass spectrometry; HPLC–MS, high-performance liquid chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; NMRS, nuclear magnetic resonance spectroscopy; UHPLC–Q-TOF/MS, ultra-high-performance liquid chromatography–quadrupole time-of-flight–mass spectrometry; UPLC–MS/MS, ultra-performance liquid chromatography tandem mass spectrometry.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 4 Biomarkers common to both the semen and seminal plasma in infertile male patients.

Biomarkers	OA	AS	TZ	AZ	UMI	ncHH	VC	Reference
Myo-inositol	↓		↑					141,150
Glutamate	↓	↑						142,145
Methionine	↓	↓	↑					142,144,151
Choline	↑	↑	↑					141,145,150
Phosphocholine and phosphatidylcholines	↓	↑				↓		142,145,160
Tryptophan	↓	↓	↑ or ↓		↓	↓		160,161
Proline	↓		↑		↓	↓		142,150,151,156,160
Alanine and phenylalanine	↓	↓	↑		↓	↓		142,144,145,150,156,160,161
Lactic acid		↑	↑					144,150
Citrate		↑					↑	145,152,157
Cholesterol		↑	↓					145,150
Glutamine		↑	↑		↑		↓	145,150–152
Cysteine		↑	↓ or ↑					145,151
Histidine		↑	↑					145,151
Taurine		↑	↓					145,149,150
Valine		↑ or ↓	↑ or ↓		↓	↓	↑	145,146,148 150–152,156,157,160
Uridine		↑					↓	149,157
Succinic acid		↓	↑					144,151
Citric acid		↓	↑					144,150
Tyrosine		↓	↑			↓	↓	145,150,152,157,160,161
Leucine and isoleucine			↑		↓	↓	↓	150,156,157,160
Lysine			↑		↓			148,150–152
Threonine			↑		↓	↓		150,156,160
Glutamine		↑	↑		↑		↓	145,150–152,156,157
Cysteine		↑	↑ or ↓					145,151,152
Fructose	↑	↓		↓	↓			141,148
Acylcarnitines/carnitine/ L-palmitoylcarnitine	↓	↓						142,143,149
Guanidoacetate		↓		↓				148
Glycine			↑ or ↓		↓	↓		151,152,156,160
Glutamic			↓		↓			147,150,156
Serine					↓	↓		156,160

Abbreviations: AS, asthenozoospermia; AZ, azoospermia; ncHH, normosmic congenital hypogonadotropic hypogonadism; OA, oligoasthenospermia; TZ, teratozoospermia; UMI, unexplained male infertility; VC, varicocele.

biomarkers were mainly enriched in pathways associated with energy metabolism, oxidative stress, carnitine metabolism, fat metabolism, and amino acid metabolism. Therefore, these biomarkers may serve as a hub-linking environmental and poor lifestyle factors to MI, which will provide new insights into the pathogenesis of MI caused by non-genetic factors.

5.1 | Future perspectives

By providing an overall "fingerprint" of metabolite alterations in multiple biofluids and tissues, metabolomics provides a significant opportunity for further understanding the pathogenesis of MI. However, the application of metabolomics in MI is still in its infancy. First, the levels of metabolites are prone to fluctuations due to changes in the organism's environment. Altered metabolites can cause disease, and disease can also alter metabolites. Therefore, metabolites detected at incorrect time points cannot accurately reflect the true pathogenesis of MI. Second, there are significant differences in genotype, medical history, disease process, and lifestyle habits among individuals, which affect the metabolomes of the subjects and mask the metabolic effects of the disease and, therefore, need special attention. Third, different study designs can produce differing results, due to variations in sample preparation, the analytical techniques used, and the methods

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 5 Metabolomics findings act as a hub linking poor lifestyle to male infertility.

Categories	Study design	Subjects	Biological matrix	Technique	Discriminant metabolites or findings	Pathways	Reference
High-fat diet (HFD)	Porto, Portugal animal models	12 HFD mice 12 controls	Testis (mouse)	Untargeted ^{1}H NMR	(+): AMP, succinate, glutamate, glutamine, betaine, and glutathione (−): adenosine, leucine, acetate, glutamine	1. Energy production 2. Amino acid metabolism 3. Oxidative stress 4. Purine metabolism 5. Ammonia recycling	Crisóstomo et al.[118]
	Shandong, China animal models	8 HFD mice 8 controls	Testis, serum, and primary Sertoli Cells (mouse)	Real-time PCR Western Blotting	(+): total cholesterol, high-density lipoprotein-cholesterol and low-density lipoprotein-cholesterol; lactate; ROS, MDA (−): ATP	1. Glycolysis 2. Oxidative stress 3. Lipid metabolism	Luo et al.[162]
	Beijing, China animal models	30 HFD mice 30 controls	Testis and plasma (mouse)	Untargeted LC–MS/MS	(+): total glycerides, total cholesterol, bile acids (−): retinal, retinol and retinoic acid; unsaturated acids (DHA); testosterone; SOD, GPX, and HSP70	1. Bile secretion and primary bile acid synthesis 2. Lipid metabolism 3. Choline metabolism 4. Energy production 5. Oxidative stress 6. Hormones biosynthesis and metabolism	Hao et al.[163]
	Tabriz, Iran; Alexandria, Egypt; Sohag, Egypt animal models	HFD mice controls	Blood and testis (mouse)	ELISA Western Blotting	(+): MDA, iNOS, NO; LH, FSH, and leptin; (−): GPX, SOD and catalase activities; testosterone, adiponectin	1. Oxidative stress 2. Hormones biosynthesis and metabolism	Hamed et al.[164]; Eman et al.[165]; Samer et al.[166]
Obesity	Nanjing, China case–control	20 obesity patients with abnormal semen quality (OA) 20 controls	Seminal plasma (human)	Untargeted GC–MS	(−): glucose, fructose, glucopyranose, citric acid, succinic acid; valine, glycine, serine, leucine, phenylalanine, alanine, threonine, proline, glutamic acid, citrulline, lysine and tyrosine	1. Energy production 2. Amino acid metabolism	Zhou et al.[167]
	Nanjing, China meta-analysis	Obesity patients with abnormal semen quality (OA) controls	Seminal plasma (human)		Spermidine and spermine were significantly decreased in OA group.	Spermine metabolism	Guo et al.[168]

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 5 (Continued)

Categories	Study design	Subjects	Biological matrix	Technique	Discriminant metabolites or findings	Pathways	Reference
Caloric restriction (CR)	Porto, Portugal animal models	6 CR mice 6 controls	Testis (mouse)	Untargeted ^{1}H NMR	(+): GLP-1, ghrelin; phosphocholine and hypoxanthine (−): leptin; alanine, glutamate, proline, and ethanolamine; carbonyl, nitro-tyrosine	1. Hormone metabolism 2. Energy production 3. Amino acid metabolism 4. Oxidative stress	Martins et al.[121]
Circadian rhythm disorder	Badajoz, Spain; Buenos Aires, Argentina case−control	rotating shift workers controls	Urinary and serum (human)	Targeted ELISA	(+): 5-hydroxyindoleacetic acid (−): melatonin	1. Hormone metabolism 2. Oxidative stress	Ortiz et al.[169]; Rossi et al.[170]
Sleep deprivation (SD)	Londrina, UEL; Menoumia, Egypt; Osun State, Nigeria; Paulo, Brazil; Bucheon, Korea animal models	SD mice controls	Blood and testis (mouse)	ELISA Western blotting	(+): corticosterone, cortisol; malondialdehyde; uric acid; gamma glutamyl transferase and lactate dehydrogenase activities (−): oestrogen, LH, FSH, testosterone; glutamine, glutathione; lactate and glucose; NO, cGMP	1. Hormone metabolism 2. Oxidative stress	Siervo et al.[171]; Nermin et al.[172]; Hamed et al.[173]; Alvarenga et al.[169]; Ji et al.[174]
Smoking	Leipzig, Germany case–control	10 smokers 10 controls	Sperm and seminal plasma (human)	Untargeted LC–MS/MS	(+): aspartate, glutamine, glycine, phenylalanine, and valine; carnitines (C2 and C3); NO (−): asymmetric dimethylarginine; serotonin; acylcarnitines; phenylalanine hydroxylation and tryptophan	1. Energy production 2. Amino acid metabolism 3. Oxidative stress 4. Acylcarnitine metabolism 5. Hormone metabolism	Kathrin et al.[129]
	Casablanca, Morocco; Galveston	smokers controls	Semen and serum (human)	Western blotting	(+): CAT, SOD, and MDA; ascorbic acid	1. Oxidative stress	Aboulmaouahib et al.[170]; Dawson et al.[175]
	East Jerusalem, Palestine animal models	8 mice exposure to cigarette smoke 8 controls	Testis and serum (mouse)	ELISA Western blotting	(+): lactate dehydrogenase activity; 8-hydroxy-2′-deoxyguanosine (−): sorbitol dehydrogenase activity	1. Energy production 2. Oxidative stress	Rula et al.[176]

Abbreviations: ATP, adenosine triphosphate; CAT, catalase; DHA, docosahexaenoic acid; FSH, follicle stimulating hormone; GC−MS, gas chromatography−mass spectrometry; GLP-1, glucagon-like peptide 1; GPX, glutathione peroxidase; iNOS, inducible nitric oxide synthase; LC−MS− liquid chromatography−mass spectrometry; AMP, adenine ribonucleotide; LH, luteinizing hormone; MDA, malondialdehyde; NMRS, nuclear magnetic resonance spectroscopy; NO, nitric oxide; ROS, reactive oxygen species; SOD: superoxide dismutase.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

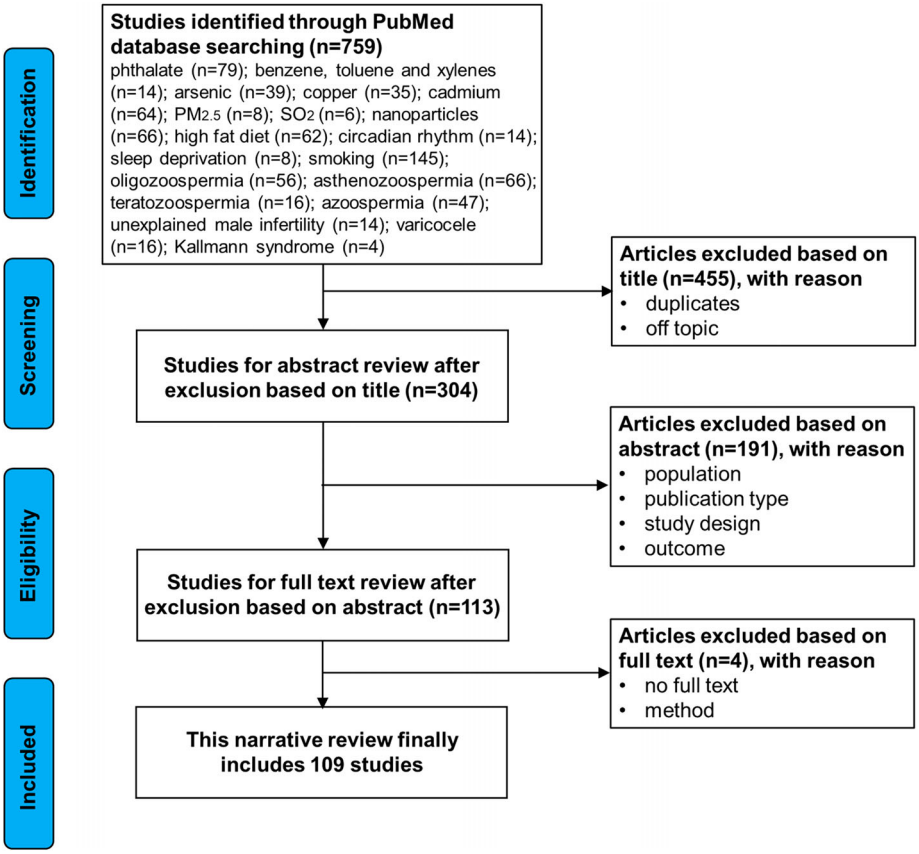


FIGURE 1 Search and screening process for the application of metabolomics in both general male infertility and infertility caused by non-genetic factors.

used for data analysis. Fourth, the public and commercial databases of metabolites are still limited and incomplete, and very few biomarkers associated with MI have been identified. Finally, the identification of unknown metabolites and the analysis of related pathways are major challenges, and there are yet un-annotated metabolites without identification available on databases such as The Human Metabolome Database (HMDB).

In response to the known unresolved issues and gaps in the application of metabolomics in MI, several aspects that future research in this area should focus on are presented in Figures 1 and 2. Specifically, they are: (1) to understand the critical development window of a disease of concern or adverse health outcome.[177] The critical window can represent the important stages in the development of one disease or other health outcomes. In contrast with the lifelong concept, metabolomics can be applied to studies of causal events occurring during relatively short periods or its critical window. The metabolomic profile preceding the disease is more likely to be the cause of the disease rather than the result; (2) the potential biomarkers and metabolic pathways revealed by current metabolomics studies in MI need to be validated in independent large-scale populations. Prospective longitudinal studies should also be conducted to understand the causality of metabolites as most of the present studies are cross-sectional and it is difficult to determine whether the observed metabolic changes are related to etiological mechanisms or the downstream effects of metabolomic derangements in MI patients; (3) MI has a multifactorial pathology and can be affected by two or more factors together, such as obesity that exacerbates spermatogenic damage by BPA[178] and the combined effects of polychlorinated biphenyls and PAEs on sperm viability.[179] Thus, by assessing the combined effects of multiple factors, the pathogenesis of MI can be more fully elucidated; (4) study designs should be comprehensively optimized to ensure the quality and accuracy of the data obtained, including subject and sample collection, sample size, sample processing and storage conditions, preparation, and data analysis; (5) metabolomics is a powerful tool to fingerprint metabolic profiles in multiple matrices, and the combination of metabolomics with other techniques, such as proteomics, transcriptomics, and analysis of the microbiota may lead to greater insight and better characterization of disease. Consequently, we expect that metabolomics and its integration with other omics approaches will be increasingly applied to the study of MI in the coming years. This will increase the understanding of its pathogenesis and provide individualized treatments.

6 | CONCLUSIONS

Metabolomics offers valuable information on MI-related physiological processes, molecular interactions, and metabolic pathways. Through the metabolomic profiling of MI caused by these non-genetic risk

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

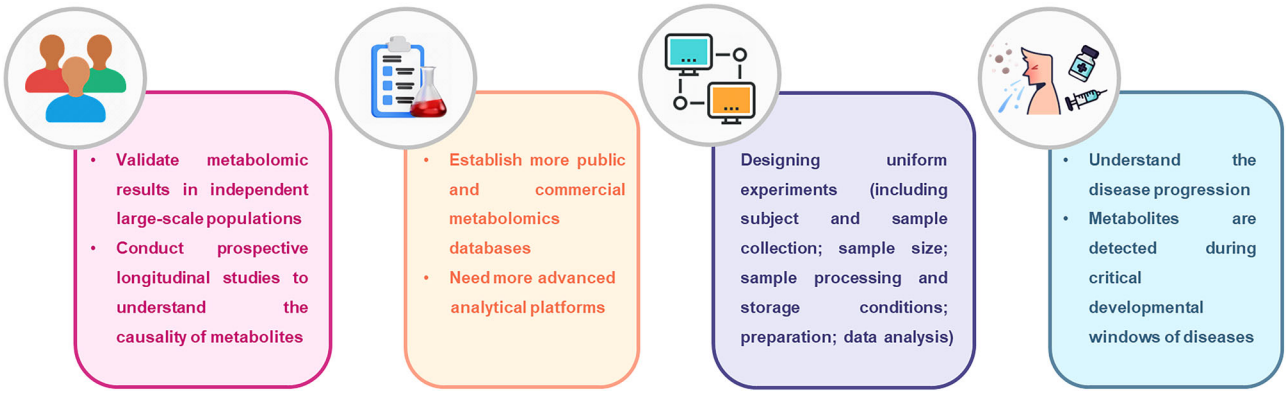


FIGURE 2 Future applications of metabolomics to the study of male infertility (MI) should pay attention to these aspects. Metabolomics fingerprints provide valuable information on MI-related physiological processes, molecular interactions, and metabolic pathways, which might offer novel diagnostic, prognostic, and precise treatment approaches to this anxiety-inducing disease. To achieve this goal, consideration should be given to the design of standardized experiments, together with the prioritization of exposure studies, biomarker databases, analytical platforms, statistical analysis, computational toxicity, experimental validation, and policy evaluation.

factors and fertile controls, various biomarkers have been separated and identified. These biomarkers include mainly amino acids, lipids, fatty acids, acylcarnitines, purines, organic acids, sugars, and other compounds. They were often enriched in metabolic pathways such as oxidative stress, energy metabolism, hormone metabolism, amino acid metabolism, and lipid metabolism, among which oxidative stress was the most common in MI.

AUTHOR CONTRIBUTIONS

J. X. and R.H. designed the review. W.Y. and R.H. searched literature and wrote the manuscript. R.H. and X.H. critically revised the manuscript. All the authors have seen and approved the final version.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data underlying this paper will be shared on reasonable request to the corresponding author.

ORCID

Rong Hua https://orcid.org/0000-0001-9607-6605

REFERENCES

1. Vander Borght M, Wyns C. Fertility and infertility: definition and epidemiology. *Clin Biochem*. 2018;62:2-10.
2. Anderson JE, Farr SL, Jamieson DJ, et al. Infertility services reported by men in the United States: national survey data. *Fertil Steril*. 2009;91(6):2466-2470.
3. Zegers-Hochschild F, Adamson GD, Dyer S, et al. The international glossary on infertility and fertility care. *Hum. Reprod*. 2017;32(9):1786-1801.
4. Leaver RB. Male infertility: an overview of causes and treatment options. *Br J Nurs*. 2016;25(18):S35-S40.
5. Krausz C, Riera-Escamilla A. Genetics of male infertility. *Nat Rev Urol*. 2018;15(6):369-384.
6. Gunes S, Esteves SC. Role of genetics and epigenetics in male infertility. *Andrologia*. 2021;53(1):e13586.
7. Salonia A, Bettocchi C, Boeri L, et al. European Association of Urology Guidelines on Sexual and Reproductive Health-2021. *Eur Urol*. 2021;80(3):333-357.
8. Ma Y, He X, Qi K, et al. Effects of environmental contaminants on fertility and reproductive health. *J Environ Sci*. 2019;77:210-217.
9. Taha EA, Ez-Aldin AM, Sayed SK, Ghandour NM, Mostafa T. Effect of smoking on sperm vitality, DNA integrity, seminal oxidative stress, zinc in fertile men. *Urology*. 2012;80(4):822-825.
10. Sharma R, Harlev A, Agarwal A, Esteves SC. Cigarette smoking and semen quality: a new meta-analysis examining the effect of the 2010 World Health Organization Laboratory methods for the examination of human semen. *Eur Urol*. 2016;70(4):635-645.
11. Ricci E, Al Beitawi S, Cipriani S, et al. Semen quality and alcohol intake: a systematic review and meta-analysis. *Reprod Biomed Online*. 2017;34(1):38-47.
12. Eisenberg ML, Kim S, Chen Z, et al. The relationship between male BMI and waist circumference on semen quality: data from the LIFE study. *Hum Reprod*. 2015;30(2):493-494.
13. Trummer H, Habermann H, Haas J, Pummer K. The impact of cigarette smoking on human semen parameters and hormones. *Hum Reprod*. 2002;17(6):1554-1559.
14. Li Y, Lin H, Ma M, et al. Semen quality of 1346 healthy men, results from the Chongqing area of southwest China. *Hum Reprod*. 2009;24(2):459-469.
15. Jensen CFS, Østergren P, Dupree JM, et al. Varicocele and male infertility. *Nat Rev Urol*. 2017;14(9):523-533.
16. Tournaye H, Krausz C, Oates RD. Concepts in diagnosis and therapy for male reproductive impairment. *Lancet Diabetes Endocrinol*. 2017;5(7):554-564.
17. Yang C, Li P, Li Z. Clinical application of aromatase inhibitors to treat male infertility. *Hum Reprod Update*. 2021;28(1):30-50.
18. Cannarella R, Calogero AE, Condorelli RA, et al. Non-hormonal treatment for male infertility: the potential role of Serenoa repens, selenium and lycopene. *Eur Rev Med Pharmacol Sci*. 2019;23(7):3112-3120.
19. Smits RM, Mackenzie-Proctor R, Yazdani A, Stankiewicz MT, Jordan V, Showell MG. Antioxidants for male subfertility. *Cochrane Database Syst Rev*. 2019;3(3):CD007411.
20. Ali M, Martinez M, Parekh N. Are antioxidants a viable treatment option for male infertility? *Andrologia*. 2021;53(1):e13644.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

21. Guzick DS, Overstreet JW, Factor-Litvak P, et al. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med*. 2001;345(19):1388-1393.
22. Huang Q, Liu L, Wu Y, et al. Seminal plasma metabolites mediate the associations of multiple environmental pollutants with semen quality in Chinese men. *Environ Int*. 2019;132:105066.
23. Lin S, Qiao N, Chen H, et al. Integration of transcriptomic and metabolomic data reveals metabolic pathway alteration in mouse spermatogonia with the effect of copper exposure. *Chemosphere*. 2020;256:126974.
24. Maleki BH, Tartibian B. High-intensity exercise training for improving reproductive function in infertile patients: a randomized controlled trial. *J Obstet Gynaecol Canada*. 2017;39(7):545-558.
25. Vertika S, Singh KK, Rajender S. Mitochondria, spermatogenesis, and male infertility—an update. *Mitochondrion*. 2020;54:26-40.
26. Ding G, Huang H-F, Liu Y, et al. The effects of diabetes on male fertility and epigenetic regulation during spermatogenesis. *Asian J Androl*. 2015;17(6):948-953.
27. Crisóstomo L, Jarak I, Rato LP, et al. Inheritable testicular metabolic memory of high-fat diet causes transgenerational sperm defects in mice. *Sci Rep*. 2021;11(1):9444.
28. Patti GJ, Yanes O, Siuzdak G. Innovation: metabolomics: the apogee of the omics trilogy. *Nat Rev Mol Cell Biol*. 2012;13(4):263-269.
29. Laíns I, Gantner M, Murinello S, et al. Metabolomics in the study of retinal health and disease. *Prog Retin Eye Res*. 2019;69:57-79.
30. Rinschen MM, Ivanisevic J, Giera M, Siuzdak G. Identification of bioactive metabolites using activity metabolomics. *Nat Rev Mol Cell Biol*. 2019;20(6):353-367.
31. Zhao Q, Huang J-F, Cheng Y, et al. Polyamine metabolism links gut microbiota and testicular dysfunction. *Microbiome*. 2021;9(1):224.
32. Nguyen T, Sedghi L, Ganther S, Malone E, Kamarajan P, Kapila YL. Host-microbe interactions: profiles in the transcriptome, the proteome, and the metabolome. *Periodontology*. 2000;82(1): 2020.
33. Nayor M, Brown KJ, Vasan RS. The molecular basis of predicting atherosclerotic cardiovascular disease risk. *Circ Res*. 2021;128(2):287-303.
34. Jin Q, Ma RCW. Metabolomics in diabetes and diabetic complications: insights from epidemiological studies. *Cells*. 2021;10(11):2832.
35. Khoramipour K, Sandbakk Ø, Keshteli AH, Gaeini AA, Wishart DS, Chamari K. Metabolomics in exercise and sports. *Sports Med*. 2022;52(3):547-583.
36. Bory C, Boulieu R, Chantin C, Mathieu M. Diagnosis of alcaptonuria: rapid analysis of homogentisic acid by HPLC. *Clin Chim Acta*. 1990;189(1):7-11.
37. Johnson CH, Ivanisevic J, Siuzdak G. Metabolomics: beyond biomarkers and towards mechanisms. *Nat Rev Mol Cell Biol*. 2016;17(7):451-459.
38. Fiehn O. Metabolomics—the link between genotypes and phenotypes. *Plant Mol Biol*. 2002;48(1-2):155-171.
39. Mumcu A, Karaer A, Dogan B, Tuncay G. Metabolomics analysis of seminal plasma in patients with idiopathic Oligoasthenoteratozoospermia using high-resolution NMR spectroscopy. *Andrology*. 2020;8(2):450-456.
40. Ma P, Zhang Z, Zhou X, Luo J, Lu H, Wang Y. Characterizing semen abnormality male infertility using non-targeted blood plasma metabolomics. *PloS One*. 2019;14(7):e0219179.
41. Zhang J, Huang Z, Chen M, et al. Urinary metabolome identifies signatures of oligozoospermic infertile men. *Fertil Steril*. 2014;102(1):44-53. e12.
42. Zhang Z, Zhang Y, Liu C, et al. Serum metabolomic profiling identifies characterization of non-obstructive azoospermic men. *Int J Mol Sci*. 2017;18(2):238.
43. Chen L, Jing T, Kastenmüller G, et al., Metabolomic and transcriptomic responses of mouse testis to the dextran sulfate sodium induced colitis. *Reproductive toxicology* (Elmsford, N.Y.), 2022;108: p. 35–42.
44. Zierer, J., Jackson MA, Kastenmüller G, et al., The fecal metabolome as a functional readout of the gut microbiome. *Nature genetics*. 2018;50(6): p. 790–795.
45. Bogdanov M, Matson WR, Wang L, et al. Metabolomic profiling to develop blood biomarkers for Parkinson's disease. *Brain*. 2008;131(2):389-396.
46. Boguenet M, Bocca C, Bouet P, et al. Metabolomic signature of the seminal plasma in men with severe oligoasthenospermia. *Andrology*. 2020;8(6):1859-1866.
47. Murgia F, Corda V, Serrenti M, et al. Seminal fluid metabolomic markers of oligozoospermic infertility in humans. *Metabolites*. 2020;10(2):64.
48. Chen L, Wen C, Deng M, et al. Metabolic and transcriptional changes in seminal plasma of asthenozoospermia patients. *Biomed Chromatogr*. 2020;34(3):e4769.
49. Mehrparvar B, Chashmniam S, Nobakht F, et al. Metabolic profiling of seminal plasma from teratozoospermia patients. *J Pharmaceut Biomed Anal*. 2020;178:112903.
50. Qiao S, Wu W, Chen M, et al. Seminal plasma metabolomics approach for the diagnosis of unexplained male infertility. *PloS One*. 2017;12(8):e0181115.
51. Minai-Tehrani A, Jafarzadeh N, Gilany K. Metabolomics: a state-of-the-art technology for better understanding of male infertility. *Andrologia*. 2016;48(6):609-616.
52. Emwas AM. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Methods Mol Biol*. 2015;1277:161-193.
53. Zhang J, Mauzerall DL, Zhu T, Liang S, Ezzati M, Remais JV, et al. Environmental health in China: progress towards clean air and safe water. *Lancet*. 2010;375(9720):1110-1119.
54. He Z, Li Z, Zhang Q, Wei Z, Duo J, Pan X. Simultaneous remediation of As(III) and dibutyl phthalate (DBP) in soil by a manganese-oxidizing bacterium and its mechanisms. *Chemosphere*. 2019;220:837-844.
55. Huang G, Zhang Q, Wu H, et al. Sperm quality and ambient air pollution exposure: a retrospective, cohort study in a Southern province of China. *Environ Res*. 2020;188:109756.
56. Caporossi L, Alteri A, Campo G, et al. Cross-sectional study on exposure to bpa and phthalates and semen parameters in men attending a fertility Center. *Int J Environ Res Public Health*. 2020;17(2):489.
57. Xu S, Wu Y, Chen Y, et al. Environmental metal exposure, seminal plasma metabolome and semen quality: evidence from Chinese reproductive-aged men. *Sci Total Environ*. 2022;838(2): 155860.
58. Manouchehri A, Shokri S, Pirhadi M, et al. The effects of toxic heavy metals lead, cadmium and copper on the epidemiology of male and female infertility. *JBRA Assist Reprod*. 2022;26(4):627-630.
59. Agarwal A, Baskaran S, Parekh N, et al. Male infertility. *Lancet*. 2021;397(10271):319-333.
60. Wang Yi-X, Wu Y, Chen H-G, et al. Seminal plasma metabolome in relation to semen quality and urinary phthalate metabolites among Chinese adult men. *Environ Int*. 2019;129:354-363.
61. Liu L, Wang H, Tian M, et al. Phthalate metabolites related to infertile biomarkers and infertility in Chinese men. *Environ Pollut (Barking, Essex: 1987)*. 2017;231(1):291-300.
62. Tielemans E, Burdorf A, Te Velde ER, et al. Occupationally related exposures and reduced semen quality: a case–control study. *Fertil Steril*. 1999;71(4):690-696.
63. Xiao GB, Pan CB, Cai YZ, Lin H, Fu ZM. Effect of benzene, toluene, xylene on the semen quality and the function of accessory gonad of exposed workers. *Ind Health*. 2001;39(2):206-210.
64. Wu Y, Ding R, Zhang Xi, et al. Meet-in-metabolite analysis: a novel strategy to identify connections between arsenic exposure and male infertility. *Environ Int*. 2021;147:106360.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

65. Huang Q, Luo L, Alamdar A, et al. Integrated proteomics and metabolomics analysis of rat testis: mechanism of arsenic-induced male reproductive toxicity. *Sci Rep*. 2016;6:32518.
66. Gad El-Hak HN, Mobarak YM. Copper oxychloride-induced testicular damage of adult albino rats and the possible role of curcumin in healing the damage. *Environ Sci Pollut Res Int*. 2020;27(11):11649-11662.
67. Sarawi WS, Alhusaini AM, Fadda LM, et al. Nano-curcumin prevents copper reproductive toxicity by attenuating oxidative stress and inflammation and improving Nrf2/HO-1 signaling and pituitary–gonadal axis in male rats. *Toxics*. 2022;10(7):356.
68. Mandil R, Rahal A, Prakash A, Garg SK, Gangwar NK, Swain DK. Ameliorative potential of α-tocopherol against flubendiamide and copper-induced testicular-insult in Wistar rats. *Chem Biol Interact*. 2016;260:91-101.
69. Elmallah M, Elkhadragy M, Al-Olayan E, Abdel Moneim A. Protective effect of fragaria ananassa crude extract on cadmium-induced lipid peroxidation, antioxidant enzymes suppression, and apoptosis in rat testes. *Int J Mol Sci*. 2017;18(5):957.
70. Zeng L, Zhou J, Wang X, Zhang Y, Wang M, Su P. Cadmium attenuates testosterone synthesis by promoting ferroptosis and blocking autophagosome-lysosome fusion. *Free Radic Biol Med*. 2021;176:176-188.
71. Faraji T, Momeni HR, Malmir M. Protective effects of silymarin on testis histopathology, oxidative stress indicators, antioxidant defence enzymes and serum testosterone in cadmium-treated mice. *Andrologia*. 2019;51(5):e13242.
72. Venditti M, Ben Rhouma M, Romano MZ, Messaoudi I, Reiter RJ, Minucci S. Evidence of melatonin ameliorative effects on the blood-testis barrier and sperm quality alterations induced by cadmium in the rat testis. *Ecotoxicol Environ Safety*. 2021;226:112878.
73. Shi F, Zhang Z, Wang J, et al. Analysis by metabolomics and transcriptomics for the energy metabolism disorder and the aryl hydrocarbon receptor activation in male reproduction of mice and GC-2spd cells exposed to PM(2.5). *Front Endocrinol*. 2021;12:807374.
74. Zhou L, Su X, Li B, et al. PM2.5 exposure impairs sperm quality through testicular damage dependent on NALP3 inflammasome and miR-183/96/182 cluster targeting FOXO1 in mouse. *Ecotoxicol Environ Safety*. 2019;169:551-563.
75. Yang Y, Yang T, Liu S, et al. Concentrated ambient PM(2.5) exposure affects mice sperm quality and testosterone biosynthesis. *PeerJ*. 2019;7:e8109.
76. Qiu L, Chen M, Wang X, et al. Exposure to concentrated ambient PM2.5 compromises spermatogenesis in a mouse model: role of suppression of hypothalamus–pituitary–gonads axis. *Toxicol Sci*. 2018;162(1):318-326.
77. Du Y, Hou L, Chu C, Jin Y, Sun W, Zhang R. Characterization of serum metabolites as biomarkers of carbon black nanoparticles-induced subchronic toxicity in rats by hybrid triple quadrupole time-of-flight mass spectrometry with non-targeted metabolomics strategy. *Toxicology*. 2019;426:152268.
78. Ye BiSu, Leung AOiW, Wong MH. The association of environmental toxicants and autism spectrum disorders in children. *Environ Pollut (Barking, Essex : 1987)*. 2017;227:234-242.
79. Schettler T. Human exposure to phthalates via consumer products. *Int J Androl*. 2006;29(1):134-139. discussion 181–5.
80. Monneret C. What is an endocrine disruptor? *C R Biol*. 2017;340(9-10):403-405.
81. Cherry N, Moore H, Mcnamee R, et al. Occupation and male infertility: glycol ethers and other exposures. *Occup Environ Med*. 2008;65(10):708-714.
82. Liu XD, et al. Toluene and Xylene combined exposure based on ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi*. 2021;39(4):248-252.
83. Wang Yi-X, Wang P, Feng W, et al. Relationships between seminal plasma metals/metalloids and semen quality, sperm apoptosis and DNA integrity. *Environ Pollut (Barking, Essex: 1987*. 2017;224:224-234.
84. Uriu-Adams JY, Keen CL. Copper, oxidative stress, and human health. *Mol Aspects Med*. 2005;26(4-5):268-298.
85. Chabchoub I, Nouioui MA, Araoud M, et al. Effects of lead, cadmium, copper and zinc levels on the male reproductive function. *Andrologia*. 2021;53(9):e14181.
86. Garza-Lombó C, Pappa A, Panayiotidis MI, Gonsebatt ME, Franco R. Arsenic-induced neurotoxicity: a mechanistic appraisal. *J Biol Inorg Chem*. 2019;24(8):1305-1316.
87. Garbinski LD, Rosen BP, Chen J. Pathways of arsenic uptake and efflux. *Environ Int*. 2019;126:585-597.
88. Renu K, Madhyastha H, Madhyastha R, Maruyama M, Vinayagam S, Valsala Gopalakrishnan A. Review on molecular and biochemical insights of arsenic-mediated male reproductive toxicity. *Life Sci*. 2018;212:37-58.
89. Bost M, Houdart S, Oberli M, Kalonji E, Huneau J-F, Margaritis I. Dietary copper and human health: current evidence and unresolved issues. Dietary copper and human health: current evidence and unresolved issues. *J Trace Elem Med Biol*. 2016;35:107-115.
90. Bhardwaj JK, Paliwal A, Saraf P. Effects of heavy metals on reproduction owing to infertility. *J Biochem Mol Toxicol*. 2021;35(8):e22823.
91. Bhardwaj JK, Panchal H, Saraf P. Cadmium as a testicular toxicant: a review. *J Appl Toxicol*. 2021;41(1):105-117.
92. Kumar S, Sharma A. Cadmium toxicity: effects on human reproduction and fertility. *Rev Environ Health*. 2019;34(4):327-338.
93. Zhu Q, Li X, Ge R-S. Toxicological effects of cadmium on mammalian testis. *Front Genet*. 2020;11:527.
94. Elmallah M, Elkhadragy M, Al-Olayan E, Abdel Moneim A. Protective effect of fragaria ananassa crude extract on cadmium-induced lipid peroxidation, antioxidant enzymes suppression, and apoptosis in rat testes. *Int J Mol Sci*. 2017;18(5):957.
95. Mahmoudi R, Azizi A, Abedini S, Hemayatkhah Jahromi V, Abidi H, Jafari Barmak M. Green tea improves rat sperm quality and reduced cadmium chloride damage effect in spermatogenesis cycle. *J Med Life*. 2018;11(4):371-380.
96. Asadi MH, Zafari F, Sarveazad A, et al. Saffron improves epididymal sperm parameters in rats exposed to cadmium. *Nephro-urol Mon*. 2014;6(1):e12125.
97. Khushboo M, Murthy MK, Devi MS, et al. Testicular toxicity and sperm quality following copper exposure in Wistar albino rats: ameliorative potentials of L-carnitine. *Environ Sci Pollut Res Int*. 2018;25(2):1837-1862.
98. Ulfanov O, Cil N, Adiguzel E. Protective effects of vitamin E on aluminium sulphate-induced testicular damage. *Toxicol Ind Health*. 2020;36(4):215-227.
99. Acharya UR, Mishra M, Patro J, Panda MK. Effect of vitamins C and E on spermatogenesis in mice exposed to cadmium. *Reprod Toxicol*. 2008;25(1):84-88.
100. Krzastek SC, Farhi J, Gray M, Smith RP. Impact of environmental toxin exposure on male fertility potential. *Transl Androl Urol*. 2020;9(6):2797-2813.
101. Vecoli C, Montano L, Andreassi MG. Environmental pollutants: genetic damage and epigenetic changes in male germ cells. *Environ Sci Pollut Res Int*. 2016;23(23):23339-23348.
102. Jurewicz J, Dziewirska E, Radwan M, Hanke W. Air pollution from natural and anthropic sources and male fertility. *Reprod Biol Endocrinol*. 2018;16(1):109.
103. Baysal A, Saygin H, Ustabasi GS. Interaction of PM2.5 airborne particulates with ZnO and TiO(2) nanoparticles and their effect on bacteria. *Environ Monit Assess*. 2017;190(1):34.
104. Wei Yi, Cao Xi-N, Tang X-L, et al. Urban fine particulate matter (PM2.5) exposure destroys blood-testis barrier (BTB) integrity

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

through excessive ROS-mediated autophagy. *Toxicol Mech Methods*. 2018;28(4):302-319.
105. Wang L, Luo D, Liu X, et al. Effects of PM(2.5) exposure on reproductive system and its mechanisms. *Chemosphere*. 2021;264(1):128436.
106. Cao Xi-N, Shen L-J, Wu S-D, et al. Urban fine particulate matter exposure causes male reproductive injury through destroying blood–testis barrier (BTB) integrity. *Toxicol Lett*. 2017;266:1-12.
107. Cao X-N, Yan C, Liu D-Y, et al. Fine particulate matter leads to reproductive impairment in male rats by overexpressing phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. *Toxicol Lett*. 2015;237(3):181-190.
108. Carré J, Gatimel N, Moreau J, Parinaud J, Léandri R. Does air pollution play a role in infertility? A systematic review. *Environ Health*. 2017;16(1):82.
109. Lu S-Y, Li Y-Xi, Zhang J-Q, et al. Associations between polycyclic aromatic hydrocarbon (PAH) exposure and oxidative stress in people living near e-waste recycling facilities in China. *Environ Int*. 2016;94:161-169.
110. Jin H, Yan M, Pan C, et al. Chronic exposure to polystyrene microplastics induced male reproductive toxicity and decreased testosterone levels via the LH-mediated LHR/cAMP/PKA/StAR pathway. *Part Fibre Toxicol*. 2022;19(1):13.
111. Zhang G, Jiang F, Chen Q, et al. Associations of ambient air pollutant exposure with seminal plasma MDA, sperm mtDNA copy number, and mtDNA integrity. *Environ Int*. 2020;136:105483.
112. Sokol RZ, Kraft P, Fowler IM, Mamet R, Kim E, Berhane KT. Exposure to environmental ozone alters semen quality. *Environ Health Perspect*. 2006;114(3):360-365.
113. Meng Z, Bai W. Oxidation damage of sulfur dioxide on testicles of mice. *Environ Res*. 2004;96(3):298-304.
114. Li X, Yi H, Wang H. Sulphur dioxide and arsenic affect male reproduction via interfering with spermatogenesis in mice. *Ecotoxicol Environ Safety*. 2018;165:164-173.
115. Zhang H, Zhang Z, Cao J, et al. Ambient ozone pollution is associated with decreased semen quality: longitudinal analysis of 8945 semen samples from 2015 to 2018 and during pollution-control period in Beijing. *China Asian J Androl*. 2019;21(5):501-507.
116. Bisht S, Faiq M, Tolahunase M, Dada R. Oxidative stress and male infertility. *Nat Rev Urol*. 2017;14(8):470-485.
117. Suliga E, Głuszek S. The relationship between diet, energy balance and fertility in men. *Int J Vitam Nutr Res*. 2020;90(5-6):514-526.
118. Crisóstomo L, Rato L, Jarak I, et al. A switch from high-fat to normal diet does not restore sperm quality but prevents metabolic syndrome. *Reproduction*. 2019;158(4):377-387.
119. Palmer NO, Bakos HW, Owens JA, Setchell BP, Lane M. Diet and exercise in an obese mouse fed a high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol Endocrinol Metab*. 2012;302(7):E768-E780.
120. Salas-Huetos A, Bulló M, Salas-Salvadó J. Dietary patterns, foods and nutrients in male fertility parameters and fecundability: a systematic review of observational studies. *Hum Reprod Update*. 2017;23(4):371-389.
121. Martins AD, Jarak I, Morais T, et al. Caloric restriction alters the hormonal profile and testicular metabolome, resulting in alterations of sperm head morphology. *Am J Physiol Endocrinol Metab*. 2020;318(1):E33-E43.
122. Lateef OM, Akintubosun MO. Sleep and reproductive health. *J Circadian Rhythms*. 2020;18:1.
123. Palnitkar G, Phillips CL, Hoyos CM, Marren AJ, Bowman MC, Yee BJ. Linking sleep disturbance to idiopathic male infertility. *Sleep Med Rev*. 2018;42:149-159.
124. Alves MG, Neuhaus-Oliveira A, Moreira PI, Socorro S, Oliveira PF. Exposure to 2,4-dichlorophenoxyacetic acid alters glucose metabolism in immature rat Sertoli cells. *Reprod Toxicol*. 2013;38:81-88.
125. Ortiz Á, Espino J, Bejarano I, et al. The correlation between urinary 5-hydroxyindoleacetic acid and sperm quality in infertile men and rotating shift workers. *Reprod Biol Endocrinol*. 2010;8:138.
126. Frungieri MB, Zitta K, Pignataro OP, Gonzalez-Calvar SI, Calandra RS. Interactions between testicular serotoninergic, catecholaminergic, and corticotropin-releasing hormone systems modulating cAMP and testosterone production in the golden hamster. *Neuroendocrinology*. 2002;76(1):35-46.
127. Rossi SP, Windschuettl S, Matzkin ME, et al. Melatonin in testes of infertile men: evidence for anti-proliferative and anti-oxidant effects on local macrophage and mast cell populations. *Andrology*. 2014;2(3):436-449.
128. Rizk NI, Rizk MS, Mohamed AS, Naguib YM. Attenuation of sleep deprivation dependent deterioration in male fertility parameters by vitamin C. *Reprod Biol Endocrinol*. 2020;18(1):2.
129. Choi JiHo, Lee SH, Bae JH, et al. Effect of sleep deprivation on the male reproductive system in rats. *J of Korean Med Sci*. 2016;31(10):1624-1630.
130. Alvarenga TA, Hirotsu C, Mazaro-Costa R, Tufik S, Andersen ML. Impairment of male reproductive function after sleep deprivation. *Fertil Steril*. 2015;103(5):1355-1362. e1.
131. Boeri L, Capogrosso P, Ventimiglia E, et al. Heavy cigarette smoking and alcohol consumption are associated with impaired sperm parameters in primary infertile men. *Asian J Androl*. 2019;21(5):478-485.
132. Engel KM, Baumann S, Blaurock J, et al. Differences in the sperm metabolomes of smoking and nonsmoking men. *Biol Reprod*. 2021;105(6):1484-1493.
133. Dawson EB, Harris WA, Teter MC, Powell LC. Effect of ascorbic acid supplementation on the sperm quality of smokers. *Fertil Steril*. 1992;58(5):1034-1039.
134. Kumosani TA, Elshal MF, Al-Jonaid AA, Abduljabar HS. The influence of smoking on semen quality, seminal microelements and Ca^{2+}-ATPase activity among infertile and fertile men. *Clin Biochem*. 2008;41(14-15):1199-1203.
135. Ghaffari MA, Rostami M. The effect of cigarette smoking on human sperm creatine kinase activity: as an ATP buffering system in sperm. *Int J Fertil Steril*. 2013;6(4):258-265.
136. Jandíková H, Dušková M, Stárka L. The influence of smoking and cessation on the human reproductive hormonal balance. *Physiol Res*. 2017;66(3):S323-S331.
137. Wang W, Yang X, Liang J, et al. Cigarette smoking has a positive and independent effect on testosterone levels. *Comp Study*. 2013;12(4):567-577.
138. Ramlau-Hansen CH, Thulstrup AM, Aggerholm AS, Jensen MS, Toft G, Bonde JP. Is smoking a risk factor for decreased semen quality? A cross-sectional analysis. *Hum Reprod*. 2007;22(1):188-196.
139. Park MGu, Ko KiW, Oh MiMi, Bae JH, Kim JeJ, Moon DuG. Effects of smoking on plasma testosterone level and erectile function in rats. *J Sex Med*. 2012;9(2):472-481.
140. Bundhun PK, Janoo G, Bhurtu A, et al. Tobacco smoking and semen quality in infertile males: a systematic review and meta-analysis. *BMC Public Health*. 2019;19(1):36.
141. Murgia F, Corda V, Serrenti M, et al. Seminal fluid metabolomic markers of oligozoospermic infertility in humans. *Metabolites*. 2020;10(2):64.
142. Boguenet M, Bocca C, Bouet P-E, et al. Metabolomic signature of the seminal plasma in men with severe oligoasthenospermia. *Andrology*. 2020;8(6):1859-1866.
143. Serri O, Boguenet M, Chao De La Barca JM, et al. A metabolomic profile of seminal fluid in extremely severe oligozoopermia suggesting an epididymal involvement. *Metabolites*. 2022;12(12):1266.
144. Chen L, Wen C-W, Deng M-J, et al. Metabolic and transcriptional changes in seminal plasma of asthenozoospermia patients. *Biomed Chromatogr*. 2020;34(3):e4769.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

145. Zhang X, Diao R, Zhu X, Li Z, Cai Z. Metabolic characterization of asthenozoospermia using nontargeted seminal plasma metabolomics. *Clin Chim Acta*. 2015;450:254-261.
146. Tang B, Shang X, Qi H, et al. Metabonomic analysis of fatty acids in seminal plasma between healthy and asthenozoospermic men based on gas chromatography mass spectrometry. *Andrologia*. 2017;49(9):e12744.
147. Vashisht A, Ahluwalia PK, Gahlay GK. A comparative analysis of the altered levels of human seminal plasma constituents as contributing factors in different types of male infertility. *Curr Issues Mol Biol*. 2021;43(3):1307-1324.
148. Jayaraman V, Ghosh S, Sengupta A, Srivastava S, Sonawat HM, Narayan PK. Identification of biochemical differences between different forms of male infertility by nuclear magnetic resonance (NMR) spectroscopy. *J Assist Reprod Genet*. 2014;31(9):1195-1204.
149. Li Li, Hao X, Chen H, et al. Metabolomic characterization of semen from asthenozoospermic patients using ultra-high-performance liquid chromatography-tandem quadrupole time-of-flight mass spectrometry. *Biomed Chromatogr*. 2020;34(9):e4897.
150. Mehrparvar B, Chashmniam S, Nobakht F, et al. Metabolic profiling of seminal plasma from teratozoospermia patients. *J Pharm Biomed Anal*. 2020;178:112903.
151. Hosseini E, Amirjannati N, Henkel R, Bazrafkan M, Moghadasfar H, Gilany K. Targeted amino acids profiling of human seminal plasma from teratozoospermia patients using LC–MS/MS. *Reprod Sci*. 2023.
152. Mumcu A, Karaer A, Dogan B, Tuncay G. Metabolomics analysis of seminal plasma in patients with idiopathic Oligoasthenoteratozoospermia using high-resolution NMR spectroscopy. *Andrology*. 2020;8(2):450-456.
153. Gilany K, Mani-Varnosfaderani A, Minai-Tehrani A, et al. Untargeted metabolomic profiling of seminal plasma in nonobstructive azoospermia men: a noninvasive detection of spermatogenesis. *Biomed Chromatogr*. 2017;31(8):3931.
154. Gilany K, Jafarzadeh N, Mani-Varnosfaderani A, et al. Metabolic fingerprinting of seminal plasma from non-obstructive azoospermia patients: positive versus negative sperm retrieval. *J Reprod Infertil*. 2018;19(2):109-114.
155. Jafarzadeh N, Mani-Varnosfaderani A, Minai-Tehrani A, Savadi-Shiraz E, Sadeghi MR, Gilany K, et al. Metabolomics fingerprinting of seminal plasma from unexplained infertile men: a need for novel diagnostic biomarkers. *Mol Reprod Dev*. 2015;82(3):150.
156. Qiao S, Wu W, Chen M, et al. Seminal plasma metabolomics approach for the diagnosis of unexplained male infertility. *PloS On*. 2017;12(8):e0181115.
157. Neto FTL, Marques RA, De Freitas Cavalcanti Filho A, et al. 1H NMR-based metabonomics for infertility diagnosis in men with varicocele. *J Assist Reprod Genet*. 2020;37(9):2233-2247.
158. Li X, Wang Xi, Li H, Li Y, Guo Ye. Seminal plasma lipidomics profiling to identify signatures of Kallmann syndrome. *Front Endocrinol*. 2021;12:692690.
159. Guo Y, Li X, Yan S, Li Y, et al. Metabolomic alterations associated with Kallmann syndrome. *Ann Transl Med*. 2020;8(8):529.
160. Li X, Guo Ye, Wang Xi, et al. Seminal plasma metabolomics signatures of normosmic congenital hypogonadotropic hypogonadism. *Heliyon*. 2023;9(4):e14779.
161. Chen X, Hu C, Dai J, Chen L. Metabolomics analysis of seminal plasma in infertile males with kidney-yang deficiency: a preliminary study. *J Evid Based Complement Alternat Med*. 2015;2015:892930.
162. Luo D, Zhang M, Su X, et al. High fat diet impairs spermatogenesis by regulating glucose and lipid metabolism in Sertoli cells. *Life Sci*. 2020;257:118028.
163. Hao Y, Feng Y, Yan X, et al. Gut microbiota–testis axis: fMT mitigates high-fat diet-diminished male fertility via improving systemic and testicular metabolome. *Microbiol sSpec*. 2022;10(3):e0002822.
164. Heydari H, Ghiasi R, Hamidian G, Ghaderpour S, Keyhanmanesh R. Voluntary exercise improves sperm parameters in high fat diet receiving rats through alteration in testicular oxidative stress, mir-34a/SIRT1/p53 and apoptosis. *Horm Mol Biol Clin Investig*. 2021;42(3):253-263.
165. Allam EA, Ibrahim HF, Abdulmalek SA, Abdelmeniem IM, Basta M. Coenzyme Q(10) alleviates testicular endocrine and spermatogenic dysfunction induced by high-fat diet in male Wistar rats: role of adipokines, oxidative stress and MAPK/ERK/JNK pathway. *Andrologia*. 2022;54(10):e14544.
166. El-Sawy SA, Amin YA, El-Naggar SA, Abdelsadik A. *Artemisia annua* L. (Sweet wormwood) leaf extract attenuates high-fat diet-induced testicular dysfunctions and improves spermatogenesis in obese rats. *J Ethnopharmacol*. 2023;313:116528.
167. Zhou Y-F, Hou Yu-Y, Ban Q, et al. Metabolomics profiling of seminal plasma in obesity-induced asthenozoospermia. *Andrology*. 2023;11(7):1303-1319.
168. Guo D, Wu W, Tang Q, et al. The impact of BMI on sperm parameters and the metabolite changes of seminal plasma concomitantly. *Oncotarget*. 2017;8(30):48619-48634.
169. Alvarenga TA, Hirotsu C, Mazaro-Costa R, Tufik S, Andersen ML. Impairment of male reproductive function after sleep deprivation. *Fertil Steril*. 2015;103(5):1355-1362. el.
170. Aboulmaouahib S, Madkour A, Kaarouch I, et al. Impact of alcohol and cigarette smoking consumption in male fertility potential: looks at lipid peroxidation, enzymatic antioxidant activities and sperm DNA damage. *Andrologia*. 2018;50(3).
171. Siervo GEML, Ogo FM, Staurengo-Ferrari L, et al. Sleep restriction during peripuberty unbalances sexual hormones and testicular cytokines in rats. *Biol Reprod*. 2019;100(1):112-122.
172. Rizk NI, Rizk MS, Mohamed AS, Naguib YM. Attenuation of sleep deprivation dependent deterioration in male fertility parameters by vitamin C. *Reprod Biol Endocrinol*. 2020;18(1):2.
173. Hamed MA, Akhigbe TM, Akhigbe RE, et al. Glutamine restores testicular glutathione-dependent antioxidant defense and upregulates NO/cGMP signaling in sleep deprivation-induced reproductive dysfunction in rats. *Biomed Pharmacother*. 2022;148:112765.
174. Choi JiHo, Lee SH, Bae JH, et al. Effect of sleep deprivation on the male reproductive system in rats. *J Korean Med Sci*. 2016;31(10):1624-16s30.
175. Dawson EB, Harris WA, Teter MC, Powell LC. Effect of ascorbic acid supplementation on the sperm quality of smokers. *Fertil Steril*. 1992;58(5):1034-1039.
176. Abdul-Ghani R, Qazzaz M, Dabdoub N, Muhammad R, Abdul-Ghani AS. Studies on cigarette smoke induced oxidative DNA damage and reduced spermatogenesis in rats. *J Environ Biol*. 2014;35(5):943-947.
177. Fang M, Hu L, Chen D, et al. Exposome in human health: utopia or wonderland. *Innovation*. 2021;2(4):100172.
178. Hu W, Dong T, Wang L, et al. Obesity aggravates toxic effect of BPA on spermatogenesis. *Environ Int*. 2017;105:56-65.
179. Hauser R, Williams P, Altshul L, Calafat AM. Evidence of interaction between polychlorinated biphenyls and phthalates in relation to human sperm motility. *Environ Health Perspect*. 2005;113(4):425-430.

How to cite this article: Yang W, Hua R, Cao Y, He X. A metabolomic perspective on the mechanisms by which environmental pollutants and lifestyle lead to male infertility. *Andrology*. 2024;12:719–739.
https://doi.org/10.1111/andr.13530

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13530 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 23 August 2023 | Revised: 24 September 2023 | Accepted: 29 September 2023

DOI: 10.1111/andr.13543

ANDROLOGY

WILEY

REVIEW ARTICLE

Vasovasostomy: A systematic review and meta-analysis comparing macroscopic, microsurgical, and robot-assisted microsurgical techniques

Ishith Seth[1,2,3] | Damien Gibson[4] | Gabriella Bulloch[3] | Konrad Joseph[5] | Jevan Cevik[1,2] | Kirby R. Qin[6] | Shekib Shahbaz[7] | Warren M. Rozen[1,2,3]

[1]Department of Plastic Surgery, Peninsula Health, Melbourne, Victoria, Australia

[2]Department of Surgery, Central Clinical School at Monash University, The Alfred Centre, Melbourne, Victoria, Australia

[3]Department of Surgery, Faculty of Medicine, The University of Melbourne, Melbourne, Victoria, Australia

[4]Department of Surgery, Faculty of Medicine, University of New South Wales, University of New South Wales, Sydney, Australia

[5]Department of Surgery, Faculty of Medicine, The University of Wollongong, New South Wales, Australia

[6]Department of Urology, Bendigo Hospital, Melbourne, Victoria, Australia

[7]Department of Urology, Monash Hospital, Melbourne, Victoria, Australia

Correspondence
Damien Gibson, Faculty of Medicine, University of New South Wales, University of New South Wales, Sydney 2200, Australia.
Email: Damien.p.gibson@gmail.com

Abstract

Background and objectives: Vasovasostomy is a cost-effective procedure for the reversal of vasectomy. A water-tight adequately blood-supplied mucosal anastomosis is required for better outcomes. This review aimed to compare the outcome of vaso-vasostomy performed by three different techniques: macroscopic, pure microsurgical, and robot-assisted microsurgical techniques.

Methods: Scopus, Web of Science, PubMed, Embase, and Cochrane library databases were searched for relevant studies from January 1901 to June 2023. We conducted our quantitative syntheses using the inverse variance method in OpenMeta software. The study's protocol was registered on PROSPERO.

Results: This review involved 95 studies of different designs, with a total sample size of 48,132. The majority of operations were performed bilaterally, and participants were monitored for up to 10 years. The pooled patency rate was the highest following robot-assisted vasovasostomy (94.4%), followed by pure microsurgical vasovasostomy (87.5%), and macroscopic vasovasostomy (83.7%). The pooled pregnancy rate following purely microsurgical vasovasostomy was higher than that of macroscopic vasovasostomy (47.4 vs. 43.7%). Definitive pregnancy rates in robotic vasovasostomy are yet to be determined.

Conclusion: Patency outcomes for vasovasostomy were best with robot-assisted microsurgical technique, followed by pure microsurgical technique, and conventional macroscopic technique. Further investigations of robot-assisted microsurgical vaso-vasostomy outcomes and randomized control trials are required to support this evidence.

KEYWORDS
macroscopic, meta-analysis, microsurgical, robot, systematic review, vasovasostomy

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2023 The Authors. *Andrology* published by Wiley Periodicals LLC on behalf of American Society of *Andrology* and European Academy of *Andrology*.

1 | INTRODUCTION

Vasectomy is a commonly used contraceptive method for males that involves a combination of cutting, tying, and/or sealing the vas deferens to interfere with the passage of spermatozoa from the testes to the urethra.[1,2] About 6–8% of couples around the globe favor vasectomy for contraception (42–60 million men annually).[3] However, within 10 years, about 6–10% of those men seek a vasectomy reversal for various reasons.[4] Motives can be broadly summarized as desiring paternity (fertility reasons) and pain (non-fertility reasons). Men may reconsider natural paternity due to events like child/spouse death, divorce, remarriage, or evolving beliefs.[3,5] Other men undergo vasectomy reversal due to experiencing symptoms of vasitis or post-vasectomy pain syndrome (PVPS).[3,6] Around 5% of men experience PVPS, and quality of life is negatively impacted in 1–2%.[7–9] This pain might be attributed to direct nerve injury during the procedure, perineural fibrosis, or increased pressure within the vas deferens and epididymis.[10]

Two surgical operations were introduced 100 years ago to reverse vasectomy, namely vasovasostomy and vasoepididymostomy.[11] These operations are not only effective for reversing vasectomy but also for improving fertility in men suffering from obstructive azoospermia.[3,12–14] Currently, vasovasostomy is the most cost-effective method for reversing vasectomy.[15] It is performed through a conventional macroscopic approach, using a purely microsurgical technique, or through a robotic-assisted microsurgical technique.[16] During vasovasostomy, the tied section of the vas deferens is typically excised and the two segments are anastomosed. The procedural goal in vasovasostomy is to attain a water-tight, tension-free, and sufficiently blood-supplied mucosal anastomosis between the two segments of vas deference.

The conventional macroscopic approach was used for vasovasostomy before the introduction of the microsurgical technique and continues to be performed in some centers today. Urologists without microsurgical experience or access to costly surgical microscopes and skilled assistants operate using the macroscopic approach. The 1970s introduction of the surgical microscope enhanced visualization and precision for intricate procedures. Using a pure microsurgical technique for vasovasostomy became the standard approach.[17] This approach requires dedicated training and a competent microsurgical assistant. A drawback of the microsurgical approach is the amplifying of the surgeon's physiologic tremor, making precise micro suturing challenging despite improved visibility. The introduction of the robotic arm has since improved the practice of microsurgical operation. The robotic arm filters the operator's physiological tremors and diminishes the need for a skilled microsurgical assistant. In 2003, Schoor et al. demonstrated the feasibility of applying robotic technology to male reproductive microsurgery, finding that robot assistance facilitated accurate and comfortable operations for experienced and inexperienced microsurgeons alike.[18,19] In addition, surgeons require shorter training periods to gain adequate experience with robotic-assisted devices when compared with microsurgical training.[20]

The efficacy of vasovasostomy performed through a conventional macroscopic approach, a purely microsurgical technique, and a robot-assisted microsurgical technique has been reported in several studies. This systematic review aims to pool the most recent evidence on the efficacy of each vasovasostomy approach in terms of patency and pregnancy rates. Moreover, this review aims to summarize the evidence on factors predicting the outcome of vasovasostomy.

2 | METHODS

This systematic review and meta-analysis was conducted according to the guidance of Cochrane's handbook for systematic reviews of intervention.[21] The manuscript is reported following the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines.[22] The protocol of this meta-analysis was published online at the PROSPERO International Prospective Register of Systematic Reviews under registration number (CRD42022455797).

2.1 | Literature search

Two authors independently conducted a systematic search on PubMed, Scopus, Cochrane CENTRAL, Web of Science, and EMBASE. The search strategy consisted of the following: ("vasovasostomy" or "vasectomy reversal" or "vasal reanastomosis" or "vasal reconnection" or "reversal of vasectomy") from January 1901 to June 2023. No filters or time limits were applied to the search. Thereafter, a manual search was conducted through the reference lists of the eligible studies and previous review articles.

2.2 | Studies selection and eligibility criteria

The retrieved search results were imported to Endnote V20™ and concurrently, the duplicated results were deleted. The screening was conducted independently by two authors through the titles and abstracts of the studies for initial eligibility. Thereafter, the full texts of the initially judged eligible studies were screened for final judgment.

The inclusion criteria consisted of randomized controlled trials (RCTs), observational studies, and case series with the inclusion of any age of participants. Only primary studies that investigated the efficacy of vasovasostomy through a macroscopic approach, a purely microsurgical technique, or a robot-assisted microsurgical technique were eligible. The exclusion criteria were studies that were case reports, available only as abstracts, and in non-English languages.

Vasovasostomy is conducted with the naked eye in the traditional macroscopic method. In the microsurgical technique, a surgical microscope with multiple magnification levels is utilized. The robot-assisted microsurgical method, as defined in several studies, integrates both a surgical microscope and robotic arms to enhance surgical precision.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

These studies have consistently provided similar definitions of the robot-assisted microsurgical technique.

2.3 | Quality assessment

The quality of the included cohort, case–control, and case series studies was evaluated using the National Institutes of Health (NIH) tool.[23] The quality of RCTs quality was assessed using Cochrane's tool.[21]

2.4 | Data extraction and study outcomes

Summary data and baseline characteristics of the included subjects were extracted from the included studies. Data on each study design, sample size, operation type, magnification strength, follow-up duration, and conclusion were extracted. In addition, a description of whether the operation was conducted unilaterally or bilaterally was summarized.

The primary study outcomes were patency, pregnancy, and complication rates. The definition of patency varied across the involved studies. Some papers defined patency as sperm presence in the semen, while others required a count of up to >20 million for definition. Moreover, some studies included criteria on sperm motility and morphological appearance in the definition of potency.

The definition of pregnancy post-procedure differed, while most papers defined pregnancy as spontaneous or artificial conception at a follow-up of 1 year. Others ranged based on length of study follow-up, some up to 10 years.

Nevertheless, most of the complications that were recorded were hematoma or wound infections.

2.5 | Data synthesis

We conducted meta-analyses in the inverse variance method using OpenMeta software. Proportions were pooled and the 95% confidence interval (CI) was calculated. Three subgroups were included in each analysis: conventional macroscopic approach, purely microsurgical technique, and robot-assisted microsurgery technique. Results of the chi-squared test p value and the I-squared (I^2) test were explored for statistically significant heterogeneity. An I^2 value of ≥50 and a chi-squared p value of < 0.01 were defined as high heterogeneity. Meta-analysis was performed using a random effect model.[24]

3 | RESULTS

3.1 | Literature search

A total of 5069 studies were retrieved from the databases search, of which, 1279 studies were duplicated. We screened the titles and abstracts of 4240 studies and accordingly, 4119 studies were judged not eligible. Afterward, the full texts of 121 studies were carefully assessed for eligibility. Ultimately, 95 studies were included in this review. Eighty studies were included in the quantitative analyses,[25–104] whereas 15 studies were only included in the quantitative synthesis (Figure 1).[105–119]

3.2 | Description of the included studies

This review included 95 studies with a total sample size of 48,132 participants; five RCTs, 84 cohort studies, one case–control study, and five case series were included. The three surgical techniques were assessed for efficacy; 25 papers assessed the conventional macroscopic technique, 68 papers assessed the pure microsurgical technique, and seven studies assessed the robot-assisted microsurgical technique. The majority of the operations were performed bilaterally, and participants were monitored for up to 10 years post-operatively. Further description of the included studies is available in Table 1.

3.3 | Quality assessment

The included RCTs had a low risk of attrition and reporting bias. Generally, the risk of selection bias was moderate whereas the risk of performance and detection bias was moderate to high among the included trials (Figures S1 and S2). The quality of cohort studies was variable. Ten studies were of good quality, 57 studies were of fair quality, and 17 studies were of poor quality (Table S1). The included case–control study was of good quality (Table S2). One case series study had good quality, whereas two case series studies had fair quality and one other study had poor quality (Table S3).

3.4 | Study outcomes

3.4.1 | Patency rate

Macroscopic vasovasostomyht

Nineteen studies reported a patency rate following macroscopic vasovasostomy, with 1549 subjects involved. The patency rate ranged from 65.2 to 91.6% in the included studies. Pooled patency rate after macroscopic vasovasostomy was 83.7% (95% CI: [81.1%, 86.4%], (1288 out of 1549), ($I^2 = 42.7\%, p = 0.026$)) (Figure 2).

Microsurgical vasovasostomy

Fifty-six studies reported a patency rate following microscopic vasovasostomy, with 11,487 subjects involved. The patency rate fluctuated from 60.5 to 99.5% across the included studies. The pooled patency rate was 87.5% (95% CI: [85.6%, 89.4%], (10,314 out of 11,487), ($I^2 = 94.5\%, p < 0.001$)) (Figure 2).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Summary and baseline characteristics of the included studies.

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Aldridge 1985	Retrospective cohort	52	Microscopic	4–74 months	–	Bilateral	Presence of motile spermatozoa in an ejaculated semen specimen	"Microsurgical vasovasostomy requires special training and practice in the laboratory before operating on a patient."
Allameh 2019	RCT	22	Microscopic	6 months	–	Bilateral	Presence of spermatozoa in semen	"Intra-operative local MMC in vasovasostomy can be regarded as a safe and efficient technique that has several advantages including lower cost. Increase in sperm count is the main effect of local MMC application that is more prominent when the interval between vasectomy and reversal is 5—10 years. However, further studies should be conducted with larger sample sizes and different MMC dosages, longer durations, and multi-center sampling to attain more definite results."
Belker 1991	Retrospective cohort	1012	Microscopic	24 months	–	Bilateral	Return of spermatozoa in the semen	"After the study period, postoperative information was sought by mail and/or telephone from patients who never returned postoperatively and from those who had sperm in the semen but had not reported a pregnancy. When postoperative information became available after the conclusion of the study period, updated information was entered into the computer. However, new information (including pregnancy reports) received after mid-1987 was not included. Computer input and statistical analysis were performed in the Department of Biostatistics and Epidemiology at the Cleveland Clinic Foundation."
Busato 2009	Retrospective cohort	17	Microscopic	36 months	×12–25	Bilateral	Presence of spermatozoa in the ejaculate at 3 and 6 months post-operatively	"High patency and pregnancy rates are associated with time intervals since vasectomy of 10 years and vasectomies performed by urologists. There was no significant difference in the anastomosis time between the first 12 procedures and the next 12 procedures. "
Bash 2022	Case series	13	Microscopic	–	–	Bilateral	Presence of spermatozoa on semen analysis	"Our findings support favorable outcomes with more liberal VV indications after medical therapy in patients previously on TT that desire VR. The use of medical therapy reduced the recommended wait times for VR after TT discontinuation alone by more than half, and the high rate of VV (96%) and sustained patency despite an 11-year median obstructive interval indicates that VR in this population is very likely to succeed."
Belker 1985	Retrospective cohort	892	Microscopic	6–36 months	–	Bilateral	–	"Earlier vasovasostomy avoids the progressive rate of sperm disappearance from the vas fluid as the obstructive interval lengthens and, thus, may avoid the adverse prognosis for the fertility of intraoperative sperm absence from the vas fluid during vasovasostomy. "

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Bolduc 2013	Retrospective cohort	518	Microscopic	3 months minimal	–	Bilateral	Presence of motile spermatozoa in the ejaculate in at least 1 analysis	"We have demonstrated that microsurgical 1-layer VV has excellent patency rates and is highly successful in enabling men who have had a vasectomy to renew paternity. We also found that preoperative and intraoperative factors are significantly associated with success and fertility status after VV. The presence of anti-sperm antibodies in the ejaculate significantly reduces the likelihood of establishing a pregnancy. Finally, we have established that measurement of the concentration of AG in ejaculate following VV is useful to predict patency and pregnancy results after microsurgical repair."
Boorjian 2004	Retrospective cohort	159	Microscopic	25 months	–	Bilateral	–	"The results of our study may be useful in counseling patients seeking post-vasectomy fertility. The information here concerning the chance for successful vasectomy reversal is particularly relevant when considering the current alternative to reversal, IVF/ICSI, using aspirated sperm. Our data indicate that for obstructive intervals of less than 15 years vasectomy reversal yields much higher pregnancy rates than IVF/ICSI and even for intervals greater than 15 years reversal outcomes equal or exceed those of IVF/ICSI. Reversal is a more cost-effective option regardless of the interval since vasectomy, especially for couples seeking more than 1 child post-vasectomy."
Cerruti 1974	RCT	6	Macroscopic	–	–	–	–	"that good results can be expected from this method used on outpatients"
Cosentino 2018	Retrospective cohort	263	Microscopic	12 months	×20	Bilateral	Semen analysis (>1,000,000 spermatozoa per mL during)	"Based on our results, some factors predicting success after vasovasostomy surgery are known but others remain unknown; our predictive model may easily predict patency and success after this surgery and offers concrete assistance in counseling patients."
Crosnoe 2014	Retrospective cohort	569	Microscopic	–	–	Bilateral	A minimum of 1 × 106 spermatozoa reported on a post-operative semen analysis	"The development of an angled vas cutter provides an increased surface area for vasal wound healing to allow for larger tissue diameter for better healing, resulting in high patency rates after vasovasostomy."
Choo 2009	Retrospective cohort	69	Macroscopic	Up to 31 months	–	–	Sperm concentration of more than 10 × 106/mL on semen analysis	"The new method of vasovasostomy with the Prolene stent has good efficacy and safety. Its benefits are the maintenance of good patency, a good pregnancy rate, and a diminished operating time."
Dohle 2005	Retrospective cohort	217	Microscopic	1 year	–	–	Presence of spermatozoa in ejaculate at follow-up	"They concluded that vasectomy reversal is the treatment of choice in men with female partners older than 37 years, although pregnancy rates were low in both groups. Sperm retrieval and ICSI did not improve the outcome of these couples."

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by myram mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Eisenberg 2009	Retrospective cohort	73	Microscopic	7 months	–	Bilateral	Ejaculation of motile spermatozoa	"Viscoat is a dispersive agent that does not adversely affect surgical patency after vasovasostomy. Subjectively, it helped with visualizing the lumen of the vas deferens during urologic microsurgery."
Engelmann 1990	Retrospective cohort	2137	Macroscopic and microscopic	–	Optical magnification either with loupes or a microscope	–	–	"Our results underline the fact that there is a learning curve in vasectomy reversal; centers with a high operative frequency reported much better results than those with a comparably low frequency. The improvement in results by a more diligent microsurgical technique that currently seems to be generally accepted could not be reflected in this survey."
Fallon 1978	Retrospective cohort	35	Microscopic	–	–	Bilateral	In some milky fluid was noted to ooze from the proximal end vas and was believed to be presumptive evidence of nr, nv, w,c, 1 patency.	"It would seem that a general improvement in results may not be obtained without a widespread change to microsurgical technique"
Farber 2020	Retrospective cohort	429	Microscopic	242 days	–	Bilateral	Presence of motile spermatozoa	"Vasovasostomy remains highly efficacious in treating obstructive azoospermia. Young patients, shorter obstructive intervals, and sperm identified intraoperatively predict improved outcomes. Clinicians can expect VV patency in 3 months and late failure within the first 2 years after surgery. However, patency rates, late failure rates, and kinetics vary by definition."
Fischer 2000	Retrospective cohort	40	Microscopic	8 weeks	–	–	Spermatozoa present at follow-up	"The simpler and faster modified one-layer VV provides sufficient accuracy for successful vasectomy reversal in most cases. For most patients, both procedures have equivalent patency."
Fallon 1981	Retrospective cohort	32	Macroscopic	18 months	Loupe/×2.5	Bilateral	–	"Personal experience with a particular technique is an important factor in success. Our results are comparable to published reports of microscopic vasovasostomy. We believe that vasovasostomy with low-power magnification is a reasonable alternative technique for the urologist. "
Fox 1994	Retrospective cohort	89	Microscopic	8 months to 5 years	–	Bilateral	Presence of spermatozoa in ejaculate at follow-up	"The additional time and effort required for a microsurgical approach to vasovasostomy were worthwhile to obtain patency and fertility rates which were superior to those of other techniques."
Fuse 1995	Case series	7	Microscopic	18 months	–	Bilateral	–	"In conclusion, the modified one-layer microsurgical technique can be performed more easily and quickly and its postoperative results including patency rate are satisfactory, but further clinical experience and assessment will be required."

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Ghaed 2018	Retrospective cohort	50	Microscopic	–	–	Bilateral	–	"Various factors—such as age, the interval between procedures, smoking status, complications after surgery, and history of surgery around the inguinal area affect the success rate of microsurgical VV. Considering these factors, surgeons can estimate the likelihood of success before the surgery. It is suggested that more studies with a bigger sample size be conducted to assess the other factors affecting the success rate of the surgery."
Gilbert 2000	Case series	12	Macroscopic laser assisted	4 years	–	Bilateral	–	"Patients undergoing fertilization procedures should be informed about the possible time-dependent deterioration of their semen parameters as the semen quality may be reduced with time."
Goldstein 1998	Retrospective cohort	194	Microscopic (microdots)	1 year	–	Bilateral	Complete spermatozoa with tails present	"The microdot technique ensures precision suture placement and facilitates the anastomosis of lumens of discrepant diameters by exact mapping of each planned suture. The microdot method separates the planning from the placement. Patency rates using the microdot technique approach 100%."
Grober 2011	Retrospective cohort	99	Microscopic	11.6 months (1–26 range)	–	Bilateral	Sperm analysis	"The results of our study have shown that MIVR does not compromise patency outcomes or semen parameters compared with more traditional approaches to VR and results in less pain during the early period of recovery after surgery and quicker functional recovery."
Gudeloglu 2014	Retrospective cohort	106	Robot assisted	26 months[1–73]	×10–15	Bilateral	Spermatozoa in the ejaculate	"Robotic assistance for microsurgical procedures in male infertility and urology appears to be a possible adjunct to standard microsurgery. It has several advantages including elimination of tremors, multi-view magnification, additional instrument arms, and enhanced dexterity with articulating instrument arms. The current literature supports that these procedures appear to be safe and feasible. However, larger, prospective studies are needed to demonstrate the clinical benefits over standard microsurgery."
Gopi 2007	Retrospective cohort	70	Macroscopic	18 months	–	Bilateral	The post-operative patency rate was confirmed by semen analysis at 1 month.	"In conclusion, the macroscopic technique is cost-effective and easy to learn compared to the learning curve involved in microsurgery. For older men with a longer obstructive interval, who desire to have children, it is important to emphasize that macroscopic technique in the hands of an experienced surgeon can also produce good results and should be considered as an effective means of reestablishing fertility."
Griffiths 1987	Prospective cohort	15	Macroscopic	6–12 months	Loupe/×3.5	Bilateral	Post-operative sperm counts greater than 5 million/mL	"Older patients have a lower success rate of vasectomy reversal and it is recommended that referring Medical Officers should emphasize to the patient that the statement on the current consent form for vasectomy concerning the irreversibility of vasectomy is applicable in the majority of cases."

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Hsieh 2005	Retrospective cohort	63	Macroscopic Microscopic	–	Loupe/×3 and ×10–16 ×10–16	Bilateral	Presence of motile spermatozoa in ejaculate at follow-up	"There was no significant difference in the patency and paternity rates between loupe-assisted and microsurgical MOLV. The surgery was significantly faster with the loupe-assisted method. Because of the shorter operation duration and less expensive instruments required that should reduce the cost, the loupe-assisted MOLV should be considered as the best choice for simple vasectomy reversal"
Hartig 1982	Case–control	11	Microscopic	–	×4.5	–	–	"We believe intraoperative vasography during vasovasostomy can be done safely without fear of complication. Whether or not this procedure should be done routinely to check one's operative result remains to be seen. At any rate, if the question of anastomotic patency arises at the time of surgery, we would recommend this as a safe and relatively simple way of checking the result."
Heidenreich 2000	Retrospective cohort	157	Microscopic	24 months	–	Bilateral	–	"Even in the era of ICSI, microsurgical VVS represents the standard approach for obstructive azoospermia following vasectomy. Based on a cost-benefit analysis, VVS is more successful in terms of pregnancy rates (52 vs. 22.5%). VVS does not expose female partners to complications following treatment of male infertility. In contrast to ICSI, multiple birth rates do not increase after VVS. We conclude that MESA/ICSI should be reversed for patients who are not amenable to microsurgical reconstruction."
Hellstrom 1989	Retrospective cohort	42	Microscopic	–	–	Bilateral	–	"Flow cytometry can be performed on testicular aspirates of vasovasostomy candidates preoperatively. Based on ploidy ratios and debris components, DNA histograms can be classified as normal or abnormal. Using this method, the likelihood of the presence of sperm may be predicted."
Hertz 2020	Retrospective cohort	526	Microscopic	–	–	Bilateral	–	"Surgeons with a high volume of vasectomy reversals have outcomes on par with contemporary series regardless of fellowship training in fertility. The analysis of our large, single-institution series of 14 faculty surgeons demonstrated no difference in patency after vasectomy reversal between fellowship and non-fellowship-trained surgeons. Resident participation and PGY did not negatively affect surgical outcomes. Case volume was high for faculty (mean 37, median 17 cases) and residents (mean 38, median 37 cases) alike. Analysis of new faculty with 20 cases did not find evidence of a learning curve which we postulate was due to their microsurgical experience during training."

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Ho 2005	Prospective cohort	39	Microscopic	6.2 months	–	Bilateral and unilateral	Spermatozoa on intraoperative SA	"FGV is potentially less time-consuming than standard microsurgical vasovasostomy and may be appropriate for patients with sperm on intraoperative SA or a negative intraoperative SA, and an obstructive interval of 10 years or less. Patients without these findings should be considered for vasoepididymostomy. While the early results of FGV are promising, a larger study group with the ability to obtain longer follow-up is required to definitively establish equivalency to standard microsurgical vasovasostomy."
Holman 2001	Retrospective cohort	28,246	Microscopic	–	–	–	–	"Population rates of vasectomy are stable but the risk of seeking a reversal has increased. Outcomes after vasovasostomy have improved. Care should be taken during the counseling of men before vasectomy, and especially in those aged < 30 years."
Horovitz 2012	Retrospective cohort	14	Microscopic	40.5 months	–	Bilateral	–	"Vasovasostomy is an effective treatment modality for the post-vasectomy pain syndrome, and it can achieve robust and durable long-term improvement in pain intensity and quality of life."
Huang 2002	Retrospective cohort	42	Microscopic	–	–	–	Presence of spermatozoa in follow-up semen analysis	"The patency and pregnancy rates of vasovasostomies in CGMH were 85.7% and 50.0%, respectively. Repeat surgery could be considered an effective means of restoring fertility if an initial vasovasostomy failed. Moreover, a vasovasostomy appeared to be an effective means of treating post-vasectomy pain syndrome."
Jee 2010	Retrospective cohort	50 25	Microscopic Macroscopic	2 years	×16–25 Loupe	Bilateral	Presence of motile spermatozoa at the 6th month post-operative	"The microscopic technique yielded a higher patency rate than loupe-assisted technique, possibly by reducing the chance of postoperative vassal stricture."
Jeon 2017	Retrospective cohort	70	Macroscopic	12 weeks	Loupe	–	Patency was defined as the presence of motile spermatozoa in the ejaculate. Men who did not undergo semen analyses but had a successful natural pregnancy were also regarded as having patent anastomosis.	"Loupe-assisted vasovasostomy using the Prolene stent can be an alternative method when microscopy is unavailable. This may enable more urologists to perform vasovasostomy."

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Jokelainen 2000	Retrospective cohort	25	Macroscopic	–	Loupe	–	–	"Macroscopic vasovasostomy is an effective means of re-establishing fertility in vasectomized men."
Jenkins 1979	Retrospective cohort	17	Microscopic	18 months	–	–	Improvement in sperm density, viability, and motility	"The high success rate in the present series it is considered that the enforced bed-rest is important to minimize the strain on the anastomosis and the risk of sperm leakage into the surrounding tissue."
Kabalin 1991	Retrospective cohort	111	Macroscopic	–	Loupe/×4	Bilateral	Presence of spermatozoa in follow-up semen analysis	"Microscopic technique requires a more trained operator and the operating urologist must choose the technique that he finds more comfortable."
Kessler 1981	Retrospective cohort	83	Macroscopic	–	–	Bilateral	–	"Statistically significant correlation was found between postoperative motility and pregnancy rate but none between sperm count and pregnancy rate."
Kumar 2010	Prospective cohort	6	Microscopic	–	–	Bilateral	–	"Two-layer vasovasostomy is a simple technique that can be performed in quick time with excellent results. It may allow a common ground between the complex microdot two-layer technique and the over-simplified single-layer procedure."
Kavoussi 2018	Retrospective cohort	100	Robot assisted	6 months	–	–	Sperm concentration	"High-percentage patency rates are achievable very early in the transition from pure microsurgical vasovasostomy to RAVV. Postoperative mean sperm concentrations in the initial semen analyses after RAVV are consistent over time. For a single microsurgeon not formally trained in robotic surgery, 75 RAVV cases were required to optimize and plateau in anastomosis times and 75 cases were required to optimize operative times."
Kaye 1983	Retrospective cohort	21	Microscopic	43 months (range 20–80)	–	Bilateral	Sperm counts > 20 million	"Microsurgical vasovasostomy performed under local anesthesia through an infra pubic incision appears to be safe and economical, with little morbidity. The early results are comparable to those obtained with more complex and expensive vasovasostomy procedures."
Kolettis 2002	Retrospective cohort	74	Microscopic	12 months	–	Unilateral and bilateral	The presence of spermatozoa in the semen.	"Our data add to the published studies supporting the application of vasectomy reversal, even in cases of longer obstructive intervals. At some obstructive interval, depending on each center's success rates, the success rate for ICSI will surpass that of vasectomy reversal. Where the obstructive interval exceeds this threshold, these couples may be better served with ICSI and may select this option. As with all couples with infertility, a collaborative approach between the urologists and gynecologists is essential to providing the least invasive, most cost-effective care."

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Kolettis 2005	Retrospective cohort	245	Microscopic	9.0 (1–60 range) months	–	Unilateral and bilateral	The presence of spermatozoa in at least one post-operative semen sample.	"The results of our study demonstrated that secondary azoospermia after vasovasostomy is rare. It is more common in unilateral cases and the obstructive interval for transiently patent cases is longer. Sperm cryopreservation, when motile sperm appear in the semen postoperatively, can circumvent the problem of secondary azoospermia, but most men will not need the frozen sperm."
Kolettis 2006	Retrospective cohort	34	Microscopic	10 months	–	Unilateral and bilateral	Patency was defined as the presence of motile spermatozoa in at least 1 post-operative SA	"The patency rate for VV when only sperm parts are present in the vas fluid was lower than previously reported patency rates with complete sperm but still comparable with most surgeons' experience with VE. The pregnancy rate was also less than previously reported pregnancy rates with complete sperm."
Kuang 2004	Retrospective cohort	10	Microscopic	NA	×10–30	–	–	"Use of RAVV in this human ex vivo vas model was feasible. While RAVV took longer to perform and was associated with adverse haptic events, elimination of tremors and comparable patency rates suggest that it may be a viable surgical alternative for microsurgical vasovasostomy."
Lee 1980[1]	Retrospective cohort	66	Macroscopic	1 year	–	–	Motile spermatozoa in ejaculate at 1 year post-operative	"In conclusion, we have found that there is very little difference in the patency and pregnancy rates resulting from macroscopic and microscopic anastomoses of the vas at our institution. Presently we favor the use of the microscope so that the convoluted vas and epididymis may be explored in cases where the lumen in the cut end of the vas is not enlarged or where no sperm are found in the fluid from the cut end of the vas. We believe that in the future this technique may provide better pregnancy rates."
Lee 1986	Retrospective cohort	592	Macroscopic Microscopic	1 year	Loupe/×2–4 ×15	Bilateral	The appearance of normal viable spermatozoa in counts of more than 10 × 106	"The discrepancy between anatomical and functional success rates may be explained by 2 major factors. 1) Oligospermia persists owing to depressed sperm production, 16, maturation, and sperm senescence resulting from testicular and epididymal environmental changes after vasectomy. 2) Initially good sperm counts after vasovasostomy subsequently may decrease. This condition probably is caused by secondary scarring at the anastomotic site before the wife becomes pregnant. "

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Lee 1980	Retrospective cohort	315	Macroscopic Microscopic	–	Loupe ×2 –	Bilateral	–	"We have found that there is very little difference in the patency and pregnancy rates resulting from macroscopic and microscopic anastomoses of the vas at our institution. Presently we favor the use of the microscope so that the convoluted vas and epididymis may be explored in cases where the lumen in the cut end of the vas is not enlarged or where no sperm are found in the fluid from the cut end of the vas. We believe that in the future this technique may provide better pregnancy rates. "
Lee 2012	Retrospective cohort	32	Microscopic	3.22 years	–	Bilateral	–	"VR is an option for PVPS when first-line conservative management fails. In this study, VR was effective in reducing pain for such patients, generating uniformly high-level satisfaction with surgical outcomes. Present data also underscore a significant therapeutic difference, depending on whether or not patency is preserved. VR should therefore be considered in patients with PVPS who are young, wish to recover their fertility, and are of shorter postvasectomy duration."
Majzoub 2017	Case report	171	Microscopic	18 months	Magnifying surgical microscope	Unilateral and bilateral	Presence of any spermatozoa in the ejaculate	"Vasectomy reversal remains a highly effective procedure capable of restoring male fertility after elective sterilization. VV in the presence of sperm or sperm parts from intraoperative vassal fluid samples was associated with a higher likelihood of pregnancy. Although postoperative semen parameters were significantly higher in patients who successfully conceived, most pregnancies were documented in patients with significantly lower semen parameter reference ranges than those described by the WHO manual. Development of VR-specific reference ranges such as those in the present paper would allow surgeons to better counsel patients regarding the potential for spontaneous conception after post-vasectomy reconstruction."
Marshall 2016	Retrospective cohort	79	Robot assisted	–	–	–	–	"Robot-assisted vasovasostomy with a single-layer vasal anastomosis has similar outcomes when compared to the published results of the two largest studies which have evaluated MVV. Although there are many limitations to our study, we have demonstrated that sRAVV is feasible and may prove to be an alternate approach to vasectomy reversal that can be performed successfully by urologists trained in robotic surgery. Further study is needed looking specifically at pregnancy data and cost efficiency."
Moon 2015	Retrospective cohort	263	Microscopic	–	×10–15	Bilateral	Presence of motile spermatozoas	"Ambulatory mini-incision microsurgical vasectomy reversal using Moon's clamp and under local anesthesia is a surgically feasible option that offers the advantages of a low-risk operation. It also achieves successful vasovasostomy without other accessory devices and allows patients to return to their daily activities quickly with minimal complications."

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Mason 1997	Retrospective cohort	66	Macroscopic	6 weeks	No magnification	Bilateral	Semen count of >20 million spermatozoa/mL, progressive motility of 50%, and morphologically normal forms in at least 30	"We feel that macroscopic techniques have a place in the reversal of vasectomy, as it offers satisfactory results and is an easier procedure for the surgeon dealing with relatively few cases. Good counseling to prospective patients detailing the possibility of success is necessary. A cost-saving benefit is also apparent in units where the hardware for microscopic surgery is not available."
Mechlin 2013	Retrospective cohort	6	Robot assisted	7 weeks	–	Bilateral	–	"Because the follow-up was too short, the modified intussusception vasoepididymostomy has not shown evident superiority compared to two-suture intussusception vasoepididymostomy, however, further studies with a large sample and long-term follow-up are needed to testify the effectiveness of this technique."
Middleton 1987	Retrospective cohort	73	Macroscopic	6 months	No magnification	Bilateral	Any spermatozoa in ejaculate at 2, 4, and/or 6 months post-operative	"Our results indicate that the majority of the first pregnancies after vasectomy reversal occur within 1 year. However, it is noteworthy that there still are patients who do not achieve the first pregnancy until 3 or 4 years after the reversal."
Nalesnik 2003	Retrospective cohort	73	Microscopic	41 months	–	–	–	"After vasovasostomy in patients with a vasal obstructive interval of 7 years, up to 45% of couples may achieve pregnancy and up to 35% could go on to conceive a second child. Long-term anastomosis patency after the procedure is estimated to be approximately 60%. Maternal age is a significant factor in predicting a couple's success in conceiving a child. Vasovasostomy remains a highly effective option for restoration of fertility in vasectomy patients while offering the opportunity for multiple pregnancies with only a single intervention."
Ostrowski 2015	Retrospective cohort	2947	Microscopic	–	–	Unilateral and bilateral	–	"The presence of motile sperm at vasectomy reversal approaches statistical significance on univariate analysis of intraoperative factors that affect clinical pregnancy rates regardless of the surgery performed. On multivariate analysis, female partner age independently predicts clinical pregnancy rates, as does the presence of no sperm or only sperm heads on light microscopy. These findings on light microscopy are poor prognostic indicators. These data can be used to better guide the clinical counseling of couples after vasectomy reversal."
Owen 1977	Retrospective cohort	475	Microscopic	–	–	Unilateral	–	"A reliable technique of vasectomy repair has been described using a meticulous technique which avoids the complications caused by damage to the vas or its blood supply. The repair has been so successful that only one side need be operated upon."

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Pai 1973	Retrospective cohort	36	Microscopic	10 years	–	Bilateral	–	"Epididymovasostomies showed very poor results, and the reason may have been that postvasectomy inflammation extended to the epididymis, and The success rate in vasovasostomy was found to have no relation to age, length of use of splints, or time interval between vasectomy and vas reconstruction."
Parekattil 2010	Retrospective cohort	20	Robot assisted	Up to 22 months	×15–20	Bilateral	–	"The use of robotic assistance in microsurgical vasovasostomy may have potential benefits over MVV with regards to decreasing operative duration and significantly improving early semen analysis measures. Further evaluation and longer follow-up are needed to assess its clinical potential."
Parekattil 2012	RCT	110	Robot assisted Microscopic	17 months (1–52 range)	×16–20 and ×40–100	Unilateral and bilateral	–	"The use of robotic assistance in microsurgical vasovasostomy and vasoepididymostomy may have potential benefits over MVV and MVE with regards to decreasing operative duration and improving the rate of recovery of postoperative total motile sperm counts. The advantages of a stable microsurgical platform, ergonomic surgeon instrument controls, elimination of tremors, magnified immersive 3D vision, and simultaneous tri-view ability all contribute to improved surgical efficiency. Further evaluation and longer follow-up are needed to assess its clinical potential and the true cost-benefit ratio. However, the preliminary results are quite promising."
Patel 2008	Retrospective cohort	106	Microscopic	–	(Using 400 wet mount light microscopy)	Bilateral	Patency was defined as any spermatozoa in the post-operative semen specimen.	"Although vasovasostomy in the convoluted vas deferens is considered technically more challenging than in the straight vas deferens, patency rates and postoperative semen analysis parameters for convoluted vasovasostomy and straight vasovasostomy are comparable."
Paul 2021	Retrospective cohort	35	Microscopic	9 months	×10–15	Bilateral	Spermatozoa present in the analysis	"In this cohort, the length of the excised obstructed vas deferens at VV was associated with improved sperm motility at 3 and 9 months postoperatively but not with pregnancy outcomes."
Pendas 2013	Prospective cohort	68	Microscopic	20 months	×10 and ×16	Bilateral	Average sperm concentration ‡20 million/mL	"With results similar to those described in the literature, the simple biplane technique offers an easy, safe, and low-cost procedure. More studies are necessary to compare, in terms of pregnancy rates, a simple biplane with more complex techniques for the longest obstructive intervals."

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Ramasamy 2015	Retrospective cohort	1331	Microscopic	–	–	Bilateral	–	"We have performed a retrospective review of VV outcomes. There was a higher patency associated with the presence of spermatozoa in the vassal fluid compared to the absence of spermatozoa. Information from this study will aid patient counseling on outcomes after VV."
Requeda 1983	Retrospective cohort	47	40 microscopic and 7 macro-scopic	–	–	–	–	"The highest agglutinin titers (>1:128) were found exclusively in those patients who failed to impregnate their wives. Furthermore, in the six fertile patients who produced spermatozoa with a normal fertilizing capacity, the agglutinating antibodies were either absent or detected at low titers of 1:32 or less. The presence of sperm antibodies in the serum at titers of up to 1:32 is frequently encountered in vasovasostomized patients whose wives have conceived 3,5 demonstrating that low titers are compatible with fertility."
Royle 1981	RCT	130	Microscopic	>1 year	–	Bilateral	–	"Since there is evidence that leakage of sperms into the tissues at the time of vasovasostomy may stimulate antibody production and induce granuloma formation with fibrosis at the site of anastomosis, we conducted a prospective controlled trial of per-operative steroids to determine whether this therapy would prevent these effects."
Ravindraana 2020	Retrospective cohort	12	Macroscopic	3 months	Loupe/×7	–	–	"We would advocate loupe-assisted micro surgical vasovasostomies in men wanting a reversal for fertility purposes. However, a higher-powered sample and meta-analysis studies would provide stronger data for clinicians to have more knowledge on accurately counseling patients. Younger men may have less favorable outcomes and will need to be extensively counseled, however, it remains to be clear how significant this may be."
Sandlow 2005	Retrospective cohort	48	Microscopic	12 months	–	Unilateral and bilateral	Patency was defined as motile spermatozoa in the ejaculate post-operatively	"Vasovasostomy in the CVV is a technically more difficult procedure than standard VV. Nevertheless, postoperative results are typically comparable to those obtained via VV and superior to those of epididymovasostomy in many cases. Therefore, we recommend CVV using the same indications as for standard VV in the straight portion of the vas deferens. However, consideration for epididymovasostomy should be given when no sperm are seen in the vasal fluid intraoperatively."
Savage 2020	Prospective cohort	200	Microscopic	10 months	–	Bilateral	These are based on a lenient definition of >0 spermatozoa.	"Implementation of the ReVas technique resulted in significantly higher sperm concentrations, which were particularly pronounced when stricter success criteria were used. Patients were also 8.1× more likely to achieve a pregnancy within the first 2 years, confirming clinical relevance. External validation is warranted."

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Schwarzer 2012	Prospective cohort	788	Microscopic	–	×20–30	Unilateral and bilateral	Patency was demonstrated by semen analyses according to World Health Organization.	"In our opinion, it can be concluded that the main reason for the decreasing pregnancy rates lies in the decreasing sperm quality. Directly correlating individual pregnancies to individual semen analysis is limited, because for a relevant group of pregnant females (136) no semen analysis of the male partners was available. In our study, the distribution of the female age was similar in all the groups independent from the obstructive period. Besides the female factor, no other important factor was obvious to us. So we conclude that sperm quality is the most relevant factor influencing the pregnancy rates."
Sharlip 1982	Retrospective cohort	161	Microscopic	–	×400	Bilateral	–	"Conclusions for obstructive intervals over 12 years. However, because the results of vasoepididymostomy are uncertain and the results of vasovasostomy are highly reliable, the same rules should be applied for longer as for shorter obstructive intervals; i.e., vasoepididymostomy should only be done in the presence of unequivocal epididymal sclerosis."
Shin 2012	Retrospective cohort	97	Microscopic	6 months	–	–	Return of spermatozoa to the semen	"The sperm concentration after vasovasostomy was significantly influenced by the obstructive interval between vasectomy and reversal. In our study, patient age and FSH did not influence the change in postoperative sperm concentration. The natural pregnancy rates were lower in the oligospermia groups than the normal groups at serial follow-up. Although this finding did not reach statistical significance, it may be helpful to keep in mind when clinicians counsel patients who have oligospermia at 1-month follow-up about postoperative outcomes."
Shiraishi 2002	Retrospective cohort	21	Microscopic	36 months	–	Bilateral	–	"This study demonstrates the relative importance of interstitial fibrosis rather than intra-seminiferous status to achieve the irreversible damage (permanent con contraception) after vasectomy."
Sigman 2004	Retrospective cohort	53	Microscopic	–	–	Bilateral	Patency was defined as any spermatozoa in the post-operative semen specimen	"Modern microsurgical techniques yield excellent patency rates. The presence of sperm parts compared to whole sperm does not adversely affect patency rates after vasovasostomy. Vasovasostomy should be performed if any sperm parts are identified in the intravasal fluid."
Silber 1989	Retrospective cohort	126	Microscopic	10 years	–	–	Motile sperm count in men with spermatozoa	"It thus appears that the fertility rate and pregnancy rate may be higher than previously expected in patients with no epididymal blockage who undergo technically 'successful' vasovasostomy."
Sullivan 1977	Retrospective cohort	45	Microscopic	–	–	–	–	"Sperm agglutination and immobilization are merely 2 among many possible antigen-antibody reactions occurring after vasectomy and are less likely to be the cause of the functional failure of vasovasostomy than other antibodies, such as those against spermatozoal enzymes."

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Safarinejad 2013	RCT	94	Macroscopic Microscopic	2 years	Loupe/×4	Bilateral	Presence of spermatozoa (motile or non-motile) with tails in at least one post-operative semen analysis	"There were no significant differences in terms of patency and pregnancy rates between the MOLVV and TLMVV methods, but the MOLVV technique offers a decreased cost and operative time, and a simplified procedure."
Santomauro 2011	Retrospective cohort	13	Robot assisted	–	–	Bilateral	–	"Single-layer and double-layer robotic vasovasostomy are technically feasible, as demonstrated by the adequate results of follow-up semen analysis. Additional data and pregnancy rates are needed to determine its potential role in the management of iatrogenic infertility. Additionally, robot-assisted vasovasostomy may be used as an important step in the training of residents in a university, after appropriate mentorship and training using robotic simulators."
Sharlip 1981	Retrospective cohort	17	Microscopic	12.8 months	–	Bilateral	–	"That the two-layer anastomosis is superior to the conventional method."
Shessel 1981	Retrospective cohort	10	Macroscopic	2 years	Loupe	Bilateral	–	"Though most techniques for vasovasostomy have been successful in returning sperm to the ejaculate, the eventual conception of progeny occurs in only a portion of these patients. This discrepancy is multifactorial in etiology, not all of which are related to the quality of the ejaculation. Nonetheless, impregnation is the final goal of all such procedures and is the criterion by which success should be measured. Recent advances' in vasovasostomy have produced admirable results. They do, however, require elaborate equipment and specialized training in microtechniques. The ability of this simpler method using exteriorized stents to achieve similar success rates, perhaps, justifies its continued use by urologists."
Silber 2004	Retrospective cohort	1735	Microscopic	–	–	Bilateral	–	"We conclude that microsurgical vasectomy reversal is preferable to sperm retrieval and intracytoplasmic sperm injection (ICSI) since the pregnancy rate appears to be higher with this technique than with sperm retrieval and ICSI. It does not appear that sperm antibodies or testicular damage are likely to account for failure to achieve pregnancy after vasectomy reversal. Rather, it is likely to be related to partial or complete obstruction following surgery, or to the fertility of the female partner."

(Continues)

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Singh 1996	Retrospective cohort	60	Macroscopic Microscopic	9 months	No magnification ×16–25	Unilateral Unilateral	Presence of spermatozoa in ejaculate at follow-up	"We concluded that normal preoperative fine needle aspiration cytology, normal serum follicle stimulating and luteinizing hormone, and positive preoperative vasal fluid tests for sperm had been very important markers of successful reversal surgery likely to produce maximum benefit. These parameters helped to prognosticate a maximum chance of pregnancy, which could be communicated to the partner so desirous of knowing about his chances of inducing pregnancy. Perioperative vas patency testing and microscopic examination of the vasal fluid for sperms should be done in all cases as these are very important markers of successful reversal surgery."
Thomas 1981	Retrospective cohort	49	Microscopic	>1 year	–	–	–	"That the occurrence of these antibodies is not frequent enough to be considered a major contributor to the prevention of conception following vasovasostomy. Further study, with pre and postoperative sampling of larger numbers of patients, is currently in progress in our laboratory."
Urquhart-hay 1984	Retrospective cohort	125	Macroscopic	–	Loupe/×3.5	Bilateral	–	"Most wives who become pregnant do so within 12—24 months of the reversal procedure and because of the risk of cicatrization at the site of the anastomosis, I encourage regular coitus in the early days starting as soon as is comfortable after the operation."
Willscher 1980	Retrospective cohort	12	Microscopic	3–16 months	×20	–	–	"Depending on the ease of initial vas isolation, this technique can be done in one and one-half to two hours. Microscopic vasovasostomy with its exceptionally high success rate outweighs its disadvantages and is recommended over other forms of anastomosis."
Wright 1995	Retrospective cohort	51	Microscopic	–	×2.3–11.3	Bilateral	Sperm density	"In conclusion, the authors believe that vasectomy reversal should be offered to any vasectomized men who genuinely wish to have a pregnancy with their new or old partner. The high probability of success regardless of age, vasectomized interval, or sperm antibodies means that all genuine patients should have a chance at renewed fertility and no artificial exclusion criteria should be imposed. Pre-operatively, the probabilities of success can be presented using the vasectomized interval as a guides. Postoperatively, the initial sperm motility can give much better prognostic value than sperm density."

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Study ID	Study design	Total patients	Intervention type	Follow up	Magnification type/strength	Unilateral or bilateral	Definition of patency	Conclusion
Wang 2019	Retrospective cohort	42	Microscopic	2 years	Low-power magnification ×5	Bilateral	–	"One-layer mVV with proper stent provides some advantages over standard two-layer mVV; the requirement of the surgical microscope equipment is lower, performing the the procedure is considerably easier, and operative time is shorter, probably reducing the costs. The goal of mVV with a stent is not to reduce the requirements for microsurgery training but to reduce the difficulty of the surgery and make the procedure easier, even for well-trained surgeons. This could improve access to mVV to men wishing vasectomy reversal."
Vrijhof 1994	Retrospective cohort	82	Microscopic and macroscopic	54 months	–	Bilateral	The recurrence of spermatozoa in the ejaculate after vasal reconstruction	"The presence of a sperm granuloma at the site of ligation after vasectomy might be interpreted as a pressure relief valve. Those patients who underwent a reversal procedure, including removal of their granulomas before reanastomosis, had an explicit good quality sperm in their ejaculate."
Yahyazadeh 2021	Retrospective cohort	42	Microscopic	–	–	Unilateral and bilateral	Semen analysis after VV surgery	"Although our overall results are not as good as those of other advanced centers in the world with high experience of reversal vasectomy techniques, the efficacy of unilateral vasovasostomy compared with the standard bilateral surgery is not necessarily lower. Meanwhile, it can reduce the time of anesthesia and surgery and save costs and consumables. However, these findings should be confirmed in randomized studies."

Abbreviations: CVV, convoluted vasovasostomy; ICSI, intracytoplasmic sperm injection; MMC, mitomycin-C; MOLVV, macroscopic one-layer vasovasostomy; MT, medical therapy; MVE, microsurgical vasovasostomy; MVV, microscopic vasovasostomy; PVPS, post-vasectomy pain syndrome; RAVV, robot-assisted vasovasostomy; RCT, randomized controlled trial; sRAVV, single-layer anastomosis robot-assisted vasovasostomy; TLMVV, two-layer microsurgical vasovasostomy; TT, testosterone therapy; VE, vasoepididymostomy; VR, vasectomy reversal; VV, vasovasostomy.

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by myram mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

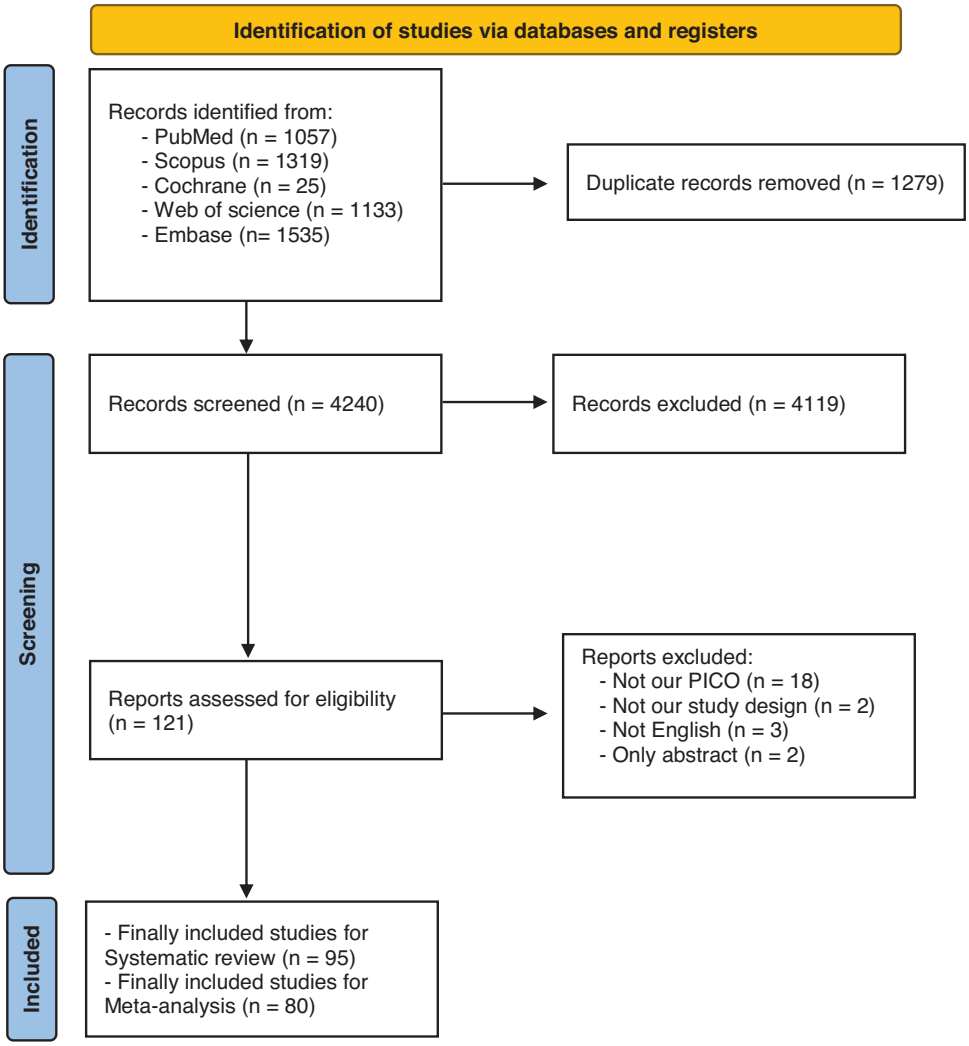


FIGURE 1 Prisma flow diagram.

Robot-assisted microsurgical vasovasostomy

The patency rate following this operation was reported in seven studies, with 397 subjects involved. The rate of patency ranged from 88.1 to 97.6% in the included studies. The patency rate following robot-assisted microsurgical vasovasostomy was homogenously pooled as 94.4% (95% CI: [92.1%, 96.6%], (371 out of 397), ($I^2 = 0\%$, $p = 0.508$)) (Figure 2).

3.4.2 | Pregnancy rate

Macroscopic vasovasostomy

Nineteen studies reported a pregnancy rate following macroscopic vasovasostomy, with 1530 subjects involved. Through the involved studies, the pregnancy rate ranged from 24.5 to 70.0%. Pooled pregnancy rate after macroscopic vasovasostomy was 43.7% (95% CI: [38.3%, 49.2%], (631 out of 1530), ($I^2 = 76.1\%$, $p < 0.001$)) (Figure 3).

Microsurgical vasovasostomy

Forty-five studies reported this outcome, with 9285 subjects involved. The pregnancy rate following microsurgical vasovasostomy ranged from 14.3 to 91.0% through the included studies. The pregnancy rate was heterogeneously pooled as 47.4% (95% CI: [39.0%, 55.7%], (5348 out of 9285), ($I^2 = 98.8\%$, $p < 0.001$)) (Figure 3).

Robot-assisted microsurgical vasovasostomy

One study reported the pregnancy rate after vasovasostomy through this technique, with a sample size of 13. Pregnancy rate was 15.3% (two out of 13) (Figure 3).

3.4.3 | Complications rate

Macroscopic vasovasostomy

Seven studies reported a complications rate following macroscopic vasovasostomy, with 204 subjects involved. Through the involved

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

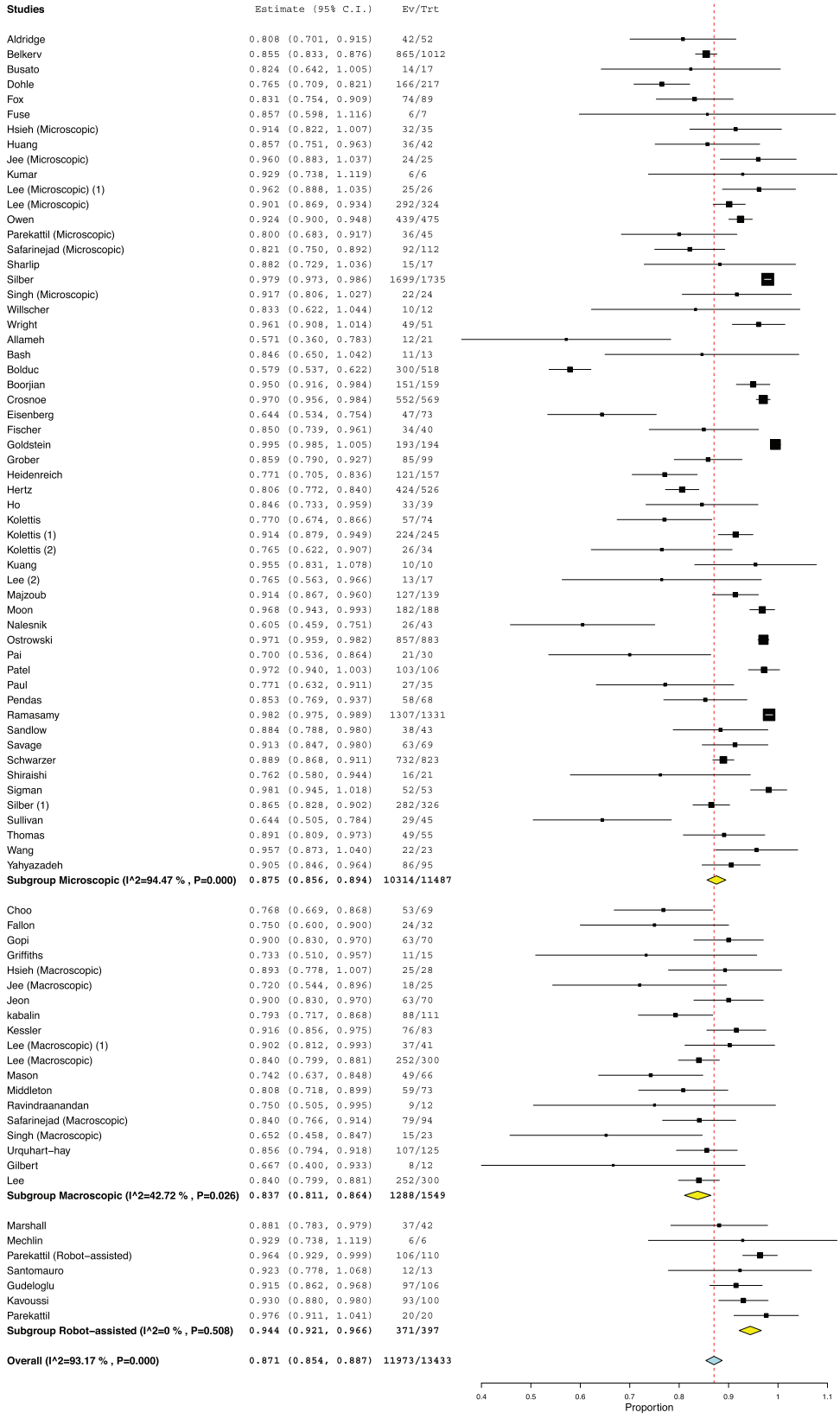


FIGURE 2 Forest plot of patency rate.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

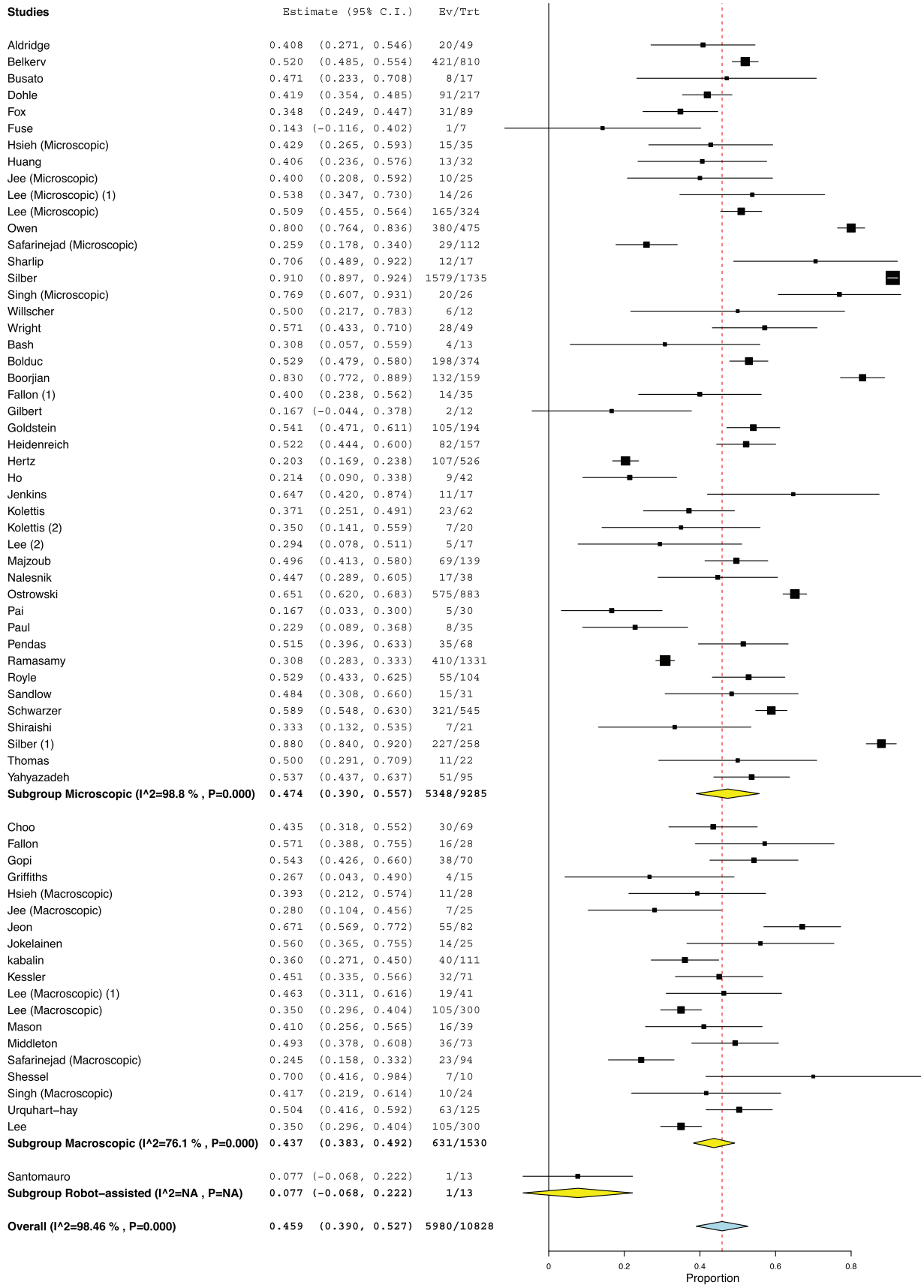


FIGURE 3 Forest plot of pregnancy rate.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

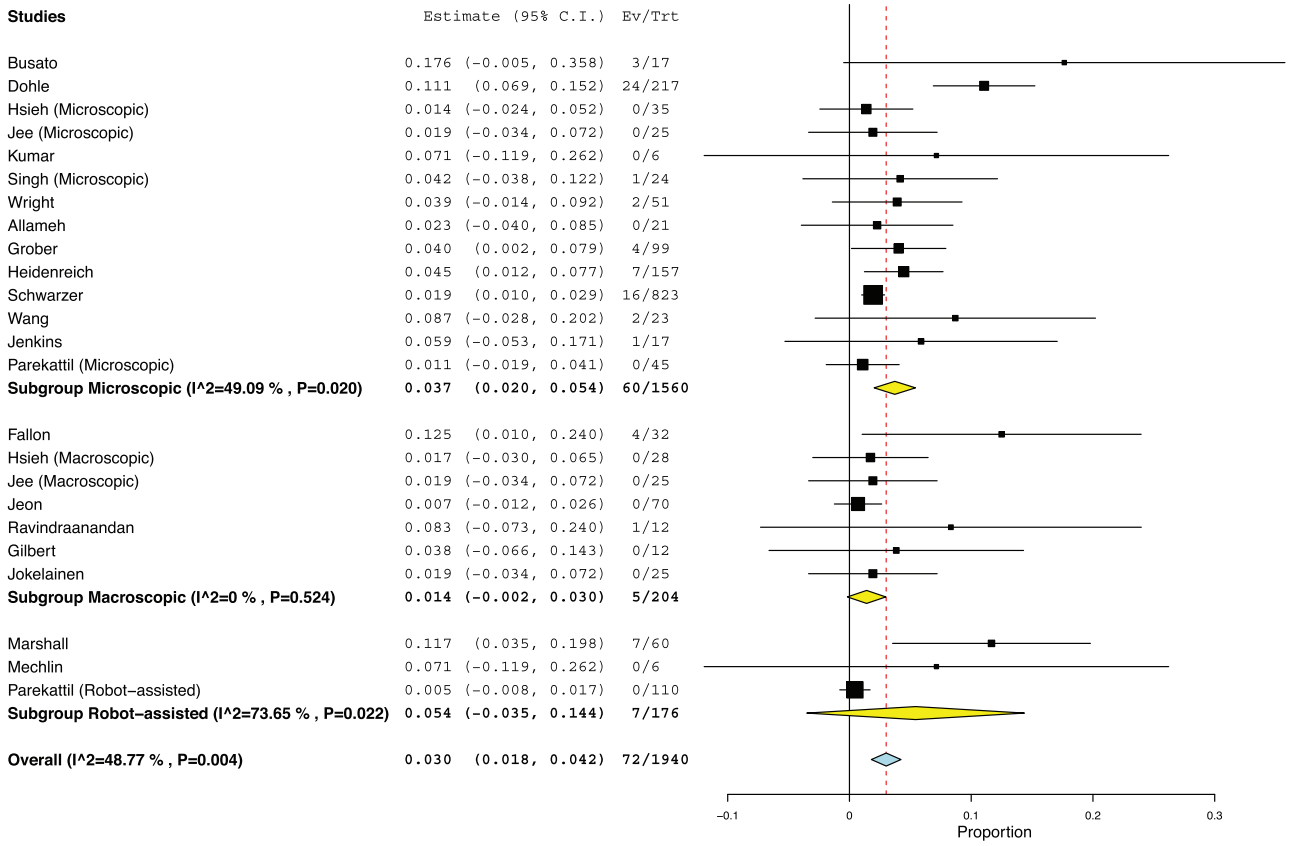


FIGURE 4 Forest plot of post-operative complications rate.

studies, the complication rate ranged from 0 to 3%. The pooled complications rate after macroscopic vasovasostomy was 1.4% (95% CI: [0, 3%], (five out of 204), ($I^2 = 0\%$, $p = 0.524$)) (Figure 4).

Microsurgical vasovasostomy

Fourteen studies reported this outcome, with 1560 subjects involved. The complications rate following microsurgical vasovasostomy ranged from 2% to 5.4% through the included studies. The complications rate was pooled as 3.4% (95% CI: [2%, 5.4%], (60 out of 1560), ($I^2 = 49.09\%$, $p = 0.02$)) (Figure 4).

Robot-assisted microsurgical vasovasostomy

Three studies reported the complications rate after vasovasostomy through this technique, with a sample size of 176. Complications rate was 5.4% (seven out of 176) (Figure 4).

3.4.4 | Factors influencing vasovasostomy outcome

The patency rate following vasovasostomy was associated with the time interval between the two operations (vasectomy and vasovasostomy). Having an obstructed vas deferens for over 10 years predicted the failure of vasovasostomy as defined by patency.[27,28,105,107] In addition, repetition of vasectomy reversal was a negative predictor for vasovasostomy success.[28] However, performing vasovasostomy bilaterally predicted increased odds of patency.[28] Likewise, bilateral detection of spermatozoa intraoperatively was a predictor for vasovasostomy success and a protective factor against late operation failure.[105] Participants with sperm granuloma or Silber grade I–III spermatozoa had a greater likelihood of vasovasostomy success.[106] Conversely, advanced age was associated with vasovasostomy failure.[107] Smoking, previous inguinal-area surgery, and post-vasovasostomy complications were predictors of vasovasostomy failure.[107] Requiring a longer duration to ejaculate semen with a motile spermatozoa concentration of more than two million per mL predicted late vasovasostomy failure.[105] Fenig et al.[120] present a mathematical tool that can assist doctors in using this nomogram to predict before the surgery if they have to do an epididymovasostomy. The tool uses two factors: the time since the previous operation and the presence of a sperm mass in the scrotum. The paper says that the tool is reliable and helpful for advising patients and sending them to experts if necessary. This helps urologists to give better information to patients about the chance of this difficult surgery and the referral to a specialist.[120]

4 | DISCUSSION

Vasovasostomy is a cost-effective solution for men seeking natural fertility following vasectomy. This operation can be performed through three techniques: macroscopic vasovasostomy, purely

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

microsurgical vasovasostomy, and robot-assisted vasovasostomy. The three techniques are currently in use; thus, we conducted this systematic review aiming to evaluate the outcomes of each technique. Ninety-five studies were involved in our review, and 85 were included in the meta-analyses. The pooled patency rate was the highest for the robot-assisted microsurgical technique (94.4%), followed by the purely microsurgical technique (87.5%) and the macroscopic technique (83.7%). The pooled pregnancy rate following purely microsurgical vasovasostomy was higher than that of macroscopic vasovasostomy (47.4 vs 43.7%). The complication rate from the different techniques was almost the same between them and it was low; macroscopic vasovasostomy was 1.4%, which was lower than purely microsurgical vasovasostomy (3.4%), and the highest one was the robot-assisted microsurgical technique with (5.4%)

Robotic vasovasostomy pregnancy data was limited to one study, encompassing 13 patients with a low reported pregnancy rate (15%). The study did not include pregnancy as a primary outcome, however at an unreported follow-up assessing patency, two patients had already conceived. The authors noted the impossibility of reporting an accurate pregnancy rate at that time. Considering this, reporting robotic vasovasostomy pregnancy rates is not feasible, and is significantly underrepresented in the literature. Patency is paramount for pregnancy and, as a surrogate, it indicates the potential for conception even if actual pregnancy rates are not provided. Efforts should be made in future research to quantify pregnancy rates in robotic vasovasostomy.

As microsurgical vasovasostomy adds more precision to the operation, it leads to better outcomes in terms of patency and conception. Moreover, the robot-assisted microsurgical technique is the most precise, offering attenuation of the surgeon's physiologic tremor, enhanced visualization of the structures, and the ability to simultaneously control three operating arms and a fourth camera.[18,19,33,47] The robot-assisted microsurgical technique uses a robot with a camera and instruments to stitch the ends of the vas deferens. The instruments are Black diamond forceps and Potts scissors, which are very precise and sharp. The surgery is faster, safer, and more comfortable than traditional microsurgery. It also has similar or better success rates in restoring fertility. Here are the steps of the technique: (1) Make a small cut on each side of the scrotum and expose the vas deferens. (2) Cut the vas deferens at the previous vasectomy site and check for fluid and spermatozoa at both ends. (3) Insert three small tubes into the abdomen and attach the robot arms with the camera and instruments. (4) Use two sutures to align the ends of the vas deferens and then use four to six sutures to join them together. (5) Close the scrotal cuts with sutures and apply a scrotal support.

These features allow for a highly precise water-tight mucosal anastomosis, and thus, better vasovasostomy outcome consistent with our findings. However, this highly precise technique has a learning curve. According to the studies included in this review, surgeons' performance—as assessed by patency rate and operative time—improved with practice. The learning curve was shorter for the pure microsurgical technique when compared with the robot-assisted microsurgical technique. Furthermore, previous experience with the microsurgical technique was not found to shorten the learning curve for the robot-assisted microsurgical technique.[28,33,41] In addition, the high cost and unavailability of the robotic arm limit its use.[41]

This study updates the results of the previous meta-analysis conducted by Duijn et al. in 2023.[121] Our review includes 95 studies that enrolled 48,069 participants, compared with 49 studies (n = 6822) in the previous review. Our findings on pooled patency and pregnancy rates were consistent with those of Duijn et al.[121] Our review is distinguished by the large number of included studies with different designs and the substantial total sample size. In addition, the majority of the included studies were of good to fair quality.

However, the results of the involved studies were heterogeneous. This heterogeneity is thought to emerge from the variation between the individual studies in outcome definitions. Some papers defined patency as the presence of spermatozoa in the ejaculated semen. Other studies required a certain number of spermatozoas (up to >20 million) to define patency. This discrepancy in defining the outcomes decreases the confidence in the pooled results. The definition of pregnancy also varied across the involved studies. Many defined pregnancies by natural or artificial means at 1 year. Others had varying lengths of follow-up, some continuing to 10 years post-procedure. This biases studies with longer follow-up and further adds to the heterogeneity. Efforts should be made to standardize the definition with the goal of accurate comparisons. Further heterogeneity can be attributed to surgical technique including variation in suture material and anastomotic technique. Furthermore, the lack of direct comparison between the surgical techniques with statistical analysis limits our confidence in favoring one technique over another. Additionally, the quality and accuracy of the procedure may depend on the experience of the surgeon, which is an important factor in the success of the procedure. However, almost all of the papers that we included in our analysis did not provide data on this factor. This is a limitation of the studies, as it may confound the outcome of the surgery. Further research is needed to compare the impact of surgeon experience on different techniques of vasovasostomy.

The findings of this study favor conducting vasovasostomy through a robot-assisted microsurgical technique over the pure microsurgical technique. Despite the absence of pregnancy data, patency rates are clear markers of technical success in the vasovasostomy procedure. The conventional macroscopic technique is the least favorable vasovasostomy technique. Studies on robot-assisted microsurgical techniques are scarce, and we recommend conducting further research on this modern technique. In addition, we recommend conducting RCTs on a large scale to compare the three surgical techniques. The characterization of long-term outcomes such as vasovasostomy late-failure rates would benefit from the extensive future investigation. Furthermore, the assessment of post-operative complication rate was not sufficient due to the lack of data in most of the included studies so we need more studies to evaluate the differences in the complication rate among the three techniques. Moreover, we need more data on the differences between the single-layer technique and the double-layer technique.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

5 | CONCLUSION

Vasovasostomy for reversal of vasectomy is an effective operation for natural fertility and reversal of post-vasectomy pain syndrome. Vasovasostomy can be performed using three techniques, conventional macroscopic technique, pure microsurgical technique, and robot-assisted microsurgical technique. Our review found improved vasovasostomy outcomes with the robot-assisted microsurgical technique, followed by the pure microsurgical technique, and lastly the conventional macroscopic technique. However, robot-assisted technique is limited by the high cost, unavailability, and the learning curve. Ongoing research investigating the robot-assisted technique outcomes and RCTs is required to further support this evidence.

AUTHOR CONTRIBUTION

I. S. performed the conceptualization, methodology, formal analysis, and writing the original draft of the paper. D. G. performed the conceptualization, methodology, and writing—review and editing. G. B. contributed with the formal analysis and writing—review and editing. K. J. performed the investigation and writing—review and editing. J. C. contributed with the investigation and writing—review and editing. K. Q. performed the investigation and writing—review and editing. S. S. helped with the supervision and writing—review and editing. W. R. carried out the supervision, conceptualization, methodology, and writing—review and editing.

ACKNOWLEDGMENTS

Open access publishing facilitated by University of New South Wales, as part of the Wiley - University of New South Wales agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

FUNDING INFORMATION

No authors have received any funding or support.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Damien Gibson https://orcid.org/0000-0003-0398-1265

REFERENCES

1. Ostrowski KA, Holt SK, Haynes B, Davies BJ, Fuchs EF, Walsh TJ. Evaluation of vasectomy trends in the United States. *Urology*. 2018;118:76-79.
2. Hamdy FC, Eardley I. *Oxford textbook of urological surgery*. Oxford University Press; 2017.
3. Patel AP, Smith RP. Vasectomy reversal: a clinical update. *Asian J Androl*. 2016;18(3):365-371.
4. Bartz D, Greenberg JA. Sterilization in the United States. *Obstet Gynecol Rev*. 2008;1(1):23-32.
5. Bernie AM, Osterberg EC, Stahl PJ, Ramasamy R, Goldstein M. Vasectomy reversal in humans. *Spermatogenesis*. 2012;2(4):273-278.
6. Jones A, Vazirian-Zadeh M, Phan YC, Mahmalji W. Post vasectomy chronic pain: are we under diagnosing vasitis? A case report and review of the literature. *ISSAM*. 2020;23(5):437-439.
7. Smith-Harrison LI, Smith RP. Vasectomy reversal for post-vasectomy pain syndrome. *Transl Androl Urol*. 2017;6(1):S10-S13. Suppl.
8. Auyeung AB, Almejally A, Alsaggar F, Doyle F. Incidence of post-vasectomy pain: systematic review and meta-analysis. *Int J Environ Res Public Health*. 2020;17(5).
9. Sharlip ID, Belker AM, Honig S, et al. Vasectomy: AUA guideline. *J Urol*. 2012;188(6):2482-2491. Suppl.
10. Sinha V, Ramasamy R. Post-vasectomy pain syndrome: diagnosis, management and treatment options. *Transl Androl Urol*. 2017;6(1):S44-S47. Suppl.
11. Kim HH, Goldstein M. History of vasectomy reversal. *Urol Clin*. 2009;36(3):359-373.
12. Jungwirth A, Diemer T, Dohle GR, Jungwirth A, Diemer T, Dohle GR, et al. *EAU guidelines on male infertility*. EAU Annual Congress; 2019.
13. Martin E, Carnett JB, Levi JV, Pennington ME. The surgical treatment of sterility due to obstruction at the epididymis; together with a study of the morphology of human spermatozoa. *Univ Pennsylvania Med Bull*. 1902;15(1):2-15.
14. Jequier AM. Edward Martin (1859–1938). The founding father of modern clinical andrology. *Int J Androl*. 1991;14(1):1-10.
15. Schroeder-Printzen I, Diemer T, Weidner W. Vasovasostomy. *Urol Int*. 2003;70(2):101-107.
16. Hayden RP, Li PS, Goldstein M. Microsurgical vasectomy reversal: contemporary techniques, intraoperative decision making, and surgical training for the next generation. *Fertil Steril*. 2019;111(3):444-453.
17. Derrick FC Jr, Yarbrough W, D'Agostino J. Vasovasostomy: results of questionnaire of members of the American Urological Association. *J Urol*. 1973;110(5):556-557.
18. Schiff J, Li PS, Goldstein M. Robotic microsurgical vasovasostomy and vasoepididymostomy: a prospective randomized study in a rat model. *J Urol*. 2004;171(4):1720-1725.
19. Schoor RA, Ross L, Niederberger C. Robotic assisted microsurgical vasal reconstruction in a model system. *World J Urol*. 2003;21(1):48-49.
20. Fleming C. Robot-assisted vasovasostomy. *Urol Clin North Am*. 2004;31(4):769-772.
21. Higgins J, Thomas J, Chandler J, et al. Cochrane Handbook for Systematic Reviews of Interventions version 6.3 (updated February 2022). 2022.
22. Page MJ, McKenzie JE, Bossuyt PM, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ (Clinical Research Ed)*. 2021;372:n71.
23. NHLBI. *Study quality assessment tools*. National Institutes of Health: Bethesda, MD, USA. 2018.
24. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ (Clinical Research Ed)*. 2003;327(7414):557-560.
25. Bash JC, Lo JO, Kapadia AA, Mason M, Hedges JC. Vasectomy reversal outcomes in men after testosterone therapy. *Sci Rep*. 2022;12(1):19496.
26. Paul CJ, West JM, Wald M. Association between the lengths of excised obstructed vas deferens segments at the time of vasovasostomy and surgical outcomes. *Turk J Urol*. 2021;47(1):3-8.
27. Yahyazadeh SR, Sadighi Gilani MA, Karimi A. Vasectomy reversal: unilateral versus bilateral vasovasostomy. *Andrologia*. 2021;53(9):e14178.
28. Hertz AM, Stamm AW, Anderson MI, Baker KC. Impact of surgical volume and resident involvement on patency rates after vasectomy reversal—a 14-year experience in an open access system. *AJUR*. 2021;8(2):197-203.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

29. Ravindraanandan M, Ong CT, Elhadi M, Mahmalji W, Akhtar M. Vasectomy reversal: a review on outcomes using a loupe-assisted vasovasostomy approach. *ISSAM*. 2020;23(5):1217-1219.
30. Savage J, Manka M, Rindels T, Alom M, Sharma KL, Trost L. Reinforcing vasal suture technique improves sperm concentration and pregnancy rates in men undergoing vasovasostomy for vasectomy reversal. *TranslAndrolUrol*. 2020;9(1):73-81.
31. Allameh F, Hosseini J, Qashqai H, Mazaherylaghab H. Efficacy of intraoperative mitomycin-C in vasovasostomy procedure: a randomized clinical trial. *Int J Fertil Steril*. 2019;13(3):240-244.
32. Wang B, Liu Z, Jiang H. Comparison of low-power magnification one-layer vasovasostomy with stent and microscopic two-layer vasovasostomy for vasectomy reversal. *Int J Impot Res*. 2020;32(6):617-622.
33. Kavoussi PK, Harlan C, Kavoussi KM, Kavoussi SK. Robot-assisted microsurgical vasovasostomy: the learning curve for a pure microsurgeon. *J Robot Surg*. 2019;13(3):501-504.
34. Jeon JC, Kwon T, Park S, Park S, Cheon SH, Moon KH. Loupe-assisted vasovasostomy using a prolene stent: a simpler vasectomy reversal technique. *World J Mens Health*. 2017;35(2):115-119.
35. Majzoub A, Tadros NN, Polackwich AS, Sharma R, Agarwal A. Vasectomy reversal semen analysis: new reference ranges predict pregnancy. *Fertil Steril*. 2017;107(4):911-915.
36. Marshall MT, Doudt AD, Berger JH, BK A, Christman MS, Choe CH. Robot-assisted vasovasostomy using a single layer anastomosis. *J Robot Surg*. 2017;11(3):299-303.
37. Moon HJ. A feasible ambulatory mini-incision microsurgical vasovasostomy under local anaesthesia using a specially designed double-ringed clamp that simplifies surgery. *Singapore Med J*. 2015;56(4):228-232.
38. Ostrowski KA, Polackwich AS, Conlin MJ, Hedges JC, Fuchs EF. Impact on pregnancy of gross and microscopic vasal fluid during vasectomy reversal. *J Urol*. 2015;194(1):156-159.
39. Ramasamy R, Mata DA, Jain L, Perkins AR, Marks SH, Lipshultz LI. Microscopic visualization of intravasal spermatozoa is positively associated with patency after bilateral microsurgical vasovasostomy. *Andrology*. 2015;3(3):532-535.
40. Crosnoe LE, Kim ED, Perkins AR, Marks MB, Burrows PJ, Marks SH. Angled vas cutter for vasovasostomy: technique and results. *Fertil Steril*. 2014;101(3):636-639.
41. Gudeloglu A, Brahmbhatt JV, Parekattil SJ. Robotic microsurgery in male infertility and urology-taking robotics to the next level. *TranslAndrolUrol*. 2014;3(1):102-112.
42. Bolduc S, Fischer MA, Deceuninck G, Thabet M. Factors predicting overall success: a review of 747 microsurgical vasovasostomies. *CUAJ*. 2007;1(4):388-394.
43. Mechlin C, McCullough A. V1591 robotic microsurgical vasectomy reversal: initial experience and surgical outcomes from a single academic center. *J Urol*. 2013;189(4S):e653-e654.
44. del Dago Pendás FJ, Hernández JRH, Fernández EML-T, del Dago Amaya PJ. Simple biplane vasovasostomy: surgical technique and results. *Journal of Men's Health*. 2013;10(2):48-53.
45. Safarinejad MR, Lashkari MH, Asgari SA, Farshi A, Babaei AR. Comparison of macroscopic one-layer over number 1 nylon suture vasovasostomy with the standard two-layer microsurgical procedure. *Hum Fertil (Camb)*. 2013;16(3):194-199.
46. Lee JY, Chang JS, Lee SH, et al. Efficacy of vasectomy reversal according to patency for the surgical treatment of postvasectomy pain syndrome. *Int J Impot Res*. 2012;24(5):202-205.
47. Parekattil SJ, Gudeloglu A, Brahmbhatt J, Wharton J, Priola KB. Robotic assisted versus pure microsurgical vasectomy reversal: technique and prospective database control trial. *J Reconstr Microsurg*. 2012;28(7):435-444.
48. Schwarzer JU. Vasectomy reversal using a microsurgical three-layer technique: one surgeon's experience over 18 years with 1300 patients. *Int J Androl*. 2012;35(5):706-713.
49. Grober ED, Jarvi K, Lo KC, Shin EJ. Mini-incision vasectomy reversal using no-scalpel vasectomy principles: efficacy and postoperative pain compared with traditional approaches to vasectomy reversal. *Urology*. 2011;77(3):602-606.
50. Santomauro MG, Choe CH, L'Esperance JO, Auge BK. Robotic vasovasostomy: description of technique and review of initial results. *J Robot Surg*. 2012;6(3):217-221.
51. Jee SH, Hong YK. One-layer vasovasostomy: microsurgical versus loupe-assisted. *Fertil Steril*. 2010;94(6):2308-2311.
52. Kumar R, Mukherjee S. "4 × 4 vasovasostomy": a simplified technique for vasectomy reversal. *IJU*. 2010;26(3):350-352.
53. Parekattil SJ, Atalah HN, Cohen MS. Video technique for human robot-assisted microsurgical vasovasostomy. *J Endourol*. 2010;24(4):511-514.
54. Busato WF Jr. Vasectomy reversal: a seven-year experience. *Urol Int*. 2009;82(2):170-174.
55. Choo HS, Cheon SH, Park S, Ji YH, Moon KH. A new method of vasovasostomy with the prolene stent. *Korean J Urol*. 2009;40(7):375-379.
56. Eisenberg ML, Walsh TJ, Turek PJ. Use of viscoelastic solution to improve visualization during urologic microsurgery: evaluation of patency after vasovasostomy. *Urology*. 2009;73(1):134-136.
57. Patel SR, Sigman M. Comparison of outcomes of vasovasostomy performed in the convoluted and straight vas deferens. *J Urol*. 2008;179(1):256-269.
58. Gopi SS, Townell NH. Vasectomy reversal: is the microscope really essential? *Scott Med J*. 2007;52(2):18-20.
59. Kolettis PN, Burns JR, Nangia AK, Sandlow JI. Outcomes for vasovasostomy performed when only sperm parts are present in the vasal fluid. *J Androl*. 2006;27(4):565-567.
60. Dohle GR, Smit M. Microsurgical vasectomy reversal: results and predictors of success. *Andrologie*. 2005;15(2):167-171.
61. Ho KL, Witte MN, Bird ET, Hakim S. Fibrin glue assisted 3-suture vasovasostomy. *J Urol*. 2005;174(4):1360-1363. Pt 1. discussion 3.
62. Hsieh ML, Huang HC, Chen Y, Huang ST, Chang PL. Loupe-assisted vs microsurgical technique for modified one-layer vasovasostomy: is the microsurgery really better? *BJU Int*. 2005;96(6):864-866.
63. Kolettis PN, Fretz P, Burns JR, D'Amico AM, Box LC, Sandlow JI. Secondary azoospermia after vasovasostomy. *Urology*. 2005;65(5):968-971.
64. Sandlow JI, Kolettis PN. Vasovasostomy in the convoluted vas deferens: indications and outcomes. *J Urol*. 2005;173(2):540-542.
65. Boorjian S, Lipkin M, Goldstein M. The impact of obstructive interval and sperm granuloma on outcome of vasectomy reversal. *J Urol*. 2004;171(1):304-306.
66. Kuang W, Shin PR, Matin S. Initial evaluation of robotic technology for microsurgical vasovasostomy. *J Urol*. 2004;171(1):300-303.
67. Sigman M. The relationship between intravasal sperm quality and patency rates after vasovasostomy. *J Urol*. 2004;171(1):307-309.
68. Silber SJ, Grotjan HE. Microscopic vasectomy reversal 30 years later: a summary of 4010 cases by the same surgeon. *J Androl*. 2004;25(6):845-859.
69. Nalesnik JG. Vasovasostomy: multiple children and long-term pregnancy rates. *Curr Surg*. 2003;60(3):348-350.
70. Huang HC, Hsieh ML, Huang ST, Tsui KH, Lai RH, Chang PL. Microsurgical vasectomy reversal: ten-years' experience in a single institute. *Chang Gung Med J*. 2002;25(7):453-457.
71. Kolettis PN, Sabanegh ES, D'Amico AM, Box L, Sebesta M, Burns JR. Outcomes for vasectomy reversal performed after obstructive intervals of at least 10 years. *Urology*. 2002;60(5):885-888.
72. Shiraishi K, Takihara H, Naito K. Influence of interstitial fibrosis on spermatogenesis after vasectomy and vasovasostomy. *Contraception*. 2002;65(3):245-249.
73. Fischer MA, Grantmyre JE. Comparison of modified one- and two-layer microsurgical vasovasostomy. *BJU Int*. 2000;85(9):1085-1088.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

74. Gilbert PTO, editor Unpredictable long-term tissue effects in laser-assisted vasovasostomy2000/05/1.
75. Heidenreich A, Altmann P, Engelmann UH. Microsurgical vasovasostomy versus microsurgical epididymal sperm aspiration/testicular extraction of sperm combined with intracytoplasmic sperm injection. A cost-benefit analysis. *Eur Urol*. 2000;37(5):609-614.
76. Jokelainen OS, Rintala E, Koskimies AI, Rannikko S. Vasovasostomy–a 15-year experience. *Scand J Urol Nephrol*. 2001;35(2):132-135.
77. Goldstein M, Li PS, Matthews GJ. Microsurgical vasovasostomy: the microdot technique of precision suture placement. *J Urol*. 1998;159(1):188-190.
78. Mason RG, Connell PG, Bull JC. Reversal of vasectomy using a macroscopic technique: a retrospective study. *Ann R Coll Surg Engl*. 1997;79(6):420-422.
79. Singh I, Kaza RC. A case in favour of one sided microscopic vasovasostomy–the New Delhi experience. *Int Urol Nephrol*. 1996;28(1):27-31.
80. Fuse H, Kimura H, Katayama T. Modified one-layer microsurgical vasovasostomy in vasectomized patients. *Int Urol Nephrol*. 1995;27(4):451-456.
81. Wright GM, Cato A, Webb DR. Microsurgical vasovasostomy in military personnel. *Aust N Z J Surg*. 1995;65(1):20-26.
82. Fox M. Vasectomy reversal–microsurgery for best results. *Br J Urol*. 1994;73(4):449-453.
83. Belker AM, Thomas AJ Jr, Fuchs EF, Konnak JW, Sharlip ID. Results of 1,469 microsurgical vasectomy reversals by the Vasovasostomy Study Group. *J Urol*. 1991;145(3):505-511.
84. Kabalin JN, Kessler R. Macroscopic vasovasostomy re-examined. *Urology*. 1991;38(2):135-138.
85. Silber SJ. Pregnancy after vasovasostomy for vasectomy reversal: a study of factors affecting long-term return of fertility in 282 patients followed for 10 years. *Hum Reprod*. 1989;4(3):318-322.
86. Griffiths CL. Reversal of vasectomy: vasovasostomy. *J R Army Med Corps*. 1987;133(2):87-88.
87. Middleton RG, Smith JA, Moore MH, Urry RL. A 15-year followup of a nonmicrosurgical technique for vasovasostomy. *J Urol*. 1987;137(5):886-887.
88. Lee HY. A 20-year experience with vasovasostomy. *J Urol*. 1986;136(2):413-415.
89. Aldridge KW, Bueschen AJ, Lloyd LK, Burns JR. Microsurgical vasovasostomy for reversal of elective bilateral segmental vasectomy. *South Med J*. 1985;78(8):967-969.
90. Urquhart-Hay D. A low-power magnification technique for the reanastomosis of the vas–further results in a personal series of 125 patients. *Aust N Z J Surg*. 1984;54(1):73-74.
91. Fallon B, Miller RK, Gerber WL. Nonmicroscopic vasovasostomy. *J Urol*. 1981;126(3):361-362.
92. Kessler R, Freiha F. Macroscopic vasovasostomy. *Fertil Steril*. 1981;36(4):531-532.
93. Royle MG, Parslow JM, Kingscott MM, Wallace DM, Hendry WF. Reversal of vasectomy: the effects of sperm antibodies on subsequent fertility. *Br J Urol*. 1981;53(6):654-659.
94. Sharlip ID. Vasovasostomy: comparison of two microsurgical techniques. *Urology*. 1981;17(4):347-352.
95. Shessel FS, Lynne CM, Politano VA. Use of exteriorized stents in vasovasostomy. *Urology*. 1981;17(2):163-165.
96. Thomas AJ, Jr, Pontes JE, Rose NR, Segal S. Microsurgical vasovasostomy: immunologic consequences and subsequent fertility. *Fertil Steril*. 1981;35(4):447-450.
97. Lee L, McLoughlin MG. Vasovasostomy: a comparison of macroscopic and microscopic techniques at one institution. *Fertil Steril*. 1980;33(1):54-55.
98. Lee HY. Members Of The Vasectomy Study Project. Observations of the results of 300 vasovasostomies. *J Androl*. 1980;1(1):11-15.
99. Willscher MK, Novicki DE. Simplified technique for microscopic vasovasostomy. *Urology*. 1980;15(2):147-149.
100. Jenkins IL, Blacklock NJ. Experience wiht vasovasostomy: operative technique and results. *Br J Urol*. 1979;51(1):43-45.
101. Fallon B, Jacobo E, Bunge RG. Restoration of fertility by vasovasostomy. *J Urol*. 1978;119(1):85-86.
102. Owen ER. Microsurgical vasovasostomy: a reliable vasectomy reversal. *Aust N Z J Surg*. 1977;47(3):305-309.
103. Sullivan MJ, Howe GE. Correlation of circulating antisperm antibodies to functional success in vasovasostomy. *J Urol*. 1977;117(2):189-191.
104. Pai MG, BT SK, Kaundinya C, Bhat HS. Vasovasostomy. A clinical study with 10 years' follow-up. *Fertil Steril*. 1973;24(10):798-801.
105. Farber NJ, Flannigan R, Srivastava A, Wang H, Goldstein M. Vasovasostomy: kinetics and predictors of patency. *Fertil Steril*. 2020;113(4):774-780.
106. Cosentino M, Peraza MF, Vives A, et al. Factors predicting success after microsurgical vasovasostomy. *Int Urol Nephrol*. 2018;50(4):625-632.
107. Ghaed MA, Mahmoodi F, HR A. Prognostic factors associated with bilateral, microsurgical vasovasostomy success. *Middle East Fertility Society Journal*. 2018;23(4):373-376.
108. Horovitz D, Tjong V, Domes T, Lo K, Grober ED, Jarvi K. Vasectomy reversal provides long-term pain relief for men with the post-vasectomy pain syndrome. *J Urol*. 2012;187(2):613-617.
109. Shin YS, Kim SD, Park JK. Preoperative factors influencing postoperative results after vasovasostomy. *World J Mens Health*. 2012;30(3):177-182.
110. Holman CD, Wisniewski ZS, Semmens JB, Rouse IL, Bass AJ. Population-based outcomes after 28,246 in-hospital vasectomies and 1,902 vasovasostomies in Western Australia. *BJU Int*. 2000;86(9):1043-1049.
111. Vrijhof HJ, Delaere KP. Vasovasostomy results in 66 patients related to obstructive intervals and serum agglutinin titres. *Urol Int*. 1994;53(3):143-146.
112. Engelmann UH, Schramek P, Tomamichel G, Deindl F, Senge T. Vasectomy reversal in central Europe: results of questionnaire of urologists in Austria, Germany and Switzerland. *J Urol*. 1990;143(1):64-67.
113. Hellstrom WJ, Deitch AD, deVere White RW. Evaluation of vasovasostomy candidates by deoxyribonucleic acid flow cytometry of testicular aspirates. *Fertil Steril*. 1989;51(3):546-548.
114. Belker AM, Fuchs EF, Konnak JW, Sharlip ID. Transient fertility after vasovasostomy in 892 patients. *J Urol*. 1985;134(1):75-76.
115. Kaye KW, Gonzalez R, Fraley EE. Microsurgical vasovasostomy: an outpatient procedure under local anesthesia. *J Urol*. 1983;129(5):992-994.
116. Requeda E, Charron J, Roberts KD, Chapdelaine A, Bleau G. Fertilizing capacity and sperm antibodies in vasovasostomized men. *Fertil Steril*. 1983;39(2):197-203.
117. Hartig PR, Meyer JJ. Vasovasostomy with use of intraoperative vasography. *Urology*. 1982;19(4):404-406.
118. Sharlip ID. The significance of intravasal azoospermia during vasovasostomy: answer to a surgical dilemma. *Fertil Steril*. 1982;38(4):496-498.
119. Cerruti RA, Jepson P, Furnas DW, Silber I. Vasovasostomy. Outpatient procedure for reversal of vasectomy. *Urology*. 1974;3(2):209-210.
120. Fenig DM, Kattan MW, Mills JN, Gisbert M, Yu C, Lipshultz LI. Nomogram to preoperatively predict the probability of requiring epididymovasostomy during vasectomy reversal. *J Urol*. 2012;187(1):215-218.
121. Duijn M, van der Zee JA, Bachour Y. Outcomes of three vasovasostomy surgical techniques in vasectomized men: a systematic review of the current literature. *SN Comprehensive Clinical Medicine*. 2023;5(1):56.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Seth I, Gibson D, Bulloch G, et al. Vasovasostomy: A systematic review and meta-analysis comparing macroscopic, microsurgical, and robot-assisted microsurgical techniques. *Andrology*. 2024;12:740–767. https://doi.org/10.1111/andr.13543

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13543 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 14 August 2023 | Revised: 29 August 2023 | Accepted: 1 September 2023

DOI: 10.1111/andr.13526

ANDROLOGY

WILEY

REVIEW ARTICLE

The chronic alcohol consumption influences the gonadal axis in men: Results from a meta-analysis

Daniele Santi[1,2,3] | Angelo Cignarelli[4] | Matteo Baldi[5] | Andrea Sansone[6] | Giorgia Spaggiari[2,3] | Manuela Simoni[1,2,3] | Giovanni Corona[7]

[1]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy

[2]Unit of Endocrinology, Department of Medical Specialties, Azienda Ospedaliero-Universitaria of Modena, Modena, Italy

[3]Unit of Andrology and Sexual Medicine of the Unit of Endocrinology, Department of Medical Specialties, Azienda Ospedaliero-Universitaria of Modena, Modena, Italy

[4]Section of Internal Medicine, Endocrinology, Andrology and Metabolic Diseases, Department of Precision and Regenerative Medicine and Ionian Area, University of Bari Aldo Moro, Bari, Italy

[5]Unit of Medicine, Azienda Ospedaliero-Universitaria Città della Salute e della Scienza di Torino, presidio Molinette, Turin, Italy

[6]Chair of Endocrinology and Medical Sexology (ENDOSEX), Department of Systems Medicine, University of Rome Tor Vergata, Rome, Italy

[7]Endocrinology Unit, Azienda USL Bologna, Bologna, Italy

Correspondence
Daniele Santi, Unit of Endocrinology, Department of Medical Specialties, Azienda Ospedaliero-Universitaria of Modena, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy.
Email: daniele.santi@unimore.it

Abstract

Background: Low testosterone concentrations affect 2–13% of adult males, with a direct association between reduction in testosterone (T) concentrations and cardiovascular events. Lifestyle habits have been linked to visceral fat accumulation and endocrine disorders like secondary hypogonadism. Alcohol intake has also been a topic of debate, with studies showing a detrimental effect on sperm production and underlying mechanisms. This meta-analysis aims to comprehensively evaluate the effect of alcohol consumption on T serum concentrations in adult men.

Methods: The literature search included only controlled clinical trials comparing men who drink alcohol to men who do not, or who assumed placebo or nonalcoholic beverages. The primary outcome was the comparison of total testosterone serum concentrations between the study and control groups. The publications were examined for publication bias using Egger's test.

Results: Twenty-one studies were included in the analysis for a total of 30 trials that examined the effects of alcohol consumption on testosterone level in 10,199 subjects. The meta-analysis showed that alcohol consumption overall is related to significant reduction in circulating concentrations of total testosterone (mean difference [MD] = −4.02; 95% CI −6.30, −1.73), free T (MD = −0.17; 95% CI −0.23, −0.12), sex hormone binding globulin (SHBG) (MD = −1.94; 95% CI −3.37, −0.48), an increase in estradiol (E2) (MD = 7.65; 95% CI 1.06, 14.23) and neutral effect on luteinizing hormone (LH) (MD = −0.15; 95% CI −0.36, 0.06), independently by age, body mass index (BMI), E2, and LH serum concentrations and alcohol intake. However, these results are evident only in healthy men exposed to chronic alcohol consumption and not in those with a recognized diagnosis of alcohol use disorder or after acute alcohol intake.

Conclusion: This study suggests how chronic alcohol consumption may inhibit the gonadal axis in healthy men, although the exact pathophysiological mechanisms connecting alcohol exposure and steroidogenesis are still not completely clarified.

KEYWORDS
alcohol, androgen, gonadotropin, SHBG, testosterone

© 2023 American Society of Andrology and European Academy of Andrology.

1 | INTRODUCTION

Low testosterone (T) serum concentrations are estimated to affect the 2–13% of adult males in the general population.[1,2] Alongside its epidemiological relevance, a T deficiency has been associated with earlier death from all causes, as well as cardiovascular diseases, and reduced quality of life.[3–6] Accordingly, several meta-analyses confirmed a direct association between T concentrations reduction and the cardiovascular events onset.[7,8]

Alongside known causes of male hypogonadism,[9] several lifestyle habits have been advocated so far in its pathogenesis. In this setting, recent findings demonstrated that obesity is frequently associated to visceral fat accumulation and, consequently, to endocrine disorders, such as secondary hypogonadism in both adults and adolescents.[10] Moreover, several experimental studies suggested that the diet itself could alter both the production and the metabolism of T.[11] Indeed, several studies reported that the vegetarian diet, characterized by low saturated fats and high fiber content, showed a reduced T production compared with omnivorous diets, at least when bioavailable T is considered.[11] However, these old studies are burdened by small sample sizes and some methodological limits.[11–14] In more recent times, many other diet components have been evaluated so far in their influence on androgen production, such as soy flavonoids,[15] coffee,[16,17] and low carbohydrate intake.[18–20] Comprehensively, which components of diet and with what weight could really influence T production in humans is still a matter of debate.

Another conflicting issue is related to the role of alcohol intake. Many studies in the literature evaluated the alcohol effects on male reproductive tract using the rat model, in order to consider both spermatogenic and steroidogenic compartments. A detrimental effect exerted by alcohol on sperm production is supported by studies on animal models, and several underlying mechanisms have been advocated, from the alcohol-induced cell necrosis and apoptosis,[21] to the increased oxidative stress,[22,23] and opioid system activation.[24] On the other hand, since the 80s, both acute and chronic alcohol exposure was demonstrated able to induce primary and secondary hypogonadism, that is, reducing hypothalamic gonadotropin releasing hormone (GnRH) and pituitary luteinizing hormone (LH) production,[25,26] but also inhibiting T secretion by the testes.[27] In addition, in vitro studies reported the ethanol capability to promote the aromatization of androgens to estrogens, although this effect in vivo remains to be confirmed.[28] Comprehensively, the exact pathophysiological mechanisms connecting alcohol exposure and steroidogenesis are not yet completely clarified.[29]

Several studies in the literature are available evaluating the correlation between T serum concentrations and alcohol consumption. However, a global view on this topic in adult men is still lacking. Thus, this meta-analysis is aimed to comprehensively evaluate the effect of alcohol consumption on T serum concentrations in adult men.

2 | MATERIALS AND METHODS

This meta-analysis was performed in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) reporting guideline and registered in the PROSPERO database for systematic reviews (ID CRD42023409978).

2.1 | Search strategy

The literature search was performed using the following strategy: ((“alcohol s”[All Fields] OR “alcoholate”[All Fields] OR “alcoholates”[All Fields] OR “alcohols”[Supplementary Concept] OR “alcohols”[All Fields] OR “alcohols”[MeSH Terms] OR “ethanol”[Supplementary Concept] OR “ethanol”[All Fields] OR “alcohol”[All Fields] OR “ethanol”[MeSH Terms] OR (“wine”[MeSH Terms] OR “wine”[All Fields]) OR (“beer”[MeSH Terms] OR “beer”[All Fields]) OR “red wine”[All Fields] OR “white wine”[All Fields] OR (“resveratrol”[Supplementary Concept] OR “resveratrol”[All Fields] OR “resveratrol”[MeSH Terms] OR “resveratrol s”[All Fields] OR “resveratrols”[All Fields])) AND (“testosterone”[Supplementary Concept] OR “testosterone”[All Fields] OR “testosteron”[All Fields] OR “testosterone”[MeSH Terms] OR “testosterones”[All Fields] OR “testosterone s”[All Fields])) OR (“androgen s”[All Fields] OR “androgene”[All Fields] OR “androgenes”[All Fields] OR “androgenic”[All Fields] OR “androgenicity”[All Fields] OR “androgenized”[All Fields] OR “androgenizing”[All Fields] OR “androgenous”[All Fields] OR “androgens”[Pharmacological Action] OR “androgens”[Supplementary Concept] OR “androgens”[All Fields] OR “androgen”[All Fields] OR “androgens”[MeSH Terms] OR “virilism”[MeSH Terms] OR “virilism”[All Fields] OR “androgenization”[All Fields]). Three datasets were queried, that is, Medline, Embase, and Cochrane library. The literature search was completed using the “hand-searching” strategies applied to relevant studies and reviews detected, to evaluate potential escaped studies from the original search.

The search, which accrued data until December 31, 2022, was restricted to English-language articles including human participants.

The identification of relevant studies was performed independently by two of the authors (D.S., G.S.), and conflicts were resolved by another investigator (A.C.).

2.2 | Study selection

We decided to consider only controlled clinical trials in which men who drink alcohol, both acutely and chronically, were compared with men (i) who do not or (ii) who assumed placebo or (iii) who assumed nonalcoholic beverages. Moreover, T serum concentrations need to be reported in all included trials, as primary or secondary outcome. On the contrary, studies presenting other study designs and/or not reporting

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

testosterone serum concentrations in both groups were excluded from the analysis. In addition, to limit the presence of possible confounders, pre-existing documented alcoholic liver dysfunction was considered as an exclusion criterion.

The Endnote software (Version 21) was used for literature management and duplication filtration and removal.

2.3 | Outcome and quality assessment

The principal outcome was the comparison of total T serum concentrations between study group, including subjects with acute or chronic alcohol intake, to control group, consisting of abstainers. According to studies description, the acute alcohol intake was defined as either a single alcohol assumption or the evaluation performed immediately after the alcohol assumption (i.e., alcohol intoxication). Otherwise, the alcohol assumption was primarily defined as chronic.

Secondary endpoints were estradiol (E2), LH, follicle stimulating hormone (FSH) and sex hormone binding globulin (SHBG) serum concentrations, when available. Moreover, for each study, the characteristics of alcohol intake was extracted, such as mode of intake (acute or chronic), type, dosage, and duration.

The quality of trials included was assessed using the Cochrane criteria.[30]

2.4 | Statistical analysis

Total T serum concentrations were converted in nmol/L when reported otherwise. When primary and secondary endpoints are differently reported, mean ± standard deviation was calculated. Free T was calculated using mean total T and SHBG serum concentrations using the Vermeulen formula and setting albumin serum concentrations to 43 g/L.[31] The mean difference (MD) was used, and the 95% confidence intervals (95%CI) was calculated for each endpoint. Values of $p < 0.05$ were considered statistically significant. The percentage of variation across studies because of heterogeneity rather than chance in continuous variables considered was assessed using I^2 statistics. Even when low heterogeneity was detected, a random-effect model was applied because the validity of heterogeneity tests can be limited with a small number of component studies. Funnel plots and the Begg adjusted rank correlation test were used to estimate possible publication or disclosure bias.

Continuous data were compared first between subjects with alcohol intake and controls. Then, sensitivity analyses were performed, performing subgroup analyses and comparing studies enrolling subjects exposed to chronic alcohol intake with studies including subjects exposed to acute alcohol intake. The effect of acute alcohol consumption was evaluated also considering continuous data before and after intake.

Meta-regression analyses were performed applying the random-effect model with Hedges method, adapted by Knapp–Hartung test.

The meta-analysis was conducted using the Review Manager (RevMan) 5.3 software (Version 5.3.5 Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). All additional analyses were performed used 'Statistical Package for the Social Sciences' software for Macintosh (version 28.0.1.0; SPSS Inc., Chicago, IL).

3 | RESULTS

A total of 162,479 manuscripts were first retrieved. Fifty-nine studies were extracted after abstract evaluation and 21 studies were finally included in the analysis,[32–52] for a total of 30 trials (Figure 1). Among studies excluded, 12 studies enrolled only females,[53–64] 10 did not provide a control group,[65–74] 7 studies did not report hormonal data within the manuscript,[75–81] 5 did not measure T serum concentrations,[82–86] and 4 were duplicates of previous publications[87–90] (Figure 1). Table 1 summarizes data of included studies, of which 4 evaluated acute alcohol intake and 17 considered chronic alcohol intake.

Comprehensively, the evaluation of total T serum concentrations was performed in 10,199 subjects (age 44.7 ± 7.3 years, body mass index [BMI] 25.0 ± 3.1 kg/m^2) under either chronic or acute alcohol intake in comparison to 6448 controls (Figure 2). When chronic alcohol intake was evaluated, the study group could be divided into two subgroups, according to the alcohol habits. Indeed, 10 trials included subjects that were chronic alcohol abuse and 15 trials healthy volunteers who were enrolled and were asked to drink specific alcohol quantity during the study protocol. On the contrary, the control group consisted in 17 studies in which men did not used alcohol and four studies in which enrolled men were assigned to either placebo (two studies) or not-alcoholic beverages (two studies) (Table 1).

3.1 | Acute alcohol intake

No significant differences in T serum concentrations were detected when acute alcohol intake group was compared with placebo (Figure 2). Among these studies, only one reported no alcohol consumption in control group,[36] thus the lack of significant difference between study and controls persisted also after removing it. Similarly, total T serum concentrations were comparable before and after acute alcohol administration (MD 0.9 nmol/L, 95%CI: −3.8, 5.6 nmol/L, $p = 0.710$).

Similarly, although only two studies evaluated the acute alcohol effect on E2, no differences were detected between alcohol and controls groups ($p = 0.730$) (Figure 3). In line with the latter observation, no differences were detected on SHBG concentrations after acute administration, although only two trials were analyzed ($p = 0.910$) (Figure 4).

3.2 | Chronic alcohol intake

I^2 calculated in trials assessing total T was 99% ($p < 0.001$). The funnel plot suggested no publication bias.

Total T serum concentrations were significantly lower in subjects with chronic alcohol intake compared with controls (without any

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

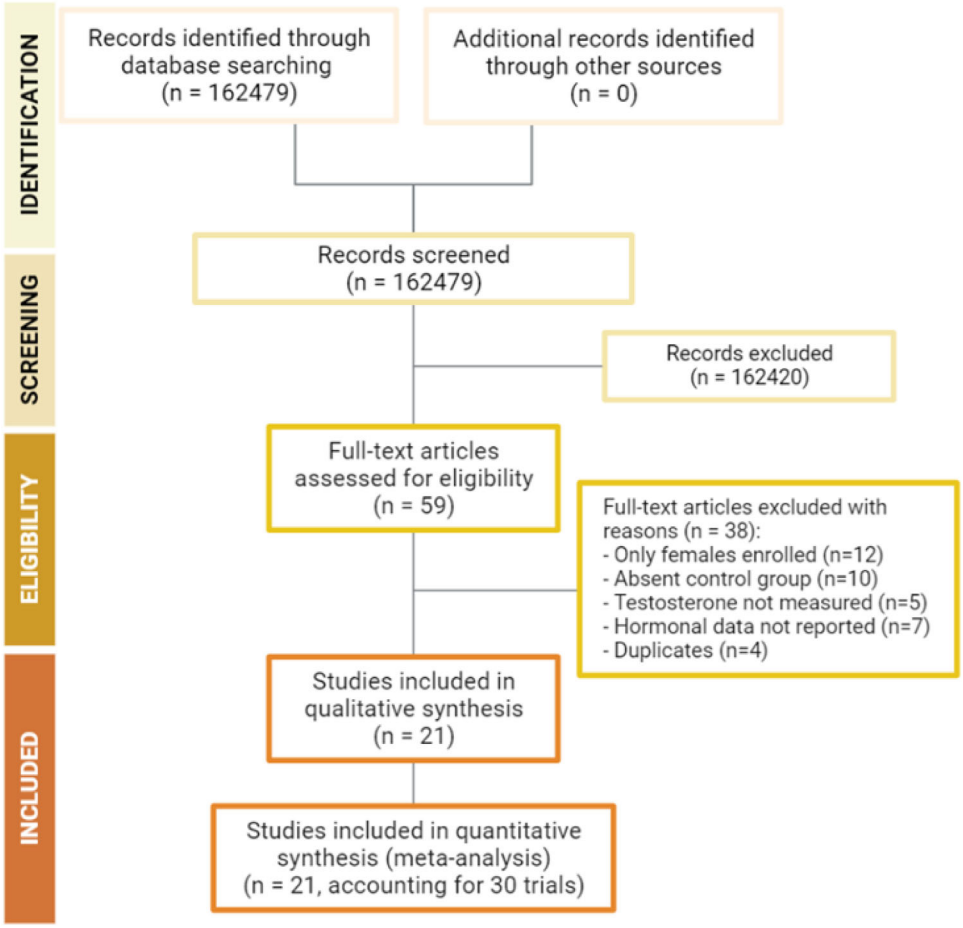


FIGURE 1 Flow chart of the literature search.

alcohol intake) ($p < 0.001$) (Figure 2). However, when data were further analyzed in detail, the latter association was confirmed only when healthy volunteers were considered (MD −6.5 nmol/L, 95%CI: −7.5, −2.3 nmol/L, $p < 0.001$) (Supplementary Figure S1). Conversely, total T serum concentrations did not differ comparing alcohol-dependent men to control subjects (MD −1.0 nmol/L, 95%CI: −5.8, 3.7 nmol/L, $p = 0.670$) (Supplementary Figure S1).

The total T serum concentrations decrease observed in chronic alcohol intake was not accompanied by any LH variation, resulting comparable in patients compared with controls (MD −0.03 IU/L, 95%CI: −0.20, 0.13 IU/L, $p = 0.720$) (Supplementary Figure S2).

Although E2 serum concentrations were reported only in a minority of trials ($n = 17$), significant higher circulating estrogens were detected in alcohol group compared with controls ($p = 0.020$) (Figure 3). Moreover, SHBG serum concentrations were significantly lower in men with chronic alcohol intake compared with controls ($p = 0.010$). Accordingly, calculated free T serum concentrations were significantly higher in controls rather than in men exposed to chronic alcohol intake (MD −0.19 nmol/L, 95%CI: −0.25, −0.13 nmol/L, $p < 0.001$) (Supplementary Figure S3). However, as well observed in total T, this difference was confirmed only considering healthy volunteers (MD −0.25 nmol/L, 95%CI: −0.34, 0.16, $p < 0.001$), but it was lost in chronic alcohol abuse (MD −0.11 nmol/L, 95%CI: −0.27, 0.04, $p = 0.150$). On the contrary, no difference in terms of free T serum concentrations was detected when acute alcohol administration was evaluated (MD −0.02 nmol/L, 95%CI: −0.12, 0.07 nmol/L, $p = 0.620$) (Supplementary Figure S3).

To evaluate whether total serum T concentrations difference in chronic alcohol intake could be related to other variables, meta-regression analyses were performed. Several parameters were considered including age (S: −6.0 [−13.9, 1.8]; I: −0.9 [−4.0, 2.2], $p = 0.118$; see also Supplementary Figure S4), BMI (S: 2.2 [−1.9, 3.8]; I: 0.1 [−1.6, 1.7], $p = 0.088$), E2 (S: −0.6 [−1.4, 0.1]; I: −0.7 [−1.6, 0.2], $p = 0.094$; see also Supplementary Figure S5), and LH serum concentrations (S: 1.6 [−3.3, 6.5]; I: −1.8 [−4.2, 0.6], $p = 0.689$; see also Supplementary Figure S6). Another meta-regression analysis considered the amount of alcohol intake, measured through the weekly units of alcohol taken in the previous week. The analysis showed no significant association (S: 5.3 [−2.1, 12.3]; I: −0.8 [−3.7, 1.9], $p = 0.997$), even when subjects affected by alcoholism were considered separately from healthy volunteers ($p = 0.911$ and $p = 0.823$, respectively).

4 | DISCUSSION

Here, we confirm that alcohol consumption influences T serum concentrations in males. This effect is not evident considering acute

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by meryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Characteristics of studies included in the meta-analysis. Data were reported as mean ± standard deviation.

					Study group					Control group			
Author	Year	Alcohol use	Inclusion criteria	Alcohol intake evaluation	*n*	Age (years)	BMI (kg/m^2)	Alcohol type	Alcohol intake	*n*	Age (years)	BMI (kg/m^2)	Placebo
Bertello	1982	Chronic	Chronic alcohol abuse	Questionnaire	12	41.3 ± 7.9	NR	Wine	2 L daily	20	NR	NR	None
Markianos	1987	Chronic	Alcohol abuse admitted for detoxication	Questionnaire	32	46.4 ± 8.1	NR	Any	NR	32	46.1 ± 8.1	NR	None
Gümüş	1988	Chronic	Chronic alcohol abuse	Questionnaire	44	44.6 ± 8.1	NR	Any	NR	30	44.7 ± 7.8	NR	None
Gomathi	1993	Chronic	Chronic alcohol abuse	Questionnaire	5	NR	NR	Any	NR	5	NR	NR	None
Heinz	1995	Chronic	Chronic alcohol abuse	Questionnaire	12	42.9 ± 9.5	NR	Any	256 ± 133 g daily	14	38.5 ± 7.3	NR	None
Iturriaga	1999	Chronic	Chronic alcohol abuse	Questionnaire	21	39.0 ± 5.0	22.7 ± 2.9	Any	>150 g daily	21	33.1 ± 7.6	24.8 ± 1.9	None
Frias	2002	Acute	Acute alcohol intoxication	Behavioral symptoms of drunkeness	12	NR	NR	Any	Intoxication	11	NR	NR	None
Sarkola	2003	Acute	Volunteers	Interventional trial	13	24.0 ± 3.0	23. ± 2.7	10% vodka in juice	0.5 g/kg	13	24.0 ± 3.0	23. ± 2.7	Placebo
Sierksma	2004	Chronic	Volunteers	Interventional trial	10	55.0 ± 6.0	24.9 ± 2.2	Beer	4 units daily for 3 weeks	10	55.0 ± 6.0	24.9 ± 2.2	Nonalcoholic beer
Walter	2006	Chronic	Chronic alcohol abuse	Questionnaire	51	42.8 ± 10.2	NR	Any	28.2 ± 5.0 g daily	43	44.6 ± 12.3	NR	None
Maneesh	2006	Chronic	Chronic alcohol abuse	CAGE questionnaire	46	29.6 ± 4.2	22.2 ± 0.2	Any	NR	55	26.5 ± 4.8	23.8 ± 0.2	None
Hansen	2012	Chronic	Volunteers	Questionnaire	291	NR	NR	Any	1-10 drinks within 5 days before	54	NR	NR	None
Vingren	2013	Acute	Resistance-trained men	Interventional trial	4	25.3 ± 3.2	NR	Grain ethanol	1.09 g/kg	4	25.3 ± 3.2	NR	Placebo
Jensen	2014	Chronic	Volunteers	Questionnaire	5339	NR	NR	Any	1-20 units weekly	1133	NR	NR	None
Haugvad	2014	Acute	Volunteers	Interventional trial	16	26.0 ± 4.0	NR	Vodka in 200-mL sugar-free lemonade	0.7-1.4 g/kg	8	26.0 ± 4.0	NR	300 mL sugar-free lemonade

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

Author	Year	Alcohol use	Inclusion criteria	Alcohol intake evaluation	Study group				Alcohol intake	Control group			
					n	Age (years)	BMI (kg/m^2)	Alcohol type		*n*	Age (years)	BMI (kg/m^2)	Placebo
Jensen	2014	Chronic	Volunteers	Questionnaire	951	NR	NR	Any	1-40 units weekly	243	NR	NR	None
Rao	2015	Chronic	Volunteers	Questionnaire	2982	54.7 ± 11.0	NR	Any	>12 units weekly	1260	54.7 ± 11.0	NR	None
Heberlein	2016	Chronic	Chronic alcohol abuse	CAGE questionnaire	81	42.2 ± 7.8	24.6 ± 3.6	Any	194.9 ± 83.4 g daily	17	44.4 ± 9.6	26.4 ± 4.6	None
Kumari	2016	Chronic	Volunteers	AUDIT for assessment of alcohol abuse	12	40.5 ± 3.8	30.9 ± 3.5	Any	0.5 g/kg/body weight daily	14	40.5 ± 3.8	30.9 ± 3.5	None
Ho	2019	Chronic	Chronic alcohol abuse	Penn Alcohol Craving Scale questionnaire	29	46.1 ± 16.9	NR	Any	NR	29	46.4 ± 16.9	NR	None
Koh	2022	Chronic	Healthy subjects	Questionnaire	236	55.7 ± 9.5	24.5 ± 3.0	Any	9.9 ± 11.9 g daily	78	60.6 ± 8.7	24.0 ± 2.8	None

Note: Volunteers identified man that voluntarily decided to participate in the original trial, without any health alcohol-related disease.
AUDIT: alcohol use disorders identification test; BMI: body mass index; CAGE: cutting down, annoyed by criticism, guilty feeling, and eye-opener; *n*: number; NR: not reported.

alcohol intake, but it becomes clinically relevant after chronic alcohol exposure. Indeed, acute alcohol intake does not influence total T concentrations, neither comparing with placebo groups, nor considering pre- and postalcohol consumption. On the other hand, a reduction of total T serum concentrations of an average 4.86 nmol/L (95%CI: 7.46, 2.26 nmol/L) is evident subjects exposed to chronic alcohol intake compared with abstainers subjects overall. Similar results were observed considering both calculated free T and SHBG. Nevertheless, no relevant modification in sex hormones was evident comparing subjects affected by alcoholism.

The negative impact of chronic alcohol consumption on total T serum concentrations is here confirmed in a large sample size, accounting for more than 16,000 subjects. This result is in line with recent meta-analysis, showing that alcohol consumption affected semen volume and reproductive hormones.[91] In our study, we focused on T serum concentrations, showing the alcohol effect occurs in healthy men and not in those subjects with a recognized diagnosis of alcohol use disorder, according to the International Statistical Classification of Diseases and Related Health Problems 11th (ICD-11) and to the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-V).[92,93]This result should be carefully considered in light of the potential liver dysfunction typical of chronic alcohol consumers. Indeed, whether it is true that we excluded a priori studies enrolling alcoholic men with a diagnosed liver dysfunction or insufficiency, we cannot rule out a hidden liver dysfunction in the study group when chronic alcohol consumption is considered. The direct correlation between amount of alcohol consumed and liver dysfunction, fibrosis, cirrhosis and related mortality is strongly demonstrated in large observational studies.[94,95] Thus, we cannot establish whether chronic alcohol consumers included in the study group display a normal liver function. This is extremely relevant also for our aim, because a chronic liver disease is characterized by progressive necrosis of hepatocytes and development of liver dysfunction, which may remarkably affect the metabolism of various substances including proteins, sex hormones and lipids.[96] In particular, the liver produces proteins essential for the transport and the homeostasis of steroid hormones, such as SHBG. It is well known how liver dysfunction could lead to an increase of SHBG with consequent increase in total T serum concentrations and a reduction in the amount of bioavailable T for the target tissues. In addition, chronic alcohol intake can promote the accumulation of lipids in the liver, leading to protein synthesis reduction, including SHBG, with consequent total T serum concentrations decrease.[97] Accordingly, several studies in men have already suggested that SHBG production by the liver is related to intrahepatic fat[98,99] rather than fasting blood glucose, glycated hemoglobin (HbA1c), or circulating lipid concentrations. In particular, it has been estimated that a reduction in intrahepatic lipid content could be responsible for a 20% increase in SHBG concentrations,[100] as recently confirmed in a small prospective study enrolling obese male patients treated with very low-caloric ketogenic diet.[19] Alongside a significant and persistent amelioration of the body weight and body composition parameters, a significant increase of total T concentrations was observed, mainly because of a significant increase in SHBG serum concentrations.[19] The interaction between

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

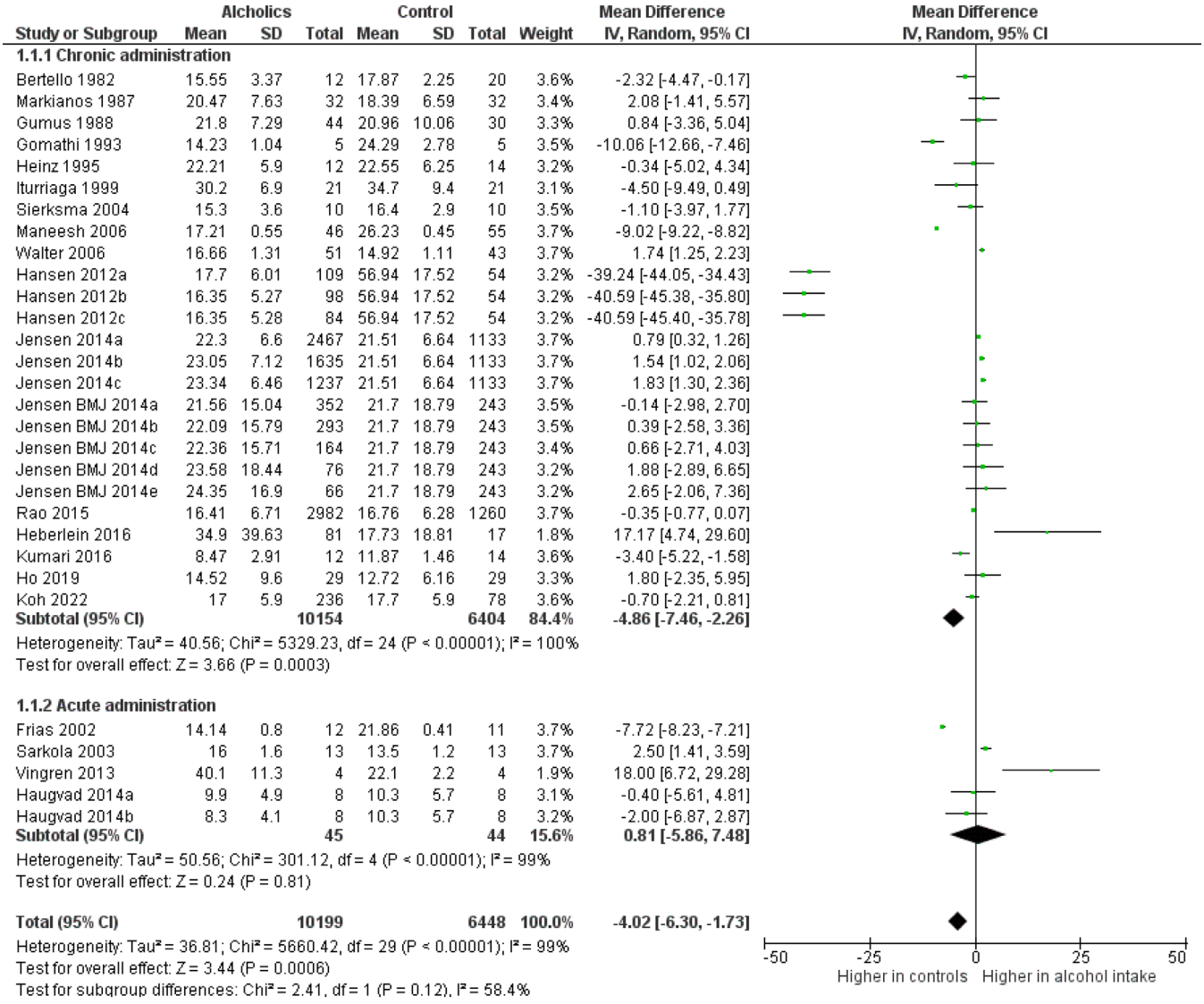


FIGURE 2 Forrest plot showing testosterone serum concentrations between alcohol abuse men (chronic administration) or subjects treated with alcohol (acute administration) and controls.

liver function/dysfunction, total T serum concentrations and SHBG contributes to a complicated balance which could mask the comprehensive effect of alcohol on T serum concentrations in alcoholic men. Interestingly, the chronic alcohol intake on T serum concentrations here highlighted is independent from patients' age and alcohol amount assumed, suggesting that several other unknown factors should be considered in this interrelationship.

The mechanism linking chronic alcohol consumption and T decrease is still not completely defined, so far. Experimental studies in animal models demonstrated an alcohol-related T decrement in male rats,[72,101–106] possibly justified through actions at multiple sites.[107] Here, we demonstrated an overall total T reduction in chronic alcohol users compared with a control group not accompanied by a compensatory increase in LH. These results could be explained by a potential action of alcohol mainly on the hypothalamus-pituitary gonadal (HPG) axis, rather than to the gonad itself. Indeed, reinforcing this hypothesis, a compensatory increase in LH concentrations was not evident even in presence of a reduction of free T in the study group. However, the alcohol effect on LH concentrations and pulsatility is still unclear in the literature. Whether the majority of in vivo studies on the topic showed an alcohol-related LH decrease,[27,101,103,106,108,109] confirming our hypothesis, others experiments detected a gonadotropins increase[26] or unchanged concentrations[72] after alcohol administration. Thus, studies available failed to clarify the primary site of the actions of alcohol on HPG axis. In vitro studies showed alcohol-related effects in both isolated testicular tissue,[110–116] pituitary gonadotropes,[117,118] and hypothalamic preparations.[119–121] On the other hand, alcohol-exposed patients showed comprehensively higher concentrations of estrogens compared with controls; thus, the overall feedback effect on LH because of T and E2 could result neutral. The E2 serum concentrations increase during chronic alcohol consumption has been studied in females, in which alcohol consumption has been evaluated among

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

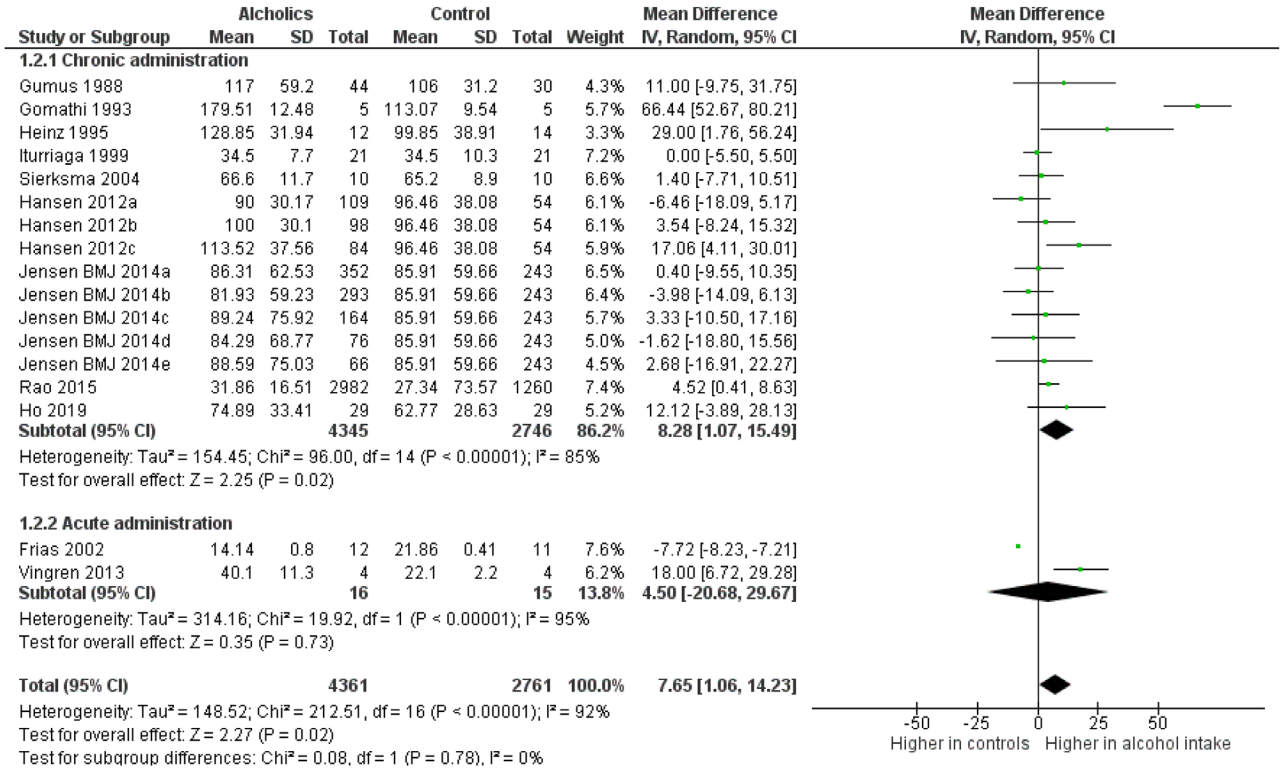


FIGURE 3 Forrest plot showing estradiol serum concentrations between alcohol abuse men (chronic administration) or subjects treated with alcohol (acute administration) and controls.

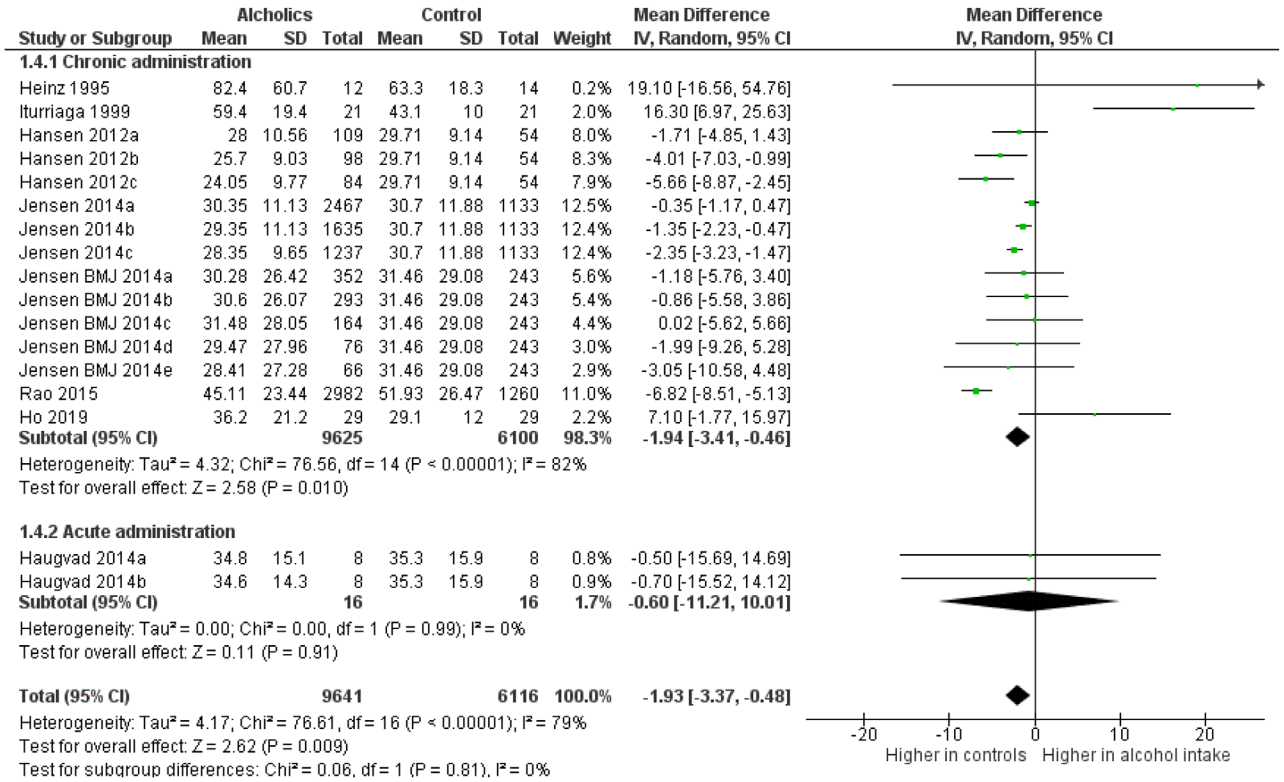


FIGURE 4 Forrest plot showing sex hormone binding globulin (SHBG) serum concentrations between alcohol abuse men (chronic administration) or subjects treated with alcohol (acute administration) and controls.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

risk factors for cancer development.[122] Because circulating estrogens are associated to an increased breast cancer risk in women,[123–125] and this cancer is related to alcohol intake,[126,127] the alcohol effect on estrogens has been suggested.[128] However, less is known in males, in which the E2 measurement suffers of important methodological pitfalls.[129–132] In this setting, we could speculate that alcohol intake could influence aromatase activity, throughout both direct and indirect effects. From one side, several studies demonstrated that some component of alcoholic beverages, such as procyanidin B and phytoestrogens (i.e., flavones and isoflavones) could bind the active site of aromatase, leading to functional dysregulations.[133–135] On the other side, alcohol could increase circulating sex hormones through an increased hepatic redox state and the inhibition of the activity of sulfotransferase and 2-hydroxylase, resulting in a decrease in steroid degradation.[136] Thus, we cannot rule out that the alcohol-related E2 increase that we detected in alcohol abuse are potentially explained by an increased aromatization.

Our results should be carefully considered. Indeed, net of the large number of subjects evaluated thanks to the meta-analytic approach, our study has important limitations to be taken into consideration. First, the high heterogeneity among studies remains the most important issue. This variability reflects a great heterogeneity for patient inclusion criteria in each study, different methodological steroids measurements and various ways to evaluate alcohol consumption. This latter is probably the main source of bias in our meta-analysis. In particular, the amount of chronic alcohol intake is approximately quantified by self-filled questionnaires and not accurately determined. Similarly, the time span of chronic alcohol intake, although used in our meta-regression analysis, is scanty reported and not all trials evaluated this important variable. Moreover, the association between BMI and both T and SHBG is well known. However, the majority of studies included did not report BMI among variables, thus limiting the evaluation of this potential confounding factor. Nevertheless, the lack of potential confounders in all the studies included in our study preventing the analysis with an eventual adjustment for several parameters.

In conclusion, further proper designed studies are needed to clarify the connection between alcohol intake and sex steroids homeostasis, because this association appears evident from the meta-analytic approach.

AUTHOR CONTRIBUTIONS

DS and GS conceived the studies and extracted data from the literature. DS performed statistical analyses. DS, AC, MB, AS, GS, MS, and GC drafted and final approved the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

FUNDING INFORMATION

No funding was provided for the study's conduction.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available because of privacy or ethical restrictions.

ORCID

Angelo Cignarelli https://orcid.org/0000-0001-6477-9031
Andrea Sansone https://orcid.org/0000-0002-1210-2843
Giovanni Corona https://orcid.org/0000-0002-9894-2885

REFERENCES

1. Zarotsky V, Huang MY, Carman W, et al. Systematic literature review of the risk factors, comorbidities, and consequences of hypogonadism in men. *androl*. 2014;2(6):819-834.
2. Araujo AB, Esche GR, Kupelian V, et al. Prevalence of symptomatic androgen deficiency in men. *J Clin Endocrinol Metab*. 2007;92(11):4241-4247.
3. Fallara G, Pozzi E, Corsini C, et al. Morbidity and mortality in men: role of androgens. *Best Pract Res Clin Endocrinol Metab*. 2022;36(4):101662.
4. Watts EL, Appleby PN, Perez-Cornago A, et al. Low free testosterone and prostate cancer risk: a collaborative analysis of 20 prospective studies. *Eur Urol*. 2018;74(5):585-594.
5. Wallis CJD, Lo K, Lee Y, et al. Survival and cardiovascular events in men treated with testosterone replacement therapy: an intention-to-treat observational cohort study. *Lancet Diabetes Endocrinol*. 2016;4(6):498-506.
6. Isidori AM, Aversa A, Calogero A, et al. Adult- and late-onset male hypogonadism: the clinical practice guidelines of the Italian Society of Andrology and Sexual Medicine (SIAMS) and the Italian Society of Endocrinology (SIE). *J Endocrinol Invest*. 2022;45(12):2385-2403.
7. Corona G, Rastrelli G, Di Pasquale G, Sforza A, Mannucci E, Maggi M. Testosterone and cardiovascular risk: meta-analysis of interventional studies. *J Sex Med*. 2018;15(6):820-838.
8. Corona G, Rastrelli G, Di Pasquale G, Sforza A, Mannucci E, Maggi M. Endogenous testosterone levels and cardiovascular risk: meta-analysis of observational studies. *J Sex Med*. 2018;15(9):1260-1271.
9. Santi D, Corona G. Primary and secondary hypogonadism. In: Simoni M, Huhtaniemi I, eds. *Endocrinology of the Testis and Male Reproduction*. Springer; 2017. Endocrinology.
10. Handakas E, Lau CH, Alfano R, et al. A systematic review of metabolomic studies of childhood obesity: state of the evidence for metabolic determinants and consequences. *Obes Rev*. 2022;23:e13384. Suppl 1.
11. Bélanger A, Locong A, Noel C, et al. Influence of diet on plasma steroids and sex hormone-binding globulin levels in adult men. *J Steroid Biochem*. 1989;32(6):829-833.
12. Deslypere JP, Vermeulen A. Leydig cell function in normal men: effect of age, life-style, residence, diet, and activity. *J Clin Endocrinol Metab*. 1984;59(5):955-962.
13. Pusateri DJ, Roth WT, Ross JK, Shultz TD. Dietary and hormonal evaluation of men at different risks for prostate cancer: plasma and fecal hormone-nutrient interrelationships. *Am J Clin Nutr*. 1990;51(3):371-377.
14. Key TJ, Roe L, Thorogood M, Moore JW, Clark GM, Wang DY. Testosterone, sex hormone-binding globulin, calculated free testosterone, and oestradiol in male vegans and omnivores. *Br J Nutr*. 1990;64(1):111-119.
15. Hamilton-Reeves JM, Vazquez G, Duval SJ, Phipps WR, Kurzer MS, Messina MJ. Clinical studies show no effects of soy protein or

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

isoflavones on reproductive hormones in men: results of a meta-analysis. *Fertil Steril*. 2010;94(3):997-1007.
16. Wedick NM, Mantzoros CS, Ding EL, et al. The effects of caffeinated and decaffeinated coffee on sex hormone-binding globulin and endogenous sex hormone levels: a randomized controlled trial. *Nutrition journal*. 2012;11:86.
17. Aprioku JS, Boms R, Ife-Tubiyele DO. Effect of simultaneous coffee/caffeine and ethanol administration on sperm quality and reproductive hormones: an experimental study in Sprague Dawley rats. *Drug Chem Toxicol*. 2022;45(3):1012-1020.
18. da Silva Schmitt C, da Costa CM, Souto JCS, et al. The effects of a low carbohydrate diet on erectile function and serum testosterone levels in hypogonadal men with metabolic syndrome: a randomized clinical trial. *BMC Endocr Disord*. 2023;23(1):30.
19. Cignarelli A, Santi D, Genchi VA, et al. Very low-calorie ketogenic diet rapidly augments testosterone levels in non-diabetic obese subjects. *androl*. 2023;11(2):234-244.
20. Furini C, Spaggiari G, Simoni M, Greco C, Santi D. Ketogenic state improves testosterone serum levels-results from a systematic review and meta-analysis. *Endocrine*. 2022;79(2):273-282.
21. Sinha Hikim AP, Swerdloff RS. Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod*. 1999;4(1):38-47.
22. Chiao YB, Van Thiel DH. Biochemical mechanisms that contribute to alcohol-induced hypogonadism in the male. *Alcohol Clin Exp Res*. 1983;7(2):131-134.
23. Adams ML, Nock B, Truong R, Cicero TJ. Nitric oxide control of steroidogenesis: endocrine effects of NG-nitro-L-arginine and comparisons to alcohol. *Life Sci*. 1992;50(6):PI35-P140.
24. Gianoulakis C. Characterization of the effects of acute ethanol administration on the release of beta-endorphin peptides by the rat hypothalamus. *Eur J Pharmacol*. 1990;180(1):21-29.
25. Cicero TJ. Alcohol-induced deficits in the hypothalamic-pituitary-luteinizing hormone axis in the male. *Alcohol Clin Exp Res*. 1982;6(2):207-215.
26. Salonen I, Pakarinen P, Huhtaniemi I. Effect of chronic ethanol diet on expression of gonadotropin genes in the male rat. *J Pharmacol Exp Ther*. 1992;260(2):463-467.
27. Little PJ, Adams ML, Cicero TJ. Effects of alcohol on the hypothalamic-pituitary-gonadal axis in the developing male rat. *J Pharmacol Exp Ther*. 1992;263(3):1056-1061.
28. Kasahara Y, Kishi H, Yokomizo R, Okamoto A. Ethanol potentiates follicle-stimulating hormone action in ovarian granulosa cells. *J Endocrinol*. 2023;257(1).
29. Emanuele MA, Emanuele N. Alcohol and the male reproductive system. *Alcohol Res Health*. 2001;25(4):282-287.
30. Higgins JP, Altman DG, Gøtzsche PC, et al. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ (Clinical research ed)*. 2011;343:d5928.
31. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab*. 1999;84(10):3666-3672.
32. Jensen TK, Swan S, Jørgensen N, et al. Alcohol and male reproductive health: a cross-sectional study of 8344 healthy men from Europe and the USA. *Hum Reprod*. 2014;29(8):1801-1809.
33. Sierksma A, van der Gaag MS, Kluft C, Hendriks HF. Moderate alcohol consumption reduces plasma C-reactive protein and fibrinogen levels; a randomized, diet-controlled intervention study. *Eur J Clin Nutr*. 2002;56(11):1130-1136.
34. Vingren JL, Kraemer WJ, Hatfield DL, et al. Effect of resistance exercise on muscle steroidogenesis. *J Appl Physiol (Bethesda, Md : 1985)*. 2008;105(6):1754-1760.
35. Sarkola T, Eriksson CJ. Testosterone increases in men after a low dose of alcohol. *Alcohol Clin Exp Res*. 2003;27(4):682-685.
36. Frias J, Torres JM, Miranda MT, Ruiz E, Ortega E. Effects of acute alcohol intoxication on pituitary-gonadal axis hormones, pituitary-adrenal axis hormones, beta-endorphin and prolactin in human adults of both sexes. *Alcohol Alcohol*. 2002;37(2):169-173.
37. Koh K, Kim SS, Kim JS, et al. Relationship between alcohol consumption and testosterone deficiency according to facial flushes among middle-aged and older Korean men. *Korean J Fam Med*. 2022;43(6):381-387.
38. Walter M, Gerhard U, Gerlach M, Weijers HG, Boening J, Wiesbeck GA. Controlled study on the combined effect of alcohol and tobacco smoking on testosterone in alcohol-dependent men. *Alcohol Alcohol*. 2007;42(1):19-23.
39. Markianos M, Moussas G, Lykouras LL. Normal testosterone plasma levels in non-abstinent alcoholics. *Drug Alcohol Depend*. 1987;20(1):81-85.
40. Haugvad A, Haugvad L, Hamarsland H, Paulsen G. Ethanol does not delay muscle recovery but decreases testosterone/cortisol ratio. *Med Sci Sports Exerc*. 2014;46(11):2175-2183.
41. Gomathi C, Balasubramanian K, Bhanu NV, Srikanth V, Govindarajulu P. Effect of chronic alcoholism on semen–studies on lipid profiles. *Int J Androl*. 1993;16(3):175-181.
42. Gümüş B, Yiğitoğlu MR, Lekili M, Uyanik BS, Müezzinoğlu T, Büyüksu C. Effect of long-term alcohol abuse on male sexual function and serum gonadal hormone levels. *Int Urol Nephrol*. 1998;30(6):755-759.
43. Bertello P, Agrimonti F, Gurioli L, Frairia R, Fornaro D, Angeli A. Circadian patterns of plasma cortisol and testosterone in chronic male alcoholics. *Alcohol Clin Exp Res*. 1982;6(4):475-481.
44. Heinz A, Rommelspacher H, Gräf KJ, Kürten I, Otto M, Baumgartner A. Hypothalamic-pituitary-gonadal axis, prolactin, and cortisol in alcoholics during withdrawal and after three weeks of abstinence: comparison with healthy control subjects. *Psychiatry Res*. 1995;56(1):81-95.
45. Ho AM, Geske JR, Bakalkin G, Winham SJ, Karpyak VM. Correlations between sex-related hormones, alcohol dependence and alcohol craving. *Drug Alcohol Depend*. 2019;197:183-190.
46. Heberlein A, Lenz B, Opfermann B, et al. Association of testosterone and BDNF serum levels with craving during alcohol withdrawal. *Alcohol*. 2016;54:67-72.
47. Kumari S, Mittal A, Dabur R. Moderate alcohol consumption in chronic form enhances the synthesis of cholesterol and C-21 steroid hormones, while treatment with *Tinospora cordifolia* modulate these events in men. *Steroids*. 2016;114:68-77.
48. Rao M, Zuo LD, Fang F, et al. Association of alcohol consumption with markers of prostate health and reproductive hormone profiles: a multi-center study of 4,535 men in China. *PLoS One*. 2015;10(11):e0142780.
49. Jensen TK, Gottschau M, Madsen JO, et al. Habitual alcohol consumption associated with reduced semen quality and changes in reproductive hormones; a cross-sectional study among 1221 young Danish men. *BMJ open*. 2014;4(9):e005462.
50. Hansen ML, Thulstrup AM, Bonde JP, Olsen J, Håkonsen LB, Ramlau-Hansen CH. Does last week's alcohol intake affect semen quality or reproductive hormones? A cross-sectional study among healthy young Danish men. *Reprod Toxicol (Elmsford, NY)*. 2012;34(3):457-462.
51. Maneesh M, Dutta S, Chakrabarti A, Vasudevan DM. Alcohol abuse-duration dependent decrease in plasma testosterone and antioxidants in males. *Indian J Physiol Pharmacol*. 2006;50(3):291-296.
52. Iturriaga H, Lioi X, Valladares L. Sex hormone-binding globulin in non-cirrhotic alcoholic patients during early withdrawal and after longer abstinence. *Alcohol Alcohol*. 1999;34(6):903-909.
53. Hirko KA, Spiegelman D, Willett WC, Hankinson SE, Eliassen AH. Alcohol consumption in relation to plasma sex hormones, prolactin, and sex hormone-binding globulin in premenopausal women. *Cancer Epidemiol Biomarkers Prev*. 2014;23(12):2943-2953.
54. Onland-Moret NC, Peeters PH, van der Schouw YT, Grobbee DE, van Gils CH. Alcohol and endogenous sex steroid levels in

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

postmenopausal women: a cross-sectional study. *J Clin Endocrinol Metab*. 2005;90(3):1414-1419.
55. García-Closas M, Herbstman J, Schiffman M, Glass A, Dorgan JF. Relationship between serum hormone concentrations, reproductive history, alcohol consumption and genetic polymorphisms in pre-menopausal women. *Int J Cancer*. 2002;102(2):172-178.
56. Sarkola T, Adlercreutz H, Heinonen S, Eriksson CJ. Alcohol intake, androgen and glucocorticoid steroids in premenopausal women using oral contraceptives: an interventional study. *J Steroid Biochem Mol Biol*. 2001;78(2):157-165.
57. Sarkola T, Fukunaga T, Mäkisalo H, Peter Eriksson CJ. Acute effect of alcohol on androgens in premenopausal women. *Alcohol Alcohol*. 2000;35(1):84-90.
58. Shufelt C, Merz CN, Yang Y, et al. Red versus white wine as a nutritional aromatase inhibitor in premenopausal women: a pilot study. *J Womens Health (Larchmt)*. 2012;21(3):281-284.
59. Cigolini M, Targher G, Bergamo Andreis IA, et al. Moderate alcohol consumption and its relation to visceral fat and plasma androgens in healthy women. *Int J Obes Relat Metab Disord*. 1996;20(3):206-212.
60. Weinland C, Mühle C, Kornhuber J, Lenz B. Progesterone serum levels correlate negatively with craving in female postmenopausal in-patients with alcohol use disorder: a sex- and menopausal status-separated study. *Prog Neuropsychopharmacol Biol Psychiatry*. 2021;110:110278.
61. Tin Tin S, Key TJ, Reeves GK. Alcohol intake and endogenous hormones in pre- and postmenopausal women: findings from the UK Biobank. *Cancer Epidemiol Biomarkers Prev*. 2021;30(12):2294-2301.
62. Peltier MR, Verplaetse TL, Roberts W, et al. Changes in excessive alcohol use among older women across the menopausal transition: a longitudinal analysis of the Study of Women's Health Across the Nation. *Biol Sex Differ*. 2020;11(1):37.
63. Assi N, Rinaldi S, Viallon V, et al. Mediation analysis of the alcohol-postmenopausal breast cancer relationship by sex hormones in the EPIC cohort. *Int J Cancer*. 2020;146(3):759-768.
64. Frydenberg H, Flote VG, Larsson IM, et al. Alcohol consumption, endogenous estrogen and mammographic density among premenopausal women. *Breast Cancer Res.: BCR*. 2015;17(1):103.
65. Couwenbergs CJ. Acute effects of drinking beer or wine on the steroid hormones of healthy men. *J Steroid Biochem*. 1988;31(4a):467-473.
66. Mühle C, Barry B, Weinland C, Kornhuber J, Lenz B. Estrogen receptor 1 gene variants and estradiol activities in alcohol dependence. *Prog Neuropsychopharmacol Biol Psychiatry*. 2019;92:301-307.
67. Meyer RE, Dolinsky ZS. Ethanol beverage anticipation: effects on plasma testosterone and luteinizing hormone levels–a pilot study. *J Stud Alcohol*. 1990;51(4):350-355.
68. Gordon GG, Altman K, Southren AL, Rubin E, Lieber CS. Effect of alcohol (ethanol) administration on sex-hormone metabolism in normal men. *N Engl J Med*. 1976;295(15):793-797.
69. Yadav P, Yadav B, Chakrabarty BK, et al. Analysis of relationship of hormonal, hematological and biochemical parameters in Indian alcoholics with severity of alcohol dependence. *Med J Armed Forces India*. 2022;78(3):308-315.
70. Persky H, O'Brien CP, Fine E, Howard WJ, Khan MA, Beck RW. The effect of alcohol and smoking on testosterone function and aggression in chronic alcoholics. *Am J Psychiatry*. 1977;134(6):621-625.
71. Bannister P, Handley T, Chapman C, Losowsky MS. LH pulsatility following acute ethanol ingestion in men. *Clin Endocrinol (Oxf)*. 1986;25(2):143-150.
72. Ida Y, Tsujimaru S, Nakamaura K, et al. Effects of acute and repeated alcohol ingestion on hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal functioning in normal males. *Drug Alcohol Depend*. 1992;31(1):57-64.
73. Mendelson JH, Mello NK, Ellingboe J. Effects of acute alcohol intake on pituitary-gonadal hormones in normal human males. *J Pharmacol Exp Ther*. 1977;202(3):676-682.
74. Iranmanesh A, Veldhuis JD, Samojlik E, Rogol AD, Johnson ML, Lizarralde G. Alterations in the pulsatile properties of gonadotropin secretion in alcoholic men. *J Androl*. 1988;9(3):207-214.
75. Murphy AP, Snape AE, Minett GM, Skein M, Duffield R. The effect of post-match alcohol ingestion on recovery from competitive rugby league matches. *J Strength Cond Res*. 2013;27(5):1304-1312.
76. Danel T, Vantyghem MC, Touitou Y. Responses of the steroid circadian system to alcohol in humans: importance of the time and duration of intake. *Chronobiol Int*. 2006;23(5):1025-1034.
77. Röjdmark S, Calissendorff J, Brismar K. Alcohol ingestion decreases both diurnal and nocturnal secretion of leptin in healthy individuals. *Clin Endocrinol (Oxf)*. 2001;55(5):639-647.
78. Heikkonen E, Ylikahri R, Roine R, Välimäki M, Härkönen M, Salaspuro M. The combined effect of alcohol and physical exercise on serum testosterone, luteinizing hormone, and cortisol in males. *Alcohol Clin Exp Res*. 1996;20(4):711-716.
79. Ylikahri RH, Huttunen MO, Härkönen M, et al. Acute effects of alcohol on anterior pituitary secretion of the tropic hormones. *J Clin Endocrinol Metab*. 1978;46(5):715-720.
80. Välimäki MJ, Härkönen M, Eriksson CJ, Ylikahri RH. Sex hormones and adrenocortical steroids in men acutely intoxicated with ethanol. *Alcohol*. 1984;1(1):89-93.
81. Rodrigues R, Franke RA, Teixeira BC, et al. Combined and isolated effects of alcohol intake and one night of sleep deprivation on mood states, hormonal and inflammatory responses in healthy male adults: a crossover randomized controlled trial. *Chin J Physiol*. 2017;60(6):327-337.
82. Lenz B, Bouna-Pyrrou P, Mühle C, Kornhuber J. Low digit ratio (2D:4D) and late pubertal onset indicate prenatal hyperandrogenziation in alcohol binge drinking. *Prog Neuropsychopharmacol Biol Psychiatry*. 2018;86:370-378.
83. Lenz B, Weinland C, Bach P, et al. Oxytocin blood concentrations in alcohol use disorder: a cross-sectional, longitudinal, and sex-separated study. *Eur Neuropsychopharmacol*. 2021;51:55-67.
84. Rubin E, Lieber CS, Altman K, Gordon GG, Southren AL. Prolonged ethanol consumption increases testosterone metabolism in the liver. *Science*. 1976;191(4227):563-564.
85. De Sousa Fernandes Perna EB, Theunissen EL, Kuypers KP, Toennes SW, Ramaekers JG. Subjective aggression during alcohol and cannabis intoxication before and after aggression exposure. *Psychopharmacology (Berl)*. 2016;233(18):3331-3340.
86. Sperling W, Biermann T, Bleich S, et al. Non-right-handedness and free serum testosterone levels in detoxified patients with alcohol dependence. *Alcohol Alcohol*. 2010;45(3):237-240.
87. Frias J, Rodriguez R, Torres JM, Ruiz E, Ortega E. Effects of acute alcohol intoxication on pituitary-gonadal axis hormones, pituitary-adrenal axis hormones, beta-endorphin and prolactin in human adolescents of both sexes. *Life Sci*. 2000;67(9):1081-1086.
88. Mendelson JH, Ellingboe J, Mello NK, Kuehnle J. Effects of alcohol on plasma testosterone and luteinizing hormone levels. *Alcohol Clin Exp Res*. 1978;2(3):255-258.
89. Gordon GG, Southren AL, Lieber CS. The effects of alcoholic liver disease and alcohol ingestion on sex hormone levels. *Alcohol Clin Exp Res*. 1978;2(3):259-263.
90. Ellingboe J. Acute effects of ethanol on sex hormones in non-alcoholic men and women. *Alcohol Alcohol Suppl*. 1987;1:109-116.
91. Nguyen-Thanh T, Hoang-Thi AP, Anh Thu DT. Investigating the association between alcohol intake and male reproductive function: a current meta-analysis. *Heliyon*. 2023;9(5):e15723.
92. Slade T, Mewton L, O'Dean S, et al. DSM-5 and ICD-11 alcohol use disorder criteria in young adult regular drinkers: lifetime prevalence and age of onset. *Drug Alcohol Depend*. 2021;229:109184. Pt B.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

93. Lago L, Bruno R, Degenhardt L. Concordance of ICD-11 and DSM-5 definitions of alcohol and cannabis use disorders: a population survey. *The Lancet Psychiatry*. 2016;3(7):673-684.
94. Bosetti C, Levi F, Lucchini F, Zatonski WA, Negri E, La Vecchia C. Worldwide mortality from cirrhosis: an update to 2002. *J Hepatol*. 2007;46(5):827-839.
95. Mathurin P, Bataller R. Trends in the management and burden of alcoholic liver disease. *J Hepatol*. 2015;62(1):S38-S46. Suppl.
96. Zang G, Sun X, Sun Y, et al. Chronic liver diseases and erectile dysfunction. *Front Public Health*. 2022;10:1092353.
97. Kley HK, Nieschlag E, Wiegelmann W, Solbach HG, Krüskemper HL. Steroid hormones and their binding in plasma of male patients with fatty liver, chronic hepatitis and liver cirrhosis. *Acta Endocrinol (Copenh)*. 1975;79(2):275-285.
98. Bonnet F, Velayoudom Cephise FL, Gautier A, et al. Role of sex steroids, intrahepatic fat and liver enzymes in the association between SHBG and metabolic features. *Clin Endocrinol (Oxf)*. 2013;79(4):517-522.
99. Tchernof A, Labrie F, Bélanger A, et al. Relationships between endogenous steroid hormone, sex hormone-binding globulin and lipoprotein levels in men: contribution of visceral obesity, insulin levels and other metabolic variables. *Atherosclerosis*. 1997;133(2):235-244.
100. Telgenkamp I, Kusters Y, Schalkwijk CG, et al. Contribution of liver fat to weight loss-induced changes in serum hepatokines: a randomized controlled trial. *J Clin Endocrinol Metab*. 2019;104(7):2719-2727.
101. Salonen I, Huhtaniemi I. Effects of chronic ethanol diet on pituitary-testicular function of the rat. *Biol Reprod*. 1990;42(1):55-62.
102. Adams ML, Little PJ, Bell B, Cicero TJ. Alcohol affects rat testicular interstitial fluid volume and testicular secretion of testosterone and beta-endorphin. *J Pharmacol Exp Ther*. 1991;258(3):1008-1014.
103. Cicero TJ, Meyer ER, Bell RD. Effects of ethanol on the hypothalamic-pituitary-luteinizing hormone axis and testicular steroidogenesis. *J Pharmacol Exp Ther*. 1979;208(2):210-215.
104. Rivier C, Vale W. Influence of ethanol on reproductive functions of the adult male rat as a function of body weight. *Alcohol Clin Exp Res*. 1983;7(2):210-212.
105. Gonzalez-Reimers E, Martinez-Riera A, Santolaria-Fernandez F, et al. Relative and combined effects of ethanol and protein deficiency on gonadal function and histology. *Alcohol*. 1994;11(5):355-360.
106. Cicero TJ, Bernstein D, Badger TM. Effects of acute alcohol administration on reproductive endocrinology in the male rat. *Alcohol Clin Exp Res*. 1978;2(3):249-254.
107. Ogilvie KM, Rivier C. Effect of alcohol on the proestrous surge of luteinizing hormone (LH) and the activation of LH-releasing hormone (LHRH) neurons in the female rat. *J Neurosci*. 1997;17(7):2595-2604.
108. Dees WL, Mc Arthur NH, Farr KL, Culler MD, Harms PG. Effects of ethanol on rat hypothalamic luteinizing hormone releasing hormone. A study utilizing radioimmunoassay. *Biol Reprod*. 1983;28(5):1066-1070.
109. Adams ML, Cicero TJ. Effects of alcohol on beta-endorphin and reproductive hormones in the male rat. *Alcohol Clin Exp Res*. 1991;15(4):685-692.
110. Cobb CF, Ennis MF, van Thiel DH, Gavaler JS, Lester R. Isolated testes perfusion: a method using a cell- and protein-free perfusate useful for the evaluation of potential drug and/or metabolic injury. *Metabolism*. 1980;29(1):71-79.
111. Van Thiel DH, Cobb CF, Herman GB, Perez HA, Estes L, Gavaler JS. An examination of various mechanisms for ethanol-induced testicular injury: studies utilizing the isolated perfused rat testes. *Endocrinology*. 1981;109(6):2009-2015.
112. Cicero TJ, Bell RD. Ethanol-induced reductions in testicular steroidogenesis: major differences between in vitro and in vitro approaches. *Steroids*. 1982;40(5):561-568.
113. Santucci L, Graham TJ, Van Thiel DH. Inhibition of testosterone production by rat Leydig cells with ethanol and acetaldehyde: prevention of ethanol toxicity with 4-methylpyrazole. *Alcohol Clin Exp Res*. 1983;7(2):135-139.
114. Widenius TV. Ethanol-induced inhibition of testosterone biosynthesis in vitro: lack of acetaldehyde effect. *Alcohol Alcohol*. 1987;22(1):17-22.
115. Akane A, Fukushima S, Shiono H, Fukui Y. Effects of ethanol on testicular steroidogenesis in the rat. *Alcohol Alcohol*. 1988;23(3):203-209.
116. Orpana AK, Härkönen M, Eriksson CJ. Ethanol-induced inhibition of testosterone biosynthesis in rat Leydig cells: role of mitochondrial substrate shuttles and citrate. *Alcohol Alcohol*. 1990;25(5):499-507.
117. Schade RR, Bonner G, Gay VC, Van Thiel DH. Evidence for a direct inhibitory effect of ethanol upon gonadotropin secretion at the pituitary level. *Alcohol Clin Exp Res*. 1983;7(2):150-152.
118. Uddin S, Emanuele MA, Emanuele NV, Reda D, Kelley MR. The effect of in vitro ethanol exposure on luteinizing hormone and follicle stimulating hormone mRNA levels, content, and secretion. *Endocr Res*. 1994;20(2):201-217.
119. Emanuele MA, Tentler J, Reda D, Kirsteins L, Emanuele NV, Lawrence AM. The effect of in vitro ethanol exposure on LHRH release from perifused rat hypothalami. *Endocr Res*. 1990;16(3):313-321.
120. Inukai T, Wang X, Greer MA, Greer SE. Isotonic but not hypertonic ethanol stimulates LHRH secretion from perifused rat median eminence. *Neuroendocrinology*. 1993;58(2):258-262.
121. Canteros G, Rettori V, Franchi A, et al. Ethanol inhibits luteinizing hormone-releasing hormone (LHRH) secretion by blocking the response of LHRH neuronal terminals to nitric oxide. *Proc Nat Acad Sci USA*. 1995;92(8):3416-3420.
122. Alcohol use and burden for 195 countries and territories, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet (London, England)*. 2018;392(10152):1015-1035.
123. Key TJ, Appleby PN, Reeves GK, et al. Sex hormones and risk of breast cancer in premenopausal women: a collaborative reanalysis of individual participant data from seven prospective studies. *Lancet Oncol*. 2013;14(10):1009-1019.
124. Key TJ, Appleby PN, Reeves GK, et al. Steroid hormone measurements from different types of assays in relation to body mass index and breast cancer risk in postmenopausal women: reanalysis of eighteen prospective studies. *Steroids*. 2015;99:49-55. Pt A.
125. Tin Tin S, Reeves GK, Key TJ. Endogenous hormones and risk of invasive breast cancer in pre- and post-menopausal women: findings from the UK Biobank. *Br J Cancer*. 2021;125(1):126-134.
126. Hamajima N, Hirose K, Tajima K, et al. Alcohol, tobacco and breast cancer–collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. *Br J Cancer*. 2002;87(11):1234-1245.
127. Allen NE, Beral V, Casabonne D, et al. Moderate alcohol intake and cancer incidence in women. *J Natl Cancer Inst*. 2009;101(5):296-305.
128. Kopp TI, Jensen DM, Ravn-Haren G, et al. Alcohol-related breast cancer in postmenopausal women—effect of CYP19A1, PPARG and PPARGC1A polymorphisms on female sex-hormone levels and interaction with alcohol consumption and NSAID usage in a nested case-control study and a randomised controlled trial. *BMC Cancer*. 2016;16:283.
129. Mezzullo M, Pelusi C, Fazzini A, et al. Female and male serum reference intervals for challenging sex and precursor steroids by liquid chromatography—tandem mass spectrometry. *J Steroid Biochem Mol Biol*. 2020;197:105538.
130. Smy L, Straseski JA. Measuring estrogens in women, men, and children: recent advances 2012–2017. *Clin Biochem*. 2018;62:11-23.
131. De Vincentis S, Decaroli MC, Milic J, et al. Determinants of sexual function in men living with HIV younger than 50 years old: focus on

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

organic, relational, and psychological issues. *androl*. 2022;11(6):954-969.
132. Santi D, Madeo B, Carli F, et al. Serum total estradiol, but not testosterone is associated with reduced bone mineral density (BMD) in HIV-infected men: a cross-sectional, observational study. *Osteoporos Int*. 2016;27(3):1103-1114.
133. Eng ET, Ye J, Williams D, et al. Suppression of estrogen biosynthesis by procyanidin dimers in red wine and grape seeds. *Cancer Res*. 2003;63(23):8516-8522.
134. Kijima I, Phung S, Hur G, Kwok SL, Chen S. Grape seed extract is an aromatase inhibitor and a suppressor of aromatase expression. *Cancer Res*. 2006;66(11):5960-5967.
135. Kao YC, Zhou C, Sherman M, Laughton CA, Chen S. Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: a site-directed mutagenesis study. *Environ Health Perspect*. 1998;106(2):85-92.
136. Yao X, Xia F, Tang W, Xiao C, Yang M, Zhou B. Isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomics for the investigation of the effect of Hugan Qingzhi on non-alcoholic fatty liver disease in rats. *J Ethnopharmacol*. 2018;212:208-215.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Santi D, Cignarelli A, Baldi M, et al. The chronic alcohol consumption influences the gonadal axis in men: Results from a meta-analysis. *Andrology*. 2024;12:768–780. https://doi.org/10.1111/andr.13526

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13526 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 23 February 2024 | Accepted: 3 March 2024

DOI: 10.1111/andr.13633

ANDROLOGY WILEY

SOCIETY NEWS

Leading at the vanguard of andrology: The Network for Young Researchers in Andrology joins forces with the European Academy of Andrology

Alberto de la Iglesia[1,2] | **Dorte L. Egeberg**[1,3] | **Daniel Marcu**[1,4] | **Guillaume Richer**[1,5] | **Brendan J. Houston**[1,6] | **Omar Ammar**[1,7,8] | **Gülizar Saritas**[1,9] | **Emily Delgouffe**[1,5] | **Davor Jezek**[10,11] | **Csilla Krausz**[10,12] | **Ewa Rajpert-De Meyts**[9,10] | **Hermann M. Behre**[10,13]

[1]Network for Young Researchers in Andrology (NYRA), Barcelona, Spain

[2]Department of Cellular Plasticity and Reproduction, Université Paris Cité, INSERM, CNRS, Institut Cochin, Paris, France

[3]European Sperm Bank, Copenhagen, Denmark

[4]School of Biological Science, University of East Anglia, Norwich, UK

[5]Genetics, reproduction, and development (GRAD) research group, Biology of the Testis (BITE) laboratory, Vrije Universiteit Brussel, Brussels, Belgium

[6]School of BioSciences, The University of Melbourne, Parkville, Australia

[7]Ar-Razzi Hospital, Ramadi, Iraq

[8]Department of Obstetrics and Gynaecology, College of Medicine, University of Anbar, Ramadi, Iraq

[9]Department of Growth and Reproduction, Copenhagen University Hospital, Copenhagen, Denmark

[10]European Academy of Andrology (EAA), Münster, Germany

[11]Department for Transfusion Medicine and Transplantation Biology, Reproductive Tissue Bank, University Hospital Zagreb, Zagreb, Croatia

[12]Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy

[13]Center for Reproductive Medicine and Andrology, University Hospital, Martin Luther University Halle-Wittenberg, Halle, Germany

Correspondence
Alberto de la Iglesia, Network for Young Researchers in Andrology (NYRA), 08021, Barcelona, Spain.
Email: alberto.iglesia@inserm.fr

KEYWORDS
andrology, early career researchers, European Academy of Andrology, Network for Young Researchers in Andrology

The Network for Young Researchers in Andrology (NYRA) was founded in 2006 during the 14th European Testis Workshop held in Bavaria, Germany, by a group of enthusiastic young scientists with a strong interest in male reproductive health.[1] From the beginning, NYRA's main goal has been to create safe spaces where early-stage researchers feel confident to openly exchange scientific advances, while actively promoting networking to foster their careers, develop their soft skills, and facilitate international collaboration. While the aim has remained the same, during these 17 years, the organization has proven to be highly dynamic. Being founded as Young "Testis Club", the network was renamed to "International Network for Young Researchers in Male Fertility" in 2008.[2] Finally, in 2017, it rebranded to NYRA, its current name. This dynamism is not only reflected in terms of name, but also of board members, geographical distribution of activities, and ways of communicating with its audience. To date, more than 40 young scientists from different parts of the world have been part of the NYRA board, each contributing in a unique way to the development of the organization and the growth of its activities. Past board members have successfully moved into their careers in or outside academia, highlighting their passion and motivation to develop themselves professionally in the field.

On a yearly basis, the NYRA Board organizes an international NYRA Meeting, which is undoubtedly NYRA's main and most fruitful

© 2024 American Society of Andrology and European Academy of Andrology.

FIGURE 1 Alberto de la Iglesia (Network for Young Researchers in Andrology [NYRA] President) and Ewa Rajpert-De Meyts (European Academy of Andrology [EAA] Secretary) introducing the NYRA-EAA merger proposal during the EAA General Assembly 2022.

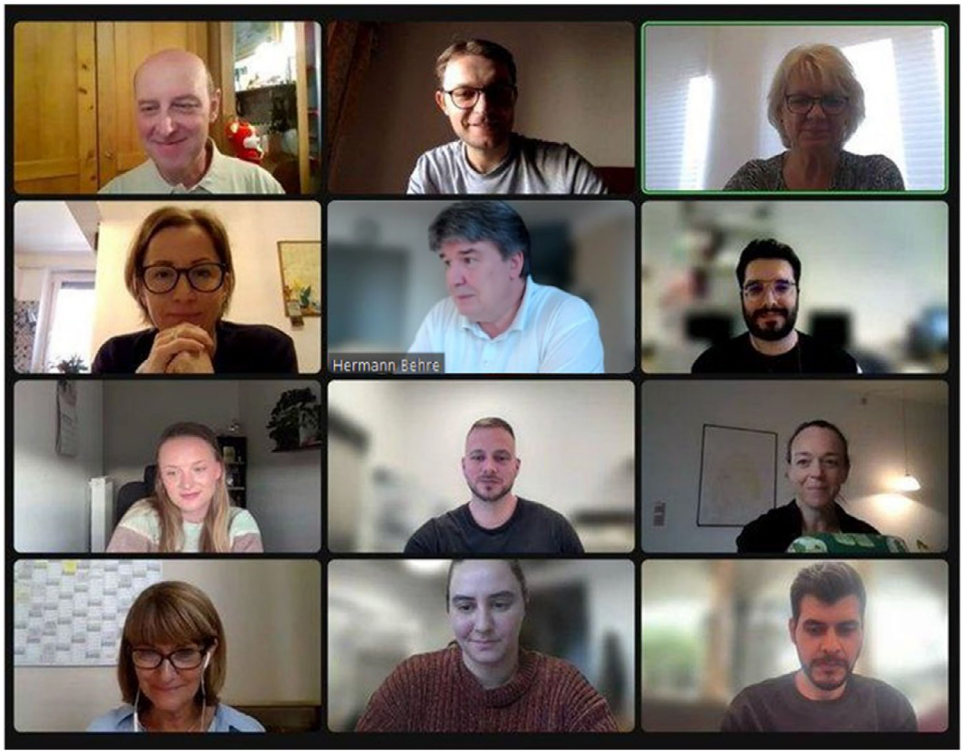


FIGURE 2 Network for Young Researchers in Andrology-European Academy of Andrology (NYRA-EAA) first online meeting developing the practical details to implement the merger. From upper left to down right, Davor Jezek (EAA Treasurer), Daniel Marcu (NYRA Treasurer), Ewa Rajpert-De Meyts (EAA Secretary), Kriszti Jeszenoi (EAA Office), Hermann M. Behre (EAA President), Alberto de la Iglesia (NYRA President), Emily Delgouffe (NYRA Board), Guillaume Richer (NYRA Board), Dorte Egeberg (NYRA Secretary), Krisztina Csongradi (EAA Office), Gülizar Saritas (NYRA Board), and Omar Ammar (NYRA Board).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13633 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

activity.[2–5] Nowadays, NYRA Meetings are a reference hub for top-level science and scientific discussions on male (in)fertility, bringing together all stages of early career researchers (MSc students, PhD candidates, and early post-doctoral researchers) with internationally renowned group leaders, company CEOs, and professionals of leadership and self-awareness. As a non-profit organization, the development of NYRA activities heavily relies on the support from collaborating societies and sponsors. Historically, NYRA has remained particularly close to the European Academy of Andrology (EAA), a collaborating society that has strongly promoted all NYRA-endorsed events in both economic and promotional ways. In addition to the direct generous support for NYRA activities, the European Congress of Andrology, organized by the EAA, hosts a "NYRA Session" within its program, and former NYRA board members have become prominent EAA members after their time in NYRA.

In the context of a global decline in fertility rates, in which the male contribution still remains overlooked, it is critical to build strongly structured networks to guarantee the development of educational and scientific activities to forge new frontiers in the andrology field. Despite male factors contributing to 50% of infertility cases, major attention is paid to the examination of women.[6,7] Moreover, the exact causes of male infertility remain elusive in half of the cases due to its heterogeneous and complex etiology.[8] There is thus a strong need to promote basic, translational, and clinical research.[9] Of equal importance is to support the new generations of basic and clinical scientists in andrology, who hold future milestones in their hands and are responsible for the development of the field. Therefore, in such a social and scientific scenario, and based on the previous good and strong relationship between NYRA and EAA, a merger between both organizations arose naturally, incorporating NYRA as the 'young arm' of the EAA.

While the two organizations share similar backgrounds as international non-profit societies gathering members with a focus on andrology, important differences between them still remain, indicating that this merger will lead to a unique synergy and mutual benefits for the field. The EAA was established in 1992 in Germany by a group of prominent European andrologists, most of them heading established groups of clinicians and scientists.[10] Since then, the EAA has developed a well-functioning society focusing on improving education in andrology through several means, such as congresses (ECA), EAA Schools, webinars, and the specialist scientific journal *International Journal of Andrology*, which in the spirit of global collaboration in 2012 merged with the American counterpart, *Journal of Andrology*, to form *Andrology* (https://onlinelibrary.wiley.com/journal/20472927), jointly owned by the EAA and the American Society of Andrology.[11] Similarly, NYRA was founded by a group of young scientists with a strong interest in male reproduction, as a more informal means to develop projects and career goals through networking. NYRA has always been open and free to all young scientists in the field and is orchestrated by a board formed by early-stage researchers. While EAA mainly engages senior-level clinicians, NYRA predominantly consists of younger basic scientists. Therefore, the synergy this collaboration will bring is evident and it will lead to years-long mutual support between the two organizations. Within this merger, NYRA will hold a semi-autonomous status, maintaining its own name, board, website, and communication systems, as well as the ability to plan NYRA-endorsed activities and establish relationships with collaborating societies. Independently of the NYRA-EAA membership, the subscription to the NYRA newsletter remains open and free of charge, to stay updated with NYRA's latest news.

This initiative was worked through during the second term of the EAA presidency of Csilla Krausz and pre-approved during the EAA General Assembly on October 20, 2022, held as part of the 12th ECA in Barcelona (Spain). It was the first time NYRA had direct participation in the biennial EAA General Assembly, the most important internal event of the EAA, in which Ewa Rajpert-De Meyts (EAA Secretary) and Alberto de la Iglesia (NYRA President) introduced the proposal of the merger to the EAA members (Figure 1). The official approval of the Memorandum of Understanding for the implementation of this merger took place on May 27, 2023, and the document was signed by Hermann M. Behre (EAA President), Ewa Rajpert-De Meyts (EAA Secretary), Alberto de la Iglesia (NYRA President) and Dorte Egeberg (NYRA Secretary), on behalf of the members of the EAA Executive Council and the NYRA Board. From that moment, NYRA had a direct representation in the EAA Executive Council, with the NYRA President becoming an ex-officio member of the EAA Executive Council. The communication between the two organizations is fluid and NYRA is receiving direct support from the EAA Office in the development of new activities (Figure 2).

This merger has already brought important benefits to the young andrology community. A new NYRA-EAA registration yearly fee at a reduced cost was introduced to facilitate the incorporation of younger members to the EAA, allowing them to enjoy EAA membership benefits. They include the possibility to actively participate in EAA Committees and educational activities; priority access to EAA Schools, webinars, and courses; priority access to EAA travel grants to attend any EAA-endorsed meetings announced in EAA newsletters and website; access to fellowships in EAA-certified training centers; access to monthly EAA literature alerts; the possibility to participate in EAA-organized meetings with discounted fees for EAA members; and membership to the International Society of Andrology. All information and most recent updates can be found on the EAA and NYRA websites (www.andrologyacademy.net and www.nyra-youngresearch.eu, respectively).

Both the NYRA Board and EAA Executive Council are excited to see what is about to come after this merger, hoping it marks a new beginning for the andrology community. New activities are in development under the new NYRA-EAA umbrella, keeping the same goal in mind: improving andrology research and clinical practice by investing in the education and career development of the next generation of scientists and clinicians.

ACKNOWLEDGMENTS

NYRA acknowledges Juan Manuel Paturlanne, Isidora Kjezelj, Andjela Kovacevic, and Avinash Gaikwad, NYRA Board members incorporated after the merger took place, for revising this text and actively participating in the development of new NYRA-EAA activities. NYRA and EAA

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13633 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

acknowledge the assistance of the EAA Office (Krisztina Csongradi and Kriszti Jeszenoi) for their administrative support.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ORCID

Alberto de la Iglesia https://orcid.org/0000-0003-3475-2086
Guillaume Richer https://orcid.org/0000-0003-2888-9078
Brendan J. Houston https://orcid.org/0000-0002-1078-756X
Omar Ammar https://orcid.org/0000-0001-8048-9747
Ewa Rajpert-De Meyts https://orcid.org/0000-0002-5946-7559

REFERENCES

1. Gromoll J, Michel C, Kostova E, et al. Minutes of the 14th European workshop on molecular and cellular endocrinology of the testis. *Mol Cell Endocrinol*. 2006;257-258:1-5.
2. Tüttelmann F, de Gendt K, Amaral A, et al. The future of testis research is turning 6! Six years of international network for young researchers in male fertility. *Int J Androl*. 2012;35(2):211-213.
3. Amaral A, Wahlgren A, Tüttelmann F, et al. Minutes of the 5th meeting of the International Network for Young Researchers in Male Fertility. *Asian J Androl*. 2012;14(5):796.
4. Marcu D, Egeberg D, Richer G, et al. Shaping the future of male reproductive health: fostering talent at the 14th Network of Young Researchers in Andrology meeting. *Biol Open*. 2023;12(5):5. doi:10.1242/bio.059901
5. Marcu D, Egeberg DL, Richer G, et al. Empowering tomorrow's leaders: the impact of the 15th Network of Young Researchers in Andrology (NYRA) meeting on male reproductive health and interdisciplinary collaboration. *Biol Open*. 2024;13(1):bio060178.
6. Levine H, Jørgensen N, Martino-Andrade A, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis of samples collected globally in the 20th and 21st centuries. *Hum Reprod Update*. 2022;29(2):157-176.
7. de Jonge CJ, Gellatly SA, Vazquez-Levin MH, Barratt CLR, Rautakallio-Hokkanen S. Male attitudes towards infertility: results from a Global Questionnaire. *World J Mens Health*. 2023;41(4):204-214.
8. Aitken RJ. The changing tide of human fertility. *Hum Reprod*. 2022;37(4):629-638.
9. Kimmins S, Anderson RA, Barratt CLR, et al. Frequency, morbidity and equity—the case for increased research on male fertility. *Nat Rev Urol*. 2024;21(2):102-124. doi:10.1038/s41585-023-00820-4
10. Nieschlag E, Forti G, Wu F, Toppari J, Krausz C. History of the European Academy of Andrology. *Andrology*. 2022;10(2):4-9. Suppl.
11. Rajpert-De Meyts E, Carrell DT, Simoni M, Giwercman A, Hofmann MC. Marking the first decade of andrology—conception and early development of the journal. *Andrology*. 2022;10(2):139-143. Suppl.

How to cite this article: de la Iglesia A, Egeberg DL, Marcu D, et al. Leading at the vanguard of andrology: The Network for Young Researchers in Andrology joins forces with the European Academy of Andrology. *Andrology*. 2024;12:781–784. https://doi.org/10.1111/andr.13633

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13633 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 21 June 2023 | Revised: 8 September 2023 | Accepted: 9 September 2023

DOI: 10.1111/andr.13533

ANDROLOGY WILEY

ORIGINAL ARTICLE

A real-world pharmacovigilance study of FDA adverse event reporting system events for sildenafil

Yan Wang[1] | Bin Zhao[2,3] | Haiyan Yang[4] | Zheng Wan[5]

[1]Medical Reproductive Center, People's Hospital of Jiuquan City, Jiuquan, Gansu, China

[2]Xiamen Health and Medical Big Data Center, Xiamen, China

[3]Xiamen Medicine Research Institute, Xiamen, China

[4]Department of Gynecological Oncology, The Fourth Affiliated Hospital of Guangxi Medical University, Liuzhou, China

[5]Department of Minimally Invasive and Interventional Therapy for Cancer, School of Medicine, Zhongshan Hospital of Xiamen University, Xiamen University, Xiamen, China

Correspondence
Haiyan Yang, Department of Gynecological Oncology, The Fourth Affiliated Hospital of Guangxi Medical University, Liuzhou, China.
Email: haiyanzheda@163.com

Zheng Wan, Department of Minimally Invasive and Interventional Therapy for Cancer, Zhongshan Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen, China.
Email: wanzheng0626@xmu.edu.cn

Funding information
Natural Science Foundation of Fujian Province, Grant/Award Number: 2022J05298; Guangxi Medical University Youth Science Fund Project, Grant/Award Number: GXMUYSF 202370; Natural Science Foundation of Xiamen, China,, Grant/Award Number: 3502Z20227155; Scientific Research Project of Health and Family Planning Commission of GuangXi Zhuang Autonomous Region,, Grant/Award Number: 20180513

Abstract

Background: Sildenafil, a selective inhibitor of phosphodiesterase type 5 (PDE5), is widely used for the treatment of erectile dysfunction (ED). However, the safety profile of sildenafil, including adverse event (AEs), requires comprehensive evaluation.

Methods: This retrospective pharmacovigilance study aimed to evaluate AEs linked to sildenafil by analyzing data sourced from the FDA Adverse Event Reporting System (FAERS) database. A case/non-case design was utilized, and various algorithms including the reporting odds ratio (ROR), the proportional reporting ratio (PRR), the Bayesian confidence propagation neural network (BCPNN), and the multiitem gamma Poisson shrinker (MGPS) were employed to measure the signals indicating the presence of sildenafil-related AEs.

Results: Among 339,230 reports, 33,692 specifically mentioned sildenafil use. Most of AEs occurred in males over 60 years old. The United States accounted for the highest proportion of reported AEs. Severe outcomes, including death, disability, and life-threatening events, were reported. Significant system organ class (SOC) included "Reproductive system and breast disorders" (SOC: 10038604), "Neoplasms benign, malignant and unspecified" (SOC: 10038738), "Vascular disorders" (SOC: 10047065), and "Blood and lymphatic system disorders" (SOC: 10005329). Noteworthy preferred terms (PTs) associated with sildenafil included "Vision blurred," "Flushing," "sudden hearing loss," "Painful erection," and "Priapism." Unexpected AEs, such as "Malignant melanoma," "Pulmonary hypertension," "Malignant melanoma in situ," "Pulmonary arterial hypertension," "Metastatic malignant melanoma," "Malignant melanoma stage III," "Malignant melanoma stage II," "Acquired hemophilia," "Aortic dissection rupture," and "Intracranial artery dissection" were also identified.

Conclusions: These findings emphasize the importance of monitoring and understanding the potential risks associated with sildenafil. Further investigation is warranted to validate these associations and address previously unrecognized safety concerns.

KEYWORDS
adverse event, data mining, FAERS database, pharmacovigilance, sildenafil

Yan Wang and Bin Zhao contributed to the work equally and should be regarded as co-first authors.

© 2023 American Society of Andrology and European Academy of Andrology.

1 | INTRODUCTION

Erectile dysfunction (ED), often colloquially termed impotence, refers to the inability of males to achieve or maintain a sufficiently rigid penile erection during sexual stimulation, thereby hindering satisfactory sexual intercourse.[1] ED can result from a multitude of factors, including physiological, psychological, and environmental elements.[2] Within the spectrum of etiological factors, phosphodiesterase 5 (PDE5) plays a crucial role in the erectile function.[3] In typical physiological conditions, sexual arousal initiates a series of biochemical reactions, in which the release of a signaling molecule known as nitric oxide (NO) plays a pivotal role.[4] NO activates an enzyme called guanylate cyclase within the penile corpus cavernosum, leading to an increase in intracellular cyclic guanosine monophosphate (cGMP) levels. Elevated cGMP levels subsequently induce relaxation of penile smooth muscles and increased blood flow, resulting in the occurrence of penile erection.[3] However, when ED occurs, increased PDE5 activity leads to cGMP degradation, ultimately hindering penile erection. To address this issue, many PDE5 inhibitors have been extensively utilized for the therapeutic management of ED.[5] Among these PDE5 inhibitors, sildenafil stands out as the most prominently recognized therapeutic agent.[6] Since its approval for treating ED in 1998,[7] sildenafil has gained increasing popularity among a multitude of individuals.

Sildenafil is widely recognized for its intrinsic safety, and its potential adverse events (AEs) are frequently disregarded in clinical assessment and practice. Although some common AEs such as headaches,[7] and more severe AEs like heart attacks[8] have been documented, these data primarily originate from succinct clinical trials, case studies, and meta-analyses, often constrained by specific systems due to rigorous diagnostic and selection criteria.[9–11] Furthermore, the sample size is relatively modest, and limitations exist in terms of follow-up duration. Prolonged sildenafil usage could reveal previously unnoticed or significant safety considerations. In addition, it is vulnerable to improper utilization, encompassing both the erroneous administration of dosages intended for sexual potency and its misuse to augment athletic performance.[12,13] These actions subsequently elevate the probability of experiencing adverse reactions. Hence, a meticulous scrutiny of sildenafil-associated AEs is imperative and essential.

The FDA Adverse Event Reporting System (FAERS) database is a publicly available voluntary reporting system that contains a wide range of records documenting adverse drug incidents reported by medical practitioners, pharmacists, manufacturers, and other individuals.[14] As the largest pharmacovigilance database globally, FAERS serves as a valuable tool for identifying AEs associated with medication use.[15,16] This study aimed to analyze data from the FAERS database and perform post-marketing surveillance to assess the AEs linked with sildenafil.

2 | PATIENTS AND METHODS

2.1 Data source and collection

To evaluate the safety of sildenafil in the post-marketing phase, a retrospective pharmacovigilance study was conducted. Data were obtained from the publicly available FAERS database, covering a substantial timeframe from Q1 2004 to Q4 2021. The FAERS database comprises seven comprehensive datasets, providing diverse information on different aspects of drug usage and adverse events. These datasets include demographic and administrative information (DEMO), drug-related details (DRUG), reports on adverse drug reactions (REAC), patient outcomes (OUTC), sources of the reports (RPSR), drug therapy duration (THER), and indications for use or diagnosis (INDI). In adherence to the FDA's guidelines, our study implemented a rigorous process to identify and eliminate duplicate reports. We selected the most recent FDA_DT entry with the same CASEID.[14] In cases where the CASEID and FDA_DT were identical, we prioritized the higher PRIMARYID to ensure the exclusion of redundant data. This meticulous approach aimed to enhance the accuracy and reliability of our analysis by avoiding duplicate entries and ensuring a comprehensive assessment of the safety profile of sildenafil.

2.1 | Adverse event and drug identification

The AEs reported in FAERS are classified according to the Medical Dictionary for Regulatory Activities (MedDRA) terminology, specifically using the Preferred Terms (PTs). To ensure a comprehensive assessment of the toxicity spectrum, we meticulously identified individual AE reports associated with sildenafil at both the system organ class (SOC) and PT levels. It is important to note that FAERS allows for the reporting of adverse events related to various drug names. The role of the reported drug in each event is indicated by specific codes, including preferred suspect (PS), secondary suspect (SS), concomitant (C), and interacting (I).

In our study, we focused on analyzing the target drugs, namely sildenafil (the generic name) and Viagra (the trade name), as documented in the DRUG file. To ensure consistency, we specifically selected the role code as "PS" to designate the reported drug as the PS in the adverse event. By utilizing this approach, we aimed to obtain a comprehensive understanding of the specific AEs associated with sildenafil, considering its generic and trade name variants.

2.2 | Data mining algorithm and statistical analysis

Our study follows a case/non-case design, which can be considered as a case–control study. We specifically focused on analyzing AEs attributed to the investigated drugs rather than those caused by underlying disease states. To explore the relationship between a specific drug and an adverse event (AE), both Frequentist and Bayesian techniques were utilized in the disproportionality analysis. The measures employed in this study included the reporting odds ratio (ROR),[17] the proportional reporting ratio (PRR),[18] the Bayesian confidence propagation neural network (BCPNN),[19] and the multiitem gamma Poisson shrinker (MGPS).[20] Prior to performing calculations for ROR and PRR, it is imperative to acquire the values of variables *a*, *b*, *c*, and *d*. Here, variable '*a*' signifies the count of individuals encountering the desired

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13533 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Table matrix.

	Sildenafil	Non-sildenafil
Target AEs	*a*	*C*
Non-target AEs	*b*	*d*
$N = a+b+c+d$		

Abbreviation: AE, adverse event.

AEs subsequent to sildenafil exposure; variable '*b*' signifies the count of individuals experiencing non-target adverse events post sildenafil exposure; variable '*c*' denotes the count of individuals facing the target adverse event subsequent to non- sildenafil exposure; and variable '*d*' represents the count of individuals experiencing non-target adverse events following non-sildenafil exposure. The total count *N* is given by the sum of *a*, *b*, *c*, and *d* ($N = a + b + c + d$, refer to Table 1). The specific formulas for the four algorithms are outlined below:

(i) ROR algorithm

$$\mathrm{ROR} = (ad) / (bc)$$

$$95\%\,\mathrm{CI} = e^{\ln(\mathrm{ROR}) \pm 1.96\sqrt{\frac{1}{a}+\frac{1}{b}+\frac{1}{c}+\frac{1}{d}}}.$$

The criteria of positive safety signal detection: the lower limit of 95% CI > 1, $N \geq 3$;

(ii) PRR algorithm

$$\mathrm{PRR} = [a(c+d)] / [c(a+b)]$$

$$\chi 2 = \frac{(a+b+c+d)(ad-bc)^2}{(a+b)(c+d)(a+c)(b+d)}.$$

The criteria of positive safety signal detection: PRR≥2, $\chi 2 \geq 4$, $N \geq 3$;

(iii) BPCNN algorithm

$$\mathrm{IC} = \log_2 \frac{a(a+b+c+d)}{(a+b)(a+c)}$$

$$95\%\,\mathrm{CI} = E(\mathrm{IC}) \pm 2 \times \sqrt{V(\mathrm{IC})}.$$

The criteria of positive safety signal detection: IC025 > 0 (IC025: the lower bound of 95% CI);

(iv) EBGM algorithm

$$\mathrm{EBGM} = (aN) / [(a+b)(a+c)]$$

$$95\%\,\mathrm{CI} = e^{\ln(\mathrm{EGBM}) \pm 1.96\sqrt{\frac{1}{a}+\frac{1}{b}+\frac{1}{c}+\frac{1}{d}}}.$$

The criteria of positive safety signal detection: EBGM05 > 2 (EBGM05: the lower bound of 95% CI).

In our study, we defined "cases" as patients who were treated with a drug and reported a specific AE. Conversely, all other possible pairs were considered "non-cases." In our analysis, we also considered unexpected AEs, which refer to significant AEs that were not listed in the FDA drug labeling. These unexpected AEs could potentially indicate previously unrecognized or significant safety concerns associated with the use of sildenafil. To perform the data processing and statistical analyses, we employed the R software (version 4.0.2), which enabled us to conduct rigorous and comprehensive analyses of the collected data.

TABLE 2 Characteristics of reports associated with sildenafil from 2004 Q1–2021 Q4.

	Case Number	Percentage
Total counts	33692	
Gender		
Male	24279	72.06%
Female	5879	17.45%
Unknown	3534	10.49%
Age		
<20	656	1.95%
20–29	492	1.46%
30–39	879	2.61%
40–49	1878	5.57%
50–59	3766	11.18%
60–69	4953	14.70%
70–79	3459	10.27%
>80	1559	4.63%
Unknown	16050	47.64%
Reported Countries (Top five ranked)		
US (United States)	27657	82.09%
CA (Canada)	738	2.19%
UK (United Kingdom)	738	2.19%
JP (Japan)	476	1.41%
Germany	409	1.21%
Serious outcomes		
Death	4613	13.69%
Disability	517	1.53%
Hospitalization	4008	11.90%
Life-threatening	345	1.02%

3 | RESULTS

3.1 | General characteristics

During the study period, which spanned from the first quarter of 2004 to the last quarter of 2021, a total of 339,230 reports were submitted to the FAERS database. Out of these, 33,692 reports specifically mentioned the use of sildenafil. Table 2 provides a comprehensive overview of the clinical characteristics associated with sildenafil-related events. Notably, a larger proportion of AEs were reported in males (72.06%)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13533 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

compared to females. This observation aligns with the intended usage of sildenafil, primarily for the treatment of ED, although it is also utilized for pulmonary hypertension (PHT) in female patients.

In terms of age distribution, most of patients experiencing AEs were aged over 60 years, which is notably higher than the median age typically seen in clinical trials. It is worth noting that in a separate clinical study, the clearance rate of sildenafil was found to be reduced in healthy elderly volunteers (over 65 years old), which could potentially explain the higher incidence of AEs observed in elderly individuals.

Geographically, the United States accounted for the highest proportion of reported AEs, comprising 82.09% of all cases. Among the reported outcomes, death was the most frequently mentioned severe outcome, occurring in 13.69% of cases. Additionally, disability and life-threatening events were reported in 517 (1.53%) and 345 (1.02%) cases, respectively.

3.2 | Signal values associated with sildenafil ranked by reporting odds ratio algorithm

Table 3 displays the significant SOCs identified in our analysis. The noteworthy SOCs were "Reproductive system and breast disorders" (SOC: 10038604), "Neoplasms benign, malignant and unspecified" (SOC: 10038738), "Vascular disorders" (SOC: 10047065), and "Blood and lymphatic system disorders" (SOC: 10005329). The section addressing "Reproductive system and breast disorders" encompasses a total of 5,918 cases. The relative reporting odds (ROR) along with its 95% confidence interval (CI) were calculated to be 51.74 (95% CI: 50.22–53.31). The proportional reporting ratio (PRR) was determined as 42.83 (95% CI: 41.76–43.92). The Chi-square value (χ^2) yielded a substantial result of 210,810.61. Additionally, the information component (IC) at IC025 was computed as 5.22 (95% CI: 5.12). Moreover, the Empirical Bayes Geometric Mean (EBGM) at EBGM05 stood at 37.3 (95% CI: 36.21). The category "Neoplasms benign, malignant, and unspecified" comprised a total of 1,117 cases. The ROR along with its 95% CI was calculated as 8.03 (7.56–8.53). Similarly, the PRR with a 95% CI was found to be 7.8 (7.36–8.27). The χ^2 reached 6,470.14, and the IC at IC025 stood at 2.93 (2.73). Furthermore, the EBGM calculated at EBGM05 was determined to be 7.62 (7.17). The category "Vascular disorders" encompassed a total of 144 cases. The ROR with a 95% CI was calculated to be 9.15 (7.74–10.8), while the PRR, also with a 95% CI, yielded a value of 9.11 (7.72–10.75). Furthermore, the χ^2 reached 1007.63, and the IC at the 0.25 quantile (IC025) was determined to be 3.15 (2.59). Notably, the EBGM at the 0.05 quantile (EBGM05) exhibited a score of 8.86 (7.5). The section on "Blood and Lymphatic System Disorders" encompassed seven cases. The ROR with a 95% CI was calculated at 5.76 (2.72–12.17), while the PRR, also with a 95% CI, was 5.76 (2.72–12.17). The Chi-square value was found to be 26.97. Additionally, the IC025 indicated a value of 2.5 (0.31), and the EBGM05 was determined to be 5.66 (2.68). These findings provide valuable insights into the specific areas where adverse events associated with sildenafil were frequently reported.

To further investigate the safety profile of sildenafil, we examined the PTs that exhibited significant associations with the use of this medication. Table S1 presents all the AEs that emerged from our study. Among these, we observed the common AEs including "Vision blurred," "Flushing," "sudden hearing loss," "Painful erection," and "Priapism." Furthermore, our study uncovered unexpected adverse events that had not been previously documented extensively. These top 10 unexpected AEs include "Malignant melanoma," "Pulmonary hypertension," "Malignant melanoma in situ," "Pulmonary arterial hypertension," "Metastatic malignant melanoma," "Malignant melanoma stage III," "Malignant melanoma stage II," "Acquired hemophilia," "Aortic dissection rupture," and "Intracranial artery dissection" (Table 4).

4 | DISCUSSION

This study analyzed a total of 33,692 reports that were specifically linked to AEs attributed to the use of sildenafil. Notably, there was a predominant occurrence of these reported AEs that was observed among male patients, which aligns with sildenafil's primary indication for treating ED. Given that sildenafil's primary prescription is for addressing ED-related concerns, a higher proportion of reported AEs from male users is expected. It is important to acknowledge, however, that sildenafil also finds application in treating other medical conditions such as PHT,[21] cancer,[22] and ophthalmology.[23] For example, sildenafil has demonstrated efficacy and good tolerability for persistent PHT of the newborn,[24] particularly in cases where alternative therapies like inhaled nitric oxide and extracorporeal membrane oxygenation are not available.[25] Consequently, reports from female patients were also included in this analysis.

The distribution of reported AEs linked to sildenafil demonstrates a significant bias toward individuals aged 60 years and older, suggesting a heightened occurrence of AEs within this specific age cohort. This particular observation gives rise to apprehensions concerning the safety and tolerability of sildenafil among the elderly population, as physiological changes associated with aging can exert influence over drug metabolism and elimination processes.[11] Age-related alterations in hepatic and renal functions, coupled with changes in drug absorption and distribution, can give rise to disparities in drug clearance and pharmacokinetics within the elderly population.[9,26] These physiological changes may affect the metabolism and elimination of sildenafil, potentially leading to a higher risk of AEs.

Specifically, our findings highlighted significant links between sildenafil and the SOCs of "Eye disorders." Traditionally, the visual disturbances associated with sildenafil use were attributed to its inhibition of phosphodiesterase 6 (PDE6) in the photoreceptor cells.[27] However, recent investigations have revealed that sildenafil also affects bipolar cells, expanding our understanding of its impact on the visual function.[28] Additionally, emerging evidence suggests that sildenafil may exert beneficial effects on ocular health, such as preventing thickening of the Bruch membrane and promoting regeneration of the ellipsoid zone in cases of dry age-related macular degeneration.[29] These novel findings underscore the complex interplay between

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13533 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 Signal strength of AEs of sildenafil at the System Organ Class (SOC) level in Food and Drug Administration Adverse Event Reporting System (FAERS) database.

SOC Code	SOC	Case reports	ROR (95% CI)	PRR (95% CI)	$\chi 2$	IC (IC025)	EBGM (EBGM05)
10018065	General disorders and administration site conditions	10637	5.29 (5.17–5.41)	3.93 (3.87–4)	24972.82	1.96 (1.89)	3.89 (3.8)
10038604	Reproductive system and breast disorders*	5918	51.74 (50.22–53.31)	42.83 (41.76–43.92)	210810.61	5.22 (5.12)	37.3 (36.21)
10015919	Eye disorders	1893	6.72 (6.42–7.05)	6.4 (6.13–6.69)	8514.24	2.65 (2.49)	6.28 (5.99)
10022117	Injury, poisoning and procedural complications	1884	2.93 (2.8–3.07)	2.82 (2.7–2.95)	2240.63	1.49 (1.33)	2.8 (2.68)
10029205	Nervous system disorders	1862	4.81 (4.59–5.04)	4.6 (4.4–4.81)	5223.56	2.18 (2.02)	4.54 (4.33)
10029104	Respiratory, thoracic and mediastinal disorders	1466	55.64 (52.56–58.89)	53.26 (50.42–56.26)	63267.08	5.49 (5.3)	44.94 (42.45)
10038738	Neoplasms benign, malignant and unspecified (incl cysts and polyps)*	1117	8.03 (7.56–8.53)	7.8 (7.36–8.27)	6470.14	2.93 (2.73)	7.62 (7.17)
10013993	Ear and labyrinth disorders	573	4.43 (4.08–4.81)	4.37 (4.03–4.74)	1473.22	2.11 (1.83)	4.32 (3.98)
10077536	Product issues	309	32.98 (29.3–37.12)	32.69 (29.07–36.76)	8506.84	4.88 (4.48)	29.39 (26.11)
10022891	Investigations	262	12.95 (11.43–14.66)	12.86 (11.36–14.54)	2741.03	3.62 (3.21)	12.34 (10.9)
10042613	Surgical and medical procedures	196	7.54 (6.54–8.69)	7.5 (6.51–8.64)	1076.92	2.87 (2.4)	7.33 (6.36)
10007541	Cardiac disorders	182	7.52 (6.49–8.72)	7.49 (6.47–8.67)	997.35	2.87 (2.38)	7.32 (6.32)
10047065	Vascular disorders*	144	9.15 (7.74–10.8)	9.11 (7.72–10.75)	1007.63	3.15 (2.59)	8.86 (7.5)
10037175	Psychiatric disorders	112	13.7 (11.33–16.56)	13.66 (11.3–16.5)	1253.21	3.71 (3.08)	13.07 (10.81)
10040785	Skin and subcutaneous tissue disorders	107	7.51 (6.19–9.1)	7.49 (6.18–9.07)	586.09	2.87 (2.24)	7.32 (6.04)
10010331	Congenital, familial, and genetic disorders	43	20.22 (14.83–27.56)	20.19 (14.82–27.51)	731.95	4.24 (3.24)	18.91 (13.87)
10041244	Social circumstances	25	11.28 (7.56–16.82)	11.27 (7.56–16.81)	224.97	3.44 (2.17)	10.87 (7.29)
10021881	Infections and infestations	24	8.51 (5.67–12.77)	8.5 (5.67–12.76)	154.24	3.05 (1.76)	8.28 (5.52)
10017947	Gastrointestinal disorders	11	22.11 (11.97–40.85)	22.1 (11.97–40.83)	205.48	4.36 (2.49)	20.56 (11.13)
10036585	Pregnancy, puerperium, and perinatal conditions	10	8.74 (4.66–16.4)	8.74 (4.66–16.39)	66.44	3.09 (1.18)	8.5 (4.53)
10005329	Blood and lymphatic system disorders*	7	5.76 (2.72–12.17)	5.76 (2.72–12.17)	26.97	2.5 (0.31)	5.66 (2.68)
10014698	Endocrine disorders	5	13.4 (5.46–32.86)	13.4 (5.46–32.85)	54.74	3.68 (1.15)	12.83 (5.23)
10021428	Immune system disorders	5	14.5 (5.9–35.63)	14.5 (5.9–35.62)	59.76	3.79 (1.25)	13.84 (5.63)
10019805	Hepatobiliary disorders	3	21.1 (6.53–68.21)	21.1 (6.53–68.19)	53.43	4.3 (1.21)	19.7 (6.09)
10038359	Renal and urinary disorders	3	17.58 (5.48–56.45)	17.58 (5.48–56.44)	44.15	4.05 (0.99)	16.61 (5.17)

Note: *indicates that the SOC is not marked on the instruction manual.

Abbreviations: CI, confidence interval; ROR, reporting odds ratio; PRR, proportional reporting ratio; $\chi 2$, chi-squared; IC, information component; EBGM, empirical Bayesian geometric mean; BCPNN, Bayesian confidence propagation neural network.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13533 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 4 Top 10 unexpected adverse event of sildenafil at the preferred terms level in Food and Drug Administration Adverse Event Reporting System (FAERS) database.

Preferred terms	Case reports	ROR (95% CI)	PRR (95% CI)	IC (IC025)	EBGM (EBGM05)
Malignant melanoma	979	50.79 (47.42–54.41)	49.34 (46.14–52.77)	5.4 (5.17)	42.13 (39.33)
Pulmonary hypertension	547	17.53 (16.07–19.12)	17.26 (15.84–18.8)	4.03 (3.74)	16.32 (14.96)
Malignant melanoma in situ	240	148.46 (126.94–173.62)	147.4 (126.14–172.26)	6.6 (6.11)	97.06 (83)
Pulmonary arterial hypertension	117	5.01 (4.17–6.02)	5 (4.17–6)	2.3 (1.7)	4.93 (4.1)
Metastatic malignant melanoma	76	21.8 (17.25-27.53)	21.75 (17.23-27.46)	4.34 (3.57)	20.26 (16.04)
Malignant melanoma stage III	28	87.59 (57.3–133.87)	87.52 (57.27–133.73)	6.07 (4.73)	66.99 (43.83)
Malignant melanoma stage II	21	67.94 (42.18–109.43)	67.9 (42.17–109.34)	5.78 (4.28)	54.89 (34.08)
Acquired hemophilia	7	5.76 (2.72–12.17)	5.76 (2.72–12.17)	2.5 (0.31)	5.66 (2.68)
Aortic dissection rupture	6	51.15 (21.43–122.09)	51.15 (21.43–122.06)	5.44 (2.9)	43.43 (18.2)
Intracranial artery dissection	4	225.06 (60.43–838.18)	225.04 (60.43–838.01)	6.97 (3.5)	125.47 (33.69)

Abbreviations: CI, confidence interval; EBGM, empirical Bayesian geometric mean; EBGM, empirical Bayesian geometric; EBGM05, the lower limit of 95%; IC, information component; IC025, the lower limit of 95% CI of the IC; PRR, proportional reporting ratio; ROR, reporting odds ratio.

sildenafil and ocular physiology, warranting close monitoring of ocular manifestations in patients undergoing sildenafil therapy. Beyond ocular effects, our analysis identified significant associations between sildenafil and adverse events related to the nervous system and general disorders. Therefore, it is crucial to diligently monitor patients receiving sildenafil for any neurological manifestations, as well as general AEs that may arise during treatment.

Moreover, our analysis at the PT level revealed additional noteworthy AEs associated with sildenafil use. Consistent with previous clinical trials and known risks, we observed expected AEs such as "Cardiac failure acute" and "myocardial fibrosis.[11]" These findings further substantiate the existing knowledge regarding the potential cardiovascular effects of sildenafil. In a recent multicenter, double-blind, randomized clinical trial, researchers investigated the efficacy of sildenafil in patients with persistent pulmonary hypertension (PH) following the successful correction of valvular heart disease (VHD). Surprisingly, the study found that treatment with sildenafil (40 mg three times daily) in this specific patient population was associated with worse clinical outcomes.[21] Therefore, caution should be exercised when considering the off-label use of sildenafil for treating PH secondary to left heart disease. These findings emphasize the importance of tailored and evidence-based treatment strategies for patients with different etiologies of PH.

Regarding the "Cerebral small vessel ischemic disease," a study examined the infusion of sildenafil and its impact on cerebral perfusion. Interestingly, despite a significant reduction in cerebral perfusion pressure, the study did not observe any changes in global or regional perfusion.[30] This suggests that the proximal vasodilatory effects of sildenafil may not effectively alter brain perfusion. However, it is important to note that the cerebral autoregulatory function remained intact in this group, highlighting the potential preservation of the brain's ability to regulate blood flow. Future investigations should explore whether sildenafil has the potential to restore or enhance autoregulation in individuals affected by subarachnoid hemorrhage, presenting an intriguing avenue for further research.

In our analysis, the most frequently documented severe outcome was death, indicating the significance of closely monitoring and addressing potential risks associated with sildenafil use. Furthermore, disability and life-threatening events were also reported, underscoring the importance of comprehensive surveillance and management of AEs in patients receiving sildenafil therapy. These findings provide valuable insights into the cardiovascular effects of sildenafil and highlight the need for cautious monitoring, especially in patients with preexisting hypertension or concurrent use of antihypertensive drugs.

In addition to the anticipated AEs commonly associated with sildenafil use, our study uncovered several unexpected AEs that warrant further investigation and evaluation. These unexpected AEs include "Acquired hemophilia," "Malignant melanoma," "Aortic dissection rupture," and "Intracranial artery dissection."

Acquired hemophilia, a rare autoimmune disorder characterized by the development of autoantibodies against clotting factors,[31] is an unexpected AE that was reported in association with sildenafil use. Thrombocytopenia subsequent to the administration of sildenafil has been documented in the existing literature. However, no mention of the utilization of sildenafil as a potential instigator of acquired hemophilia has been found in ref.[32] The potential link between sildenafil and acquired hemophilia raises questions about the underlying mechanisms and whether there is a causal relationship. Further research is needed to explore this association and determine the extent of the risk.

Another unexpected AE that emerged from our analysis is malignant melanoma, a type of skin cancer.[33] While previous studies have investigated the relationship between sildenafil and melanoma, the findings have been inconclusive.[34] The research by Li et al. demonstrated that sildenafil facilitated melanoma cell invasion, shedding light on potential factors contributing to the differences in survival rates between sexes.[35] Arozarena et al. demonstrated that PDE5A was downregulated in numerous melanoma cell lines expressing oncogenic BRAF.[36] This observation suggests that this intrinsic phenotype may

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13533 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

potentially serve as a biomarker for increased invasiveness and poorer prognosis. Although there exists a theoretical possibility for PDE5A-targeting drugs to facilitate melanoma metastasis, the administration of sildenafil did not lead to an augmentation in melanoma cell lung colonization in mouse models.[36] Our study adds to the existing evidence, suggesting the need for continued surveillance and exploration of this potential association.

Aortic dissection rupture[37] and intracranial artery dissection[38] are vascular complications that have not been reported in association with sildenafil use. Some studies revealed an occurrence of cerebral hemorrhage subsequent to the administration of sildenafil.[39–41] However, it solely observed the phenomenon without conducting a comprehensive causal analysis. These unexpected AEs raise concerns regarding the impact of sildenafil on vascular integrity and the potential risk for severe cardiovascular events. Further investigation is necessary to elucidate the underlying mechanisms and assess the magnitude of the risk in individuals using sildenafil.

Our study has some inherent limitations that must be acknowledged. First, the voluntary nature of reporting to the FAERS database makes it susceptible to underreporting, resulting in potentially incomplete data. Less severe or common adverse events may be underrepresented, while more serious or rare events could be overreported. Second, subjectivity and reporter biases may influence the reporting process, potentially introducing reporting bias. Third, the FAERS reports vary in quality and level of detail. Some reports may lack sufficient granularity, which could hinder robust analysis and interpretation of the events.

5 | CONCLUSIONS

This study has revealed a considerable occurrence of acquired hemophilia, malignant melanoma, aortic dissection rupture, and intracranial artery dissection among the reported adverse events linked to sildenafil use. This implies a possible connection between sildenafil and these conditions that warrants further investigation. More research is needed to confirm these associations and examine potential safety issues that may have been previously missed regarding sildenafil.

AUTHOR CONTRIBUTIONS

Yan Wang and Bin Zhao designed the study. Yan Wang, Haiyan Yang and Bin Zhao performed data analysis. Zheng Wan and Bin Zhao wrote the draft. All members participated in discussion. All authors approved the final manuscript.

ACKNOWLEDGMENTS

This study was performed using the FDA Adverse Event Reporting System (FAERS) source that was provided by the FDA. The information, results, or interpretation of the current study do not represent any opinion of the FDA.

We thank Zhao's team (Official WeChat Account: SCIPhD) of Sheng Xin Zhu Shou for their suggestions and language editing of this paper. Natural Science Foundation of Fujian Province, Grant No.: 2022J05298; the Guangxi Medical University Youth Science Fund Project, Grant No.: GXMUYSF 202370; the Natural Science Foundation of Xiamen, China, Grant No.: 3502Z20227155; the self-funded Scientific Research Project of Health and Family Planning Commission of GuangXi Zhuang Autonomous Region, Grant No.: 20180513.

CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Zheng Wan https://orcid.org/0000-0001-8455-3478

REFERENCES

1. Shamloul R, Ghanem H. Erectile dysfunction. *Lancet*. 2013;381(9861):153-165. doi:10.1016/S0140-6736(12)60520-0
2. Muneer A, Kalsi J, Nazareth I, Arya M. Erectile dysfunction. *BMJ*. 2014;348:g129. doi:10.1136/bmj.g129
3. Mobley DF, Khera M, Baum N. Recent advances in the treatment of erectile dysfunction. *Postgrad Med J*. 2017;93:679-685. doi:10.1136/postgradmedj-2016-134073
4. Najari BB, Kashanian JA. Erectile dysfunction. *JAMA*. 2016;316(17):1838. doi:10.1001/jama.2016.12284
5. Andersson KE. PDE5 inhibitors—pharmacology and clinical applications 20 years after sildenafil discovery. *Br J Pharmacol*. 2018;175(13):2554-2565. doi:10.1111/bph.14205
6. Goldstein I, Burnett AL, Rosen RC, Park PW, Stecher VJ. The serendipitous story of sildenafil: an unexpected oral therapy for erectile dysfunction. *Sex Med Rev*. 2019;7(1):115-128. doi:10.1016/j.sxmr.2018.06.005
7. Goldstein I, Lue TF, Padma-Nathan H, Rosen RC, Steers WD, Wicker PA. Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group. *N Engl J Med*. 1998;338(20):1397-1404.
8. Chetan C, Suryawanshi P, Patnaik S, et al. Oral versus intravenous sildenafil for pulmonary hypertension in neonates: a randomized trial. *BMC Pediatr*. 2022;22(1):311. doi:10.1186/s12887-022-03366-3
9. Kaminetsky JC, Stecher V, Tseng L-J. Quality of erections by age group in men with erectile dysfunction. *Int J Clin Pract*. 2017;71(10). doi:10.1111/ijcp.12976
10. Meuleman E, Cuzin B, Opsomer RJ, et al. A dose-escalation study to assess the efficacy and safety of sildenafil citrate in men with erectile dysfunction. *BJU Int*. 2001;87(1):75-81.
11. Zusman RM, Morales A, Glasser DB, Osterloh IH. Overall cardiovascular profile of sildenafil citrate. *Am J Cardiol*. 1999;83(5A):35C-44C.
12. Swearingen SG, Klausner JD. Sildenafil use, sexual risk behavior, and risk for sexually transmitted diseases, including HIV infection. *Am J Med*. 2005;118(6):571-577.
13. Mathur R, Parmar A, Rajpurohit SS, Tundup T, Bhad R, Rao R. Sildenafil misuse for its psychoactive properties? A case report. *J Addict Med*. 2021;15(6):522-524. doi:10.1097/ADM.0000000000000805
14. Yu RJ, Krantz MS, Phillips EJ, Stone CA. Emerging causes of drug-induced anaphylaxis: a review of anaphylaxis-associated reports in the FDA Adverse Event Reporting System (FAERS). *J Allergy Clin Immunol Pract*. 2021;9(2):819-829. doi:10.1016/j.jaip.2020.09.021
15. Anand K, Ensor J, Trachtenberg B, Bernicker EH. Osimertinib-induced cardiotoxicity: a retrospective review of the FDA Adverse Events

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13533 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Reporting System (FAERS). *JACC CardioOncol*. 2019;1(2):172-178. doi:10.1016/j.jaccao.2019.10.006
16. Yang H, Wan Z, Chen M, Zhang X, Cui W, Zhao B. A real-world data analysis of topotecan in the FDA Adverse Event Reporting System (FAERS) database. *Expert Opin Drug Metab Toxicol*. 2023;19(4):217-223. doi:10.1080/17425255.2023.2219390
17. Rothman KJ, Lanes S, Sacks ST. The reporting odds ratio and its advantages over the proportional reporting ratio. *Pharmacoepidemiol Drug Saf*. 2004;13(8):519-523.
18. Evans SJ, Waller PC, Davis S. Use of proportional reporting ratios (PRRs) for signal generation from spontaneous adverse drug reaction reports. *Pharmacoepidemiol Drug Saf*. 2001;10(6):483-486.
19. Bate A, Lindquist M, Edwards IR, et al. A Bayesian neural network method for adverse drug reaction signal generation. *Eur J Clin Pharmacol*. 1998;54(4):315-321.
20. Simms KM, Kortepeter C, Avigan M. Lamotrigine and aseptic meningitis. *Neurology*. 2012;78(12):921-927. doi:10.1212/WNL.0b013e31824c4634
21. Bermejo J, Yotti R, García-Orta R, et al. Sildenafil for improving outcomes in patients with corrected valvular heart disease and persistent pulmonary hypertension: a multicenter, double-blind, randomized clinical trial. *Eur Heart J*. 2018;39(15):1255-1264. doi:10.1093/eurheartj/ehx700
22. Iratni R, Ayoub MA. Sildenafil in combination therapy against cancer: a literature review. *Curr Med Chem*. 2021;28(11):2248-2259. doi:10.2174/0929867327666200730165338
23. Arora S, Surakiatchanukul T, Arora T, Cagini C, Lupidi M, Chhablani J. Sildenafil in ophthalmology: an update. *Surv Ophthalmol*. 2022;67(2):463-487. doi:10.1016/j.survophthal.2021.06.004
24. Pierce CM, Zhang MH, Jonsson B, et al. Efficacy and safety of IV sildenafil in the treatment of newborn infants with, or at risk of, persistent pulmonary hypertension of the newborn (PPHN): a multicenter, randomized, placebo-controlled trial. *J Pediatr*. 2021;237:154-161. doi:10.1016/j.jpeds.2021.05.051
25. Imam SS, El-Farrash RA, Taha AS, Saleh GA. Milrinone versus sildenafil in treatment of neonatal persistent pulmonary hypertension: a randomized control trial. *J Cardiovasc Pharmacol*. 2022;80(5):746-752. doi:10.1097/FJC.0000000000001332
26. Graziano S, Montana A, Zaami S, et al. Sildenafil-associated hepatoxicity: a review of the literature. *Eur Rev Med Pharmacol Sci*. 2017;21(1):17-22. Suppl.
27. Foresta C, Caretta N, Zuccarello D, et al. Expression of the PDE5 enzyme on human retinal tissue: new aspects of PDE5 inhibitors ocular side effects. *Eye*. 2008;22(1):144-149.
28. Jägle H, Jägle C, Sérey L, et al. Visual short-term effects of Viagra: double-blind study in healthy young subjects. *Am J Ophthalmol*. 2004;137(5):842-849.
29. Coleman DJ, Lee W, Chang S, et al. Treatment of macular degeneration with sildenafil: results of a two-year trial. *Ophthalmologica*. 2018;240(1):45-54. doi:10.1159/000486105
30. Dhar R, Washington C, Diringer M, et al. Acute effect of intravenous sildenafil on cerebral blood flow in patients with vasospasm after subarachnoid hemorrhage. *Neurocrit Care*. 2016;25(2):201-204. doi:10.1007/s12028-016-0243-0
31. Kruse-Jarres R, Kempton CL, Baudo F, et al. Acquired hemophilia A: updated review of evidence and treatment guidance. *Am J Hematol*. 2017;92(7):695-705. doi:10.1002/ajh.24777
32. Philip A, Ramchandani S, Dorrance K, Dorrance C. Sildenafil-induced thrombocytopenia. *Ann Intern Med*. 2008;149(6):437-439.
33. Moran B, Silva R, Perry AS, Gallagher WM. Epigenetics of malignant melanoma. *Semin Cancer Biol*. 2018;51:80-88. doi:10.1016/j.semcancer.2017.10.006
34. Stehle D, Barresi M, Schulz J, Feil R. Heterogeneity of cGMP signalling in tumour cells and the tumour microenvironment: challenges and chances for cancer pharmacology and therapeutics. *Pharmacol Ther*. 2023;242:108337. doi:10.1016/j.pharmthera.2023.108337
35. Li W-Q, Qureshi AA, Robinson KC, Han J. Sildenafil use and increased risk of incident melanoma in US men: a prospective cohort study. *JAMA Intern Med*. 2014;174(6):964-970. doi:10.1001/jamainternmed.2014.594
36. Arozarena I, Sanchez-Laorden B, Packer L, et al. Oncogenic BRAF induces melanoma cell invasion by downregulating the cGMP-specific phosphodiesterase PDE5A. *Cancer Cell*. 2011;19(1):45-57. doi:10.1016/j.ccr.2010.10.029
37. Mussa FF, Horton JD, Moridzadeh R, Nicholson J, Trimarchi S, Eagle KA. Acute aortic dissection and intramural hematoma: a systematic review. *JAMA*. 2016;316(7):754-763. doi:10.1001/jama.2016.10026
38. Debette S, Compter A, Labeyrie M-A, et al. Epidemiology, pathophysiology, diagnosis, and management of intracranial artery dissection. *Lancet Neurol*. 2015;14(6):640-654. doi:10.1016/S1474-4422(15)00009-5
39. Hellstrom WJG. Visual field defect and intracerebral hemorrhage associated with use of vardenafil (Levitra). *Neurology*. 2006;66(2):293-294.
40. Alpsan MH, Bebek N, Ciftci FD, Coban O, Bahar S, Tuncay R. Intracerebral hemorrhage associated with sildenafil use: a case report. *J Neurol*. 2008;255(6):932-933. doi:10.1007/s00415-008-0618-z
41. Ayberk G, Ozveren MF, Yaman ME, Tosun H. Intracerebral hemorrhage after sildenafil citrate use: an incidental association? *Urol J*. 2014;11(2):1524-1526.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Wang Y, Zhao B, Yang H, Wan Z. A real-world pharmacovigilance study of FDA adverse event reporting system events for sildenafil. *Andrology*. 2024;12:785–792. https://doi.org/10.1111/andr.13533

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13533 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 26 June 2023 | Revised: 9 August 2023 | Accepted: 9 September 2023

DOI: 10.1111/andr.13534

ANDROLOGY

WILEY

ORIGINAL ARTICLE

Genetically proxied intestinal microbiota and risk of erectile dysfunction

Fuxun Zhang[1] | Yang Xiong[2] | Yangchang Zhang[3] | Kan Wu[2] | Bo Zhang[1]

[1]Department of Urology, Tangdu Hospital, Air Force Medical University, Xi'an, Shaanxi, China

[2]Department of Urology, Institute of Urology, West China Hospital, Sichuan University, Chengdu, Sichuan, China

[3]Department of Public Health, Capital Medical University, Beijing, China

Correspondence
Bo Zhang, Department of Urology, Tangdu Hospital, Air Force Medical University, Xi'an, Shaanxi, China.
Email: zhangbo_tduro@163.com, zhangbo@fmmu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81872077

Abstract

Background: The interaction between intestinal microbiota and erectile dysfunction (ED) is less investigated. This study was performed to explore the association between intestinal microbiota and ED.

Methods: In this two-sample Mendelian randomization (MR) study, genetic variants of gut microbiota were obtained from MiBioGen consortium containing 18,340 individuals. Six methods including inverse variance weighting (IVW), MR-Egger, weighted median, maximum likelihood, MR robust adjusted profile score, and MR pleiotropy residual sum and outlier were used to investigate the causal links between intestinal microbiota and ED. Furthermore, reverse MR analysis was performed to exclude the causal impact of ED on gut microbiota.

Results: As revealed by the IVW estimator, the risks of ED were raised by genetically proxied *Lachnospiraceae* (OR: 1.27), *Lachnospiraceae NC2004 group* (OR: 1.17), *Oscillibacter* (OR: 1.20), *Senegalimassilia* (OR: 1.32) (All $P < 0.05$) and *Tyzzerella-3* (OR: 1.14, $P < 0.05$). It was observed that *Ruminococcaceae UCG013* exerted protective effect against ED (OR: 0.77, $P < 0.05$). These results were consistent with other estimators in sensitivity analyses. In reverse MR analyses, genetic liability to ED did not alter the abundances of *Lachnospiraceae*, *Lachnospiraceae NC2004 group*, *Oscillibacter*, *Senegalimassilia*, *Tyzzerella-3*, and *Ruminococcaceae UCG013* (All $P > 0.05$). No heterogeneity and pleiotropy were detected by Cochran's Q-test, MR-Egger, and global test (All $P > 0.05$).

Conclusions: This study provided novel evidence that genetically proxied *Lachnospiraceae*, *Lachnospiraceae NC2004 group*, *Oscillibacter*, *Senegalimassilia*, *Tyzzerella-3*, and *Ruminococcaceae UCG013* had potentially causal effects on ED. Further studies are needed to clarify the biological mechanisms linking intestinal microbiota to ED.

KEYWORDS
causal association, erectile dysfunction, genome-wide association study, intestinal microbiota, Mendelian randomization

Fuxun Zhang and Yang Xiong contribute equally to this work.

© 2023 American Society of Andrology and European Academy of Andrology.

1 | BACKGROUND

Erectile dysfunction (ED) is defined as the inability to achieve or maintain sufficient erection for satisfactory sexual performance. This non-lethal condition is prevalent globally and seriously affects men's physical and psychological health.[1] According to the report from European Male Ageing Study (EMAS), the average prevalence of ED was 30% in men aged 40–79 years.[2] Another milestone study, Massachusetts Male Ageing Study (MMAS), revealed a prevalence of 52% in men aging from 40 to 70 years.[3] Meanwhile, the high prevalence of ED should not be attributed to aging exclusively. Several studies from different geographical regions reported a prevalence of ED ranging from 5% to 30% among men below 40 years.[4,5] ED is usually considered as a mixed organic and psychogenic nature, and associated with multiple risk factors such as aging, metabolic dysfunction, depression, and so on.[6] In all, many risk factors are modifiable and have been investigated for a long time. However, few studies focus on the role of gut microbiota in the onset of ED.

It has been well documented that human health and conditions can be maintained or mediated by microbial communities.[7] For example, aberrant composition and function of intestinal microbiota have been detected in metabolic or allergic diseases.[8,9] Moreover, in non-metabolic conditions such as cancers, intestinal microbiota can be considered as a risk or preventive element.[10] However, limited attention was paid on the role of gut microbiota in ED in the past. It has been reported that gut microbiota diversity of ED patients was significantly different with healthy controls.[11,12] Moreover, a cross-sectional study found that *Alistipes* and *Clostoridium XVIII* were associated with ED through sequencing technologies.[13] Additionally, metabolites of altered gut microbiota may participate in the development of ED through immune-inflammatory axis or endothelial injury.[14,15] Nonetheless, given the cross-sectional design and limited sample size of previous studies, the causal link between gut microbiota and ED still remains unclear and needs further investigation.

In recent years, Mendelian randomization (MR) has been extensively used to evaluate the causal association of intestinal microbiota with diseases, such as metabolic and cardiovascular diseases.[16] To control the biases from limited sample size and cross-sectional design, we used MR to investigate the role of intestinal microbiota in ED. MR is an epidemiological approach using single nucleotide polymorphisms (SNPs) as instrumental variables (IVs) to replace exposures (i.e., intestinal microbiota) and outcomes (i.e., ED).[17] SNPs are assigned randomly when forming zygote, which are not affected by postnatal confounding factors. Therefore, under MR framework, causal inference can be yielded. In this study, we used summary statistics from MiBioGen consortium and Medical Research Council Integrative Epidemiology Unit (MRC-IEU) to perform two-sample MR analysis, which provided novel evidence linking gut microbiota to ED.

2 | METHODS

2.1 | Data sources and selection of instrumental variable

Summary statistics of human gut microbiota were obtained from MiBioGen consortium.[15] This study included 18,340 individuals from 24 cohorts. The microbial composition and relative abundances of gut microbiota were detected using 16S ribosomal RNA gene sequencing. Microbiota quantitative trait loci mapping analysis was performed to identify genetic variants of the host and evaluate the association between human genetic variants and intestinal microbiota. In this study, the lowest taxonomic level was genus. Summary statistics of ED were retrieved from one previous genome wide association study by Bovijn et al.[18] This study included 223,805 male participants (6,175 ED cases and 217,630 controls) to identify the closely associated SNPs of ED. All the participants were from European descent.

The flowchart of this study is showed in Figure 1. To obtain adequate IVs and increase the statistical power, IVs were filtered from the identified SNPs at a genome-wide statistical significance of $P < 1 \times 10^{-5}$. The left SNPs were further pruned if the linkage disequilibrium r^2 were ≥ 0.01 at a window size of 10,000 kb. SNPs with minor allele frequency (MAF) < 0.01 are generally accepted as rare SNPs, which have limited impact on the traits. Therefore, only SNPs with MAF ≥ 0.01 were reserved.

2.2 | Statistical analysis

Six methods were used to investigate the effects of gut microbiota on ED, including inverse variance weighting (IVW), MR-Egger, weighted median, maximum likelihood (ML), MR robust adjusted profile score (MR.RAPS) and MR pleiotropy residual sum and outlier (MR-PRESSO). In IVW models, all the IVs are assumed as valid IVs and then combined using a meta-technique. This method is used as the main analyses. When heterogeneity existed, the random effects' IVW model was used; otherwise, fixed effects' IVW estimator was deemed as the main analysis. To produce unbiased results even when pleiotropy existed, MR-Egger and weighted median methods relax this precondition (have invalid IVs) and were used as sensitivity analyses. In addition, given the minimal bias in limited sample sizes, ML method was also used . The MR.RAPS method could produce consistent results when weak and pleiotropic SNPs exist. After excluding pleiotropic outliers, MR-PRESSO estimator combined the effects from IVs as the IVW model. This estimator could be used to detect the presence of pleiotropy.

Cochran *Q*-test was applied to evaluate the heterogeneity of IVs. *Q*-statistics with *P*-value < 0.05 indicated the presence of heterogeneity, and the random-effects' IVW method was used to generate more

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13534 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

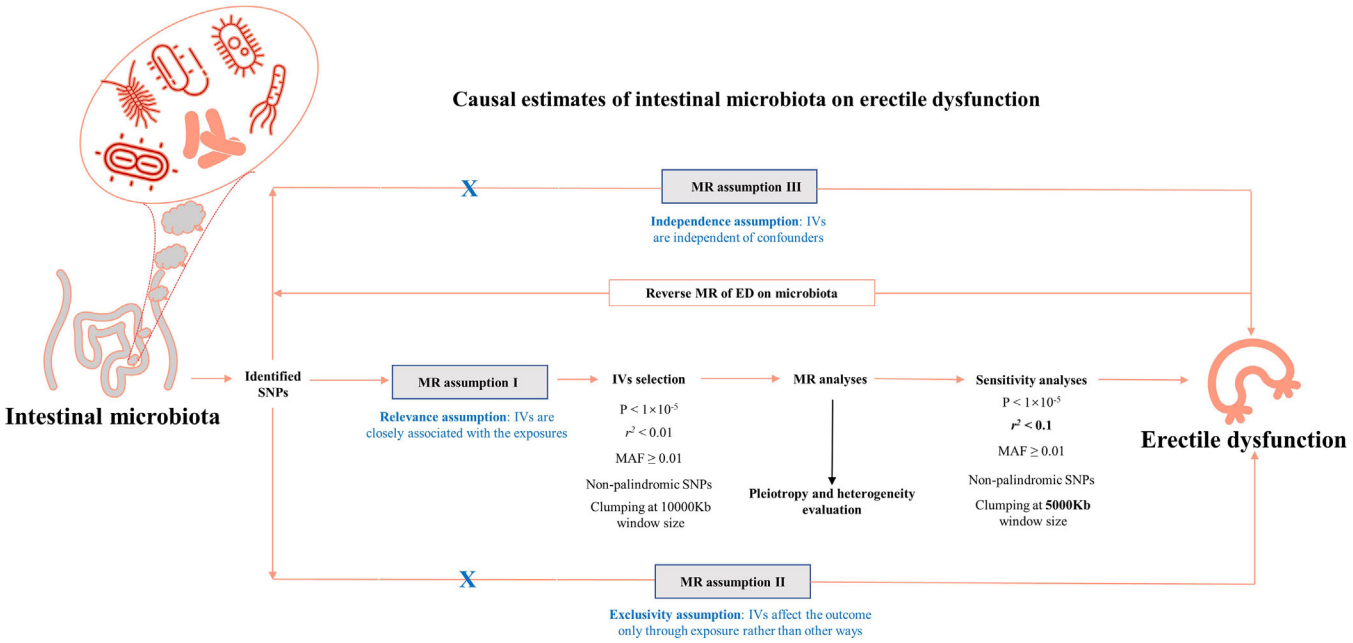


FIGURE 1 Study design and flow chart.
Abbreviations: ED, erectile dysfunction; IVs, instrumental variables; MR, Mendelian randomization; MAF, minor allele frequency; SNPs, single nucleotide polymorphisms.

conservative but robust estimates. To assess the horizontal pleiotropy, MR-Egger intercept term and global test from MR-PRESSO estimator were used. Furthermore, to verify the absence of pleiotropy and reverse causality, reverse MR analysis was conducted using ED as the exposure and intestinal microbiota as the outcome. The strength of SNPs was quantified via calculating *F*-statistics of each bacterial taxon as previously reported.[19] The *F*-statistic greater than 10 indicated less likelihood of weak instrumental bias.

All statistical analyses were conducted using R 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). "TwoSampleMR," "MR-PRESSO," and "MendelianRandomization" packages were used for data analyses. "forest" package was used to draw the forest plot.

3 | RESULTS

3.1 | Genetically proxied *lachnospiraceae* and *lachnospiraceae nc2004 group* increased the risk of erectile dysfunction

A total of 17 SNPs were used as IVs of *Lachnospiraceae* with an average *F*-statistic of 20.98 (Table 1). Genetically proxied *Lachnospiraceae* could increase the risk of ED as revealed by the IVW estimator (OR: 1.27, 95% CI: 1.05–1.52, $P = 0.012$) (Figure 2). This finding remained significant in different estimators (All $P < 0.05$), except the MR-Egger method. However, the direction and effect size of MR-Egger estimator were similar to other methods (OR: 1.50, 95% CI: 0.95–2.36, $P = 0.099$). The scatter plots displayed an increasing risk of ED with the increase of SNP effect on *Lachnospiraceae* (Figure 3A). No pleiotropy was identified by the global test and the MR-Egger method (both $P > 0.05$) (Table 1). Meanwhile, no heterogeneity was detected according to the funnel plot ($P > 0.05$) (Table 2 and Figure 4A).

Lachnospiraceae NC2004 group is a genus of *Lachnospiraceae* family. Similar to *Lachnospiraceae*, *Lachnospiraceae NC2004 group* could increase the risk of ED as revealed by the IVW method (OR: 1.17, 95% CI: 1.01–1.37, $P < 0.05$) (Figure 2). The direction and effect size of sensitivity analyses were in line with the IVW method (Figure 2). With the increase of the SNP effect on *Lachnospiraceae NC2004 group*, the effect of SNP on ED also ascended (Figure 3B). In all analyses, no pleiotropy and heterogeneity were identified (All $P > 0.05$) (Tables 1 and 2 and Figure 4B).

3.2 | Genetically proxied *oscillibacter* increased the risk of erectile dysfunction

Oscillibacter is a genus of *Oscillospiraceae* family. A total of 13 SNPs were used as IVs of *Oscillibacter* with an average *F*-statistic of 22.33 (Table 1). In line with the sensitivity analyses, IVW estimator revealed an increased risk of ED (OR: 1.20, 95% CI: 1.04–1.39, $P < 0.05$) (Figure 2). Genetically predicted *Oscillibacter* abundance was positively associated with the risk of ED (Figure 3C). There were no heterogeneity and pleiotropy in MR analyses, supporting the robustness of results (All $P > 0.05$) (Tables 1 and 2 and Figure 4C).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13534 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Strength and pleiotrophy of instrumental variables.

Family/Genus	Number of SNPs	*F*-statistics	*P* for Global test	Intercept for Egger regression	*P* for Egger
Lachnospiraceae	17	20.98	0.411	−0.0125	0.431
Lachnospiraceae NC2004 group	9	20.72	0.585	−0.0290	0.449
Oscillibacter	13	22.33	0.531	−0.0224	0.456
Ruminococcaceae UCG013	12	21.82	0.307	0.0278	0.251
Senegalimassilia	5	20.94	0.683	0.0005	0.990
Tyzzerella-3	13	22.84	0.558	0.0057	0.905

Abbreviations: ED, erectile dysfunction; MR, Mendelian randomization; SNPs, single nucleotide polymorphisms.

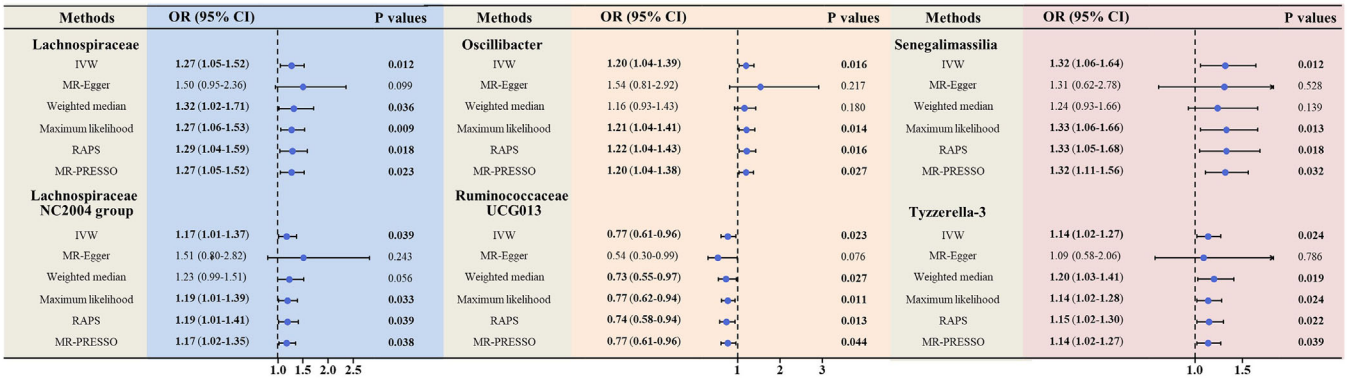


FIGURE 2 The results of MR estimating the causal association between intestinal microbiota and ED.
Abbreviations: CI, confidence interval; ED, erectile dysfunction; IVW, inverse variance weighted; MR, Mendelian randomization; OR, odds ratio; PRESSO, pleiotropy residual sum and outlier; RAPS, robust adjusted profile score.

TABLE 2 Detailed results for heterogeneity of instrumental variables.

	Q-values			*P*-values		
Family/Genus	MR Egger	IVW	ML	MR Egger	IVW	ML
Lachnospiraceae	16.535	17.256	17.001	0.347	0.369	0.386
Lachnospiraceae NC2004 group	6.509	7.151	6.995	0.482	0.520	0.537
Oscillibacter	10.476	11.073	10.849	0.488	0.523	0.542
Ruminococcaceae UCG013	12.101	13.899	13.595	0.278	0.239	0.256
Senegalimassilia	2.452	2.452	2.315	0.484	0.653	0.678
Tyzzerella-3	11.249	11.264	11.101	0.423	0.506	0.520

Abbreviations: IVW, inverse variance weighting; ML, maximum likelihood; MR: Mendelian randomization.

3.3 | Genetically proxied *Ruminococcaceae UCG013* decreased the risk of erectile dysfunction

Ruminococcaceae UCG013 is a genus of *Ruminococcaceae* family. There were 12 SNPs used as IVs of *Ruminococcaceae UCG013* with an average *F*-statistic of 21.82 (Table 1). Of note, this germ was the only genus that exhibited protective effect against ED in the gut microbiota. All the methods yielded consistent causal estimates (OR for IVW: 0.77; OR for MR-Egger: 0.54; OR for weighted median: 0.73; OR for ML: 0.77; OR for RAPS: 0.74; and OR for MR-PRESSO: 0.77) All *P* values were < 0.05 except MR-Egger estimator ($P = 0.076$). The marginal significance of MR-Egger may be attributed to the relatively low statistical power of this method for valid IVs. Genetically proxied *Ruminococcaceae UCG013* abundance was negatively associated with the risk of ED (Figure 3D). No heterogeneity and pleiotropy for IVs were detected (All $P > 0.05$) (Tables 1 and 2 and Figure 4D).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13534 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

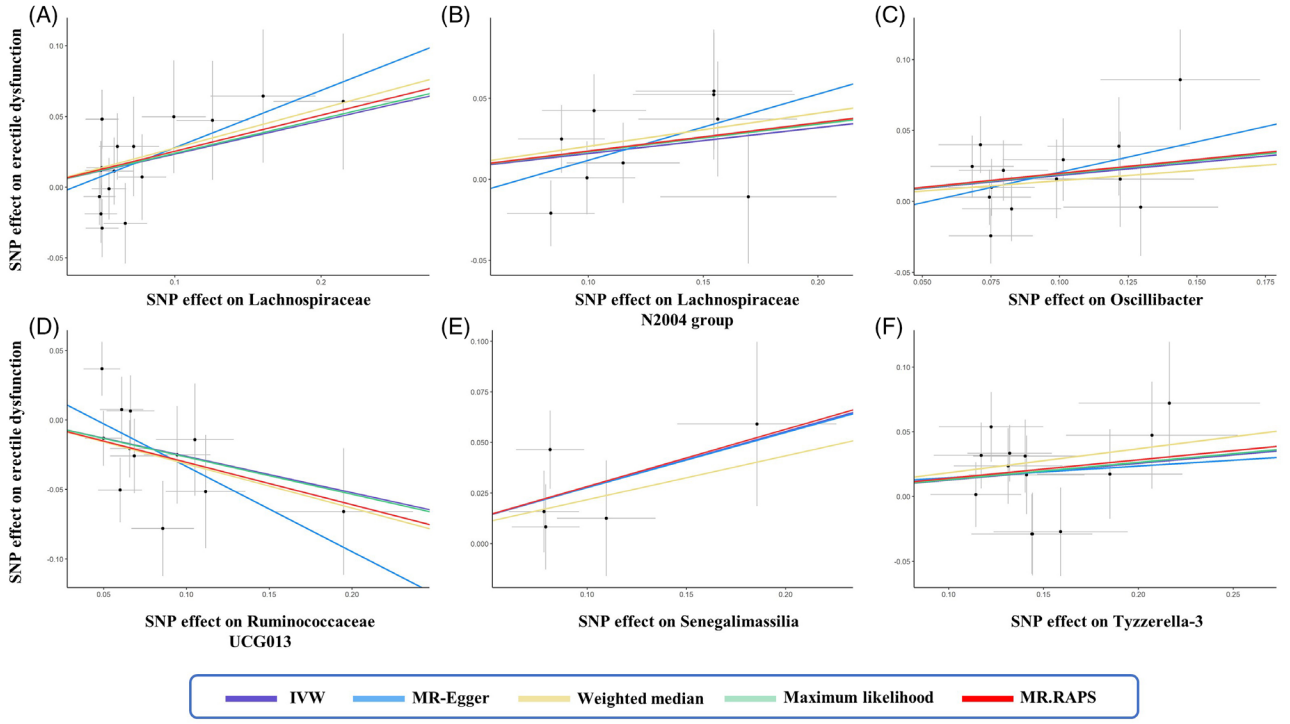


FIGURE 3 Scatter plots for the causal effects of gut microbiota on ED.
Abbreviations: ED, erectile dysfunction; IVW, inverse variance weighted; RAPS: robust adjusted profile score.

3.4 | Genetically proxied *senegalimassilia* increased the risk of erectile dysfunction

Senegalimassilia is a genus of *Coriobacteriaceae* family. A total of five SNPs were used as IVs of *Senegalimassilia* with an average *F*-statistic of 20.94 (Table 1). IVW estimator disclosed a causality of *Senegalimassilia* for ED (OR: 1.32, 95% CI: 1.06–1.64, $P < 0.05$) (Figure 2). The direction and effect size of causal estimates were accordant in sensitivity analyses. The scatter plots showed that with the increment of SNP effect on *Senegalimassilia* abundance, the SNP effect on ED also elevated (Figure 3E). No heterogeneity and pleiotropy for IVs were detected (All $P > 0.05$ in Tables 1 and 2 and Figure 4E).

3.5 | Genetically proxied *tyzzerella-3* increased the risk of erectile dysfunction

There were 13 SNPs used as IVs of *Tyzzerella-3* with an average *F*-statistic of 22.84 (Table 1). A 1.14-fold risk of ED (OR: 1.14, 95% CI: 1.02-1.27, $P < 0.05$) was detected for genetically proxied *Tyzzerella-3* by the IVW method (Figure 2). This finding was also accordant with the results of sensitivity analyses. In Figure 3F, genetically predicted *Tyzzerella-3* abundance was positively associated with the risk of ED. No heterogeneity and pleiotropy were detected (All $P > 0.05$) (Tables 1 and 2 and Figure 4F).

3.6 | Reverse Mendelian randomization analyses

To further exclude reverse causality, we extracted IVs from ED genome-wide association study (GWAS) as an exposure to explore the influence of ED on intestinal microbiota (Figure 5). Due to only one SNP (rs57989773) was extracted, the Wald ratio method was used to calculate the ORs. Reverse MR analyses did not support the causal effects of genetically proxied ED on the identified six germs. The ORs were 1.12 (95% CI: 0.98–1.59, $P = 0.101$) for *Lachnospiraceae*, 0.90 (95% CI: 0.71–1.14, $P = 0.370$) for *Lachnospiraceae NC2004 group*, 1.13 (95% CI: 0.93–1.38, $P = 0.229$) for *Oscillibacter*, 1.02 (95% CI: 0.88–1.18, $P = 0.795$) for *Ruminococcaceae UCG013*, 1.07 (95% CI: 0.86–1.33, $P = 0.558$) for *Senegalimassilia*, and 1.05 (95% CI: 0.81–1.37, $P = 0.701$) for *Tyzzerella-3*, respectively.

4 | DISCUSSION

As the second genome of human beings, the intestinal microbiota plays an important role in the onset and progress of various diseases.[20] The gut microbiota might participate the development of diseases by generating microbial metabolites or activating downstream signaling pathways which ultimately change cellular physiology.[21] Currently, several risk factors of ED have been recognized and described.[22] For instance, diabetes and low levels of testosterone may deteriorate erectile performance through different mechanisms, including the

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13534 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

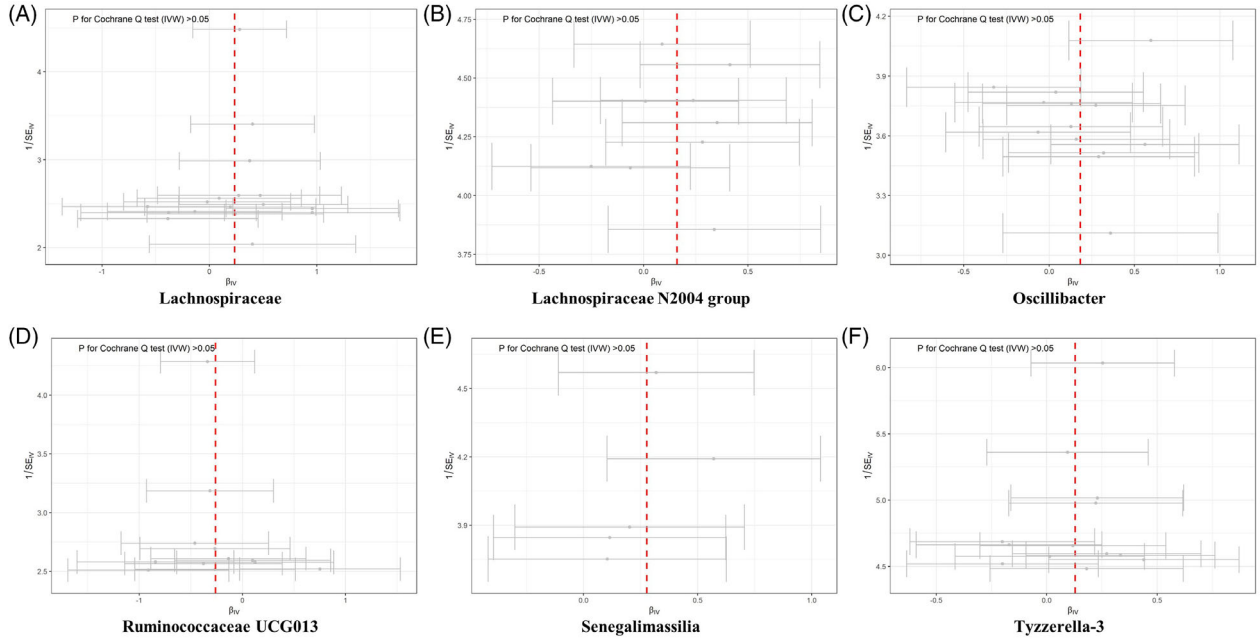


FIGURE 4 Funnel plots for the heterogeneity of instrumental variables.
Abbreviations: IVW, inverse variance weighted; SE, size effect.

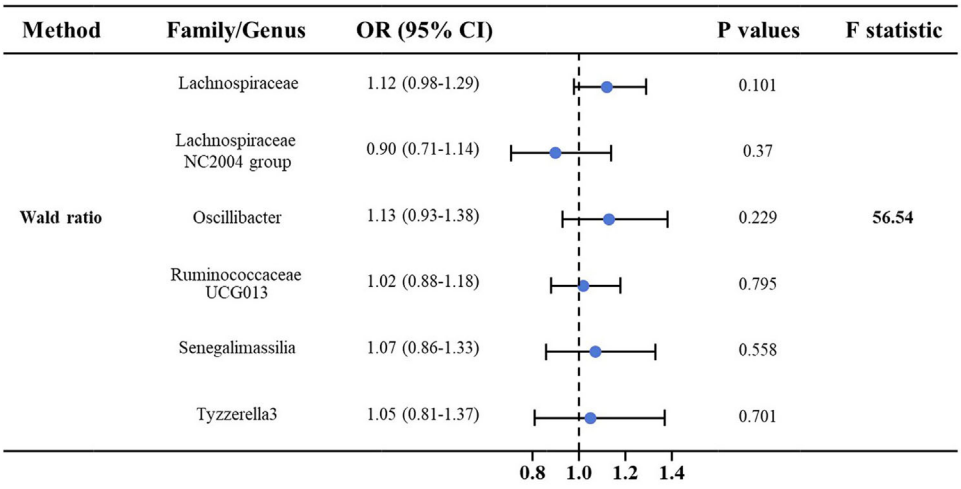


FIGURE 5 Reverse MR for the causal effects of ED on gut microbiota.
Abbreviations: ED, erectile dysfunction; MR, Mendelian randomization.

endothelial impairment of corpus cavernosum, aberrant expression and activation of phospho-diesterase type 5, and so on.[23,24] However, few studies have investigated the causal association between gut microbiota and ED.

Recently, a pilot study detected significantly altered composition of intestinal microbiota in ED patients comparing with health controls, and found that *Actinomyces* as a key pathogenic bacteria was correlated with ED.[12] Nevertheless, given the limitation of pilot design and small-sample size, this study did not revealed the causality between gut microbiota and ED. In our study, genetic data were used to explore the causal association between intestinal microbiota and ED, avoiding the bias from confounding factors. Our analysis revealed that *Lachnospiraceae*, *Lachnospiraceae NC2004 group*, *Oscillibacter*, *Senegalimassilia*, and *Tyzzerella-3* were causally associated with an increased risk of ED. Conversely, the protective effect of *Ruminococcaceae UCG013* on erectile function was also identified. These results provide novel evidence of gut microbiota on the diagnose or treatment of ED.

Lachnospiraceae can be found in the meconium of early infants, and increased abundance of *Lachnospiraceae* in the intestinal lumen was detected to be associated with several different diseases. For

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13534 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

instance, *Blautia* and *Roseburia*, the main genera of *Lachnospiraceae*, are involved in the control of atherosclerosis and maturation of the immune system, which might be mediated by the end products of *Lachnospiraceae* metabolism, toll-like receptor and arachidonic acid-dependent signaling pathway.[25,26] Meanwhile, *Lachnospiraceae* family appears to be correlated with inflammatory conditions such as inflammatory bowel disease (IBD), and chronic kidney disease (CKD), in which nuclear factor kappa-B (NF-κB) signaling is involved.[27] In this study, we found that *Lachnospiraceae* and *Lachnospiraceae N2004 group* might be causally associated with ED. Thus, we hypothesized that the dysbiosis of *Lachnospiraceae* may induce low-grade systemic inflammation through toll-like receptor, arachidonic acid or NF-κB signaling, consequently harming the vascular system and then triggering ED.

Although genus *Oscillibacter* was identified many years ago, the infection of *Oscillibacter* in human was rarely described.[28] As in a previous study explored the relationship between gut microbiome and blood metabolites, increased abundances of fecal *Oscillibacter* showed causality with decreased concentration of triglyceride, which seems to be beneficial to the erectile function.[29] However, it is established that the metabolic end product of *Oscillibacter* is homologous to the neurotransmitter γ-aminobutyric acid (GABA), and one clade consisting of five Operational Taxonomic Units within *Oscillibacter* showed a significant association with depression.[30] Meanwhile, high abundance of genus *Oscillibacter* has been found in patients with depression.[31] Thus, *Oscillibacter* may incur depressive disorders and subsequently aggravate ED, which might be mediated by neurotransmitter-like metabolite of *Oscillibacter* and GABA signaling pathway in the central nervous system.

Ruminococcaceae, belong to *Firmicutes*, canproduce several short-chain fatty acids (SCFAs) by hydrolyzing starch or sugars.[32] It is reported that SCFAs are major source of nutrition for epithelial cells in colon, and are conducive to improve the epigenetic states of histone and decrease the expression of inflammatory markers in the hosts.[33] Meanwhile, SCFAs produced by *Ruminococcaceae* may mediate the proliferation of intestinal stem cells through activating the Wnt/β-catenin signaling pathway, suggesting the possibility of *Ruminococcaceae* as a preventative agent for inflammatory diseases.[34] On the other hand, the effects of fatty acids on the erectile function are complicated, as well as the deranged metabolism of fatty acids may represent a marker of vasogenic ED.[35,36] Our results demonstrated the protective effect of *Ruminococcaceae UCG013* against ED, which may attribute to the role of SCFAs in the inhibition of inflammatory response and the regulation of fatty acids metabolism.

Genus *Senegalimassilia* were reported to be significantly associated with the production of enterolactone.[37] Meanwhile, *Senegalimassilia* might be a protective factor for hypertension as previous study reported.[38] However, *Senegalimassilia* was causally linked with ED in our study, which is inconsistent with the results of this study. Due to little information of *Senegalimassilia* was disclosed, more evidence of causal association between *Senegalimassilia* and ED development is still needed. Moreover, we found that *Tyzzerella-3* was a risk factor for ED in this MR study. Previous evidence showed that genus *Tyzzerella* of *Firmicutes* was associated with incident diabetes and abundant production of aromatic amines.[8,39] Meanwhile, it is reported that lower levels of *Tyzzerella-3* were associated with acute myocardial infarction, in which the SCFAs pathway was involved.[40] Thus, it seems that dysbiosis of *Tyzzerella-3* may mediate the endocrine or endothelial dysfunction through the SCFAs' pathway, which produce adverse effects on the erectile function.

Several limitations in our study should be noted for interpreting our results. First, the original dataset collected data from 24 multi-ethnic cohorts in order to expand the sample size to 18,340 individuals. Although we have adopted this biggest dataset of gut microbiome to date, the sample size still needs to be enlarged, which could further strengthen the statistical power. Second, the multi-ethnic genetic background may generate bias into the genetic association between gut microbiota and host genetic variations. Therefore, further GWAS study with larger sample sizes, simpler ethnic background, and unified sequencing techniques should be considered. Third, due to the usage of summary-level data, the nonlinear association between the germs' abundances and ED risk cannot be explored. Fourth, our results were not adjusted for documented risk factors of ED, such as metabolic conditions and testosterone abnormality. Additionally, we did not stratify outcome as psychogenic, vasculogenic, and other etiology in analysis. All those limitations should be evaluated and addressed in future studies.

5 | CONCLUSIONS

In this two-sample MR study, we found that genetically proxied *Lachnospiraceae*, *Lachnospiraceae NC2004 group*, *Oscillibacter*, *Senegalimassilia*, *Tyzzerella-3*, and *Ruminococcaceae UCG013* had potentially causal effects on ED. Further studies are needed to investigate the biological mechanisms linking gut microbiota to the ED.

AUTHOR CONTRIBUTIONS

Conception and design: FZ, YX, BZ. *Administrative support*: BZ. *Provision of study materials or patients*: FZ, YX, KW, YZ. *Collection and assembly of data*: FZ, YX, KW. *Data analysis and interpretation*: FZ, YX, YZ. *Manuscript writing*: FZ, YX. *Final approval of manuscript*: FZ, YX, YZ, KW, BZ.

ACKNOWLEDGMENTS

All authors appreciate the participants and investigators of the FinnGen study. The authors also are grateful to the MiBioGen consortium for releasing the GWAS summary statistics of intestinal microbiota, the National Natural Science Foundation of China, Grant No.: 81872077

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13534 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

ORCID

Bo Zhang https://orcid.org/0009-0004-2979-1683

REFERENCES

1. Yafi FA, Jenkins L, Albersen M, et al. Erectile dysfunction. *Nat Rev Dis Primers*. 2016;2:16003.
2. Corona G, Lee DM, Forti G, et al. Age-related changes in general and sexual health in middle-aged and older men: results from the European Male Ageing Study (EMAS). *J Sex Med*. 2010;7(4):1362-1380. Pt 1.
3. Feldman HA, Goldstein I, Hatzichristou DG, et al. Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. *J Urol*. 1994;151(1):54-61.
4. Mialon A, Berchtold A, Michaud PA, et al. Sexual dysfunctions among young men: prevalence and associated factors. *J Adolesc Health*. 2012;51(1):25-31.
5. Rastrelli G, Maggi M. Erectile dysfunction in fit and healthy young men: psychological or pathological? *Transl Androl Urol*. 2017;6(1):79-90.
6. Shamloul R, Ghanem H. Erectile dysfunction. *Lancet*. 2013;381(9861):153-165.
7. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. *N Engl J Med*. 2016;375(24):2369-2379.
8. Ruuskanen MO, Erawijantari PP, Havulinna AS, et al. Gut microbiome composition is predictive of incident type 2 diabetes in a population cohort of 5,572 Finnish adults. *Diab Care*. 2022;45(4):811-818.
9. Xiong Y, Hu S, Zhou H, et al. High-throughput 16S rDNA sequencing of the pulmonary microbiome of rats with allergic asthma. *Genes Dis*. 2019;7(2):272-282.
10. Long Y, Tang L, Zhou Y, et al. Causal relationship between gut microbiota and cancers: a two-sample Mendelian randomisation study. *BMC Med*. 2023;21(1):66.
11. Geng Q, Chen S, Sun Y, et al. Correlation between gut microbiota diversity and psychogenic erectile dysfunction. *Transl Androl Urol*. 2021;10(12):4412-4421.
12. Kang J, Wang Q, Wang S, et al. Characteristics of gut microbiota in patients with erectile dysfunction: a Chinese pilot study. *World J Mens Health*. 2023; Epub ahead of print.
13. Okamoto T, Hatakeyama S, Imai A, et al. The association between gut microbiome and erectile dysfunction: a community-based cross-sectional study in Japan. *Int Urol Nephrol*. 2020;52(8):1421-1428.
14. Andersen ML, Gozal D, Pires GN, et al. Exploring the potential relationships among obstructive sleep apnea, erectile dysfunction, and gut microbiota: a narrative review. *Sex Med Rev*. 2023; Epub ahead of print.
15. Wang Y, Xie Z. Exploring the role of gut microbiome in male reproduction. *Andrology*. 2022;10(3):441-450.
16. Ai S, Zhang J, Zhao G, et al. Causal associations of short and long sleep durations with 12 cardiovascular diseases: linear and nonlinear Mendelian randomization analyses in UK Biobank. *Eur Heart J*. 2021;42(34):3349-3357.
17. Greenland S. An introduction to instrumental variables for epidemiologists. *Int J Epidemiol*. 2000;29(4):722-729.
18. Bovijn J, Jackson L, Censin J, et al. GWAS identifies risk locus for erectile dysfunction and implicates hypothalamic neurobiology and diabetes in etiology. *Am J Hum Genet*. 2019;104(2):157-163.
19. Xiong Y, Zhang F, Zhang Y, et al. Insights into modifiable risk factors of erectile dysfunction, a wide-angled Mendelian Randomization study. *J Adv Res*. 2023; Epub ahead of print.
20. Human Microbiome Jumpstart Reference Strains Consortium, KE Nelson, Weinstock GM, Highlander SK, et al, Human Microbiome Jumpstart Reference Strains Consortium. A catalog of reference genomes from the human microbiome. *Science*. 2010;328(5981):994-999.
21. Parker A, Romano S, Ansorge R, et al. Fecal microbiota transfer between young and aged mice reverses hallmarks of the aging gut, eye, and brain. *Microbiome*. 2022;10(1):68.
22. Corona G, Cucinotta D, Di Lorenzo G, et al. The Italian Society of Andrology and Sexual Medicine (SIAMS), along with ten other Italian Scientific Societies, guidelines on the diagnosis and management of erectile dysfunction. *J Endocrinol Invest*. 2023;46(6):1241-1274.
23. Cignarelli A, Genchi VA, D'Oria R, et al. Role of glucose-lowering medications in erectile dysfunction. *J Clin Med*. 2021;10(11):2501.
24. Morelli A, Filippi S, Mancina R, et al. Androgens regulate phosphodiesterase type 5 expression and functional activity in corpora cavernosa. *Endocrinology*. 2004;145(5):2253-2263.
25. Kasahara K, Krautkramer KA, Org E, et al. interactions between Roseburia intestinalis and diet modulate atherogenesis in a murine model. *Nat Microbiol*. 2018;3(12):1461-1471.
26. Wang Y, Spatz M, Da Costa G, et al. Deletion of both Dectin-1 and Dectin-2 affects the bacterial but not fungal gut microbiota and susceptibility to colitis in mice. *Microbiome*. 2022;10(1):91.
27. Vacca M, Celano G, Calabrese FM, et al. The controversial role of human gut Lachnospiraceae. *Microorganisms*. 2020;8(4):573.
28. Sydenham TV, Arpi M, Klein K, et al. Four cases of bacteremia caused by Oscillibacter ruminantium, a newly described species. *J Clin Microbiol*. 2014;52(4):1304-1307.
29. Liu X, Tong X, Zou Y, et al. Mendelian randomization analyses support causal relationships between blood metabolites and the gut microbiome. *Nat Genet*. 2022;54(1):52-61.
30. Naseribafrouei A, Hestad K, Avershina E, et al. Correlation between the human fecal microbiota and depression. *Neurogastroenterol Motil*. 2014;26(8):1155-1162.
31. Barandouzi ZA, Starkweather AR, Henderson WA, et al. Altered composition of gut microbiota in depression: a systematic review. *Front Psychiatry*. 2020;11:541.
32. D'Amato A, Di Cesare Mannelli L, Lucarini E, et al. Faecal microbiota transplant from aged donor mice affects spatial learning and memory via modulating hippocampal synaptic plasticity- and neurotransmission-related proteins in young recipients. *Microbiome*. 2020;8(1):140.
33. Sun M, Wu W, Liu Z, et al. Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. *J Gastroenterol*. 2017;52(1):1-8.
34. Xie J, Li LF, Dai TY, et al. Short-chain fatty acids produced by Ruminococcaceae mediate α-linolenic acid promote intestinal stem cells proliferation. *Mol Nutr Food Res*. 2022;66(1):e2100408.
35. Ben Khedher MR, Bouhajja H, Haj Ahmed S, et al. Role of disturbed fatty acids metabolism in the pathophysiology of diabetic erectile dysfunction. *Lipids Health Dis*. 2017;16(1):241.
36. Olabiyi AA, Ajayi K. Diet, herbs and erectile function: a good friendship!. *Andrologia*. 2022;54(6):e14424.
37. McCann SE, Hullar MAJ, Tritchler DL, et al. Enterolignan production in a flaxseed intervention study in postmenopausal US women of African ancestry and European ancestry. *Nutrients*. 2021;13(3):919.
38. Li Y, Fu R, Li R, et al. Causality of gut microbiome and hypertension: a bidirectional Mendelian randomization study. *Front Cardiovasc Med*. 2023;10:1167346.
39. Sugiyama Y, Mori Y, Nara M, et al. Gut bacterial aromatic amine production: aromatic amino acid decarboxylase and its effects on peripheral serotonin production. *Gut Microbes*. 2022;14(1):2128605.
40. Han Y, Gong Z, Sun G, et al. Dysbiosis of gut microbiota in patients with acute myocardial infarction. *Front Microbiol*. 2021;12:680101.

How to cite this article: Zhang F, Xiong Y, Zhang Y, Wu K, Zhang B. Genetically proxied intestinal microbiota and risk of erectile dysfunction. *Andrology*. 2024;12:793–800. https://doi.org/10.1111/andr.13534

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13534 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 27 April 2023 | Revised: 18 July 2023 | Accepted: 28 August 2023

DOI: 10.1111/andr.13524

ANDROLOGY WILEY

ORIGINAL ARTICLE

Prevalence, lifestyle, and risk factors of erectile dysfunction, premature ejaculation, and low libido in middle-aged men: first results of the Bavarian Men's Health-Study

Kathleen Herkommer[1] | Valentin H. Meissner[1] | Andreas Dinkel[2] | Matthias Jahnen[1] | Stefan Schiele[1] | Martina Kron[3] | Donna P. Ankerst[4] | Jürgen E. Gschwend[1]

[1]Department of Urology, School of Medicine, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany

[2]Department of Psychosomatic Medicine and Psychotherapy, School of Medicine, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany

[3]Institute of Epidemiology and Medical Biometrics, University of Ulm, Ulm, Germany

[4]Departments of Mathematics and Life Science Systems, Munich Data Science Institute, Technical University of Munich, Garching, Germany

Correspondence
Kathleen Herkommer, Department of Urology, Klinikum rechts der Isar, Technical University of Munich, Ismaninger Str. 22, 81675 Munich, Germany.
Email: kathleen.herkommer@tum.de

Abstract

Background: Erectile dysfunction (ED), premature ejaculation (PE), and low libido (LL) are reported as the most common male sexual dysfunctions.

Objective: To evaluate the prevalence of ED, PE, and LL and associations with lifestyle risk factors and comorbidities in middle-aged men.

Materials and methods: This study included a population-based random sample of 2500 50-year-old men who completed validated questionnaires, including the International Index of Erectile Function, the Erection Hardness Score, the Sexual Complaints Screener, and further questionnaires. Multiple logistic regression of outcomes ED, PE, and LL was used to model the association with explanatory factors.

Results: The prevalence of at least one sexual dysfunction was 30%. 21%, 5.2%, and 7.2% of men had ED, PE, and LL, respectively. The risk of ED increased with PE (odds ratio [OR]: 1.94, 95% confidence interval [95%CI]: 1.22–3.08), LL (OR: 2.04, 95%CI: 1.26–3.29), higher waist circumference (OR: 2.23, 95%CI: 1.67–2.96), and lower urinary tract symptoms (LUTS) (OR: 1.88, 95%CI: 1.39–2.55), partnership was associated with a lower risk (OR: 0.57, 95%CI: 0.39–0.85). The risk of PE increased with ED (OR: 1.94, 95%CI: 1.23–3.07), partnership (OR:5.42, 95%CI: 1.30–22.60), depression (OR: 2.37, 95%CI: 1.09–5.14), and LUTS (OR: 2.42, 95%CI: 1.52–3.87), and decreased with physical activity (OR: 0.44, 95%CI: 0.21–0.93). The risk of LL increased with ED (OR: 2.09, 95%CI: 1.31–3.34) and poorer self-rated health (OR: 2.97, 95%CI: 1.54–5.71).

Discussion and conclusions: Roughly one in three 50-year-old men experience some form of sexual dysfunction and risk factors identified in this study underline the multifactorial nature of ED, PE, and LL. Many risk factors are modifiable which underlines the role of patient education. Modifiable risk factors should be addressed in patient education and men should take active measures to remove the risk posed by these factors.

Kathleen Herkommer and Valentin H. Meissner contributed equally.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2023 The Authors. *Andrology* published by Wiley Periodicals LLC on behalf of American Society of *Andrology* and European Academy of *Andrology*.

KEYWORDS
BMH-Study, erectile dysfunction, low libido, premature ejaculation, risk factors

1 | INTRODUCTION

Erectile dysfunction (ED), premature ejaculation (PE), and low libido (LL) are frequently reported as the most common male sexual dysfunctions observed in the general population.[1–3]

ED is defined as the inability to achieve or maintain an erection sufficient for satisfactory sexual performance.[4] It can be estimated that the prevalence, although presenting a great deal of variation according to the age of the population studied and the diagnostic tool used, ranges from 15% to 50% in middle-aged men in the general population.[4–6] The most often used diagnostic tool is the International Index of Erectile Function-Erectile Function (IIEF-EF) domain.[7] However, the IIEF-EF domain does not capture men without sexual intercourse, few sexual attempts or non-heterosexual orientation identity. Therefore, a considerable number of men are not captured by this tool. To capture all men independent of their sexual orientation identity and sexual activity further additional tools such as the Erection Hardness Score (EHS)[8] could be used.

PE is a three-dimensional condition that includes (I) short intravaginal ejaculation latency time (IELT) within about one minute (lifelong PE) or within about 3 min (acquired PE), (II) inability to delay ejaculation, and (III) personal distress and/or avoidance of sexual intimacy.[9] Next to ED, PE is the most common type of male sexual dysfunction, with a prevalence rate ranging from 3.4% to 31% in men aged 40—80 years.[10–12]

LL is defined as a "deficiency or absence of sexual fantasies or desire for sexual activity" and additionally requires "marked distress or interpersonal difficulty".[13] A Danish population-based study published in 2007 revealed that the range of men reporting LL varied, depending on age, between 2.3% and 7.7%.[14] A large German population-based survey showed similar results with a prevalence rate between 0.5% and 4.2% in men aged 41—50 years.[2]

The causes of the aforementioned three male sexual dysfunctions are physical, psychological, or a combination of these factors. They occur most often due to comorbidities (diabetes, hypertension, endocrine alterations, urogenital tumors, urinary infections, incontinence, and surgical damage to nerves and organs), psychological problems (depression and anxiety), and psychoactive and antihypertensive drug use.[15] Lifestyle (smoking, alcohol abuse, inactivity, and obesity) and sociodemographic factors (age, income, education, and employment status) may also be associated with male sexual dysfunction.[16,17] Sexual dysfunction can impact the quality of life of affected men and their partners.[18] Given the frequent concomitant occurrence of ED, PE, and LL and their burden on affected men, it is important to detect their prevalence and associations with potential modifiable lifestyle risk factors and comorbidities in middle-aged men. While there is some evidence regarding the interplay between ED and PE,[3] little is known about interactions between all these three aforementioned and most common sexual dysfunctions, especially in population-based samples of middle-aged men.

Thus, the objectives of this analysis were first, to investigate the prevalence of ED, PE, and LL in a large community-based sample of 50-year-old men, and secondly, to determine the association between these sexual dysfunctions and lifestyle, comorbidities, and other potentially modifiable risk factors.

2 | MATERIALS AND METHODS

2.1 | Bavarian Men's Health Study

The Bavarian Men's Health Study (BMH-Study) is an ongoing study that focuses on various aspects of male physical, mental, and sexual health in a large, population-based random sample of 50-year-old Bavarian men concomitantly participating in a prostate cancer screening trial. Men were invited from the local population registers within a radius of 100 km around the study center by using simple random sampling. A short postal inquiry and invitation to participate in the trial were sent. Address information was provided by local registration offices. To obtain a representative overview of the population, we decided to include all participants without applying any exclusion criteria. The study protocol was reviewed and approved by the ethical review committee of the Technical University of Munich. All participants are informed about study procedures and provide written informed consent. At three time-points, comprising study entry, 5 and 10 years afterward, participants visit the clinical center for an interview and brief physical examination, where they complete standardized questionnaires. The report here contains the cross-sectional results of the baseline measures for men enrolled between April 2020 and April 2021.

3 | PARTICIPANTS AND MEASURES

3.1 | Sociodemographic, lifestyle, and psychosocial factors, comorbidities, and sexual behavior

A self-report sheet was used to assess sociodemographic variables, including being in a partnership, duration of the partnership, living with the partner, having children, level of education, employment status, and self-perceived economic situation. Lifestyle factors included among others, smoking, alcohol consumption, physical activity, and waist circumference (in cm, measured by an instructed study physician). Lower urinary tract symptoms (LUTS) were assessed with the

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13524 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

validated German version of the International Prostate Symptom Score (IPSS).[19] All further comorbidities (hypertension, diabetes mellitus, dyslipidemia, depression, etc.) and current medication were assessed in the clinical interview. Self-rated health was measured with the first question of the Short Form-36 Health Survey.[20] Anxiety and depression were assessed using the German version of the two-question screening tool Generalized Anxiety Disorder-2 and the Patient Health Questionnaire-2.[21] Both measures are rated on a 4-point Likert scale (0–3). A summary score ≥3 indicates clinical levels of anxiety and depression, respectively. These measures have proven reliable and valid in previous studies.[21] Sexual behavior included among others, the six items: sexual orientation identity, partnered sexual activity/solo masturbation within the past 3 months, the importance of sexuality, sexual satisfaction, and number of lifetime sexual partners. Details of group categorizations are provided in Table 1.

3.2 | Erectile dysfunction

The presence of ED was assessed using the German version of the IIEF-EF[7] and the Erection Hardness Score (EHS).[8] Severity of ED was classified as mild (IIEF-EF score 22—25), mild to moderate (IIEF-EF score 17—21), moderate (IIEF-EF score 11—16), or severe (IIEF-EF score 6—10). Since men with failed sexual intercourse, few sexual attempts, or non-heterosexual orientation identity are not suitable to be evaluated by the IIEF-EF domain the EHS was additionally asked.[22] The EHS is a validated single-item patient-reported outcome (PRO) on the hardness of erection ranging from 0 (penis does not enlarge) to 4 (completely hard and fully rigid). The presence of ED was defined as an IIEF-EF score ≤25 or an EHS score ≤3.[7,8]

3.3 | PE and delayed ejaculation

PE and delayed ejaculation (DE) were assessed using the Sexual Complaints Screener for Men (SCS-M),[23] an evidence-based screening tool estimating men's sexual complaints during the past 6 months. The SCS-M was developed based on expert opinion to assess sexual problems in a medical practice setting, with its validity and reliability recently established for Turkish men.[24] Participants were classified into three groups according to the International Society for Sexual Medicine Ad Hoc Committee for the Definition of Premature Ejaculation[9] (no PE vs. lifelong/acquired PE vs. variable/subjective PE) and into two groups for DE (no DE vs. DE). The screening measure comprised the following two items.

(I) "Some men cannot control their sexual excitement so that they cum (ejaculate) before or shortly (within approximately 2 min) after penetration. Has this happened to you during the last 6 months?" Response options were "no sexual activity"; "never/almost never"; "rarely"; "sometimes"; "often"; "almost all the time/almost always".

(II) "Has this been a personal problem for you?" Here, response options were "not at all a problem"; "a very small problem"; "some problem"; "a considerable problem"; "a very great problem".

TABLE 1 Baseline characteristics of the study sample ($n = 2500$). The numbers indicated are among the completed entries and not always adding up to the total sample size. Percent refers to the observed data and does not include missing data.

	n	%
Sexual dysfunctions		
IIEF-EF domain		
no (26–30)	1458	63
mild to moderate, mild (17–25)	386	17
moderate, severe (≤16)	460	20
EHS		
0	12	0.5
1	23	1.0
2	55	2.3
3	612	25
4	1708	71
Erectile dysfunction		
No	1929	79
Yes (IIEF-EF score ≤25 or EHS score ≤3)	504	21
Premature ejaculation		
No	1762	84
Yes (lifelong/acquired)	109	5.2
Yes (variable/subjective)	240	11
Delayed ejaculation		
No	2195	99
Yes	27	1.2
Low libido		
No	2254	93
Yes	175	7.2
Sociodemographic factors		
Partnership	2173	87
Duration of partnership (months)		
0	319	13
1–60	201	8.2
>60	1931	79
Living with the partner	2025	84
Children		
0	758	30
1	494	20
≥2	1248	50
Level of education		
low	255	10
intermediate	514	21
high	1731	69
Employment status		
unemployed	74	3.0
employed part-time	110	4.4
employed full-time	2299	93

(Continues)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13524 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 (Continued)

	n	%
Self-perceived economic situation		
poor	48	1.9
satisfactory	307	12
good	2133	86
Lifestyle factors		
Smoking		
non-smoker	1449	58
former smoker	717	29
current smoker	334	13
Alcohol consumption		
low	414	17
moderate	1885	77
excessive	148	6.1
Physical activity		
≤1 time/week	354	14
2–5 times/week	1519	61
≥6 times/week	622	25
Waist circumference (cm)		
≤94	1048	42
>94–≤102	692	28
>102	744	30
Comorbidities		
Hypertension	412	17
Diabetes mellitus	58	2.3
Dyslipidemia	123	4.9
Depression	105	4.2
Lower urinary tract symptoms (IPSS)		
no/mild (≤7)	2087	85
moderate, severe (>7)	381	15
Psychosocial factors		
Self-rated health		
good	2332	94
poor	148	6.0
Depression (PHQ-2)		
<3	2372	96
≥3	107	4.3
Anxiety (GAD-2)		
<3	2380	96
≥3	101	4.1
Sexual behavior		
Sexual orientation identity		
heterosexual	2349	94
homosexual	126	5.0
bisexual	25	1.0
Partnered sexual activity within the past 3 months		
none	374	15
up to one time/month	512	21
a few times/month to one time/week	1110	45
2–3 times/week	389	16
≥4 times/week	82	3.3
Solo masturbation within the past 3 months		
none	221	9.3
up to one time/month	430	22
a few times/month to one time/week	916	38
2–3 times/week	532	22
≥4 times/week	188	7.9
Importance of sexuality		
very unimportant	18	0.7
unimportant	125	5.1
more or less important	826	34
important	1128	46
very important	352	14
Sexual satisfaction		
very unsatisfied	148	6.1
unsatisfied	328	14
more or less satisfied	854	35
satisfied	907	37
very satisfied	202	8.3
Number of lifetime sexual partners		
0	16	0.7
1	193	8.0
2–10	1455	61
11–30	531	22
>30	205	8.5

Abbreviations: EHS, Erection Hardness Score; GAD-2, Generalized Anxiety Disorder-2; IIEF-EF, International Index of Erectile Function-Erectile Function; IPSS, International Prostate Symptom Score; PHQ-2, Patient Health Questionnaire-2.

Participants were classified into two groups according to the ISSM definition of PE: "no PE" and "lifelong/acquired PE". They were identified as having "no PE", if they answered the first question with "never/almost never" and the second question with "not at all a problem". In contrast, participants were identified as having lifelong/acquired PE when answering the first question with "often" or "almost all the time/almost always" and the second question with "some problem", "a considerable problem" or "a very great problem". If participants answered the first question with "never/almost never", "rarely" or "sometimes" and the second question with "some problem", "a considerable problem" or "a very great problem" they were identified as having variable/subjective PE.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13524 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

3.4 | Low libido

The presence of LL was assessed with the question "How often have you felt sexual desire during the past 4 weeks?" (very frequently, frequently, occasionally, rarely, very rarely/never), which was adopted from a study of men and women in Germany.[2] The latter two answer options were defined as LL.

3.5 | Statistical analysis

Descriptive statistics were used to summarize participant characteristics and simple logistic regression to explore individual characteristic associations with the outcomes of ED, PE, and LL. Three separate multiple logistic regression models with backward selection were performed for the outcomes ED, PE, and LL to measure associations with lifestyle factors and comorbidities. For each specific outcome modeled, partnership, lifestyle factors, comorbidities, psychosocial factors, and the other two outcomes were included as explanatory variables. Results were reported in terms of odds ratios (ORs), along with 95% confidence intervals (CIs). All statistical tests were two-sided, exploratory, and performed at the 0.05 level of significance. Data analyses were conducted using the Statistical Analysis System (SAS), version 9.4 (SAS Institute Inc.).

4 | RESULTS

4.1 | Study population

Note that, 2500 Bavarian men with a mean age of 50.4 ± 0.8 years were included in the BMH-Study between April 2020 and April 2021. The overall response rate was 95% (2500/2637). The overall prevalence of at least one sexual dysfunction was 30% (677/2254). The prevalence of ED (IIEF-EF score ≤25 or EHS score ≤3) was 21% (504/2433). 5.2% (109/2,111) of men had lifelong or acquired PE and 11.0% (240/2111) of men had variable or subjective PE. Few men reported delayed ejaculation (1.2%(27/2222)) and the prevalence of LL was 7.2% (175/2429). Further cohort characteristics are summarized in Table 1, and results of simple logistic regression analyses of ED, PE, and LL with characteristics in Tables S1–S3.

4.2 | Factors associated with ED, PE, and LL

PE and LL were both associated with ED (OR: 1.94, 95%CI: 1.22–3.08; OR: 2.04, 95%CI: 1.26–3.29, respectively). Men in a partnership were less likely to have ED (OR: 0.57, 95%CI: 0.39–0.85). Men with a higher waist circumference showed a higher likelihood of having ED (compared to ≤94 cm, >94–≤102 cm: OR: 1.51, 95%CI: 1.12–2.03; > 102 cm: OR: 2.23, 95%CI: 1.67–2.96). Men with LUTS had higher odds of having ED (OR: 1.88, 95%CI: 1.39–2.55). (Table 2)

TABLE 2 Multiple logistic regression analysis of factors associated with erectile dysfunction.

	OR	95% CI	*p*-value
Sexual dysfunctions			
Premature ejaculation (ref: no)	1.94	1.22–3.08	0.005
Low libido (ref: no)	2.04	1.26–3.29	0.004
Sociodemographic factors			
Partnership (ref: no)	0.57	0.39–0.85	0.005
Lifestyle factors			
Waist circumference (cm) (ref: ≤94)			<0.001
>94–≤102	1.51	1.12–2.03	
>102	2.23	1.67–2.96	
Comorbidities			
Lower urinary tract symptoms (ref: IPSS ≤7)	1.88	1.39–2.55	<0.001

Abbreviation: IPSS, International Prostate Symptom Score.

TABLE 3 Multiple logistic regression analysis of factors associated with premature ejaculation.

	OR	95% CI	*p*-value
Sexual dysfunctions			
Erectile dysfunction (ref: no)	1.94	1.23–3.07	0.005
Sociodemographic factors			
Partnership (ref: no)	5.42	1.30–22.60	0.020
Lifestyle factors			
Physical activity (ref: ≤1 time/week)			0.036
2–5 times/week	0.94	0.54–1.65	
≥6 times/week	0.44	0.21–0.93	
Comorbidities			
Lower urinary tract symptoms (ref: IPSS ≤ 7)	2.42	1.52–3.87	<0.001
Psychosocial factors			
Depression (ref: PHQ-2 < 3)	2.37	1.09–5.14	0.029

Abbreviations: IPSS, International Prostate Symptom Score; PHQ-2, Patient Health Questionnaire-2.

ED showed an association with PE (OR: 1.94, 95%CI: 1.23–3.07). Partnership was associated with 5.4-fold increased odds of having PE (OR: 5.42, 95%CI: 1.30–22.6). Men physically active ≥6 times/week were less likely to report PE (OR: 0.44, 95%CI: 0.21–0.93) and men with LUTS were more likely to report PE (OR: 2.42, 95%CI: 1.52–3.87). Regarding psychosocial factors, symptoms of depression were associated with 2.4-fold increased odds of having PE (OR: 2.37, 95%CI: 1.09–5.14). (Table 3)

ED was associated with LL (OR: 2.09, 95%CI: 1.31–3.34). Sociodemographic/lifestyle factors and other comorbidities showed no association with LL. The greatest odds ratio for having LL was allotted to men with poorer self-rated health (OR: 2.97, 95%CI: 1.54–5.71). (Table 4)

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13524 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 4 Multiple logistic regression analysis of factors associated with low libido.

	OR	95% CI	*p*-value
Sexual dysfunctions			
Erectile dysfunction (ref: no)	2.09	1.31–3.34	0.002
Psychosocial factors			
Self-rated health (ref: good)	2.97	1.54–5.71	0.001

Abbreviations: GAD-2, Generalized Anxiety Disorder-2; IPSS, International Prostate Symptom Score; PHQ-2, Patient Health Questionnaire-2.

5 | DISCUSSION

This cross-sectional study provides a basis for the understanding of men's sexual health and the most common male sexual dysfunctions in a large population-based random sample of middle-aged men. One in three middle-aged men experience some form of sexual dysfunction and risk factors identified in this study underline the multifactorial nature of ED, PE, and LL. Every fifth 50-year-old man reported being affected by ED, defined as both IIEF-EF score ≤25 and EHS ≤3. A previously published US-American study assessing ED in prostate cancer patients before treatment reported a comparable prevalence of 20% in healthy 50-year-old men.[6] ED prevalence was higher in other large European studies including more than 3000 and about 2000 German men, respectively, for example, 29.5% in 40–59-year-old men living in a heterosexual, well-established relationship,[25] and among sexually active German men, 17% and 31% in 40–49-year-old men and 50–59-year-old men, respectively, versus among all men, 28% in 40–49-year-old men and 45% in 50–59-year-old men, respectively.[26] However, both studies were hampered by low response rates of 31% and 32%, respectively. Further, when applying the same ED definition (IIEF-EF score ≤25) used in the abovementioned studies, ED prevalence in the cohort of this study is comparable at 37%.[25,26] Notably, the IIEF-EF-domain does not capture men without sexual intercourse, few sexual attempts, or non-heterosexual orientation identity. Adding the EHS in this study captures all men independent of their sexual orientation identity and sexual activity.

The results observed underline the well-known link between ED and PE.[3] However, men complaining of both ED and PE should be considered and treated primarily as men with ED and treatment should be predominantly focused on the erectile problem.[27] Men with ED had likewise higher odds of reporting LL, which is in line with previous studies.[28] ED leads to low self-confidence and avoidance behavior that might result in LL.[28]

From the investigated lifestyle factors and comorbidities only higher waist circumference and LUTS were associated with ED. A waist circumference >102 cm was associated with 2.2-fold increased odds of having ED. Previous studies reported that the relative risk of ED was nearly twice as high (risk ratio: 1.9) in obese men compared with men with normal BMI,[29] and that the prevalence of ED increased from 32.1% to 74.5% with increasing waist circumference.[30] The Cologne Male Survey described the relationship of LUTS and ED,[31] and this analysis showed likewise a 1.8-fold increased odds of ED in men with LUTS (IPSS > 7).

Contrary to the results of previous studies,[6,32] ED was not associated with other lifestyle factors and comorbidities. In simple logistic regression, less physical activity, hypertension, diabetes, and depression were associated with ED, but the effect failed to reach significance when controlling for concurrent confounders in the multiple regression analyses. Notably, the aforementioned studies showing an effect of these risk factors investigated mainly older men. Shorter existence and lower severity of symptoms caused by these risk factors might mitigate ED severity, however, with increasing age symptoms of ED might aggravate. The correlation between ED and cardiovascular events is an issue of major interest warranting further studies on older high-risk populations.

In the present analysis, the prevalence of lifelong/acquired PE was 5.2%, and 11% of men had variable/subjective PE. This is consistent with findings from two large observational studies, which also used the ISSM evidence-based definition of PE. In these studies, the prevalence of lifelong/acquired PE was 6.2% and 8.0%, respectively; however, the prevalence of variable/subjective PE was higher in these samples (14% and 18%, respectively).[33,34] This underlines the assumption that the higher prevalence of subjective PE might partly be due to cultural reasons,[35] since these samples were from Turkey and China, respectively.

Regarding lifestyle factors, physically active men had lower odds of having PE. This was consistent with previous findings, which reported that engaging in physical exercise is associated with lower levels of PE symptoms.[36] Physical activity is attributed to have a regulating effect on the serotonergic neurotransmission, which activates the ejaculatory reflex.[37]

The prevalence of LL was 7.2% in this analysis which is comparable to other large, population-based surveys. The prevalence of LL in men aged 41—50 years increased from 0.5% to 4.2% in two comparable population-based German samples in 2005 and 2016 comprising 1106 and 1095 men, respectively.[2] In a large nationally representative Danish population ($N = 10,458$, response rate 84.8%), a prevalence of 7.7% in men aged 45–66 years was reported.[14]

Interestingly, an association between LL and various lifestyle factors/comorbidities could not be found in the multiple analyses. In fact, when assessing the factors separately, an association of LL with less physical activity, alcohol consumption, depression, and LUTS was found, whereas higher waist circumference, smoking, hypertension, diabetes, and dyslipidemia showed no association. Studies investigating older men showed that age itself, low physical activity, obesity, depression, and diabetes were associated with lower sexual desire.[14,38]

The findings of this study provide a good generalizability due to the large, population-based, and randomly selected sample of 50-year-old men. Additionally, instructed physicians surveyed all men; thus detailed medical history was obtained and a physical examination was conducted. Furthermore, sexual dysfunctions (i.e., ED and PE) were assessed using validated questionnaires, allowing comparison of results. The BMH-Study will also provide longitudinal data in

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13524 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

the future, which will enable a more profound understanding of male sexual health and sexual dysfunctions.

However, several limitations of the current analysis should be mentioned. Data from the BMH-Study are collected from men who are attending a prostate cancer screening trial and might include more health-concerned men, as well as men who are suffering from prostate symptoms resulting in a recruitment bias. Patients with controlled comorbidities who are under specific therapies were not distinguished from patients with uncontrolled comorbidities without therapy and duration of comorbidities or lifestyle factors were not considered. However, these factors have various severity levels. For instance, the risk of ED is different between men with severe or mild cases of diabetes, and between patients with controlled and uncontrolled diabetes or different length of time. Further limitations include the lack of serum testosterone levels and the use of a single item to assess LL. Therefore, data do not necessarily correspond to a clinical diagnosis. Lastly, outcome measures are subjective and self-reported and are therefore at risk for exaggeration.

6 | CONCLUSIONS

Results of the current analysis provide a basis for the understanding of men's sexual health and the most common male sexual dysfunctions in a large population-based random sample of middle-aged men. ED measured with both the IIEF-EF domain and the EHS was more precise and independent of men's sexual orientation identity. Factors associated included not only typical risk factors and comorbidities but also other sexual dysfunctions and LUTS, underlining the multifactorial nature of these sexual dysfunctions. Most associated risk factors and comorbidities are modifiable, which should be addressed in patient education as well as in men's health campaigns and men should take active measures to remove the risk posed by these factors.

AUTHOR CONTRIBUTIONS

In addition to being the corresponding author, Kathleen Herkommer's contribution to this manuscript was research design and oversight, acquisition of data analysis and interpretation, and drafting of the manuscript. Valentin Meissner helped with the analysis and interpretation of data and drafted the manuscript. Stefan Schiele helped with the statistical analysis and interpretation of the data. Jürgen Gschwend helped with additional administrative support. All authors revised critically the manuscript for important intellectual content.

ACKNOWLEDGMENTS

Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Kathleen Herkommer https://orcid.org/0000-0002-0339-3394

REFERENCES

1. Salonia A, Bettocchi C, Boeri L, et al. European Association of Urology Guidelines on Sexual and Reproductive Health-2021 update: Male sexual dysfunction. *Eur Urol*. 2021;80(3):333-357. doi:10.1016/j.eururo.2021.06.007
2. Beutel ME, Burghardt J, Tibubos AN, Klein EM, Schmutzer G, Brähler E. Declining sexual activity and desire in men-findings from representative German surveys, 2005 and 2016. *J Sex Med*. 2018;15(5):750-756. doi:10.1016/j.jsxm.2018.03.010
3. Corona G, Rastrelli G, Limoncin E, Sforza A, Jannini EA, Maggi M. Interplay between premature ejaculation and erectile dysfunction: A systematic review and meta-analysis. *J Sex Med*. 2015;12(12):2291-2300. doi:10.1111/jsm.13041
4. NIH Consensus Conference. Impotence. NIH consensus development panel on impotence. *JAMA*. 1993;270(1):83-90.
5. Corona G, Lee DM, Forti G, et al. Age-related changes in general and sexual health in middle-aged and older men: results from the European Male Ageing Study (EMAS). *J Sex Med*. 2010;7(4 Pt 1):1362-1380. doi:10.1111/j.1743-6109.2009.01601.x
6. Pellegrino F, Sjoberg DD, Tin AL, et al. Relationship between age, comorbidity, and the prevalence of erectile dysfunction. *Eur Urol Focus*. 2022. doi:10.1016/j.euf.2022.08.006
7. Rosen RC, Riley A, Wagner G, Osterloh IH, Kirkpatrick J, Mishra A. The international index of erectile function (IIEF): A multidimensional scale for assessment of erectile dysfunction. *Urology*. 1997;49(6):822-830. doi:10.1016/s0090-4295(97)00238-0
8. Mulhall JP, Goldstein I, Bushmakin AG, Cappelleri JC, Hvidsten K. Validation of the erection hardness score. *J Sex Med*. 2007;4(6):1626-1634. doi:10.1111/j.1743-6109.2007.00600.x
9. Serefoglu EC, McMahon CG, Waldinger MD, et al. An evidence-based unified definition of lifelong and acquired premature ejaculation: Report of the second international society for sexual medicine ad hoc committee for the definition of premature ejaculation. *Sex Med*. Jun 2014;2(2):41-59. doi:10.1002/sm2.27
10. Laumann EO, Nicolosi A, Glasser DB, et al. Sexual problems among women and men aged 40-80 y: prevalence and correlates identified in the Global Study of Sexual Attitudes and Behaviors. *Int J Impot Res*. 2005;17(1):39-57. doi:10.1038/sj.ijir.3901250
11. Porst H, Montorsi F, Rosen RC, Gaynor L, Grupe S, Alexander J. The Premature Ejaculation Prevalence and Attitudes (PEPA) survey: Prevalence, comorbidities, and professional help-seeking. *Eur Urol*. 2007;51(3):816-823. doi:10.1016/j.eururo.2006.07.004. discussion 824.
12. McCabe MP, Sharlip ID, Lewis R, et al. Incidence and prevalence of sexual dysfunction in women and men: A consensus statement from the Fourth International Consultation on Sexual Medicine 2015. *J Sex Med*. 2016;13(2):144-152. doi:10.1016/j.jsxm.2015.12.034
13. Parish SJ, Hahn SR. Hypoactive sexual desire disorder: A review of epidemiology, biopsychology, diagnosis, and treatment. *Sex Med Rev*. Apr 2016;4(2):103-120. doi:10.1016/j.sxmr.2015.11.009
14. Eplov L, Giraldi A, Davidsen M, Garde K, Kamper-Jørgensen F. Sexual desire in a nationally representative Danish population. *J Sex Med*. 2007;4(1):47-56. doi:10.1111/j.1743-6109.2006.00396.x
15. Irfan M, Hussain NHN, Noor NM, Mohamed M, Sidi H, Ismail SB. Epidemiology of male sexual dysfunction in Asian and European Regions: A systematic review. *Am J Mens Health*. 2020;14(4):1557988320937200. doi:10.1177/1557988320937200
16. Donnelly DW, Donnelly C, Kearney T, et al. Urinary, bowel and sexual health in older men from Northern Ireland. *BJU Int*. 2018;122(5):845-857. doi:10.1111/bju.14182

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13524 by mryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

17. Palacios-Ceña D, Carrasco-Garrido P, Hernández-Barrera V, Alonso-Blanco C, Jiménez-García R. Fernández-de-las-Peñas C. Sexual behaviors among older adults in Spain: results from a population-based national sexual health survey. *J Sex Med*. Jan 2012;9(1):121-129. doi:10.1111/j.1743-6109.2011.02511.x
18. Verze P, Arcaniolo D, Palmieri A, et al. Premature ejaculation among Italian men: Prevalence and clinical correlates from an observational, non-interventional, cross-sectional, epidemiological study (IPER). *Sex Med*. 2018;6(3):193-202. doi:10.1016/j.esxm.2018.04.005
19. Barry MJ, Fowler FJ, jr, O'Leary MP, et al. The American Urological Association Symptom Index for benign prostatic hyperplasia. *J Urol*. 2017;197(2):S189-S197. doi:10.1016/j.juro.2016.10.071
20. Ware J, jr, Kosinski M, Keller SD. A 12-Item Short-Form Health Survey: construction of scales and preliminary tests of reliability and validity. *Med Care*. 1996;34(3):220-233. doi:10.1097/00005650-199603000-00003
21. Löwe B, Wahl I, Rose M, et al. A 4-item measure of depression and anxiety: validation and standardization of the Patient Health Questionnaire-4 (PHQ-4) in the general population. *J Affect Disord*. 2010;122(1-2):86-95. doi:10.1016/j.jad.2009.06.019
22. Rynja S, Bosch R, Kok E, Wouters G, de Kort L. IIEF-15: unsuitable for assessing erectile function of young men? *J Sex Med*. 2010;7(8):2825-2830. doi:10.1111/j.1743-6109.2010.01847.x
23. Hatzichristou D, Rosen RC, Derogatis LR, et al. Recommendations for the clinical evaluation of men and women with sexual dysfunction. *J Sex Med*. 2010;7(1Pt 2):337-348. doi:10.1111/j.1743-6109.2009.01619.x
24. Gündüz A, R KumruBayazit, İ Gündoğmuş, et al. Turkish validity and reliability of the sexual complaints screener for women. *Psychiatr Clin Psychopharmacol*. 2019;29(4):632-639. doi:10.1080/24750573.2018.1525811
25. May M, Gralla O, Knoll N, et al. Erectile dysfunction, discrepancy between high prevalence and low utilization of treatment options: results from the 'Cottbus Survey' with 10 000 men. *BJU Int*. 2007;100(5):1110-1115. doi:10.1111/j.1464-410X.2007.07101.x
26. Englert H, Schaefer G, Roll S, Ahlers C, Beier K, Willich S. Prevalence of erectile dysfunction among middle-aged men in a metropolitan area in Germany. *Int J Impot Res*. 2007;19(2):183-188. doi:10.1038/sj.ijir.3901510
27. Rastrelli G, Cipriani S, Corona G, Vignozzi L, Maggi M. Clinical characteristics of men complaining of premature ejaculation together with erectile dysfunction: a cross-sectional study. *Andrology*. 2019;7(2):163-171. doi:10.1111/andr.12579
28. Corona G, Rastrelli G, Ricca V, et al. Risk factors associated with primary and secondary reduced libido in male patients with sexual dysfunction. *J Sex Med*. 2013;10(4):1074-1089. doi:10.1111/jsm.12043
29. Bacon CG, Mittleman MA, Kawachi I, Giovannucci E, Glasser DB, Rimm EB. A prospective study of risk factors for erectile dysfunction. *J Urol*. 2006;176(1):217-221. doi:10.1016/s0022-5347(06)00589-1
30. Lee RK, Chung D, Chughtai B, Te AE, Kaplan SA. Central obesity as measured by waist circumference is predictive of severity of lower urinary tract symptoms. *BJU Int*. 2012;110(4):540-545. doi:10.1111/j.1464-410X.2011.10819.x
31. Braun MH, Sommer F, Haupt G, Mathers MJ, Reifenrath B, Engelmann UH. Lower urinary tract symptoms and erectile dysfunction: comorbidity or typical "Aging Male" symptoms? Results of the "Cologne Male Survey". *Eur Urol*. 2003;44(5):588-594. doi:10.1016/s0302-2838(03)00358-0
32. Li JZ, Maguire TA, Zou KH, Lee LJ, Donde SS, Taylor DG. Prevalence, comorbidities, and risk factors of erectile dysfunction: Results from a prospective real-world study in the United Kingdom. *Int J Clin Pract*. 2022;2022:5229702. doi:10.1155/2022/5229702
33. Serefoglu EC, Yaman O, Cayan S, et al. Prevalence of the complaint of ejaculating prematurely and the four premature ejaculation syndromes: results from the Turkish Society of Andrology Sexual Health Survey. *J Sex Med*. 2011;8(2):540-548. doi:10.1111/j.1743-6109.2010.02095.x
34. Gao J, Zhang X, Su P, et al. Prevalence and factors associated with the complaint of premature ejaculation and the four premature ejaculation syndromes: a large observational study in China. *J Sex Med*. 2013;10(7):1874-1881. doi:10.1111/jsm.12180
35. Richardson D, Goldmeier D. Premature ejaculation–does country of origin tell us anything about etiology? *J Sex Med*. 2005;2(4):508-512. doi:10.1111/j.1743-6109.2005.00074.x
36. Ventus D, Jern P. Lifestyle factors and premature ejaculation: Are physical exercise, alcohol consumption, and body mass index associated with premature ejaculation and comorbid erectile problems? *J Sex Med*. 2016;13(10):1482-1487. https://doi.org10.1016/j.jsxm.2016.08.007
37. Waldinger MD, Berendsen HH, Blok BF, Olivier B, Holstege G. Premature ejaculation and serotonergic antidepressants-induced delayed ejaculation: the involvement of the serotonergic system. *Behav Brain Res*. 1998;92(2):111-118. doi:10.1016/s0166-4328(97)00183-6
38. Martin S, Atlantis E, Wilson D, et al. Clinical and biopsychosocial determinants of sexual dysfunction in middle-aged and older Australian men. *J Sex Med*. 2012;9(8):2093-2103. doi:10.1111/j.1743-6109.2012.02805.x

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Herkommer K, Meissner VH, Dinkel A, et al. Prevalence, lifestyle, and risk factors of erectile dysfunction, premature ejaculation, and low libido in middle-aged men: first results of the Bavarian Men's Health-Study. *Andrology*. 2024;12:801–808. https://doi.org/10.1111/andr.13524

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13524 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 24 April 2023 | Revised: 7 September 2023 | Accepted: 3 October 2023

DOI: 10.1111/andr.13545

ANDROLOGY WILEY

ORIGINAL ARTICLE

Microbiome reveals inflammatory-related bacteria and putative functional pathways involved in human papillomavirus-associated penile squamous cell carcinoma

Amanda de Deus[1,2] | Gabriele Gonçalves[2] | Jenilson da Silva[1,2] | Luís Cláudio de Jesus[3] | Ana Paula S. Azevedo-Santos[4] | Hivana Dall Agnol[2,5] | Silma Regina Pereira[2]

[1]Postgraduate Program in Health Science, Federal University of Maranhão, São Luís, Brazil

[2]Laboratory of Genetics and Molecular Biology, Department of Biology, Federal University of Maranhão, São Luís, Brazil

[3]Department of Genetics, Ecology, and Evolution, Federal University of Minas Gerais, Belo Horizonte, Brazil

[4]Department of Physiological Sciences, Federal University of Maranhão, São Luís, Brazil

[5]Department of Pathology, Federal University of Maranhão, São Luís, Brazil

Correspondence
Silma Regina Pereira, Laboratory of Genetics and Molecular Biology, Department of Biology, Federal University of Maranhão, São Luís, Brazil.
Email: silma.pereira@ufma.br

Abstract

Background: Penile squamous cell carcinoma (PSCC) is a rare disease that is more prevalent in developing countries, such as Brazil, and is linked to poor genital hygiene, which promotes the proliferation of microorganisms. Dysbiosis has an effect on the local immune response, increases the risk of viral infection, and can generate inflammatory processes. Current knowledge of the microbiota found in penile tissues is limited, and the bacterial diversity of the PSCC remains unknown. In this investigation, the microbiota associated with penile cancer and its potential role in tumor development and progression were identified.

Methods: The 16S rRNA gene was analyzed by next-generation sequencing in 19 tumors and their respective non-tumor adjacent tissues to perform taxonomic classification, analysis of core microbiome, abundance, and diversity of amplicon sequence variants (ASVs) (QIIME2 v.2020.2), and in silico functional prediction (PICRUST2, $p < 0.05$).

Results: In both tissues, the phyla Proteobacteria and Firmicutes, and genera *Alcaligenes* and *Fusobaterium*, were the most prevalent. Tumors presented a greater relative abundance of Fusobacteriota, Campilobacteria, and Fusobacterium ($p = 0.04$, $p = 0.04$, and $p = 0.039$, respectively). In addition, the beta diversity analysis revealed a tendency for the formation of two distinct groups when only advanced tumors (pT2 and pT3) were considered. Further, the functional analysis identified the top 35 pathways, and 79.5% of PSCC samples contained pro-inflammatory microorganisms.

Conclusion: We describe the first microbiome of penile carcinoma, which revealed an abundant and diverse microbiota as well as inflammatory-related taxa (the phyla Proteobacteria and Firmicutes, the genera *Fusobacterium* and *Prevotella*, and the species *Finegoldia magma* and *Pseudomonas geniculata*) and molecular pathways (chitin derivates degradation, the protocatechuic acid pathway, inositol metabolism, and the

Hivana Dall Agnol and Silma Regina Pereira contributed equally to this paper.

© 2023 American Society of Andrology and European Academy of Andrology.

sucrose pathway), which have also been linked to inflammation and carcinogenesis. Moreover, we found specific and abundant ASVs in both tumor and non-tumor tissues. Our data encourage further study to better understand the role of these microorganisms in penile carcinogenesis, offering an opportunity for advances in diagnosis, prognosis, and early therapy.

KEYWORDS
human papillomavirus, inflammation, microbiome, penile cancer

1 | INTRODUCTION

In developed countries, penile carcinoma is a rare tumor that predominantly affects men in their sixth decade of life; however in developing countries, such as those in Africa and South America, the incidence is higher (5.7% and 13.8%, respectively)[1] and affects younger men.[2,3] In North America and Europe, the incidence ranges from 0.1 to 1.0, although some studies indicate an upward trend attributed to an increase in human papillomavirus (HPV) infection.[4,5] Penile cancer has a favorable prognosis when detected at an early stage, but it has a high mortality rate in economically deprived regions due to the difficulty of obtaining health care.[6] In recent years, however, the proportion of patients who died in the United States (3.6%) has increased significantly.[1]

More than 95% of penile cancer are epidermoid or squamous cell carcinoma (PSCC). The clinical and histopathological characteristics (pathological subtype, perineural invasion, lymph vascular invasion, depth of invasion and grade in the primary tumor) are directly related to the prognosis, enabling the choice of an appropriate treatment.[7] World Health Organization (WHO) classifies SCC according to the TNM system, which considers T (describe the size of the primary tumor and its invasion into adjacent tissues), N (absence or presence and the extent of metastasis in regional lymph nodes), and M (absence or presence of distant metastasis). The T1 category is subdivided into two risk groups based on the presence or the absence of lymphovascular invasion and tumor grade. T2 denotes invasion of the corpus spongiosum, whereas T3 denotes invasion of the corpora, and these two patterns are associated with distinct prognosis.[7]

The main PSCC risk factors include phimosis, poor hygiene, smoking, chronic inflammation, and HPV infection. Other tumors, such as vulva,[8] uterine cervix,[9] and anogenital region[2,10] have also been associated with HPV, and around 20% of cancer cases are strongly associated with particular viral or microbial infections.[11] Moreover, it has been established that inadequate hygiene practices in the penile region contribute to the accumulation of secretions known as smegma and the growth of bacteria, including *Mycobacterium smegmatis*.[2,10] Furthermore, Liu et al.[12] conducted a study in which they discovered a significant association between the presence of anerobic bacteria in penile tissue, increased expression of cytokines in the coronal sulcus, and an individual's vulnerability to HIV infection.

Different factors, including nutrition, lifestyle, inflammatory and infectious diseases, and pharmaceuticals, can promote an unhealthy shift in the abundance, composition, and function of microbial communities (dysbiosis). Although the abundance of intratumoral microbes is low in comparison to cancer cells, the microorganisms metabolize and produce energy, which promotes their replication and survival, influencing both immune response and cancer.[13] Studies have shown that bacteria can initiate cancer via three primary mechanisms: (1) producing oncometabolites that increase mutation rate (cancer initiation); (2) triggering sustained and chronic inflammation (cancer promotion); and (3) modeling the microenvironment of the tumor and promoting metastasis (cancer progression).[11,14] This complex interaction of microorganisms and host cells can be both a challenge and a potential therapy opportunity.[15]

Microorganism communities may differ in terms of richness (i.e., number of species) and of relative species abundance in space and time. Diversity depends not only on richness, but also on evenness, and becomes a crucial factor in evaluating the microbiota's composition, function, and dynamics. Alpha diversity measures the local diversity of samples or communities. Even though there is no consensus on which diversity index is the most effective, Shannon–Weaver and Simpson diversity indices have traditionally been used to measure community diversity, and both indices increase when the richness and evenness increase.[16] On the other hand, beta diversity measures the compositional dissimilarity across samples or communities.[17]

High-throughput sequencing of 16S rRNA genes has generated massive data on bacterial communities in different tissues with greater accuracy. In addition, microbiome-specific computational pipelines have been developed to enable a more robust analysis of the vast quantity of data produced by next-generation sequencing. Consequently, it is possible to gain a greater understanding of the role of microorganisms in the development and progression of a number of diseases, including cancer.[18] Nevertheless, it is important to note that currently there is a lack of available data regarding the microbiome of PSCC.[19] In the present study, we examined the 16S rRNA gene through high-performance sequencing to characterize the microbial profile of a subset of PSCC, previously evaluated by our group.[20] All samples were positive for HPV, most of them having a high oncogenic risk. In addition, functional prediction analysis revealed the main molecular pathways potentially involved in the genesis of PSCC.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2 | MATERIALS AND METHODS

2.1 | Study population

This study enrolled 19 pairs of tumor tissue and adjacent non-tumor tissue, a subset of a larger cohort ($n = 55$) analyzed in our previous study.[20] All patients were from the Aldenora Bello Cancer Hospital in São Luís, Maranhão, Brazil, and signed a Human Research Ethics Committee-approved consent form (UFMA; CAAE: 46371515.5.0000.5087). No patient had been treated, including with antibiotics, for at least 1 month prior to surgery. Adjacent tissues were collected 2 cm away from the primary tumor, within the surgical safety margin, and classified through histopathological and immunohistochemical analysis.[20] In addition, gene expression, miRNA expression, copy number alteration, and exome sequencing data support that penile tumor-adjacent tissues are a suitable normal control in relation to the tumors.[21–23] The cancer staging was determined using the 2016 World Health Organization tumor–node–metastasis (TNM) classification.[24,25]

The mean age of patients at the time of diagnosis was 62.6 ± 16.5 years, with a range of 32–85 years. The majority of patients (79%) had a low level of education, 50% had postectomy surgery, and 42% were smokers. The most common types of tumors were condylomatous (52.6%) and usual (31.5%). In 26.4% of cases, perineural and lymphatic invasion were observed. The majority of tumors (73%) had advanced staging (pT2 or pT3), necessitating total (21%) or partial (73%) penectomy procedures. For stratifying tumor stages, pT2 and pT3 tumors (14 samples) were categorized as advanced, while pT1 tumors (5 samples) were categorized as initial to identify a potential microbial signature according to the clinical profile of advanced and early diseases, respectively. The data were analyzed using the same criteria and are presented in Table 1.

Using a standard phenol–chloroform protocol, genomic DNA with a 260/280 ratio of ~1.8 was isolated.[26,27] HPV genotyping was performed by nested-PCR in accordance with BioGenetics Molecular Technologies (Uberlandia, Minas Gerais, Brazil, patent number BR102017004615.0), which allows identifying 40 viral subtypes classified as high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82), intermediate risk (MM7, MM8, 26, 30, (6, 11, 42, 43, 44 and 57). Briefly, the first-round amplification was performed using one consensus primer MY09[28] and nine degenerate primers modified from the MY09/11 primer to cover nonspecifically all types of HPV. The β-globin gene (BGS1 and BGA2 primers) was used as a positive internal control. The second PCR amplification was carried out by a nested multiplex reaction using 40 specific primers designed and labeled with fluorophores to identify HPV subtypes. The first amplification reaction consisted of 40 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, and 5 min at 72°C. Using the MY11/09 PCR products as a template, the second reaction was conducted for 36 cycles at 95°C for 2 min, 56°C for 1 min, 72°C for 40 s, and 72°C for 5 min. The PCR product was detected by capillary electrophoresis (MegaBase 1000, Amersham Pharmacia Biotech) using the internal molecular weight standard ETRox550 (GE Healthcare).

DNA sequencing was also used for HPV genotyping (Big Dye Kit Terminator v 3.1 method in ABI PRqual ISM 3500 Genetic Analyzer Sequencer; Applied Biosystems). MEGA 6.0 Sequences software and the GenBank/NCBI/Blast Database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were utilized for data analysis and sequencing comparison, respectively. If note, the samples were characterized for inflammatory gene alterations in our previous study.[20] Briefly, the gene expression analysis was performed by Taqman Universal PCR Mix II (2×) no UNG (Fluorochrome FAM) and amplification reactions were conducted in ABI 7500 Fast thermocycler with 100 ng of cDNA and 0.5 μL of primer and probe (KIT Taqman Gene expression assay) for target genes (EGFR, COX2, TP53, and RB1), as detailed in Table S1A.

2.2 | Bacterial 16S rRNA V3–V4 sequencing

DNA library was prepared by using MiSeq Reagent Kits v2 according to the manufacturer's instructions. The V3–V4 region of the 16S rRNA gene was amplified using primers 341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). The size, quantification, and quality control of the DNA library were evaluated on TapeStation (Agilent Technologies, Santa Clara, CA, USA). The captured DNA was subjected to high-performance sequencing; using MiSeq Sequencing System (Illumina Inc, San Diego, CA, USA) to generate 250pb paired-end reads (Neoprospecta Microbiome Technologies, Florianópolis, SC, Brazil). Further, the sequences were analyzed using the Sentinel pipeline.[29] Each tissue's DNA was sequenced at the same concentration, and the sequencing quality was determined by a Phred score greater than 30. Before analyzing microbial community diversity, we normalized the number of sequences in all samples to avoid biases in the results.[16]

2.3 | Taxonomic classification through amplicon sequence variants

Using the FastQC v0.11.8 program, Phred quality (QP) was assessed for FastQ files.[29] Low-quality primers and sequence trimming (QP < 20) were carried out on Fastq files. To exclude structures typically associated with chimera sequences, clusters with abundances < 2 were excluded from the analyses.[30] Adapters were trimmed with cutadapt (https://cutadapt.readthedocs.io/en/stable/v1.10/) utilizing the default parameters. Reads were converted into amplicon sequence variants (ASVs) by using Divisive Amplicon Denoising Algorithm-2 (DADA-2) from the QIIME2-pipeline[31] with a truncation length of 250 bp and the default parameters. The taxonomic classifier was trained on V3–V4 regions using scikitlearn[32] and the SILVA v.132 database (https://arb-silva.de/documentation/release-132/).[33]

2.4 | Diversity analysis

Microbiome differences were evaluated by comparing both alpha and beta diversity metrics (QIIME2 v.2020.2 at https://view.qiime.org).[34] The metrics observed_otus, shannon, and faith_ph were used for alpha diversity analysis, while Bray–Curtis, Jaccard, and Unifrac

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Clinical and histopathological data of patients with HPV-positive penile squamous cell carcinoma ($n = 19$).

Case	Age	Phymosis	HPV subtype	Tumor grade	Tumor stage	Tumor size (cm)	Lymphatic invasion	Perineural invasion
1	72	no	16	G2	pT3	2.5	yes	yes
2	80	ni	16	G2	pT2	10	no	no
3	81	yes	18,53	G2	pT1	2.1	no	no
4	76	yes	30	G1	pT1b	3	yes	no
5	78	yes	6,16	G3	pT1b	4	no	no
6	44	yes	16	G2	pT1	1.7	no	no
7	85	no	16	G3	pT3	4	no	yes
8	83	no	16	G3	pT3	4.5	no	yes
9	51	yes	16	G2	pT2	ni	ni	ni
10	40	no	59,66	G2	pT2	3	no	no
11	68	ni	16,66	G1	pT3	5.5	no	no
12	41	yes	56	G2	pT2	3.5	no	no
13	74	no	11,35,59	G2	pT3	9	no	no
14	65	yes	16,74	G2	pT2	3.5	no	no
15	51	yes	16,35,59	G2	pT2	3	yes	no
16	32	no	16,44,74	G2	pT2	3.7	no	no
17	71	no	16	G3	pT3	3.2	no	no
18	69	no	16,33	G1	pT2	3.5	no	no
19	73	yes	16	G2	pT1	ni	ni	ni

Abbreviation: ni, no information.

(weighted and unweighted) were used for beta diversity. Bray–Curtis index take into account occurrence and abundance data, whereas the Jaccard index does not include abundance information (i.e., presence/absence).[17,35] The Unifrac index, in turn, also incorporates the phylogenetic relatedness between organisms when quantifying compositional dissimilarity.[36] Both, alpha and beta diversity, were demonstrated at a Principal Coordinate Analysis graphic (PCoA-plot). The Plugin Qiime Feature-table Core-features were used to conduct microbiome core analyses. For relative abundance analysis and data visualization, we utilized the deseq2 and ggplot2 libraries, respectively (R environment; v4.0.2).

2.5 | Predicting the functional potential of the microbiome

PICRUST2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States)[37] was used to predict the functional composition from Metabarcoding amplicon 16S data. The metabolic pathways were identified by using the q2-picrust2 plugin from QIIME2 v.2020.2. For sequence positioning, the p-placement-tool epa-ng parameter was used.[38] Metabolic pathways were predicted based on Enzyme (EC) abundance using the MinPath program[39] to predict MetaCyc pathways (https://metacyc.org/). Beta diversity analyses based on the inferred functional profile were performed using the q2-core-metrics plugin for the Bray-Curtis and Jaccard metrics. To determine which EC numbers, KOs, and metabolic pathways would be analyzed, a *t*-test was performed between the values generated for samples treated as non-tumor versus tumor, and those with a p-value < 0.05 were selected for analysis.

2.6 | Statistical analysis

Numerical variables were presented by mean and standard deviation, while categorical variables were by frequency and percentage. The statistical tests used in the analysis of beta diversity were the Permanova and Anosim. Test *t*-student to paired samples was used. The value of $p < 0.05$ was considered statistically significant. All tests were conducted using Bioedit v7.2 software.

3 | RESULTS

The sequencing of 16S rRNA gene amplicons yielded 7,904,583 high-quality (Phred > 30, non-chimeric) reads from a total of 38 paired samples, 19 of which were representative of tumor microbiome (T1–T19) and 19 adjacent non-tumor tissue microbiomes (N1–N19). Each sample generated an average of 161,136 ± 143,236 reads, ranging between 30,053 and 645,916 reads. The sequences were classified into 4,595 ASVs.

3.1 | Alfa and beta diversity

Shannon and Simpson indices revealed no statistically significant difference between tumor and non-tumor samples in terms of alpha

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

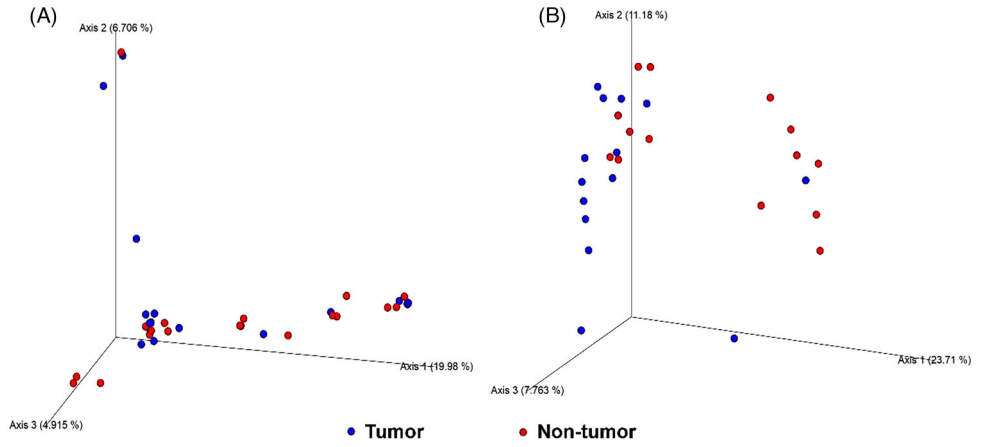


FIGURE 1 Beta-diversity of microorganisms from HPV-positive penile squamous cell carcinoma patients ($n = 38$). (A) Bray-Curtis analysis between tumor and non-tumor tissues; (B) unweighted unifrac analysis between advanced-stage tumors (pT2 and pT3) and adjacent non-tumor tissues ($p = 0.08$). HPV, human papillomavirus.

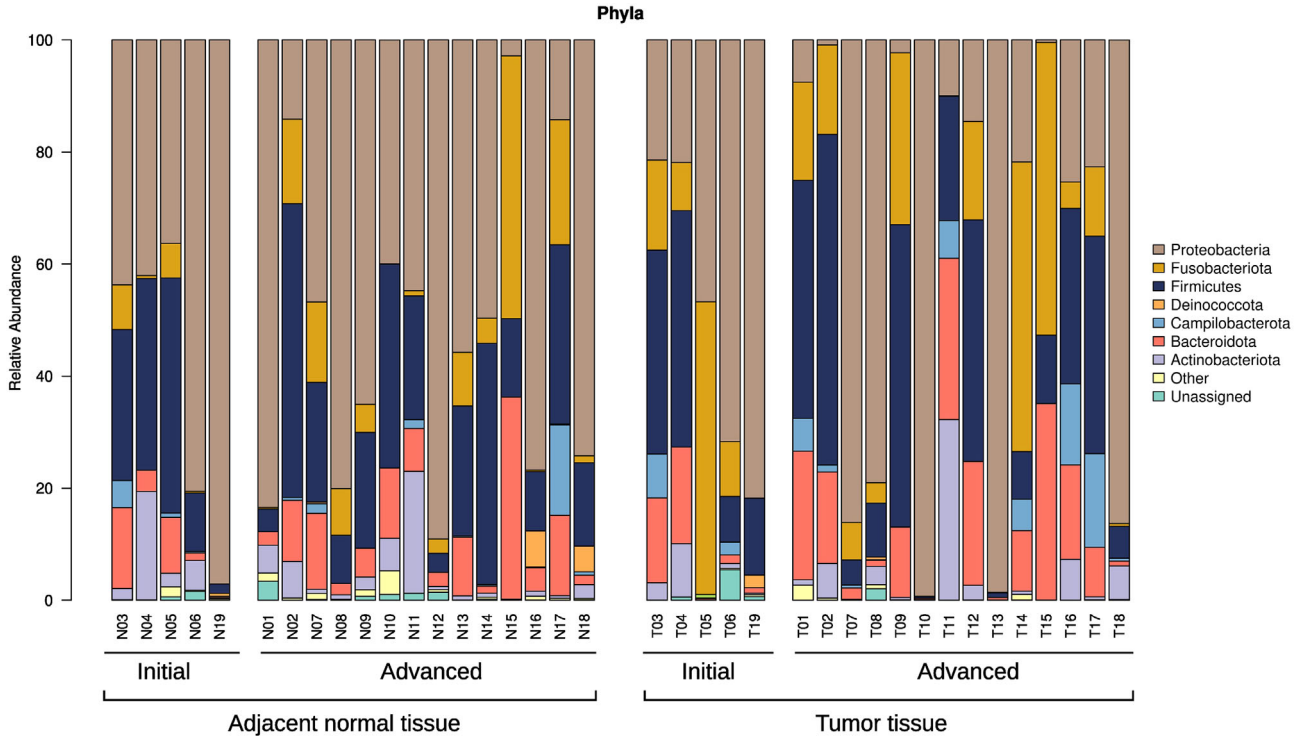


FIGURE 2 Relative abundance of phyla in non-tumor (N) and tumor (T) samples. The relative abundance (%) in the tumor (T) and non-tumor (N) samples is shown in the vertical bars. The sample groups in this study are separated into two categories: tumors and non-tumors adjacent to the primary tumor. Each of these categories is further divided based on the stage of the tumor, namely into initial tumor (pT1) and advanced tumor (pT2 or pT3). Colors indicate one of the 7 phyla presented according to the legend on the right.

diversity ($p > 0.05$). Similarly, Bray-Curtis analysis of beta diversity between tumor and non-tumor samples did not reveal a qualitative trend of divergence and formation of distinct groups (Figure 1A). However, considering only patients with advanced staging (pT2 or pT3) there was a tendency of formation of two groups in the PcoA plot (Figure 1B).

3.2 | Global analysis: penile tumor versus adjacent non-tumor tissues

In at least one of the two paired tissues, only 7 of the 22 phyla exhibited relative abundances greater than 0.5% (Figure 2). Proteobacteria and Firmicutes were the most abundant in both tumor and non-tumor

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Relative abundance of phyla in tumor and adjacent non-tumor tissue in patients with penile squamous cell carcinoma.

	Relative abundance (%)		
Phylum	Tumor	Adjacent non-tumor	p-value
Proteobacteria	42.03	54.59	0.16
Firmicutes	22.82	22.25	0.9
Fusobacteriota	15.82	7.70	0.04*
Bacteroidota	11.28	8.15	0.2
Actinobacteriota	3.89	4.12	0.82
Campilobacterota	3.26	1.43	0.04*
Deinococcota	0.15	0.64	0.26

*$p < 0.05$ (Student's *t*-test paired samples).

TABLE 3 Relative abundances of genera detected in penile squamous cell carcinoma and adjacent non-tumor tissues.

Genus	Relative abundance (%)		p-Value
	Tumor	Adjacent non-tumor	
Alcaligenes	41.80	31.05	0.57
Fusobacterium	10.50	7.87	0.039*
Prevotella	4.90	1.97	0.18
Parvimonas	2.45	0.9	0.42
Peptoniphilus	2.41	1.93	0.8
Porphyromonas	2.21	2.29	0.37
Bacteroides	0.78	2.49	0.34
Chromobacterium	0.60	2.43	0.045*,+
Anerococcus	1.84	0.99	0.5
Geobacillus	0.54	2.32	0.16
Anoxybacillus	0.06	2.15	0.16
Corynebacterium	1.38	2.01	0.23
Alcaligenaceae[1]	1.96	3.12	0.11
Eubacterium Yurii Group	1.88	0.12	0.3

*$p < 0.05$ (Student's *t*-test paired samples);.
+Analysis of four sample pairs in which the genus occurred.
[1]Identified at the family level.

samples. However, only the relative abundance of Fusobacteriota and Campylobacterota significantly differed between tumor and adjacent non-tumor tissues (Table 2), with both having a greater relative abundance in tumors.

A total of 437 genera were identified, from which the most prevalent (frequency > 1% in at least one paired tissue) are listed in Table 3. *Alcaligenes* and *Fusobacterium* were the most prevalent in both tissues (tumor and non-tumor), with a relative abundance of 41.8% and 31%, and 10.5% and 7.8%, respectively. In tumor samples, *Prevotella* (4.9%), *Parvimonas* (2.45%), *Peptoniphilus* (2.41%), *Porphyromonas* (2.21%), and *Eubacterium Yurii* group (1.88%) were also identified. Based on phosphatase and saccharolytic activities and DNA renaturation studies, this last microorganism is a subspecies, not a genus.[40] Regarding non-tumor tissues, the third ASV was only classified at the family level (*Alcaligenaceae*), but with significant incidence (3.12%). *Bacteroides* ranked fourth (2.49%), followed by *Chromobacterium* (2.43%), *Geobacillus* (2.32%), *Porphyromonas* (2.29%), *Anoxybacillus* (2.15%), *Corynebacterium* (2.01%), and *Prevotella* (1.97%). Noteworthy, there was a significant difference in the relative abundance of *Fusobacterium* and *Chromobacterium*, with *Fusobacterium* being more abundant in tumors, and *Chromobacterium* in non-tumor tissues (Table 3 and Figure 3). We highlight the fact that *Mycobacterium* was detected with a low relative abundance in both tissue samples (0.005%).

3.3 | Core microbiome analysis

The ASV core analysis performed in the advanced staging group (pT2 and pT3) revealed the presence of three distinct and abundant ASVs of the genus *Alcaligenes* in 55% of the non-tumor adjacent tissues and another ASV exclusive to the genus *Alcaligenes* in 50% of the tumor tissues. Regarding the pT1 group, no ASV core was detected in the tumor samples, whereas the bacteria *Finegoldia magma* and *Pseudomonas geniculata* were detected in 60% of the non-tumor samples.

3.4 | Differential abundance of paired amplicon sequence variants

We detected unique and abundant ASVs in both tumor and adjacent non-tumor tissues. For instance, the genera *Peptoniphilus*, *Peptostreptococcus*, and *Fusobacterium* have been identified in both tissues, albeit with different ASVs. *Alcaligenes*, *Streptobacillus*, *Bacillus*, *Anerococcus*, *Campylobacter*, *Fastidiospila*, and *Pseudoglutamicibacter* ASVs were only abundant in tumor samples, whereas ASVs from the other genera were abundant only in non-tumor tissues (Figure 4).

3.5 | Functional prediction based on the microbiome

Based on the microbiome identified in the tumor and adjacent non-tumoral tissues, 35 statistically significant ($p < 0.05$) functional pathways were predicted (Figure 5; Table S2). The relative abundances were higher in non-tumoral tissues.

4 | DISCUSSION

This is the first study to describe the microbiome in penile cancers. Globally and in pairs, we assessed tumors and their respective non-tumor tissues adjacent to the primary tumors. Analysis of alpha diversity revealed that the bacterial composition of both tissues did not differ significantly. Allali et al.[41] found similar results when comparing the microbiome of colorectal cancer (CRC) tissue with adjacent non-tumor tissue from Spanish and American patients. In addition, although

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

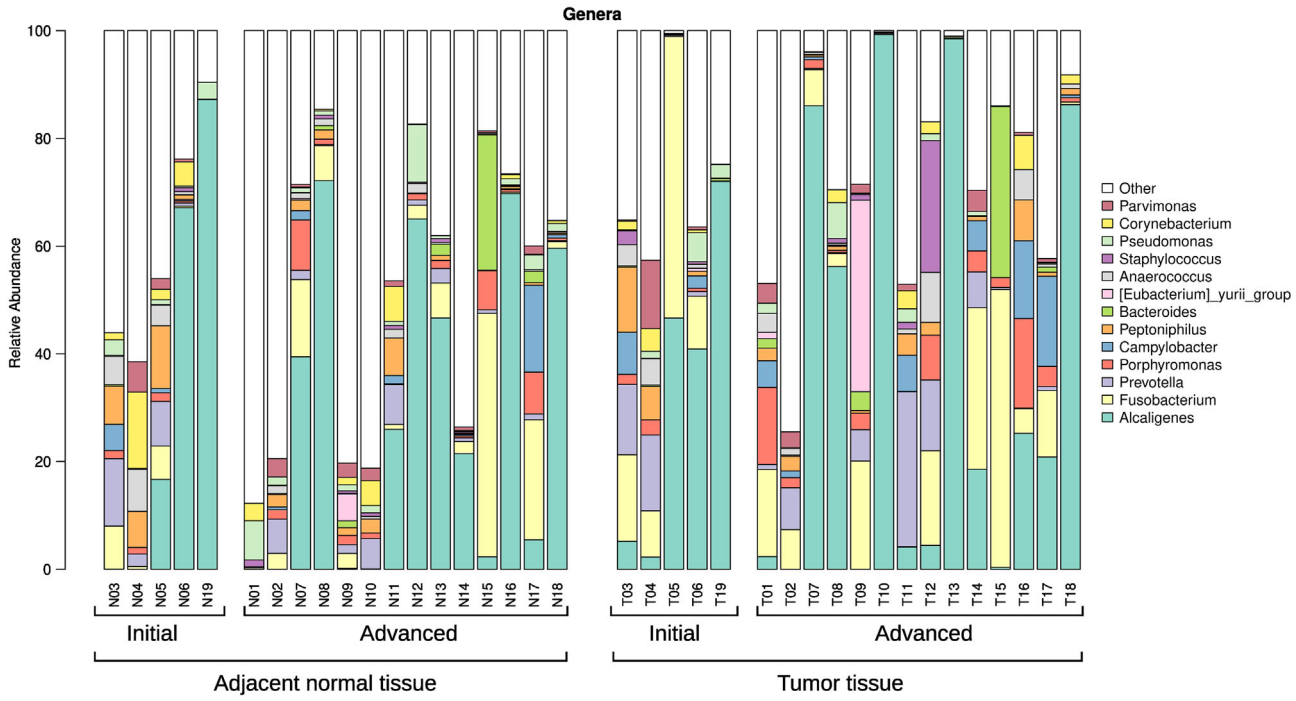


FIGURE 3 Relative abundance of genera in non-tumor (N) and tumor (T) samples. The relative abundance (%) in the tumor (T) and non-tumor (N) sample is shown in the vertical bars. The sample groups in this study are separated into two categories: tumors and non-tumors adjacent to the primary tumor. Each of these categories is further divided based on the stage of the tumor, namely into initial tumor (pT1) and advanced tumor (pT2 or pT3). Colors indicate one of the 13 genera presented according to the legend on the right.

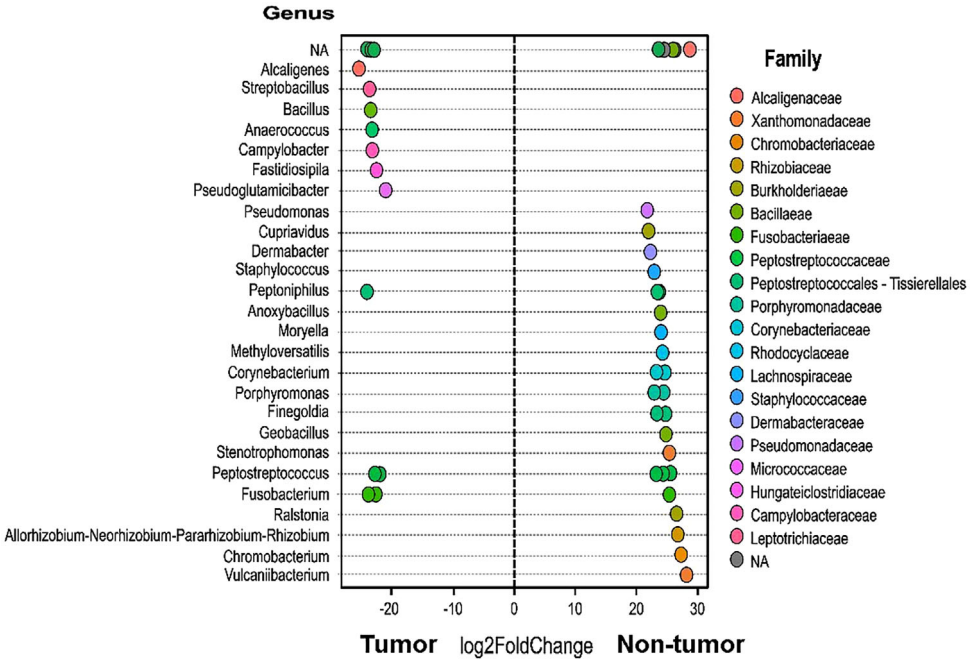


FIGURE 4 Differential abundance of ASVs in tumor and non-tumor adjacent tissues based on paired analyses. On the right (*y*) axis are the genera-level ASVs, and on the left (*y*) axis are the families (colors). On the (*x*) axis, negative "*log2 Fold Change*" values indicate a greater abundance in tumor samples, whereas positive values indicate a greater abundance in adjacent non-tumor samples ($p < 0.05$). NA, sequences identified only at the family level. ASV, amplicon sequence variant.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

FIGURE 5 Relative abundance of functional pathways in tumor (T) and non-tumor (N) samples. Circles represent the total relative abundance of microorganisms that were used to generate the possible functional pathways in tumor (T) and non-tumor (N) samples. The larger the circle, the greater the ratio (%) of microorganisms in the functional pathway, as shown in the caption on the right. The figure was generated using the R software (R Studio, vegan and ggplo2 packages).

the beta diversity analysis did not reveal a difference between tumors and non-tumor tissues, the stratified analysis of advanced tumors (pT2 and pT3) versus early tumors (pT1) revealed a trend of divergence between the two groups, suggesting a possible relationship between the microbiota and tumor prognosis.

Proteobacteria was the most abundant phylum, followed by Firmicutes in both tumor and non-tumor samples. They are considered intestinal commensals, that in some case, show pathogenic properties and have been associated with CRC associated with colitis,[42] bladder cancer,[43] HPV-positive oropharyngeal cancers,[44] bacterial vaginosis

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by meryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

associated with reduced mucosal immunity and induction of chronic inflammation.[45] According to Schwabe and Jobin,[46] Proteobacteria are related to inflammation and carcinogenesis by activating toll-like receptors or Nucleotide Oligomerization Domain (NOD), producing genotoxins, virulence factors, and tumor-promoting metabolites. Our PSCC microbiome data corroborate these findings, indicating that, in addition to inflammatory pathways disturbed by gene alterations related to the inflammatory process, such as EGFR and COX2[20]; Table S1), members of the Proteobacteria phylum may be involved in inflammation in HPV-associated PSCC.

Firmicutes, the second phylum more abundant, are gram-positive bacteria that are commonly associated with the Bacteroidetes, whose ratio between the two phyla is an indicator of dysbiosis.[47] Firmicutes has both representatives with beneficial immunomodulatory activity, and with harmful properties to the organism, and may be a biomarker for the early and non-invasive detection of CRC.[48] The relative abundances of two phyla, Fusobacteriota and Campylobacterota, were statistically different between the paired samples, with higher abundances in tumor tissues. In developed countries, Campylobacterota infection has been associated with intestinal inflammation.[49] More recently, species of the genus *Campylobacter* have been linked to esophageal cancer associated with poor oral hygiene.[50] Furthermore, co-occurrence of *Fusobacteria* and *Campylobacter spp.* has been observed in patients with CRC, with increased prevalence of *Escherichia* and *Campylobacter spp.* in tumors compared to normal adjacent tissues.[51]

Fusobacterium was more abundant in PSCC tissues than in normal tissues. The anerobic gram-negative species of this genus are typically found in the human oral cavity (*Fusobacterium spp.*), gastrointestinal system, and urogenital tract (*Leptotrichia spp.* and *Sneathia spp.*, respectively).[52] However, they are invasive, capable of releasing RNA into the cytoplasm of the host cell, stimulating the activation of NF-kB and activating inflammatory genes and oncogenes,[53] as well as promoting inflammation via the expression of IL-6, IL-8, and IL-18.[54] Rubinstein et al.[55] identified this same mechanism through FadA, a membrane adhesion protein, which binds to E-cadherin on the surface of the epithelial cell, causing its phosphorylation and internalization and activating-catenin signaling pathways. Consequently, this genus has been identified as an indicator of dysbiosis associated with CRC,[56] the prognosis of pancreatic cancer,[57] the progression of oral cancer, and the worst of gastric cancer.[58]

Alcaligenes was the second most frequent genus in both tissues. They are gram-negative bacteria whose species have a great genetic diversity and a predisposition to cause opportunistic infections in humans, but this genus is not often pathogenic.[21] On the other hand, *Chromobacterium* was more abundant in non-tumorous tissues. Interestingly, the gram-negative bacterium *Chromobacterium violaceum* produces violacein, a purple pigment that showed activity against tropical pathogens as well as bactericidal, cytotoxic, antiviral, antifungal, antioxidant, and anticancer activities.[59] In addition, *Corynebacterium*, *Prevotella*, and *Porphyromonas* were among the most abundant genera in non-tumor penile tissues, consistent with previous studies evaluating the penile microbiota of normal tissues.[60,61] Noteworthy the relative abundance of *Prevotella* in human mucosa sites has been linked to inflammatory pathologies, such as bacterial vaginosis and periodontitis, through elevation of innate cytokines (IL-1-α, IL-1-β, Il-8, and TNF-α) in cervicovaginal fluids.[62] In addition, some species of this genus are involved in immune escape mechanisms that predispose to chronic inflammation, triggering an IL-17 tumor response and tumor progression.[63] Interestingly, *Prevotella* has been found in other HPV-associated squamous cell carcinomas, such as head and neck[64] and cervix.[65]

We highlight the presence of *F. magma* and *P. geniculata* in 60% of early disease patients' non-tumor tissues. *F. magma* is an anerobic gram-positive bacterium related to inflammation through neutrophil activation,[66] whereas *Pseudomonas* is an opportunistic pathogen, with *P. aeruginosa* having more clinical significance.[67] The presence of these species in normal tissues adjacent to pT1 tumors encourages future investigation into the prognostic value of these microorganisms in penile carcinogenesis. On the other hand, unique and abundant ASVs of *Alcaligenes*, *Streptobacillus*, *Bacillus*, *Anerococcus*, *Campylobacter*, *Fastidiospila*, and *Pseudoglutamicibacter* were found in tumor samples and will be investigated further to determine their involvement in PSCC.

As mentioned previously, our results revealed the presence of phyla and genera that have been linked to inflammatory processes. However, *Mycobacterium*, which has been associated with penile cancer,[2,10] was found at a low relative abundance (0.005%). We did not expect this finding since poor genital hygiene leads to the accumulation of smegma in the foreskin, hence promoting the growth of bacteria such as *Mycobacterium smegmatis*.

A microbiota-based functional prediction analysis allowed us to identify 35 molecular pathways with significantly lower abundance in tumor tissues. We highlight some pathways already associated with cancer, such as chitin derivates degradation. Chitin and chitosan derivates can exhibit anti-tumor activity via apoptosis stimulation, immunomodulation, anti-oxidative, and anti-angiogenic mechanisms.[68,69] The protocatechuic acid pathway is especially notable since it was demonstrated that this phenolic acid can promote apoptosis in colorectal tumor cells.[70] Different diseases, including glioblastoma and medulloblastoma, have been associated with a disruption in inositol metabolism.[71] Myo-inositol and D-chiro-inositol are involved in insulin and glucose metabolism, and myo-inositol dysregulation has been linked to a number of disorders associated with diabetes or insulin resistance.[72] Both insulin resistance and IGF-1 are associated with an increased risk of cancer.[73] In addition, sucrose pathway enrichment has been identified in healthy patients, but not in patients with cervix cancer.[9] In contrast, Hale et al.[74] identified the sucrose pathway in adenomas, and it was suggested that a diet rich in sugars would affect the microbial composition of the gastrointestinal tract and, as a result, be a risk factor for CRC. Characterization of these different pathways is the first step in explaining their function in healthy and tumor tissues, but further research is required to determine the implications of the PSCC microbiome on disease development and clinical outcomes.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

5 | CONCLUSIONS

We describe for the first time the bacterial composition and diversity, as well as the 35 major metabolic pathways that may be involved in the pathogenesis of HPV-associated PSSC. All of these metabolic pathways have a lower abundance in tumor tissues and include chitin derivates degradation, the protocatechuic acid pathway, inositol metabolism, and the sucrose pathway. Moreover, the presence of bacteria related to inflammation and carcinogenesis, such as the phyla Proteobacteria and Firmicutes, the genera *Fusobacterium* and *Prevotella*, and the species *F. magma* and *P. geniculata*, encourages further study into the diagnostic and prognostic value of these microorganisms in penile carcinogenesis. In addition, the presence of specific and abundant ASVs in both tumor and adjacent non-tumor tissues drives us to investigate these potential biomarkers. The small-sample size is a limitation of this research, but based on our findings, future functional studies will be conducted to better understand the role of bacteria in this rare and severe tumor, thereby offering an opportunity for advances in diagnosis, prevention, and early therapy.

AUTHOR CONTRIBUTIONS

AD—Contextualization, methodology, formal analysis, investigation, data curation, writing—original draft, writing—review & editing and visualization. GG—Formal analysis. JS—Methodology, formal analysis, writing—review and editing. LCLJ—Writing—review & editing and visualization. APAS—Writing—review & editing and visualization. HDA—Contextualization, methodology, formal analysis, research, writing—preparation of the original manuscript, writing—review. SRP—Contextualization, methodology, formal analysis, research, writing—preparation of the original manuscript, writing—review and editing, supervision.

CONFLICT OF INTEREST STATEMENT

The authors declare that this research was conducted in the absence of any commercial or financial relationship that could be interpreted as a potential conflict of interest.

FUNDING INFORMATION

by the Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão (FAPEMA-IECT-05551/18) to Silma Regina Pereira; by Comissão de Aperfeiçoamento de Pessoal do Nível Superior (CAPES; code 001) to Jenilson da Silva, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to Gabriele Gonçalves

DATA AVAILABILITY STATEMENT

Derived data supporting the findings of this study are available from the corresponding author on reasonable request.

ORCID

Amanda de Deus https://orcid.org/0000-0001-8952-8108
Jenilson da Silva https://orcid.org/0000-0002-2636-8108

REFERENCES

1. Ferlay J, Ervik M, Lam F, et al. Global cancer observatory: cancer today. Published 2020. Accessed August 20, 2023. https://gco.iarc.fr/today
2. Flaherty A, Kim T, Giuliano A, et al. Implications for human papillomavirus in penile cancer. *Urol Oncol*. 2014;32(1):53.e1-53.e8. doi:10.1016/j.urolonc.2013.08.010
3. Chaux A, Netto GJ, Rodríguez IM, et al. Epidemiologic profile, sexual history, pathologic features, and human papillomavirus status of 103 patients with penile carcinoma. *World J Urol*. 2013;31(4):861-867. doi:10.1007/s00345-011-0802-0
4. Borque-Fernando Á, Gaya J, Esteban-Escaño L, et al. Epidemiology, diagnosis and management of penile cancer: results from the Spanish National Registry of Penile Cancer. *Cancers*. 2023;15(3):616. doi:10.3390/cancers15030616
5. Olesen TB, Sand FL, Aalborg GL, Munk C, Kjaer SK. Incidence of penile intraepithelial neoplasia and incidence and survival of penile cancer in Denmark, 1997 to 2018. *Cancer Causes Control*. 2022;33(1):117-123. doi:10.1007/s10552-021-01510-5
6. Coelho RWP, Pinho JD, Moreno JS, et al. Penile cancer in Maranhão, Northeast Brazil: the highest incidence globally? *BMC Urol*. 2018;18(1):50. doi:10.1186/s12894-018-0365-0
7. Hakenberg OW, Compérat E, Minhas S, Necchi A, Protzel C, Watkin N, The European Association of Urology (EAU) Guidelines on Penile Cancer. 2018. Accessed August 20, 2023. http://uroweb.org/guideline/penile-cancer/
8. Fatalska A, Rusetska N, Bakuła-Zalewska E, et al. Inflammatory proteins HMGA2 and PRTN3 as drivers of vulvar squamous cell carcinoma progression. *Cancers*. 2020;13(1):27. doi:10.3390/cancers13010027
9. Tango CN, Seo SS, Kwon M, Lee DO, Chang HK, Kim MK. Taxonomic and functional differences in cervical microbiome associated with cervical cancer development. *Sci Rep*. 2020;10(1):9720. doi:10.1038/s41598-020-66607-4
10. Mittal S, Banks L. Molecular mechanisms underlying human papillomavirus E6 and E7 oncoprotein-induced cell transformation. *Mutat Res*. 2017;772:23-35. doi:10.1016/j.mrrev.2016.08.001
11. El Tekle G, Garrett WS. Bacteria in cancer initiation, promotion and progression. *Nat Rev Cancer*. 2023;23(9):600-618. doi:10.1038/s41568-023-00594-2
12. Liu CM, Prodger JL, Tobian AAR, et al. Penile anaerobic dysbiosis as a risk factor for HIV infection. *mBio*. 2017;8(4):e00996. doi:10.1128/mBio.00996-17
13. Sepich-Poore GD, Zitvogel L, Straussman R, Hasty J, Wargo JA, Knight R. The microbiome and human cancer. *Science*. 2021;371(6536):eabc4552. doi:10.1126/science.abc4552
14. Chen J, Li T, Liang J, et al. Current status of intratumour microbiome in cancer and engineered exogenous microbiota as a promising therapeutic strategy. *Biomed Pharmacother*. 2022;145:112443. doi:10.1016/j.biopha.2021.112443
15. Whisner CM, Athena Aktipis C. The role of the microbiome in cancer initiation and progression: how microbes and cancer cells utilize excess energy and promote one another's growth. *Curr Nutr Rep*. 2019;8(1):42-51. doi:10.1007/s13668-019-0257-2
16. Kim BR, Shin J, Guevarra RB, et al. Deciphering diversity indices for a better understanding of microbial communities. *J Microbiol Biotechnol*. 2017;27(12):2089-2093. doi:10.4014/jmb.1709.09027
17. Anderson MJ, Crist TO, Chase JM, et al. Navigating the multiple meanings of β diversity: a roadmap for the practicing ecologist: roadmap for beta diversity. *Ecol Lett*. 2011;14(1):19-28. doi:10.1111/j.1461-0248.2010.01552.x
18. Pope JL, Tomkovich S, Yang Y, Jobin C. Microbiota as a mediator of cancer progression and therapy. *Transl Res*. 2017;179:139-154. doi:10.1016/j.trsl.2016.07.021
19. Aydin AM, Chahoud J, Adashek JJ, et al. Understanding genomics and the immune environment of penile cancer to improve therapy. *Nat Rev Urol*. 2020;17(10):555-570. doi:10.1038/s41585-020-0359-z

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

20. Macedo J, Silva E, Nogueira L, et al. Genomic profiling reveals the pivotal role of hrHPV driving copy number and gene expression alterations, including mRNA downregulation of *TP53* and *RB1* in penile cancer. *Mol Carcinog*. 2020;59(6):604-617. doi:10.1002/mc.23185
21. da Silva J, Nogueira L, Coelho R, et al. HPV-associated penile cancer: impact of copy number alterations in miRNA/mRNA interactions and potential druggable targets. *CBM*. 2021;32(2):147-160. doi:10.3233/CBM-210035
22. da Silva J, da Costa CC, de Farias Ramos I, et al. Upregulated miRNAs on the TP53 and RB1 binding seedless regions in high-risk HPV-associated penile cancer. *Front Genet*. 2022;13:875939. doi:10.3389/fgene.2022.875939
23. do Canto LM, da Silva JM, Castelo-Branco PV, et al. Mutational signature and integrative genomic analysis of human papillomavirus-associated penile squamous cell carcinomas from Latin American patients. *Cancers*. 2022;14(14):3514. doi:10.3390/cancers14143514
24. Paner GP, Stadler WM, Hansel DE, Montironi R, Lin DW, Amin MB. Updates in the eighth edition of the tumor-node-metastasis staging classification for urologic cancers. *Eur Urol*. 2018;73(4):560-569. doi:10.1016/j.eururo.2017.12.018
25. Sanchez DF, Fernandez-Nestosa MJ, Cañete-Portillo S, Cubilla AL. Evolving insights into penile cancer pathology and the eighth edition of the AJCC TNM staging system. *Urol Oncol*. 2022;40(6):215-222. doi:10.1016/j.urolonc.2020.09.010
26. Sambrook J, Fritsch ER, Maniatis T. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press; 1989.
27. Sambrook J, Russell DW. Purification of nucleic acids by extraction with phenol:chloroform. *Cold Spring Harb Protoc*. 2006;2006(1):pdbprot4455. doi:10.1101/pdb.prot4455
28. Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. In: Cancer Cells 7: Molecular Diagnostics of Human Cancer. New York, NY: Cold Spring Harbor Laboratory Press; 1989:209-214.
29. Simon A, Biggins L, Inglesfield S, Montgomery J. FastQC a quality control tool for high throughput sequence data. *Babraham Bioinform*. Published 2010. (Accessed on March 14, 2022). https://www.bioinformatics.babraham.ac.uk/index.html
30. Smyth RP, Schlub TE, Grimm A, et al. Reducing chimera formation during PCR amplification to ensure accurate genotyping. *Gene*. 2010;469(1-2):45-51. doi:10.1016/j.gene.2010.08.009
31. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13(7):581-583. doi:10.1038/nmeth.3869
32. Bokulich NA, Kaehler BD, Rideout JR, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*. 2018;6(1):90. doi:10.1186/s40168-018-0470-z
33. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2012;41(D1):D590-D596. doi:10.1093/nar/gks1219
34. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37(8):852-857. doi:10.1038/s41587-019-0209-9
35. Koleff P, Gaston KJ, Lennon JJ. Measuring beta diversity for presence-absence data. *J Anim Ecology*. 2003;72(3):367-382. doi:10.1046/j.1365-2656.2003.00710.x
36. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol*. 2005;71(12):8228-8235. doi:10.1128/AEM.71.12.8228-8235.2005
37. Douglas GM, Maffei VJ, Zaneveld JR, et al. PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol*. 2020;38(6):685-688. doi:10.1038/s41587-020-0548-6
38. Barbera P, Kozlov AM, Czech L, et al. Data from: EPA-ng: massively parallel evolutionary placement of genetic sequences. Published online 2018:16579930 bytes. doi:10.5061/DRYAD.KB505NC
39. Ye Y, Doak TG. A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. *PLoS Comput Biol*. 2009;5(8):e1000465. doi:10.1371/journal.pcbi.1000465
40. Margaret BS, Krywolap GN. Eubacterium yurii subsp. yurii sp. nov. and Eubacterium yurii subsp. margaretiae subsp. nov.: test tube brush bacteria from subgingival dental plaque. *Int J Syst Bacteriol*. 1986;36(2):145-149. doi:10.1099/00207713-36-2-145
41. Allali I, Delgado S, Marron PI, et al. Gut microbiome compositional and functional differences between tumor and non-tumor adjacent tissues from cohorts from the US and Spain. *Gut Microbes*. 2015;6(3):161-172. doi:10.1080/19490976.2015.1039223
42. Bonnet M, Buc E, Sauvanet P, et al. Colonization of the human gut by *E. coli* and colorectal cancer risk. *Clin Cancer Res*. 2014;20(4):859-867. doi:10.1158/1078-0432.CCR-13-1343
43. Liu F, Liu A, Lu X, et al. Dysbiosis signatures of the microbial profile in tissue from bladder cancer. *Cancer Med*. 2019;8(16):6904-6914. doi:10.1002/cam4.2419
44. Rajasekaran K, Carey RM, Lin X, et al. The microbiome of HPV-positive tonsil squamous cell carcinoma and neck metastasis. *Oral Oncol*. 2021;117:105305. doi:10.1016/j.oraloncology.2021.105305
45. Lin D, Kouzy R, Abi Jaoude J, et al. Microbiome factors in HPV-driven carcinogenesis and cancers. *PLoS Pathog*. 2020;16(6):e1008524. doi:10.1371/journal.ppat.1008524
46. Schwabe RF, Jobin C. The microbiome and cancer. *Nat Rev Cancer*. 2013;13(11):800-812. doi:10.1038/nrc3610
47. Eckburg PB, Bik EM, Bernstein CN, et al. Diversity of the human intestinal microbial flora. *Science*. 2005;308(5728):1635-1638. doi:10.1126/science.1110591
48. Xie YH, Gao QY, Cai GX, et al. Fecal clostridium symbiosum for non-invasive detection of early and advanced colorectal cancer: test and validation studies. *EBioMedicine*. 2017;25:32-40. doi:10.1016/j.ebiom.2017.10.005
49. Phongsisay V. The immunobiology of *Campylobacter jejuni*: innate immunity and autoimmune diseases. *Immunobiology*. 2016;221(4):535-543. doi:10.1016/j.imbio.2015.12.005
50. Poosari A, Nutravong T, Sa-ngiamwibool P, Namwat W, Chatrchaiwiwatana S, Ungareewittaya P. Association between infection with Campylobacter species, poor oral health and environmental risk factors on esophageal cancer: a hospital-based case–control study in Thailand. *Eur J Med Res*. 2021;26(1):82. doi:10.1186/s40001-021-00561-3
51. Warren RL, Freeman DJ, Pleasance S, et al. Co-occurrence of anaerobic bacteria in colorectal carcinomas. *Microbiome*. 2013;1(1):16. doi:10.1186/2049-2618-1-16
52. Brennan CA, Garrett WS. Fusobacterium nucleatum—symbiont, opportunist and oncobacterium. *Nat Rev Microbiol*. 2019;17(3):156-166. doi:10.1038/s41579-018-0129-6
53. Zhou Z, Chen J, Yao H, Hu H. Fusobacterium and colorectal cancer. *Front Oncol*. 2018;8:371. doi:10.3389/fonc.2018.00371
54. Kostic AD, Chun E, Robertson L, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe*. 2013;14(2):207-215. doi:10.1016/j.chom.2013.07.007
55. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-Cadherin/β-Catenin signaling via its FadA Adhesin. *Cell Host Microbe*. 2013;14(2):195-206. doi:10.1016/j.chom.2013.07.012
56. Ranjbar M, Salehi R, Haghjooy Javanmard S, et al. The dysbiosis signature of Fusobacterium nucleatum in colorectal cancer-cause or consequences? A systematic review. *Cancer Cell Int*. 2021;21(1):194. doi:10.1186/s12935-021-01886-z

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

57. Mitsuhashi K, Nosho K, Sukawa Y, et al. Association of *Fusobacterium* species in pancreatic cancer tissues with molecular features and prognosis. *Oncotarget*. 2015;6(9):7209-7220. doi:10.18632/oncotarget.3109
58. Şurlin P, Nicolae FM, Şurlin VM, et al. Could periodontal disease through periopathogen fusobacterium nucleatum be an aggravating factor for gastric cancer? *J Clin Med*. 2020;9(12):3885. doi:10.3390/jcm9123885
59. Durán N, Menck CFM. Chromobacterium violaceum: a review of pharmacological and industiral perspectives. *Crit Rev Microbiol*. 2001;27(3):201-222. doi:10.1080/20014091096747
60. Carda-Diéguez M, Cárdenas N, Aparicio M, Beltrán D, Rodríguez JM, Mira A. Variations in vaginal, penile, and oral microbiota after sexual intercourse: a case report. *Front Med*. 2019;6:178. doi:10.3389/fmed.2019.00178
61. Onywera H, Williamson AL, Ponomarenko J, Meiring TL. The penile microbiota in uncircumcised and circumcised men: relationships with HIV and human papillomavirus infections and cervicovaginal microbiota. *Front Med*. 2020;7:383. doi:10.3389/fmed.2020.00383
62. Anahtar MN, Byrne EH, Doherty KE, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity*. 2015;42(5):965-976. doi:10.1016/j.immuni.2015.04.019
63. Grivennikov SI, Wang K, Mucida D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature*. 2012;491(7423):254-258. doi:10.1038/nature11465
64. Kumpitsch C, Moissl-Eichinger C, Pock J, Thurnher D, Wolf A. Preliminary insights into the impact of primary radiochemotherapy on the salivary microbiome in head and neck squamous cell carcinoma. *Sci Rep*. 2020;10(1):16582. doi:10.1038/s41598-020-73515-0
65. Wu S, Ding X, Kong Y, et al. The feature of cervical microbiota associated with the progression of cervical cancer among reproductive females. *Gynecol Oncol*. 2021;163(2):348-357. doi:10.1016/j.ygyno.2021.08.016
66. Neumann A, Björck L, Frick IM. Finegoldia magna, an anaerobic gram-positive bacterium of the normal human microbiota, induces inflammation by activating neutrophils. *Front Microbiol*. 2020;11:65. doi:10.3389/fmicb.2020.00065
67. Hirai CK. Microbiologia: Burkhoderia cepacia. Published 2020. (accessed on February 17, 2022). https://revistaanalytica.com.br/microbiologia-burkhoderia-cepacia/
68. Kabir SR, Karim MR, Alam MT. Chitinase inhibits growth of human breast and colorectal cancer cells in vitro and Ehrlich ascites carcinoma cells in vivo. *Arab J Chem*. 2022;15(11):104264. doi:10.1016/j.arabjc.2022.104264
69. Cozzarini E, Bellin M, Norberto L, et al. CHIT1 and AMCase expression in human gastric mucosa: correlation with inflammation and Helicobacter pylori infection. *Eur J Gastroenterol Hepatol*. 2009;21(10):1119-1126. doi:10.1097/MEG.0b013e328329742a
70. Acquaviva R, Tomasello B, Di Giacomo C, et al. Protocatechuic acid, a simple plant secondary metabolite, induced apoptosis by promoting oxidative stress through HO-1 downregulation and p21 upregulation in colon cancer cells. *Biomolecules*. 2021;11(10):1485. doi:10.3390/biom11101485
71. Badodi S, Pomella N, Zhang X, et al. Inositol treatment inhibits medulloblastoma through suppression of epigenetic-driven metabolic adaptation. *Nat Commun*. 2021;12(1):2148. doi:10.1038/s41467-021-22379-7
72. Croze ML, Soulage CO. Potential role and therapeutic interests of myo-inositol in metabolic diseases. *Biochimie*. 2013;95(10):1811-1827. doi:10.1016/j.biochi.2013.05.011
73. Djiogue S, Nwabo Kamdje AH, Vecchio L, et al. Insulin resistance and cancer: the role of insulin and IGFs. *Endocr Relat Cancer*. 2013;20(1):R1-R17. doi:10.1530/ERC-12-0324
74. Hale VL, Chen J, Johnson S, et al. Shifts in the fecal microbiota associated with adenomatous polyps. *Cancer Epidemiol Biomarkers Prev*. 2017;26(1):85-94. doi:10.1158/1055-9965.EPI-16-0337

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: de Deus A, Gonçalves G, da Silva J, et al. Microbiome reveals inflammatory-related bacteria and putative functional pathways involved in human papillomavirus-associated penile squamous cell carcinoma. *Andrology*. 2024;12:809–820. https://doi.org/10.1111/andr.13545

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13545 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 15 May 2023 | Revised: 18 July 2023 | Accepted: 18 September 2023

DOI: 10.1111/andr.13538

ANDROLOGY WILEY

ORIGINAL ARTICLE

What do patients with Peyronie's disease expect from therapy? A prospective multi-center study

Lukas Schäfer[1] | Jann F. Cremers[2] | Bahne Witschel[1] | Victor Schüttfort[1] | Timo O. Nieder[3] | Frederik König[1] | Malte W. Vetterlein[1] | Philipp Gild[1] | Roland Dahlem[1] | Margit Fisch[1] | Sabine Kliesch[2] | Armin Soave[1]

[1]Department of Urology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

[2]Department of Clinical and Surgical Andrology, Center of Reproductive Medicine and Andrology, University Medical Center Muenster, Muenster, Germany

[3]Institute for Sex Research, Sexual Medicine and Forensic Psychiatry, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Correspondence
Armin Soave, Department of Urology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany.
Email: a.soave@uke.de

Abstract

Background: Little is known about patients' pre-treatment expectations in Peyronie's disease (PD).

Objective: To evaluate in detail patients' expectations of conservative therapy and surgery.

Patients and methods: This multi-center study prospectively enrolled 317 PD patients, who were scheduled to receive conservative therapy or surgery between 2019 and 2022 at the Department of Urology of the University Medical Center Hamburg-Eppendorf, and the Center of Reproductive Medicine and Andrology, University Medical Center Muenster, both Germany. The primary end-point was patients' pre-treatment expectations of conservative therapy and surgery, measured with the Stanford Expectations of Treatment Scale (SETS). Secondary end-points included patient-reported psychological and physical symptoms, penile pain, symptom bother and erectile function, measured with the Peyronie's disease questionnaire (PDQ) and International Index of Erectile Function Erectile Function Domain (IIEF-EF).

Results: In total, 239 (75%) and 78 (25%) patients were scheduled for the conservative therapy and surgery, respectively. Patients undergoing surgery had higher positive and negative mean SETS expectations scores (14 vs. 11, $p < 0.001$; 9.6 vs. 6.0, $p < 0.001$). In multivariable analysis, surgery was an independent predictor of positive and negative patients' pre-treatment expectations (all $p \leq 0.001$). In thematic analysis, patients undergoing surgery emphasized distinct themes of pre-treatment expectations. Patients undergoing surgery had higher mean PDQ symptom bother as well as higher psychological and physical symptom scores (14 vs. 10, $p < 0.001$; 9.2 vs. 7.1, $p = 0.001$). There were significant positive correlations between SETS negative expectation score and PDQ symptom bother ($|\rho| = 0.25$; $p < 0.001$) as well as PDQ psychological and physical symptoms score, respectively ($|\rho| = 0.21$; $p = 0.001$).

Lukas Schäfer and Jann F. Cremers denote the equal contribution.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2023 The Authors. *Andrology* published by Wiley Periodicals LLC on behalf of American Society of *Andrology* and European Academy of *Andrology*.

Conclusion: PD patients expect both more benefit and more harm from surgery. In addition, patients undergoing surgery have more psychological and physical symptoms and more symptom bother. To set realistic expectations, it is of pivotal importance to assess patients' expectations before starting treatment.

KEYWORDS
expectation, Peyronie's disease, prospective study, therapy, treatment

1 | INTRODUCTION

Peyronie's disease (PD) is an acquired chronic connective tissue disorder of the penile tunica albuginea, and may cause penile pain and plaque formation, as well as deformity, curvature, and length loss of the penis.[1,2] Consequently, PD patients may have severe impairment of sexual activity and satisfaction, and suffer from detrimental psychological sequelae, for example, depression, negative self-perceptions, and self-attitudes of sexuality, as well as emotional and relationship problems (3–5). Depending on the phase and the severity of the disease, as well as patients' preferences, the therapy of PD consists of non-surgical, that is, conservative, and surgical treatment options. Conservative treatment options include oral medications, penile traction therapy, intralesional injections, extracorporal shock wave treatment, and multimodal treatment combining different conservative therapies.[2,6] Surgical therapy, that is, penile corporoplasty with or without grafting, is the gold standard treatment for patients with severe PD, who have stable disease for three months, and allows for correction of penile curvature and deformity, but may have important side effects, including penile shortening, hypoesthesia, and erectile dysfunction (ED).[1,2] In patients with concomitant severe ED, penile prosthesis surgery represents an accepted approach.[1,2]

Patients' expectations of treatment are defined as "*the positive and negative experiences a patient believes that they will have as a result of receiving treatment*", and may be influenced by various aspects, for example, predetermined ideas, online medical content, or psychosocial factors.[7] As demonstrated in several studies, unrealistic pre-treatment expectations may impair the process of the informed consent, jeopardize the physician–patient relationship, and result in medico-legal issues.[8] Thus, a deeper understanding of patients' pre-treatment expectations may reduce barriers between patients and surgeons and improve treatment decision-making.[8] Importantly, the position statement of the European Society of Sexual Medicine (ESSM)[1] and the European Association of Urology (EAU) guidelines on Sexual and Reproductive Health[2] strongly recommend the evaluation of patients' expectations as a clinical principle. Yet, patients' expectations of conservative therapy and surgery remain undetermined in the current body of the literature.

Against this backdrop, we aimed to provide a detailed evaluation of patients' pre-treatment expectations in PD. We hypothesized that patients undergoing surgery have more positive and more negative expectations compared to patients receiving the conservative therapy.

2 | PATIENTS AND METHODS

2.1 | Patient cohort

The local ethics committee approved this prospective study (2021-100657-BO-ff). The study has been performed according to the Declaration of Helsinki. Male patients ≥18 years with PD were eligible. Exclusion criteria were psychiatric disorders or cognitive impairment, and non-German speaking patients. Between October 2019 and November 2022, we enrolled 317 PD patients from two academic centers in Germany, that is, 162 (51%) patients from the Department of Urology, University Medical Center Hamburg-Eppendorf, and 155 (49%) patients from the Department of Clinical and Surgical Andrology, Center of Reproductive Medicine and Andrology (CeRA), University Medical Center Muenster. Informed written consent was obtained from all patients.

2.2 | Patient evaluation and treatment decision-making

In both centers, the standardized pre-treatment patient evaluation included an in-depth history of onset and duration of PD-specific symptoms, prior PD-specific treatment and general medical history, as described previously.[5] The age-adjusted Charlson comorbidity index (ACCI) was calculated,[9] to evaluate the comorbid conditions present in the patients. Auto-photographic documentation determined degree, direction, shape and severity of penile curvature, as described in detail previously.[10] Physical examination and penile ultra-sonography determined location and size of plaques of the tunica albuginea of the penis.

In both centers, the standardized treatment decision-making corresponded largely to the recommendations of the Position Statement of the ESSM[1] and the EAU guidelines on Sexual and Reproductive Health.[2] In both centers, the modes of action of different conservative therapy options as well as the single steps of surgery were explained in detail to the patients in a standardized manner. In addition, the positive effects and potential side effects of different treatment options were thoroughly discussed with the patients in a standardized fashion in both centers, before a shared decision between patient and physician was made for a specific therapy. All patients underwent the recommended specific therapy.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13538 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.3 | Standardized, prospective assessment of patients' pre-treatment expectations, patient-reported psychological and physical symptoms, penile pain, symptom bother and erectile function

Patients' pre-treatment expectations of therapy, patient-reported psychological and physical symptoms, penile pain, symptom bother and erectile function were assessed in a standardized fashion in both centers before starting the treatment. Following patient evaluation, counseling about positive effects and potential side effects of different treatment options, and shared treatment decision-making, patients were asked to complete a standardized questionnaire. The questionnaire included (a) the Stanford Expectations of Treatment Scale (SETS),[7] which allows a multidimensional assessment of patient-reported positive and negative treatment outcome expectations. The six-items SETS includes two subscales, corresponding to positive and negative expectations, reaching total scores from 0 to 18, respectively. Lower and higher scores correspond to low and high levels of expectations, respectively. Finally, SETS includes two open-ended questions regarding patients' expectations of specific benefits and specific harms resulting from the recommended therapy. Patients' pre-treatment expectations of conservative therapy and surgery, measured with SETS, were the primary end-point; (b) the Peyronie's disease questionnaire (PDQ)[11]; and (c) the International Index of Erectile Function Erectile Function Domain (IIEF-EF).[12] Patient-reported psychological and physical symptoms, penile pain, symptom bother and erectile function, measured with PDQ and IIEF-EF, were secondary end-points.

2.4 | Statistical analyses

Our statistical analysis consisted of several steps. First, we performed descriptive analyses of clinical characteristics in patients stratified by the recommended treatment, that is, conservative therapy and surgery. The distribution of frequencies and proportions of categorical variables across the groups was compared using the chi-squared or Fisher's exact test, as appropriate. The normal distribution of continuous variables was tested by the Kolmogorov–Smirnov and the Shapiro–Wilk test. Means and standard deviations (SD) were compared by Student's *t*-test and Mann–Whitney *U*-test, respectively.

Second, we calculated means of the positive and negative expectation scores of SETS with SD and stratified patients by the recommended treatment, that is, conservative therapy and surgery, to compare positive and negative expectation scores between the groups. Similarly, means of PDQ subscale scores as well as the IIEF-EF score were calculated and compared between the groups.

Third, qualitative data of the answers to the open-ended questions were categorized through thematic analysis (grounded theory).[13] In the first step, five authors (AS, LS, BW, VS, JFC) independently used half of the data to generate a framework with final themes. In the second step, the remaining data were used for internal validation. All authors approved the final themes. In the third step, the final themes were stratified by the recommended treatment, that is, conservative therapy and surgery, and graphically displayed using bar charts.

Fourth, we tested for correlations between SETS positive and negative expectation scores, respectively, and PDQ subscale scores as well as the IIEF-EF score using Spearman's rank correlation. The level of correlation was interpreted according to Cohen: $|\rho| = 0.1$, $|\rho| = 0.3$, and $|\rho| = 0.5$ corresponding to weak, moderate, and strong correlation, respectively.[14]

Finally, multivariable linear regression analysis was performed to identify predictors of positive and negative pre-treatment expectations.

Reporting was performed according to the recommendations of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (15).

All tests were two-sided and $p < 0.05$ was considered statistically significant. All analyses were performed with SPSS Statistics (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0, Armonk, NY).

3 | RESULTS

3.1 | Clinical characteristics

Clinical features are presented in Table 1. Surgery was recommended in 78 (25%) patients and consisted of corporoplasty with grafting in all patients. The conservative therapy was recommended in 239 (75%) patients, and consisted of the multimodal treatment combining different conservative therapies in 190 (80%) patients (i.e., penile traction therapy combined with phosphodiesterase type 5 inhibitors (PDE5i) in 141 (59%) patients; potaba and PDE5i combined with the penile traction therapy in 34 (14%) patients; potaba and PDE5i in 13 (5.4%) patients; penile traction therapy combined with potaba in 2 (0.8%) patients), and mono-therapy in 49 (20%) patients (i.e., penile traction therapy in 23 (9.6%) patients; PDE5i in 21 (8.8%) patients; extracorporal shock wave treatment in 3 (1.3%) patients; potaba in 2 (0.8%) patients). Patients undergoing surgery had longer duration of PD and more penile curvature, compared to patients receiving the conservative therapy. In addition, patients undergoing surgery had previously received other PD-specific treatments than their counterparts receiving conservative therapy (all $p \leq 0.001$; Table 1).

3.2 | Patients' pre-treatment expectations

Patients undergoing surgery exhibited both higher positive and higher negative mean SETS expectations scores compared to patients receiving conservative therapy (all $p < 0.001$; Table 2).

Thematic analysis of patients' positive expectations revealed three major themes, including "*straightening of the penis*," "*better sex life*," and "*stopping the disease progression*" (Figure 1A). In addition, thematic analysis of patients' negative expectations revealed three major

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13538 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Clinical characteristics of 317 patients with Peyronie's disease undergoing conservative therapy and surgery.

	Overall cohort	Conservative therapy	Surgery	*p* Value
Patients; *N* (%)	317 (100)	239 (75)	78 (25)	–
Number of presentations per patient; *N* (%)				0.99
1	235 (74)	177 (74)	58 (74)	
2	70 (22)	53 (22)	17 (22)	
3	12 (3.8)	9 (3.8)	3 (3.8)	
Age (years); mean (SD)	54 (9.6)	54 (9.9)	55 (8.7)	0.38
ACCI (*N* = 316); mean (SD)	1.7 (1.5)	1.7 (1.5)	1.9 (1.7)	0.52
Duration of PD (months) (*N* = 311); mean (SD)	22 (26)	20 (27)	30 (21)	**<0.001**
Phase of PD; *N* (%)				**<0.001**
Acute	134 (42)	134 (56)	0 (0)	
Chronic	183 (58)	105 (44)	78 (100)	
Previous PD-specific treatment (*N* = 286); *N* (%)				**0.001**
None	73 (26)	63 (29)	10 (14)	
Potaba	52 (18)	43 (20)	9 (13)	
Potaba + PDE5i	30 (11)	18 (8.4)	12 (17)	
Potaba + penile traction therapy	1 (0.3)	1 (0.5)	0 (0)	
Potaba + PDE5i + penile traction therapy	14 (4.9)	9 (4.2)	5 (6.9)	
PDE5i	50 (18)	37 (17)	13 (18)	
PDE5i + penile traction therapy	45 (16)	29 (14)	16 (22)	
Penile traction therapy	5 (1.7)	0 (0)	5 (6.9)	
ESWT	2 (0.7)	2 (0.9)	0 (0)	
Surgery	12 (4.2)	10 (4.7)	2 (2.8)	
Radiotherapy	1 (0.3)	1 (0.5)	0 (0)	
Vitamin E	1 (0.3)	1 (0.5)	0 (0)	
Plaque location; *N* (%)				0.23
Dorsal	173 (55)	127 (53)	46 (59)	
Ventral	11 (3.5)	8 (3.3)	3 (3.8)	
Left	16 (5.0)	13 (5.4)	3 (3.8)	
Right	4 (1.3)	4 (1.7)	0 (0)	
None	106 (33)	84 (35)	22 (28)	
Unknown	7 (2.2)	3 (1.3)	4 (5.1)	
Maximum plaque dimension (mm) (*N* = 188); mean (SD)	15 (11)	14 (10)	16 (12)	0.54
Direction of penile curvature; *N* (%)				0.23
Dorsal	188 (59)	134 (56)	54 (69)	
Ventral	26 (8.2)	19 (7.9)	7 (9.0)	
Left	66 (21)	52 (22)	14 (18)	
Right	20 (6.3)	17 (7.1)	3 (3.8)	
None	11 (3.5)	11 (4.6)	0 (0)	
Unknown	5 (1.6)	5 (2.1)	0 (0)	
Penile curvature (degree) (*N* = 299); mean (SD)	53 (25)	46 (24)	71 (19)	**<0.001**

Abbreviations: ACCI, age-adjusted Charlson comorbidity index; ESWT, extracorporal shock wave therapy; PD, Peyronie's disease; PDE5i, phosphodiesterase type 5 inhibitor; SD, standard deviation.
Percentages may not add up to 100%, as they are rounded.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13538 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Positive and negative pre-treatment expectations in 317 patients with Peyronie's disease undergoing conservative therapy and surgery.

	Overall cohort	Conservative therapy	Surgery	*p* Value
Patients; *N* (%)	317 (100)	239 (75)	78 (25)	–
SETS; mean (SD)				**<0.001**
Positive expectations	12 (3.8)	11 (3.8)	14 (2.9)	
SETS; mean (SD)				**<0.001**
Negative expectations	6.9 (4.9)	6.0 (4.7)	9.6 (4.8)	

Abbreviations: SETS, Stanford Expectations of Treatment Scale; SD, standard deviation.

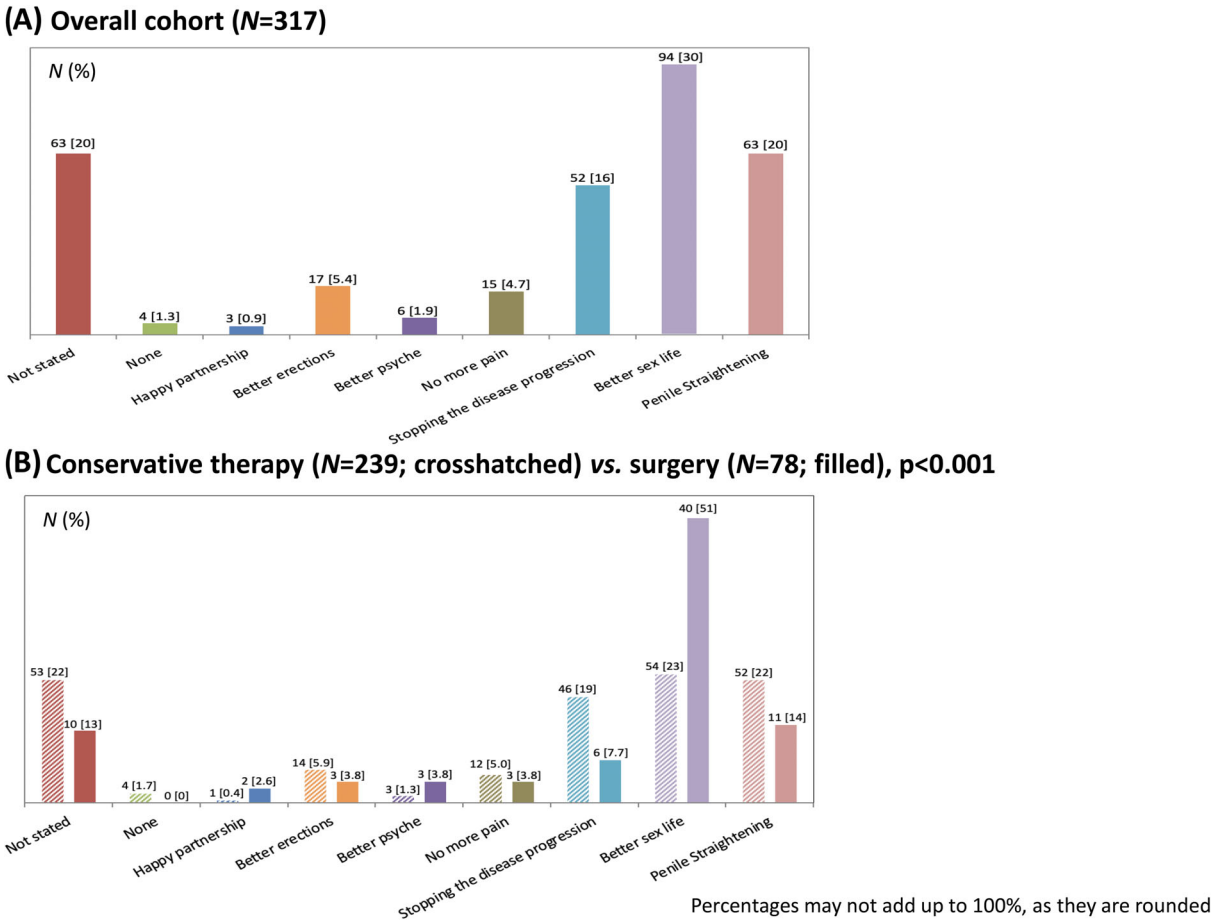


FIGURE 1 Themes of patients' positive pre-treatment expectations.

themes, including "*erectile dysfunction*," "*side effects of treatment*," and "*incomplete straightening of the penis*" (Figure 2A). Quantitative analysis revealed that the major themes differed significantly between patients undergoing surgery and conservative therapy: the most frequent major themes of positive and negative expectations in patients undergoing surgery represented "*better sex life*" and "*erectile dysfunction*", respectively. In patients receiving conservative therapy, the most frequent major themes of positive expectations represented "*better sex life*," "*straightening of the penis*," and "*stopping the disease progression*," whereas "*side effects of treatment*" and "*incomplete straightening of the penis*" represented the major themes of negative expectations (all $p < 0.001$; Figures 1B and 2B).

3.3 | Patient-reported psychological and physical symptoms, penile pain, symptom bother, and erectile function

Patients undergoing surgery had higher mean PDQ symptom bother as well as psychological and physical symptoms scores, compared to

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13538 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

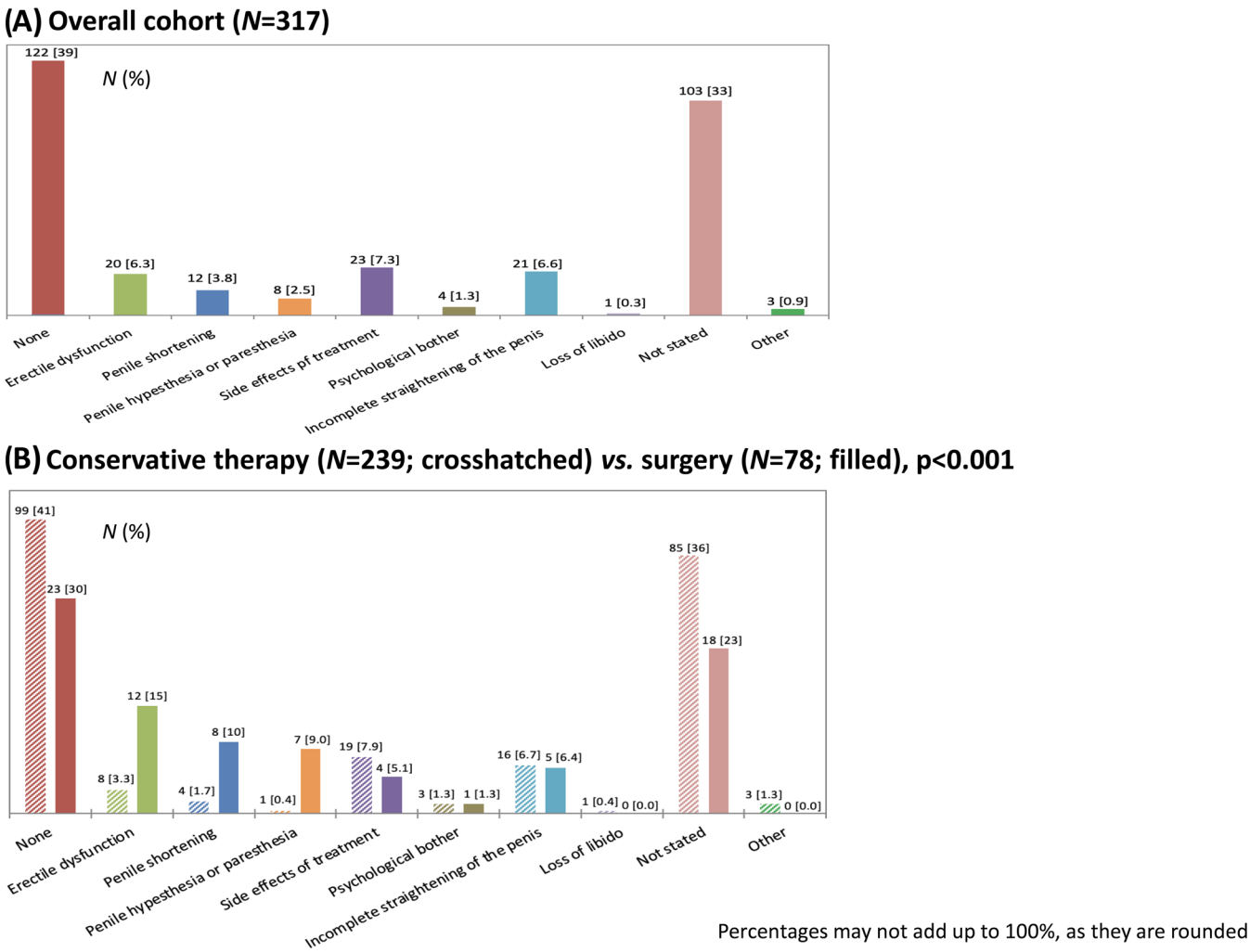


FIGURE 2 Themes of patients' negative pre-treatment expectations.

patients receiving conservative therapy (all $p \leq 0.001$; Table 3). There was no difference in the IIEF-EF score between patients undergoing surgery and conservative therapy. There was a weak negative correlation between SETS positive expectation score and PDQ penile pain score ($|\rho| = -0.15$; $p = 0.019$), as well as weak positive correlations between SETS negative expectation score and PDQ symptom bother ($|\rho| = 0.25$; $p < 0.001$) as well as PDQ psychological and physical symptoms score, respectively ($|\rho| = 0.21$; $p = 0.001$).

3.4 | Predictors of pre-treatment expectations

In multivariable linear regression analysis, surgery was an independent predictor of both positive (coefficient: 2.8; 95% confidence interval (95% CI): 1.2–4.3; $p \leq 0.001$) and negative patients' pre-treatment expectations (coefficient: 3.6; 95% CI: 1.7–5.5; $p \leq 0.001$). The remaining co-variables, that is, age, ACCI, duration and phase of PD, penile curvature, IIEF-EF score, PDQ symptom bother, penile pain as well as psychological and physical symptoms scores did not have independent effects on patients' pre-treatment expectations (all $p \geq 0.09$; Table S1).

4 | DISCUSSION

To the best of our knowledge, the present multi-center study is the first to provide a prospective meticulous evaluation of patients' pre-treatment expectations of the conservative therapy and surgery in PD. The clinical features of our patient cohort corresponded to previous reports in PD, including patients' age, comorbidities, plaque, and curvature characteristics as well as earlier treatment attempts.[16–19] We found that patients undergoing surgery had more positive and more negative expectations toward their treatment compared to their counterparts receiving conservative therapy. In addition, surgery was an independent predictor of positive and negative pre-treatment expectations. This confirms our hypothesis and corresponds to findings on pre-treatment expectations in other chronic diseases, which can be treated with either conservative therapy or surgery, for example, osteoarthritis.[20] This may be attributable to the fact that PD patients undergoing surgery may differently cope with and presumably accept and adapt to a lesser extent to the consequences of their chronic disease, as shown in other diseases.[20] Moreover, we found that patients undergoing surgery had different themes of positive and

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13538 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 Patient reported psychological and physical symptoms, penile pain, symptom bother and erectile function in 317 patients with Peyronie's disease undergoing conservative therapy and surgery.

	Overall cohort	Conservative therapy	Surgery	*p* Value
Patients; *N* (%)	317 (100)	239 (75)	78 (25)	–
PDQ score; mean (SD)				
Symptom bother (*N* = 230)	11 (5.5)	10 (5.3)	14 (5.3)	**<0.001**
Penile pain (*N* = 240)	5.7 (5.6)	5.6 (5.3)	6.2 (6.5)	0.95
Psychological and physical symptoms (*N* = 234)	7.6 (3.9)	7.1 (3.9)	9.2 (3.3)	**0.001**
IIEF-EF score (*N* = 287); mean (SD)	18 (8.6)	18 (8.8)	17 (7.9)	0.51
IIEF-EF score (*N* = 287); *N* (%)				0.13
Severe ED	70 (22)	56 (26)	14 (20)	
Moderate ED	56 (18)	39 (18)	17 (25)	
Mild to moderate ED	49 (15)	37 (17)	12 (17)	
Mild	39 (12)	25 (12)	14 (20)	
No ED	73 (23)	61 (28)	12 (17)	

Abbreviations: ED, erectile dysfunction; IIEF-EF, International Index of Erectile Function Erectile Function Domain; PDQ, Peyronie's disease questionnaire; SD, standard deviation.
Percentages may not add up to 100%, as they are rounded.

negative pre-treatment expectations, compared to patients receiving conservative therapy: "*better sex life*" represented the major theme of positive expectations in patients undergoing surgery, whereas "*better sex life*," "*straightening of the penis*," and "*stopping the disease progression*" comprised the co-major themes in patients receiving the conservative therapy. This may indicate that patients undergoing surgery do not necessarily expect to have a perfectly shaped and straight penis, but rather to be able having sexual intercourse again. In contrast, patients receiving the conservative therapy consider straightening of the penis as more important. In patients undergoing surgery, the major theme of negative expectations was "*erectile dysfunction*," whereas in patients receiving the conservative therapy, these major themes included "*side effects of treatment*" and "*incomplete straightening of the penis*." Importantly, some patients had unrealistic positive expectations, for example, "*better psyche*" and "*happy partnership*," and more patients receiving the conservative therapy, stated that they did not expect any negative effects of treatment or did not answer this open-ended question. Similarly, other authors have shown that a relevant number of patients, who were treated with different surgical techniques, had incorrect beliefs regarding the purpose of surgery in PD. For example, a retrospective study revealed that up to 18% of patients just expected plaque excision without caring about functional and cosmetic results.[18] Overall, the counseling of patients about the positive effects and potential side effects of different treatment options for PD in the present study led to realistic patients' pre-treatment expectations of the conservative therapy and surgery. However, some patients still had unrealistic positive expectations and a relevant proportion of patients did not expect any harm from treatment, although side effects have been discussed with all patients before starting conservative therapy or surgery. These findings highlight two pivotal things (a) the importance of thorough patient counseling on positive effects and potential side effects of different treatment options in PD, prior to a shared decision-making between patient and physician, in order to set realistic patients' pre-treatment expectations; (b) the importance of assessing patients' pre-treatment expectations, in order to identify and eventually modify unrealistic expectations before starting the therapy. Thus, it can be ensured that a decision for a specific therapy does not rely on patients' misconceptions of benefits and harms of different treatment options.[21]

We found that patients undergoing surgery had more symptom bother as well as psychological and physical symptoms, compared to patients receiving the conservative therapy. To the best of our knowledge, no other studies have currently compared patient-reported symptom bother in PD treated with conservative therapy and surgery. In contrast to the acute phase of PD, patients undergoing surgery have a different—chronic—disease phase, and this may contribute to a more pronounced symptom bother. In addition, more symptom bother as well as psychological and physical symptoms may be due to longer disease duration, different previous treatment attempts and patients' varying ability or willingness to adapt to and accept the PD-specific symptoms over time, as demonstrated in other chronic diseases, for example, chronic obstructive pulmonary disease or congestive heart failure.[22] Last but not the least, the higher degree of the penile curvature in patients undergoing surgery may also be a reason for more symptom bother, as other authors have previously demonstrated that the more pronounced the penile curvature, the higher the PDQ symptom bother as well as psychological and physical symptoms scores.[16,23] We found significant correlations between patients' negative expectations and symptom bother as well as psychological and physical symptoms, and a negative correlation between positive expectations and penile pain. Thus, these variables influence each other, and positive and negative treatment expectations may refer to disease-related symptoms.[24] Again, these findings highlight the importance that clinicians evaluate pre-treatment expectations in patients with PD.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13538 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Despite the strength of our study, there are some limitations that merit attention. First, SETS was initially developed for pain research,[7] not for measuring pre-treatment expectations in PD. However, currently, there are few validated multidimensional instruments available for the evaluation of patients' expectations.[24] In line with the expectancy theory,[25] SETS allows a multidimensional assessment of pre-treatment expectations, particularly with regard to negative and positive patients' expectations.[7] In addition, SETS has been successfully implemented in other contexts, for example, smoking cessation trials,[26] due to its concise and uncomplicated design. Accordingly, the present study showed that SETS is a helpful tool, and we encourage its use for the evaluation of pre-treatment expectations in PD. Second, patients receiving different conservative therapies were combined in one group. In the majority of patients, the conservative therapy consisted of multimodal treatment combining different conservative therapies. Surgery consists of corporoplasty with grafting of Biodesign four-layer small intestinal submucosa (Cook Medical LLC, Bloomington, IN, USA) at the Department of Urology, University Medical Center Hamburg-Eppendorf, and grafting of TachoSil (Corza Health Inc., San Diego, CA, USA) at the CeRA, University Medical Center Muenster. In contrast to the approach at the CeRA, corporoplasty does always include circumcision at the Department of Urology, University Medical Center Hamburg-Eppendorf. Thus, both groups, that is, patients undergoing conservative therapy and surgery, respectively, exhibit a certain heterogeneity, which may have influenced the results in the present study. In addition, none of the patients in the present study underwent penile prosthesis surgery. Therefore, it is of crucial importance that our study does not allow drawing conclusions regarding patients' expectations of penile prosthesis surgery in PD. Third, we were not able to evaluate patients who refused to participate in this study, which might have introduced an additional risk of bias. Finally, we did not analyze the impact of pre-treatment expectations on patient-reported outcome and satisfaction. Since patients' expectations do have effects on surgical outcome in other contexts, for example, bariatric,[27] cardiac and spine surgery,[28,29] we hope to report on this important issue in the near future.

In conclusion, patients with PD expect both more benefit and more harm from surgery compared to the conservative therapy. In addition, patients undergoing surgery have distinct themes of pre-treatment expectations, compared to patients receiving the conservative therapy. Moreover, patients undergoing surgery have more symptom bother as well as psychological and physical symptoms. Finally, there is a correlation between pre-treatment expectations and patient-reported symptoms and bother. The findings of the present study allow deeper understanding of patients' pre-treatment expectations in PD and underline the importance of assessing patients' expectations in clinical practice before starting the treatment.

AUTHOR CONTRIBUTIONS

All authors meet the criteria for authorship.

ACKNOWLEDGMENTS

Open access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

The authors received no financial support for the research, authorship, and/or publication of this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Sabine Kliesch https://orcid.org/0000-0002-7561-4870
Armin Soave https://orcid.org/0009-0006-4552-4934

REFERENCES

1. Osmonov D, Ragheb A, Ward S, et al. ESSM position statement on surgical treatment of Peyronie's disease. *Sex Med*. 2022;10(1):100459.
2. Salonia A, Bettocchi C, Boeri L, et al. European Association of Urology Guidelines on Sexual and Reproductive Health. 2023. Presented at the EAU Annual Congress Milan 2023. ISBN 978-94-92671-19-6.
3. Nelson CJ, Mulhall JP. Psychological impact of Peyronie's disease: a review. *J Sex Med*. 2013;10(3):653-660.
4. Nelson CJ, Diblasio C, Kendirci M, Hellstrom W, Guhring P, Mulhall JP. The chronology of depression and distress in men with Peyronie's disease. *J Sex Med*. 2008;5(8):1985-1990.
5. Soave A, Laurich S, Dahlem R, et al. Negative self-perception and self-attitude of sexuality is a risk factor for patient dissatisfaction following penile surgery with small intestinal submucosa grafting for the treatment of severe Peyronie's disease. *J Clin Med*. 2019;8(8):1121.
6. Manka MG, White LA, Yafi FA, Mulhall JP, Levine LA, Ziegelmann MJ. Comparing and Contrasting Peyronie's Disease Guidelines: points of consensus and deviation. *J Sex Med*. 2021;18(2):363-375.
7. Younger J, Gandhi V, Hubbard E, Mackey S. Development of the Stanford Expectations of Treatment Scale (SETS): a tool for measuring patient outcome expectancy in clinical trials. *Clin Trials*. 2012;9(6):767-776.
8. Waljee J, McGlinn EP, Sears ED, Chung KC. Patient expectations and patient-reported outcomes in surgery: a systematic review. *Surgery*. 2014;155(5):799-808.
9. Charlson M, Szatrowski TP, Peterson J, Gold J. Validation of a combined comorbidity index. *J Clin Epidemiol*. 1994;47(11):1245-1251.
10. Kelâmi A. Classification of congenital and acquired penile deviation. *Urol Int*. 1983;38(4):229-233.
11. Hellstrom WJ, Feldman R, Rosen RC, Smith T, Kaufman G, Tursi J. Bother and distress associated with Peyronie's disease: validation of the Peyronie's disease questionnaire. *J Urol*. 2013;190(2):627-634.
12. Cappelleri JC, Rosen RC, Smith MD, Mishra A, Osterloh IH. Diagnostic evaluation of the erectile function domain of the International Index of Erectile Function. *Urology*. 1999;54(2):346-351.
13. Walker D, Myrick F. Grounded theory: an exploration of process and procedure. *Qual Health Res*. 2006;16(4):547-559.
14. Cohen J. Statistical Power Analysis for the Behavioral Sciences. 2nd ed. *Routledge*; 1988.
15. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *Lancet*. 2007;370(9596):1453-1457.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13538 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

16. Cilio S, Fallara G, Capogrosso P, Candela L, Belladelli F, Pozzi E, et al. The symptomatic burden of Peyronie's disease at presentation according to patient age: a critical analysis of the Peyronie's disease questionnaire (PDQ) domains. *Andrology*. 2023 Mar;11(3):501-507.
17. Burri A, Porst H. The relationship between penile deformity, age, psychological bother, and erectile dysfunction in a sample of men with Peyronie's Disease (PD). *Int J Impot Res*. 2018;30(4):171-178.
18. Zheng DC, Bao JW, Guo JH, Xie MK, Li WJ, Wang Z. Effects of patients' understanding and choice of surgical types on postoperative outcomes of Peyronie's disease: a single-center retrospective study of 108 patients. *Asian J Androl*. 2021;23(5):484-489.
19. Flores JM, Nascimento B, Punjani N, Salter CA, Bernie HL, Taniguchi H, et al. Predictors of curvature improvement in men with Peyronie's disease treated with intralesional collagenase clostridium histolyticum. *J Sex Med*. 2022;19(11):1680-1686.
20. Wouters RM, Vranceanu AM, Slijper HP, et al. Patients with thumb-base osteoarthritis scheduled for surgery have more symptoms, worse psychological profile, and higher expectations than nonsurgical counterparts: a large cohort analysis. *Clin Orthop Relat Res*. 2019;477(12):2735-2746.
21. Ring D, Leopold SS. Editorial: the sacredness of surgery. *Clin Orthop Relat Res*. 2019;477(6):1257-1261.
22. Walke LM, Byers AL, McCorkle R, Fried TR. Symptom assessment in community-dwelling older adults with advanced chronic disease. *J Pain Symptom Manage*. 2006;31(1):31-37.
23. Salter CA, Nascimento B, Terrier JE, et al. Evaluating the impact of penile girth discrepancy on patient bother in men with Peyronie's disease: an observational study. *J Sex Med*. 2020;17(8):1560-1565.
24. Laferton JA, Kube T, Salzmann S, Auer CJ, Shedden-Mora MC. Patients' expectations regarding medical treatment: a critical review of concepts and their assessment. *Front Psychol*. 2017;8:233.
25. Kirsch I. Response expectancy as a determinant of experience and behavior. *Am Psychol*. 1985;40:1189-1202.
26. Ferkin AC, Tonkin SS, Maguin E, et al. A psychometric evaluation of the Stanford Expectations of Treatment Scale (SETS) in the context of a smoking cessation trial. *Nicotine Tob Res*. 2022;24(12):1914-1920.
27. van Rijswijk AS, Evren I, Geubbels N, et al. Outcome expectation and risk tolerance in patients seeking bariatric surgery. *Surg Obes Relat Dis*. 2021;17(1):139-146.
28. Saban KL, Penckofer SM. Patient expectations of quality of life following lumbar spinal surgery. *J Neurosci Nurs*. 2007;39(3):180-189.
29. Chunta KS. Expectations, anxiety, depression, and physical health status as predictors of recovery in open-heart surgery patients. *J Cardiovasc Nurs*. 2009;24(6):454-464.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Schäfer L, Cremers JF, Witschel B, et al. What do patients with Peyronie's disease expect from therapy? A prospective multi-center study. *Andrology*. 2024;12:821–829. https://doi.org/10.1111/andr.13538

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13538 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 21 January 2023 | Revised: 5 August 2023 | Accepted: 6 September 2023

DOI: 10.1111/andr.13532

ANDROLOGY

WILEY

ORIGINAL ARTICLE

Peyronie's disease response to intralesional collagenase clostridium histolyticum therapy is independent of baseline testosterone

Douglas Schneider | Mitchell O'Leary | Eliad Amini | Jake Miller | Nick Hassas | Jeanie Nguyen | Muhammed A. Moukhtar Hammad | David Barham | Faysal A. Yafi

Irvine Department of Urology, University of California, Irvine, California, USA

Correspondence
Douglas Schneider, Irvine Department of Urology, University of California, Suite 7200, 3800 W. Chapman Avenue, Irvine, CA 92868, USA.
Email: dougls1@hs.uci.edu

[Correction added on 29 September 2023, after first online publication: Figure 2 has been replaced in this version.]

Abstract

Background: Testosterone plays an important role in collagen metabolism, transforming growth factor-β1 expression, and wound healing, which are all critical factors in pathogenesis of Peyronie's disease. Some clinical studies have suggested an association between Peyronie's disease and hypogonadism.

Objective: We sought to investigate whether baseline total testosterone levels influence response to intralesional collagenase clostridium histolyticum in Peyronie's disease.

Methods: A retrospective review of patients receiving collagenase clostridium histolyticum injections with available total testosterone levels within 1 year of initial injection was conducted at a single institution. Baseline demographics, hypogonadal status, total testosterone, number of collagenase clostridium histolyticum cycles, and pre- and post-treatment degrees of curvature were collected. Hypogonadism was defined as total testosterone <300 ng/dL.

Results and discussion: Thirty-six men were included with mean age of 58.2 years (SD 10.4) and mean body mass index 26.8 (SD 3.2). The mean total testosterone was 459.2 ng/dL (SD 144.0), and four (11.1%) were hypogonadal. Mean pre-treatment curvature was 47.6°, and mean post-treatment curvature was 27.8°, with mean improvement of 19.9° (40.1%). Hypogonadal status was not significantly associated with more severe curvature, 46.4° among hypogonadal men as to 57.5° among eugonadal men ($p = 0.32$). On linear regression analysis, total testosterone did not significantly predict improvement in degrees ($\beta = -0.02$; $R^2 = 0.06$; $p = 0.14$) or percent ($\beta = 0.0$; $R^2 = 0.05$; $p = 0.18$). Improvement in neither degrees nor percent differed significantly by hypogonadal status ($p = 0.41$ and 0.82, respectively). The cycle number did significantly predict greater improvement in curvature on both univariate and multivariate analyses ($\beta = 5.7$; $R^2 = 0.34$; $p < 0.01$).

Douglas Schneider and Mitchell O'Leary contributed equally as first authors.

© 2023 American Society of Andrology and European Academy of Andrology.

Conclusion: Neither total testosterone nor hypogonadism is associated with a degree of improvement after collagenase clostridium histolyticum treatment.

KEYWORDS
hypogonadism, intralesional collagenase clostridium histolyticum, Peyronie's disease, testosterone

1 | INTRODUCTION

Peyronie's disease (PD) is a progressive fibrotic disorder involving the tunica albuginea of the penis where collagenous plaques develop.[1] PD develops secondary to the over-healing and unregulated fibrotic cascade, creating an inelastic scar on the tunica albuginea that involves the septal fibers between the corpora cavernosa.[2] Patients with PD can experience pain and discomfort in the active/acute phase of the illness, and develop various penile deformities in the chronic stages of the disease that often contribute to difficulty with intercourse and negative psychosocial outcomes in patients.[3]

Currently, treatment for PD ranges from intralesional injections to more sophisticated surgical interventions. In the realm of pharmaceuticals, collagenase clostridium histolyticum (CCh; Xiaflex, Endo International) directly targets Peyronie's plaque with the aim of decreasing curvature. CCh preferentially degrades the type I and type III collagen fibers found in PD plaques and has become increasingly prominent as the standard of care when treating PD.[3,4]

Interestingly, recent survey studies have suggested that CCh treatment outcomes are improved when given in conjunction with testosterone for PD.[5] Although there is room in the literature to suggest that testosterone can participate in anti-fibrotic signaling at the basic-science level, there is conflicting evidence in this regard, and the literature is not clear on testosterone's impact on PD treatment or severity.[6–9] Given this ambiguity in the literature, we sought to carry out an investigation to delineate the relationship between testosterone levels and the treatment of PD. Specifically, our primary aim is to evaluate whether baseline serum testosterone levels impact patients' response to CCh treatment for PD. Secondarily, we seek to better characterize the relationship between testosterone levels and PD severity.

2 | METHODS

2.1 | Experimental design

A retrospective review of patients undergoing CCh injections was conducted at the University of California, Irvine. After obtaining IRB approval, patient data were obtained from electronic medical records dating between November 2017 and October 2021. Only patients with available total testosterone (tT) levels within 1 year of initial CCh injection were included.

Curvature was assessed after in-office intracavernosal injection of vasodilatory agents using a goniometer with patient attaining at least 70% rigid erection or by patient photograph. Redosing was performed as needed to attain sufficient rigidity. Data were collected on baseline demographic characteristics (i.e., height, weight, age, body mass index [BMI]), hypogonadal status, erectile function including rigidity, tT, number of CCh cycles, and pre- and post-treatment degree of curvature. Hypogonadism was defined as a tT <300 ng/dL. Testosterone replacement therapy status was determined as of first CCh treatment. For patients who had multiplanar curvature, the more severe dimension of curvature was used in the analysis.

Xiaflex treatment followed institutional protocol based on the IMPRESS trial.[10] Xiaflex was dosed for four cycles of two injections 5–7 days apart with manual penile plaque modeling 1–3 days following injection. The dose administered was 0.90 mg for each injection. No patients in our study used a stretching device.

2.2 | Statistical analysis

All statistical analyses were performed in R (R Core Team). *t*-Tests were performed to compare continuous variables, Fisher exact tests were performed to compare categorical variables, and linear regression analysis was performed to determine the relationship between continuous variables.

3 | RESULTS

A total of 36 men were included. The mean age was 58.2 years old (SD 10.4) and the mean BMI was 26.8 (SD 3.2). The mean pre-treatment tT was 459.2 ng/dL (SD 144.0), and four (11.1%) were hypogonadal. Of eugonadal men, five (16.1%) were receiving hormonal replacement therapy, four on testosterone and one on clomiphene. Thirty (83.3%) men experienced some degree of erectile dysfunction. The mean pre-treatment curvature was 47.6° (SD 14.2) and the mean post-treatment curvature was 27.8°(SD 10.7), for a mean improvement of 19.9° (40.1%) after a median of four cycles (2–8) (Table 1). Baseline tT was not associated with baseline rigidity ($\beta = -0.01$; $R^2 = 0.02$; $p = 0.41$). Additionally, hypogonadal status was not significantly associated with more severe curvature, 57.5° among hypogonadal men as to 46.4° among eugonadal men ($p = 0.32$); similarly, baseline curvature and tT were not significantly associated ($\beta = -0.03$; $R^2 = 0.09$; $p = 0.08$).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13532 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Demographic and clinical characteristics.

Demographic characteristics	
Mean age	58.2 (SD 10.4)
Mean BMI	26.8 (SD 3.2)
Clinical characteristics	
Mean total testosterone (pre-treatment)	459.2 (SD 144.0)
Men with hypogonadism	4 (11.1%)
Men with erectile dysfunction	31 (86%)
Mean percent rigidity (pre-treatment)	73.9%[a] (SD 11.6%)
Median number of cycles	4 (2–8)
Curvature characteristics	
Mean curvature (pre-treatment)	47.6° (SD 14.2)
Mean curvature (post-treatment)	27.8° (SD 10.7)
Curvature direction	
Left	13 (36%)
Right	7 (19%)
Dorsal	24 (67%)
Ventral	3 (8%)
Multiplanar	11 (31%)

Abbreviation: BMI, body mass index.
[a]$N = 35$.

On linear regression analysis, tT did not significantly predict improvement in degrees ($\beta = -0.02$; $R^2 = 0.06$; $p = 0.14$; Figure 1) or percent ($\beta = 0.0$; $R^2 = 0.05$; $p = 0.18$; Figure 2). Similarly, improvement in neither degrees nor percent differed significantly by hypogonadal status ($p = 0.41$ and 0.82, respectively). The number of cycles significantly predicted greater improvement in curvature in both univariate and multivariate analyses ($\beta = 5.7$; $R^2 = 0.34$; $p < 0.01$).

4 | DISCUSSION

Our investigation failed to show a statistically significant association between curvature severity and hypogonadal status. The exact role testosterone plays in the severity and treatment of PD has not been definitively characterized in the literature. Although testosterone has long been suspected to participate in the pathogenesis of PD given its various physiological roles, data have been conflicting in this regard and no prospective randomized analyses exist for assessment.[6]

A compelling basic science rationale for a potential role of testosterone in PD pathogenesis does exist. Testosterone reduces collagen production, increases nitric oxide levels to antagonize fibrosis, and reduces calcium (Ca^{2+}) metabolism involved in pro-fibrotic signaling.[7] Hypogonadism also contributes to various pathologic changes in the penis, including corpora cavernosa atrophy, fibrosis, and collagen deposition.[11] Montorsi et al.[12] and Iacono et al.[13] have similarly suggested a correlation between corporal fibrosis severity and low testosterone levels, hypothesizing that fibrosis may be a consequence of the lower levels of testosterone seen in advanced age. Conversely, androgen deprivation has been shown to produce significant reduction in trabecular smooth muscle content, while increasing deposition of extracellular connective matrix.[8] Moreover, laboratory studies on rats have shown that androgen supplementation in the setting of induced androgen deficiency (i.e., castration) can restore penile nerve function and maintain the tunica albuginea.[9] Clinical evidence thus far, however, is conflicting.

Recent clinical studies have provided evidence that testosterone levels may not be associated with PD severity. Candela et al.[14] conducted a study involving 149 sexually active PD patients and found no association between serum testosterone levels and the severity of penile curvature. The study analyzed testosterone levels using LOWESS curves and linear regression models, demonstrating that neither total nor free testosterone levels were correlated with penile curvature severity. Furthermore, multivariable analysis revealed that the duration of PD remained the only significant factor associated with the magnitude of curvature. Mitsui et al.[15] similarly evaluated 109 PD patients in Japan and found an inverse correlation between penile curvature and testosterone levels.[5] However, this correlation did not remain significant after adjusting for other factors. The study demonstrated that patients with severe curvature had lower mean tT levels than those with less severe curvature, but there was no significant difference between different testosterone groups or quartiles. Logistic regression analysis indicated that testosterone levels were not the most relevant factor in predicting severe penile deformity.[15]

In addition, a study by Mulhall et al.[16] involving 184 PD subjects found no association between total or free testosterone levels and the degree of curvature. The study also showed no significant difference in mean curvature between different testosterone groups or between men with uni-planar and multiplanar curvature.

Our investigation found similar results to the above studies—neither hypogonadal status nor baseline testosterone level was associated with worse baseline curvature. While testosterone may play a role in the pathogenesis of PD, this role is not clearly seen in the clinical data.

However, testosterone has additionally been proposed as an adjunct in treatment of PD, as it could act synergistically with CCh treatment by means of contributing to collagen degradation and reducing fibrosis or by stimulating anti-fibrotic pathways itself independent of coadministration with other pharmaceuticals. Shlykova and Morgentaler[5] in 2020 showed a subjective improvement in PD symptoms after supplementation with testosterone during treatment with CCh; however, their investigation was limited by patient-reported outcomes and a lack of objective improvement measures.

Our investigation, however, demonstrated no such relationship: baseline testosterone levels were not related to treatment outcomes with CCh. From our review of the literature, we present the first data set specifically examining the relationship between testosterone levels and objective response to CCh. Moreover, we found no difference in improvement after CCh treatment based on a man's gonadal status. Predictably, we did note a significant association between number of cycles and degree of improvement, consistent with prior investigations of the efficacy of CCh.[17] Based on our findings, testosterone

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13532 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

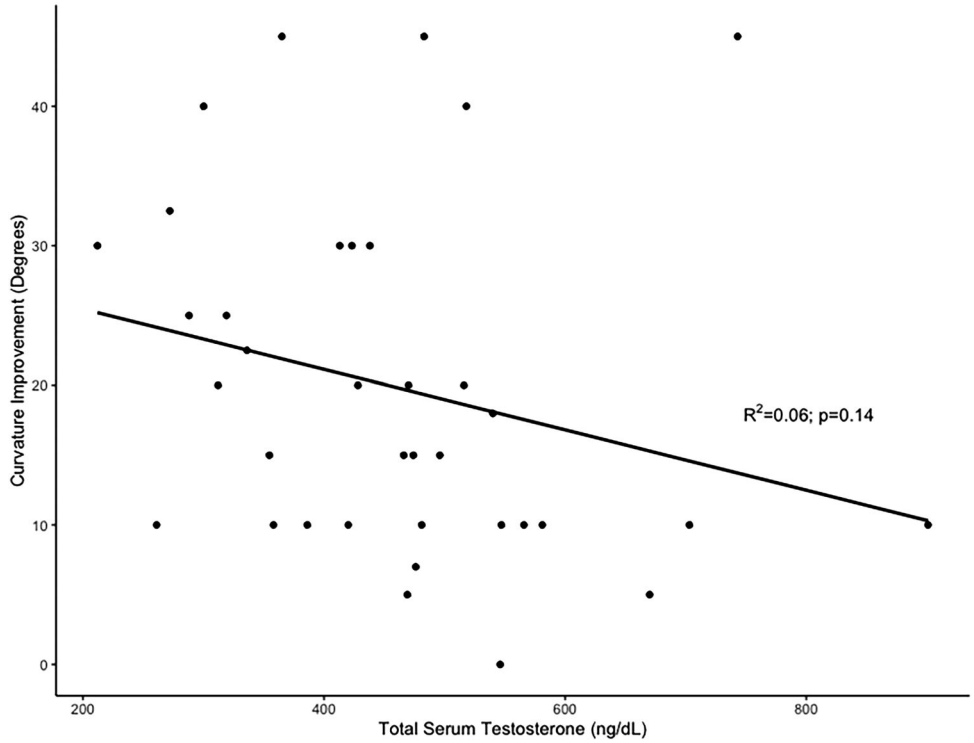


FIGURE 1 Curvature improvement by baseline serum testosterone.

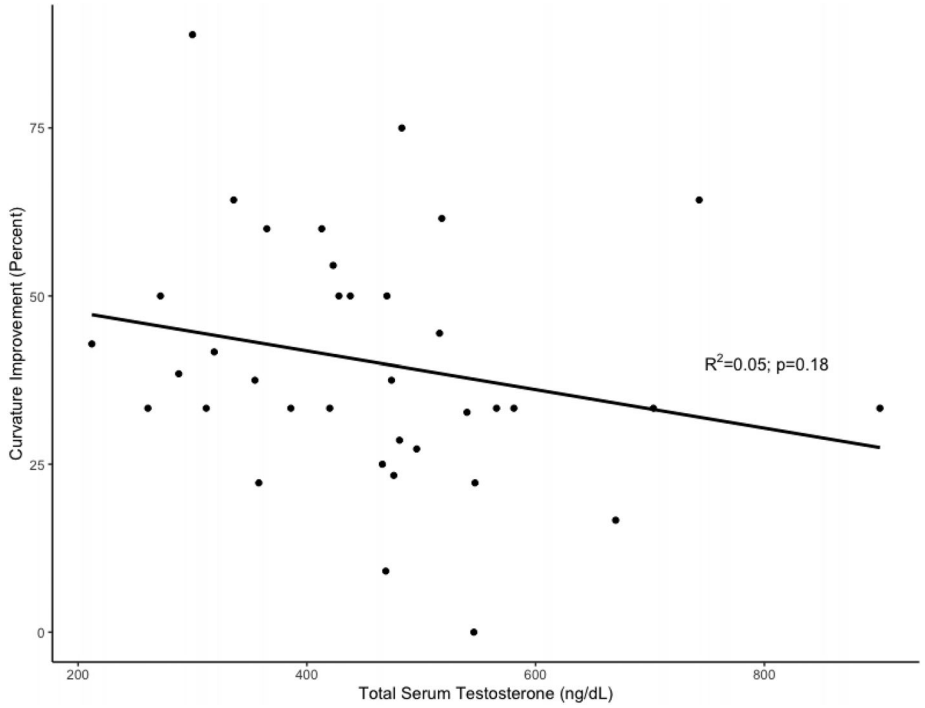


FIGURE 2 Percent curvature improvement by baseline serum testosterone.

levels should not impact the decision to treat with CCh, as both hypogonadal and eugonadal men experienced similar degrees of absolute and relative improvement after treatment.

Our analysis is limited by several factors. Notably, our investigation did not specifically assess the effect that the addition of exogenous testosterone has on PD treatment—it remains possible that

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13532 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

supplementing CCh therapy with exogenous testosterone uniquely creates a suitable microenvironment in which the anti-fibrotic properties of testosterone are appreciated and contribute to improved outcomes. Importantly, true cutoff values for defining hypogonadism remain controversial, and our study employed the cutoff of 300 ng/dL suggested by the American Urological Association guidelines.[18] While this value represents a somewhat standard cutoff, it does introduce bias in statistical analysis and subgroup definitions. Moreover, the retrospective study design of our study offers the potential for selection bias in the inclusion process. Similarly, our analysis is limited by its small sample size, heterogeneity in curvature assessment, and the inherent subjectivity of penile curvature assessment and improvement. Furthermore, we did not separate results based on degree of plaque calcification on ultrasound; nevertheless, none of the patients included had more than mild scattered calcifications. In spite of these limitations, the data described provide reassurance that intralesional CCh treatment should be offered to patients with PD irrespective of baseline tT levels.

5 | CONCLUSION

Neither total testosterone nor hypogonadism is associated with degree of improvement after collagenase clostridium histolyticum treatment. Men should be offered collagenase clostridium histolyticum therapy according to guidelines independent of gonadal status. Further large, collaborative studies are needed to confirm these findings.

AUTHOR CONTRIBUTIONS

All authors participated in the drafting, writing, and editing of the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work, ensuring integrity and accuracy.

ACKNOWLEDGMENTS

None.

CONFLICT OF INTEREST STATEMENT

Faysal A. Yafi was consultant for Coloplast, Cynosure, Antares Pharma, Clarus Pharmaceuticals, and Acerus Pharma. All authors have completed the ICMJE uniform disclosure form. The remaining authors declare they have no conflicts of interest.

FUNDING INFORMATION

The authors received no specific funding for this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available because of privacy or ethical restrictions.

ORCID

Muhammed A. Moukhtar Hammad https://orcid.org/0000-0001-5396-1364

REFERENCES

1. Peak TC, Mitchell GC, Yafi FA, et al. Role of collagenase clostridium histolyticum in Peyronie's disease. *Biol Targets Ther*. 2015;9:107-116.
2. Tsambarlis P, Levine LA. Nonsurgical management of Peyronie's disease. *Nat Rev Urol*. 2019;16:172-186.
3. Nehra A, Alterowitz R, Culkin DJ, et al. Peyronie's disease: AUA guideline. *J Urol*. 2015;194:745-753.
4. Cwikla DJ, Yafi FA. Intralesional collagenase *Clostridium histolyticum* in the management of Peyronie's disease: current best practice. *Ther Adv Urol*. 2018;10:139-153.
5. Shlykova N, Morgentaler A. Augmenting intralesional collagenase treatment with testosterone therapy provides greater self-assessed improvement in penile curvature due to Peyronie's disease. *Androg Clin Res Ther*. 2020;1:70-75.
6. Aditya I, Grober ED, Krakowsky Y. Peyronie's disease and testosterone deficiency: is there a link? *World J Urol*. 2019;37:1035-1041.
7. Krakhotkin DV, Chernylovskyi VA, Mottrie A, et al. New insights into the pathogenesis of Peyronie's disease: a narrative review. *Chronic Dis Transl Med*. 2020;6:165-181.
8. Traish AM. Androgens play a pivotal role in maintaining penile tissue architecture and erection: a review. *J Androl*. 2009;30:363-369.
9. Armagan A, Hatsushi K, Toselli P. The effects of testosterone deficiency on the structural integrity of the penile dorsal nerve in the rat. *Int J Impot Res*. 2008;20:73-78.
10. Gelbard M, Goldstein I, Hellstrom WJG, et al. Clinical efficacy, safety and tolerability of collagenase *Clostridium histolyticum* for the treatment of Peyronie disease in 2 large double-blind, randomized, placebo controlled phase 3 studies. *J Urol*. 2013;190:199-207.
11. Traish A, Kim N. The physiological role of androgens in penile erection: regulation of corpus cavernosum structure and function. *J Sex Med*. 2005;2:759-770.
12. Montorsi F, Briganti A, Salonia A, et al. The ageing male and erectile dysfunction: the ageing male and erectile dysfunction. *BJU Int*. 2003;92:516-520.
13. Iacono F, Prezioso D, Ruffo A, et al. Testosterone deficiency causes penile fibrosis and organic erectile dysfunction in aging men. Evaluating association among age, TDS and ED. *BMC Surg*. 2012;12:S24.
14. Candela L, Boeri L, Capogrosso P, et al. Serum testosterone levels are not associated with the severity of penile curvature in men with Peyronie's disease—findings from a cross-sectional study. *Int J Impot Res*. 2020;33:832-838.
15. Mitsui Y, Yamabe F, Hori S, et al. Significant inverse association of testosterone level with penile deformity severity in Japanese males with Peyronie's disease. *Int J Urol*. 2023;30:36-42.
16. Mulhall JP, Matsushita K, Nelson CJ. Testosterone levels are not associated with magnitude of deformity in men with Peyronie's disease. *J Sex Med*. 2019;16:1283-1289.
17. Anaissie J, Yafi FA, DeLay KJ, et al. Impact of number of cycles of collagenase *Clostridium histolyticum* on outcomes in patients with Peyronie's disease. *Urology*. 2017;100:125-130.
18. Mulhall JP, Trost LW, Brannigan RE, et al. Evaluation and management of testosterone deficiency: AUA guideline. *J Urol*. 2018;200:423-432.

How to cite this article: Schneider D, O'Leary M, Amini E, et al. Peyronie's disease response to intralesional collagenase clostridium histolyticum therapy is independent of baseline testosterone. *Andrology*. 2024;12:830–834. https://doi.org/10.1111/andr.13532

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13532 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 16 July 2023 | Revised: 22 August 2023 | Accepted: 2 September 2023

DOI: 10.1111/andr.13528

ANDROLOGY WILEY

ORIGINAL ARTICLE

Complications after surgical correction of penile fractures—Is there a clinical impact?

Alberto Costa Silva[1,2] | Vasco Rodrigues[1,2] | Carlos Martins Silva[1,2] | Afonso Morgado[1,2]

[1]Serviço de Urologia, Centro Hospitalar Universitário São João, Porto, Portugal

[2]Faculdade de Medicina da Universidade do Porto, Porto, Portugal

Correspondence
Alberto Costa Silva, Alameda Prof. Hernani Monteiro, 4200-319 Porto, Portugal.
Email: u016845@chsj.min-saude.pt

Abstract

Background: Surgical exploration and closure of the tunica albuginea is the recommended treatment for penile fractures. The recovery of sexual function is the main result that surgeons and patients pursue.

Objective: We sought to evaluate the sexual health effects of a surgically corrected penile fracture. Secondarily, we sought to identify risk factors that may influence long-term sexual function and their effects on genital body image satisfaction.

Methods: A retrospective analysis of patients who underwent surgical correction of penile fractures between 2007 and 2022 in a tertiary center was performed. Lesion characteristics, weeks until the resumption of sexual activity, and post-operative sexual function were recorded. The presence of glans hypoesthesia, penile deformation, penile nodule palpation, and self-satisfaction with body image were assessed.

Results: Sixty-nine patients with a mean age of 42.30 ± 12.98 years and a median follow-up of 70 (20–134) months were identified. Sexual intercourse was recorded as a percentage. Penile deformation was the most common complication, appearing in 14.5% of patients, erectile dysfunction in 5.8%, penile nodules in 4.3%, and glans hypoesthesia in 2.9%. The median post-operative International Index of Erectile Function-5 was 24 (21.5–24). Self-satisfaction with body image had a median of 9 and was negatively associated with bilateral lesions and penile deformation.

Discussion and conclusion: Distal fractures could be linked to erectile dysfunction and glans hypoesthesia. Surgical correction of penile fractures shows positive functional and self-reported outcomes, and the potential andrological complications rarely necessitate specific treatment.

KEYWORDS
erectile dysfunction, penile fracture

1 | INTRODUCTION

A penile fracture (PF) is defined as the rupture of the tunica albuginea of the corpora cavernosa, which usually happens during an erection. The classic presentation is the sudden onset of a popping sound, pain, penile detumescence, swelling, and then an eggplant deformity.[1] The etiology varies around the world; sexual intercourse and forceful manipulation or the *taqaandan* maneuver are the most common causes in Western and Eastern countries, respectively.[2–4] Surgical exploration

Alberto Costa Silva and Vasco Rodrigues contributed equally to this work

© 2023 American Society of Andrology and European Academy of Andrology.

and the closure of the tunica albuginea is the gold standard treatment for a PF because it restores the tunica albuginea's integrity and its veno-occlusive mechanism.[5] Surgical timing and its effects remain a matter of debate, with some authors saying that outcomes did not differ significantly with delayed repairs of up to 72 h.[6,7]

A full recovery of sexual function is the main goal that patients and urologists seek. Given the rarity of this pathology, there is a lack of information and evidence on the impact of surgical corruption on long-term sexual health outcomes. Penile deformation, erectile dysfunction (ED), and penile nodule sensations are reported in 4%–31%, 22%, and 19.1% of patients, respectively.[3,8] Moreover, the prevalence of glans hypoesthesia has never been reported after PF, and its impact on self-esteem and self-body image is poorly described.[9,10]

We primarily sought to evaluate the sexual health impact of a surgically corrected PF. Secondarily, we aimed to identify the risk factors that may influence long-term sexual function and their impact on genital body image satisfaction.

2 | MATERIALS AND METHODS

After approval was granted by the hospital's ethics committee (protocol 23/152), we performed a retrospective analysis of all patients who underwent the surgical correction of a PF between May 2007 and August 2022 at our tertiary referral center. Data from patient charts were collected on the trauma mechanism, use of erectogenic drugs before the episode, use of imaging for diagnosis, and time until surgery. The latter was classified as less than 6 h, between 6 and 12 h, between 12 and 18 h, and more than 18 h. From the surgical report, data were obtained on the type of incision (subcoronal, penoscrotal, or transverse over the lesion), lesion extension, laterality (right, left, or bilateral; dorsal or ventral), location of the lesion (proximal, middle, or distal third), the presence of urethral lesions, and the type of suture material used to repair the albuginea. From the routine post-operative appointment records, usually gathered 1–3 months after surgery, data were obtained on immediate complications, such as surgical site infection, suture dehiscence, necrosis of the operative wound, urethrocutaneous fistula, and the number of weeks until sexual activity was resumed.

In November 2022, all patients were assembled and, after they granted informed consent, interviewed to evaluate the long-term andrological complications they had experienced. Erectile function was assessed with the International Index of Erectile Function-5 (IIEF-5).[11,12] The evolution of erectile function was classified as worsening, maintained, or improving, according to patients' opinions. A questionnaire was used to record the presence of glans hypoesthesia, penile deformation, and penile nodule palpation, as well as the need for treatment if a penile deformation was self-reported. Self-satisfaction with genital body image was rated on a visual analog scale from 0 to 10 (0 meaning "not satisfied" and 10 meaning "totally satisfied") in response to the question "How satisfied are you with your genital body image?".

TABLE 1 Demographic data and intra-operative findings.

Trauma mechanism	
Sexual intercourse	85.5% (59)
Masturbation	2.9% (2.9)
Forceful bending (*taqaandan*)	4.3% (4.3)
Undefined	7.2% (7.2)
Lesion length[a]	20 mm (15–30 mm)
Time to surgery (h)	
<6	14.7%
6–12	19.1%
12–18	23.5%
>18	42.6%
Anatomic distribution of lesion	
Right	43.5% (30)
Left	40.6% (28)
Bilateral	15.9% (11)
Dorsal	26.1% (18)
Ventral	73.9% (51)
Proximal third	36.2% (25)
Middle third	40.6% (28)
Distal third	23.2% (16)
Urethral lesion	15.9% (11)

[a]Median (interquartile range).

2.1 | Statistical analysis

Data were collected and assessed with the Statistical Package for the Social Sciences (version 24; IBM). The Shapiro–Wilk test was used to assess parameter distribution. All continuous variables with normal distribution are expressed as the mean ± standard deviation, and non-normally distributed variables are expressed as the median (25th—75th quartiles). Categoric variables were compared with a chi-squared test, continuous variables were compared with Pearson's correlation test, and categoric and continuous variables were compared with a *t*-test or Kruskal–Wallis test, depending on normality.

3 | RESULTS

A total of 69 patients with a mean age of 42.30 ± 12.98 years at the time of the surgery were identified. Their demographic data and intra-operative findings are summarized in Table 1. Sexual intercourse was the main cause of trauma in 85.5% of patients ($n = 59$) and the diagnosis was supported by ultrasound in 62.3% of cases ($n = 43$). None of the patients reported ED or taking erectogenic drugs before the event. A subcoronal incision with degloving was the most common approach, occurring in 91.3% of cases ($n = 63$) and followed by transverse incision in 5.8% ($n = 4$) and penoscrotal incision in 2.9% ($n = 2$). Most of the patients, 65.2% ($n = 45$), underwent surgery more than 12 h after their initial presentation. Fractures were more common on the ventral

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13528 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Andrological complications of surgical correction of penile fracture.

Penile deformation	14.5% (10)
Dorsal	4.3% (3)
Ventral	2.9% (2)
Left	5.8% (4)
Right	1.4% (1)
Erectile dysfunction	5.8% (4)
Penile nodule	4.3% (3)
Glans hypoesthesia	2.9% (2)

side (73.9%, $n = 51$) and bilateral fractures were less common (15.9%, $n = 11$). Polydioxanone was the most common suture material, used in 56.0% of cases ($n = 39$), followed by polyglycolide in 30.4% ($n = 21$) and polypropylene in 13.0% ($n = 9$). None of the patients was diagnosed with a false fracture and all of the urethral lesions (15.9%, $n = 11$) were partial. None of the patients required urgent or elective surgical reintervention. The resumption of sexual activity occurred at a median of 7 (4–9) weeks and was not associated with any of the variables evaluated. None of the patients underwent penile rehabilitation or reported penis shortening.

At a median time of 70 (20–134) months after surgery, the median IIEF-5 was 24 (21–24). The prevalence of andrological complications after the surgical correction of PF is summarized in Table 2. Penile deformation was the most common complication, occurring in 14.5% of cases ($n = 10$). Of the four patients with post-operative ED, two had mild ED (IIEF-5 of 18) and the other two had mild-to-moderate ED (IIEF-5 of 15). Only one patient requested treatment for ED. Only these four patients described worsening erectile function after the event, while all others reported the maintenance of erectile function. The characteristics of patients with ED are shown in Table 3. None of the patients experienced necrosis of the operative wound, urethrocutaneous fistula, or urethral stenosis.

The statistical analysis of pre- and intra-operative findings and long-term andrological outcomes is presented in Table 4, and the analysis of andrological complications is presented in Table 5. Decreased erectile function was associated with distal lesions ($p = 0.035$), the presence of post-operative curvature ($p = 0.038$), and glans hypoesthesia ($p = 0.007$). De novo penile deformation was associated with the sensation of a penile nodule ($p = 0.009$). Glans hypoesthesia was associated with distal-third fractures ($p = 0.033$). The sensation of a penile nodule was more frequent in cases of bilateral lesions ($p = 0.039$). Self-satisfaction with body image had a median of 9 (8–9) on the visual analog scale, with lower values associated with the presence of bilateral lesions ($p = 0.024$) and penile deformation ($p < 0.001$). The type of incision and suture material did not affect any of the outcomes evaluated.

4 | DISCUSSION

Since the first report about PFs and the surgical correction of the condition, researchers have been increasingly interested in the post-operative complications and their association with pre- and intra-operative factors.[13,14] Our study sought to clarify these associations further.

Ultrasound was used to aid clinical diagnosis in nearly two-thirds of our patients, while magnetic resonance imaging (MRI) was not used, despite its proven benefits.[15,16] MRI in the emergency department is not available in this context at our center.

The tunica albuginea is thinner on the ventral part, which was also, as expected, the most common site for PFs (73.9%) in our sample. Some studies stated a predominance of lesions in the right cavernosa, but this finding is mainly reported in patients where forceful bending or *taqaandan* was performed by right-handed men.[17] As such practices are uncommon in our country, we did not find any difference in laterality. The lesion size also did not affect the complications, as other authors have reported.[18]

As in our sample, subcoronal incision with degloving and circumcision remains the most common approach worldwide because it allows for the full inspection of the corpora cavernosa and the urethra.[1,19,20] Penoscrotal incision also offers these benefits without mobilizing the neurovascular bundle.[21] Transverse incision over the lesion has been gaining appeal, particularly when the lesion's exact location is identified via pre-operative imaging.[15] These techniques resulted in no differences in complications, that is, ED and self-satisfaction with body image. The validity of each technique is still debatable as circumcision is thought to contribute to sexual dysfunction after surgery in the form of penile hypoesthesia. However, a recent study showed no differences in reported erogenous or orgasm function between circumcised and uncircumcised penises.[22]

Theoretically, non-absorbable sutures could be associated with the risk of palpable nodules.[23,24] Such sutures were only used in 13.0% of the patients in our sample. Of the three patients who reported a penile nodule, one was sutured with polydioxanone, another with polyglycolide, and the last with polypropylene; therefore, no association was found between penile nodules and suture material. Moreover, as a long follow-up was observed for a significant part of our sample, de novo Peyronie's disease that was not related to the PF event may also contribute to the presence of nodules.

Compared to Amer et al.'s systematic review,[3] our sample has a higher prevalence of ED (5.8% vs. 1.9%), more penile deformation (14.5% vs. 2.8%), and less nodule sensation (4.3% vs. 13.9%). The largest case series in that review is from Zargooshi[25] and has a lower mean population age (29.6 vs. 42.3 years old) and forceful bending (*taqaandan*) was the main cause of fracture. Age and trauma mechanism may contribute to the complications experienced, and the differences between this and our study can explain the disparities in the rate of complications with that systematic review.[20]

Glans hypoesthesia was observed in 2.9% of our patients. The main contributors to sensation in the glans are the branches of the dorsal nerve of the penis.[10] These branches are predominantly distributed along the dorsal aspect of the penis, potentially rendering them less susceptible to damage as most fractures occur on the ventral side.

We found that ED was associated with distal fractures ($p = 0.035$). Of the four patients with ED, three had distal fractures. This finding

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13528 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 Characteristics of patients with erectile dysfunction.

	Case 1	Case 2	Case 3	Case 4
Age (years)	58	46	57	48
Follow-up (months)	29	68	64	105
Time until surgery (h)	12–18	12–18	12–18	12–18
Lesion length (mm)	20	30	15	20
Incision	Subcoronal	Subcoronal	Subcoronal	Subcoronal
Urethral lesion	Yes	Yes	Yes	Yes
Trauma mechanism	Sexual intercourse	Sexual intercourse	Sexual intercourse	Sexual intercourse
Location	Right, dorsal, medium third	Left, ventral, distal third	Right, ventral, distal third	Left, ventral, distal third
Time until resumption of sexual activity (weeks)	9	3	7	4
Self-satisfaction with body image	9	7	6	7
IIEF-5	15	18	15, using PDE5i	18
Penile nodule	No	No	No	Yes
Glans hypoesthesia	No	Yes	No	Yes
Penile deformation	No	No	Yes	Yes

Abbreviations: IIEF-5, International Index of Erectile Function-5; PDE5i, phosphodiesterase 5 inhibitor.

TABLE 4 Association between pre- and intra-operative findings and andrological complications.

	Erectile function	Penile deformation	Glans hypoesthesia	Penile nodule	Self-satisfaction with body image
Patient age	0.096[a]	0.594[a]	0.166[a]	0.864[a]	0.602[b], $r = 0.064$
Trauma mechanism (sexual intercourse/masturbation/self-inflicted/undefined)	0.896[c]	0.138[c]	0.951[c]	0.912[c]	0.323[d]
Time until surgery (<6 h, 6–12 h, 12–18 h, >18 h)	0.126[c]	0.454[c]	0.428[c]	0.582[c]	0.602[d]
Type of incision (subcoronal/penoscrotal/transverse over the lesion)	0.817[c]	0.706[c]	0.907[c]	0.861[c]	0.527[d]
Lesion length	0.479[d]	0.097[d]	0.703[d]	0.832[d]	0.225[b], $r = -0.016$
Lesion location (proximal/medium/distal)	0.035[c], [e]	0.835[c]	0.033[c], [e]	0.991[c]	0.601[d]
Lesion laterality (right/left/bilateral)	0.667[c]	0.060[c]	0.221[c]	0.039[c], [e]	0.024[d], [e]
Lesion side (dorsal/ventral)	0.959[c]	0.761[c]	0.394[c]	0.770[c]	0.454[d]
Type of suture material (polydioxanone/polyglycolide/polypropylene)	0.571[c]	0.243[c]	0.453[c]	0.523[c]	0.827[d]
Time until resumption of sexual activity	0.550[d]	0.187[d]	0.060[d]	0.364[d]	0.460[b], $r = 0.090$

Note: The values correspond to the *p*-value.
[a] *t*-Test.
[b] Pearson correlation test; *r* denotes Pearson correlation coefficient.
[c] Chi-square test.
[d] Kruskal–Wallis test.
[e] Significance.

diverges from Ouanes et al.,[18] who showed an association between proximal fractures and the occurrence of post-operative ED. They categorized fracture location as distal or proximal (14.5% and 85.5%, respectively), but in our sample, even when categorizing location as either distal or non-distal (23.2% and 76.8%, respectively), a statistically significant difference remained ($p = 0.011$). Furthermore, distal fractures were associated with glans hypoesthesia ($p = 0.033$). Both patients with glans hypoesthesia had distal fractures, and when categorizing fracture location as distal or non-distal, the *p*-value was 0.09. Both patients reporting glans hypoesthesia also experienced ED, resulting in a statistically significant association ($p = 0.007$). To our knowledge, this is the first article assessing glans hypoesthesia after PF and showing its possible association with distal fractures. The surgical correction of a distal lesion may be more likely to risk affecting

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13528 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 5 Association between each andrological complication.

	Erectile function	Penile deformation	Glans hypoesthesia	Penile nodule	Self-satisfaction with body image
Erectile function	–	0.038[a,b]	0.007[a,b]	0.073[a]	0.110[c]
Penile deformation	0.038[a,b]	–	0.148[a]	0.009[a,b]	<0.001[b,c]
Glans hypoesthesia	0.007[a,b]	0.148[a]	–	0.760[a]	0.830[c]
Penile nodule	0.073[a]	0.009[a,b]	0.760[a]	–	0.030[b,c]

Note: The values correspond to the *p*-value.
[a]Chi-square test.
[b]Significance.
[c]Kruskal–Wallis test.

the neurovascular bundle as it fans out distally to encompass and enervate the glans. ED was also associated with penile deformation ($p = 0.038$). The link between these conditions is well established.[26] The cases of ED we identified were mild and only one required a phosphodiesterase 5 inhibitor. We did not find an association between age (>50 years), bilateral fracture, and the presence of ED, as other studies have reported.[5,20]

The relationship between the time until surgery and post-operative ED remains debatable. Bozzini et al.[27] stated that surgical repair performed within the first 8.23 h after admission to the ER reduces the risk of post-operative ED. A systematic review showed similar rates of ED after both immediate and delayed repair (less or more than 24 h). Even after a long surgery delay, we found no association between the time to surgery and complications. None of our patients underwent surgery more than 24 h after the event. Therefore, we used four time intervals (less than 6 h, 6–12 h, 12–18 h, and more than 18 h) for the analysis.

Penile deformation was associated with the sensation of a penile nodule ($p = 0.009$). Two of the three patients with penile nodules had curvature; this association may be because of an overlap of the concepts of penile nodules (because of suturing or local scarring) and plaque (as a result of abnormal wound healing with the remodeling of connective tissue in Peyronie's disease). Ultrasound or MRI can be useful to discriminate these etiologies.[28] According to the literature, 0%–2.2% of PF patients require surgical curvature correction, and our data showed that no patients needed such treatment.[27] The association between penile nodules and bilateral lesions ($p = 0.039$) is probably explained by a need for wider surgical exploration, with the associated trauma and increased need for suturing.

The event itself, the surgery, and its complications can affect patients' confidence and self-esteem, leading to performance anxiety and reluctance.[29,30] Nevertheless, our patients presented high levels of self-satisfaction with their body image after surgery, with lower values associated with the presence of bilateral lesions ($p = 0.024$) and penile deformation ($p = < 0.001$). This information is not new; the psychological consequences of penile deformation and bilateral lesions often result from more extensive surgical exploration that may cause more penile deformities and, therefore, impact self-satisfaction and esteem.[31]

Our study has certain limitations. This is a rare urological emergency, even at a tertiary referral hospital. We performed a retrospective analysis with a medium-sized sample and the number of events observed was low. There was no control group of conservatively treated patients, and different surgeons were involved in treatment. IIEF-5 scores before trauma were not available, and the assessment of self-image was not conducted through a validated questionnaire. In addition, glans hypoesthesia was not evaluated with an objective tool such as biothesiometry.

As a positive remark, even with a long follow-up period (median of 70 months), no patient was lost to follow-up, and all patients with surgically treated PFs were enrolled and followed an a priori protocol.

In conclusion, de novo penile deformation was the most common complication reported. Distal fractures seem to be associated with the degradation of erectile function and glans hypoesthesia. Reduced self-satisfaction with body image appears to be associated with the presence of bilateral lesions and penile deformation. Overall, the surgical treatment of a PF has good functional and self-measured outcomes, and andrological complications, such as penile deformation and palpable nodules, although not rare, seldom require treatment. To clarify some divergent results in the literature, more studies, primarily prospective and multi-centric studies such as the MARS study for priapism, are needed in this field.[32]

AUTHOR CONTRIBUTIONS

Alberto Costa Silva was responsible for the study design, data collection, and manuscript elaboration. Vasco Rodrigues was responsible for the study design and data collection. Carlos Martins Silva and Afonso Morgado were responsible for the study design and manuscript revision.

ACKNOWLEDGMENTS

None to declare.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

FUNDING INFORMATION

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13528 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are restricted by the Ethics Commission in order to protect patient privacy. The data used to support the findings of this study are available from the corresponding author upon request and approval by local ethical commission.

REFERENCES

1. Falcone M, Garaffa G, Castiglione F, Ralph DJ. Current management of penile fracture: an up-to-date systematic review. *Sex Med Rev*. 2018;6(2):253-260. doi:10.1016/j.sxmr.2017.07.009
2. Kramer AC. Penile fracture seems more likely during sex under stressful situations. *J Sex Med*. 2011;8(12):3414-3417. doi:10.1111/j.1743-6109.2011.02461.x
3. Amer T, Wilson R, Chlosta P, et al. Penile fracture: a meta-analysis. *Urol Int*. 2016;96(3):315-329. doi:10.1159/000444884
4. Zargooshi J. Penile fracture in Kermanshah, Iran: the long-term results of surgical treatment. *BJU Int*. 2002;91(3):301-302. doi:10.1046/j.1464-410X.2003.t01-1-04066.x
5. Lynch TH, Martínez-Piñeiro L, Plas E, et al. EAU guidelines on urological trauma. *Eur Urol*. 2023;47(1):1-15. doi:10.1016/j.eururo.2004.07.028
6. Kominsky H, Beebe S, Shah N, Jenkins LC. Surgical reconstruction for penile fracture: a systematic review. *Int J Impot Res*. 2020;32(1):75-80. doi:10.1038/s41443-019-0212-1
7. Wong A, Karadeniz W. Can it wait? A systematic review of immediate vs. delayed surgical repair of penile fractures. *Can Urol Assoc*. 2017;11:53-60.
8. De Luca F, Garaffa G, Falcone M, et al. Functional outcomes following immediate repair of penile fracture: a tertiary referral centre experience with 76 consecutive patients. *Scand J Urol*. 2017;51(2):170-175. doi:10.1080/21681805.2017.1280532
9. Amarenco G, Bosc S, Goldet R. La neuropathie penienne: etude clinique et electrophysiologique. A propos de 186 cas. *Neurophysiol Clin*. 1997;27(1):51-58. doi:10.1016/S0987-7053(97)89869-3
10. Šedý J, Naňka O, Špačková J, Jarolím L. Clinical implications of a close vicinity of nervus dorsalis penis/clitoridis and os pubis. *J Sex Med*. 2008;5(7):1572-1581. doi:10.1111/j.1743-6109.2008.00796.x
11. Rosen R, Cappelleri J, Smith M, Lipsky J, Peña B. Development and evaluation of an abridged, 5-item version of the International Index of Erectile Function (IIEF-5) as a diagnostic tool for erectile dysfunction. *Int J Impot Res*. 1998;11:319-326.
12. Santos Pechorro P, Martins Calvinho A, Monteiro Pereira N, Xavier Vieira R. Validação de uma versão portuguesa do Índice Internacional de Função Eréctil-5 (IIEF-5). *Rev Int Androl*. 2011;9(1):3-9. doi:10.1016/S1698-031X(11)70002-4
13. Malis J. Zur kausuistik der fractura penis. *Arch Klin Chir*. 1924;129:651.
14. Fernstrom U. Rupture of the penis. report of one operated case and review of literature. *Acta Chir Scand*. 1957;113:211-217.
15. Spiesecke P, Mang J, Fischer T, Hamm B, Lerchbaumer MH. Diagnostic performance of MRI and US in suspicion of penile fracture. *Transl Androl Urol*. 2022;11(3):377-385. doi:10.21037/tau-21-957
16. Sokolakis I, Schubert T, Oelschlaeger M, et al. The role of magnetic resonance imaging in the diagnosis of penile fracture in real-life emergency settings: comparative analysis with intraoperative findings. *J Urol*. 2019;202:552-557. doi:10.1097/JU.0000000000000211
17. Ateyah A, Mostafa T, Nasser TA, Shaeer O, Hadi AA, Al-Gabbar MA. Penile fracture: surgical repair and late effects on erectile function. *J Sex Med*. 2008;5(6):1496-1502. doi:10.1111/j.1743-6109.2007.00769.x
18. Ouanes Y, Saadi MH, Haj Alouene H, et al. Sexual function outcomes after surgical treatment of penile fracture. *Sex Med*. 2021;9(3):100353. doi:10.1016/j.esxm.2021.100353
19. Reis LO, Cartapatti M, Marmiroli R, de Oliveira Júnior EJ, Saade RD, Fregonesi A. Mechanisms predisposing penile fracture and long-term outcomes on erectile and voiding functions. *Adv Urol*. 2014;2014:1-4. doi:10.1155/2014/768158
20. Sharma G, Mandal S, Bhowmik P, Gupta P, Bahal B, Sharma PK. Sexual function outcomes and risk factors of erectile dysfunction after surgical repair of penile fracture. *Turkish J Urol*. 2021;47(2):106-112. doi:10.5152/tud.2020.20311
21. Mazaris EM, Livadas K, Chalikopoulos D, Bisas A, Deliveliotis C, Skolarikos A. Penile fractures: immediate surgical approach with a midline ventral incision. *BJU Int*. 2009;104(4):520-523. doi:10.1111/j.1464-410X.2009.08455.x
22. Zaliznyak M, Isaacson D, Duralde E, et al. Anatomic maps of erogenous sensation and pleasure in the penis: are there difference between circumcised and uncircumcised men? *J Sex Med*. 2023;20(3):253-259. doi:10.1093/jsxmed/qdac032
23. Russo G, Di Gaetano C, Costa Silva A, Morgado A. Peyronie's disease and penile curvature. In: Russo GI, Sarikaya S, Ralph D, eds. *Andrology and Sexual Medicine*. 2022:117-151. https://link.springer.com/10.1007/978-3-031-12049-7
24. Sokolakis I, Schönbauer P, Mykoniatis I, et al. Long-term results after surgical treatment of congenital penile curvature using a modified NeSBIT technique. *World J Mens Health*. 2019;37(4):564-572. doi:10.5534/WJMH.190092
25. Zargooshi J. Sexual function and tunica albuginea wound healing following penile fracture: an 18-year follow-up study of 352 patients from Kermanshah, Iran. *J Sex Med*. 2009;6(4):1141-1150. doi:10.1111/j.1743-6109.2008.01117.x
26. Muneer A, Fowler S, Ralph DJ, Summerton DJ, Rees RW. UK practice for penile prosthesis surgery: baseline analysis of the British Association of Urological Surgeons (BAUS) Penile Prosthesis Audit. *BJU Int*. 2021;127(3):326-331. doi:10.1111/bju.15219
27. Bozzini G, Albersen M, Otero JR, et al. Delaying surgical treatment of penile fracture results in poor functional outcomes: results from a large retrospective multicenter European study. *Eur Urol Focus*. 2018;4(1):106-110. doi:10.1016/j.euf.2016.02.012
28. Acikgoz A, Gokce E, Asci R, Buyukalpelli R, Yilmaz AF, Sarikaya S. Relationship between penile fracture and Peyronie's disease: a prospective study. *Int J Impot Res*. 2011;23(4):165-172. doi:10.1038/ijir.2011.24
29. Rosen R, Catania J, Lue T, et al. Impact of Peyronie's disease on sexual and psychosocial functioning: qualitative findings in patients and controls. *J Sex Med*. 2008;5(8):1977-1984. doi:10.1111/j.1743-6109.2008.00883.x
30. Bolat MS, Özen M, Önem K, Açikgöz A, Asci R. Effects of penile fracture and its surgical treatment on psychosocial and sexual function. *Int J Impot Res*. 2017;29(6):244-249. doi:10.1038/ijir.2017.31
31. Terrier JE, Nelson CJ. Psychological aspects of Peyronie's disease. *Transl Androl Urol*. 2016;5(3):290-295. doi:10.21037/tau.2016.05.14
32. Russo GI. *ManAgement of pRiapiSm and its Impact on Outcomes (MARS)*. 2021. Accessed July 17, 2023. https://classic.clinicaltrials.gov/ct2/show/NCT04932902

How to cite this article: Costa Silva A, Rodrigues V, Martins Silva C, Morgado A. Complications after surgical correction of penile fractures—Is there a clinical impact? *Andrology*. 2024;12:835–840. https://doi.org/10.1111/andr.13528

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13528 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 14 July 2023 | Revised: 18 September 2023 | Accepted: 10 October 2023

DOI: 10.1111/andr.13547

ANDROLOGY WILEY

ORIGINAL ARTICLE

Decreased grey matter volume in dorsolateral prefrontal cortex and thalamus accompanied by compensatory increases in middle cingulate gyrus of premature ejaculation patients

Songzhan Gao[1] | Jianhuai Chen[2] | Jia Liu[3] | Yichun Guan[4] | Rusheng Liu[1] | Jie Yang[5,6] | Xianfeng Yang[1]

[1]Department of Andrology, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China

[2]Department of Andrology, Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China

[3]Department of clinical laboratory, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China

[4]Department of Reproductive Medicine, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China

[5]Department of Urology, Jiangsu Provincial People's Hospital, First Affiliated Hospital of Nanjing Medical University, Nanjing, China

[6]Department of Urology, People's Hospital of Xinjiang Kizilsu Kirgiz Autonomous Prefecture, Xinjiang Uygur Autonomous Region, China

Correspondence
Dr Xianfeng Yang, Department of Andrology, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China.
Email: 15517125138@163.com

Dr Jie Yang, Department of Urology, Jiangsu Provincial People's Hospital, First Affiliated Hospital of Nanjing Medical University, Nanjing, China.
Email: yj197912@163.com

Songzhan Gao and Jianhuai Chen are the co-first authors.

Funding information
Henan Medical Science and Technology Research Program Project,, Grant/Award Number: 2022-304; Jiangsu Traditional Chinese Medicine Science and Technology Development Plan Project, Grant/Award Number: MS2022023; Excellent Young Doctor Training Program of Jiangsu Province Hospital of Chinese Medicine, Grant/Award Number: 2023QB0126; Special Project of Innovation and Development Fund of Jiangsu Province Hospital of Chinese Medicine, Grant/Award Number: Y2021CX24; Jiangsu Province Graduate Research and Practice Innovation Program Project-School Assisted General Project, Grant/Award Number: SJCX23_0804; General project of Natural Science Foundation of Xinjiang Uygur Autonomous Region, Grant/Award Number: 2022D01A23

Abstract

Introduction: The prefrontal–cingulate–thalamic areas are associated with ejaculation control. Functional abnormalities of these areas and decreased grey matter volume (GMV) in the subcortical areas have been confirmed in premature ejaculation (PE) patients. However, no study has explored the corresponding GMV changes in the prefrontal–cingulate–thalamic areas, which are considered as the important basis for functional abnormalities. This study aimed to investigated whether PE patients exhibited impaired GMV in the brain, especially the prefrontal–cingulate–thalamic areas, and whether these structural deficits were associated with declined ejaculatory control.

Methods: T1-weighted structural magnetic resonance imaging (MRI) data were acquired from 50 lifelong PE patients and 50 age-, and education-matched healthy controls (HCs). The PE diagnostic tool (PEDT) was applied to assess the subjective symptoms of PE. Based on the method of voxel-based morphometry (VBM), GMV were measured and compared between groups. In addition, the correlations between GMV of brain regions showed differences between groups and PEDT scores were evaluated in the patient group.

Results: PE patients showed decreased GMV in the right dorsolateral superior frontal gyrus (clusters = 13, peak *T*-values = −4.30) and left thalamus (clusters = 47, $T = -4.33$), and increased GMV in the left middle cingulate gyrus (clusters = 12, $T = 4.02$) when compared with HCs. In the patient group, GMV of the left thalamus were negatively associated with PEDT scores ($r = -0.35$; $P = 0.01$). Receiver operating

© 2023 American Society of Andrology and European Academy of Andrology.

characteristic (ROC) analysis showed that GMV of the right dorsolateral superior frontal gyrus (AUC = 0.71, $P < 0.01$, sensitivity = 60%, specificity = 78%), left thalamus (AUC = 0.72, $P < 0.01$, sensitivity = 92%, specificity = 46%) and middle cingulate gyrus (AUC = 0.69, $P < 0.01$, sensitivity = 50%, specificity = 90%), and the combined model (AUC = 0.84, $P < 0.01$, sensitivity = 78%, specificity = 80%) all had the ability to distinguish PE patients from HCs.

Conclusion: Disturbances in GMV were revealed in the prefrontal–cingulate–thalamic areas of PE patients. The findings implied that decreased GMV in the dorsolateral prefrontal cortex and thalamus might be associated with the central pathological neural mechanism underlying the declined ejaculatory control while increased GMV in the middle cingulate gyrus might be the compensatory mechanism underlying PE.

KEYWORDS
grey matter volume, premature ejaculation, structural magnetic resonance imaging, voxel-based morphometry

1 | INTRODUCTION

Premature ejaculation (PE) is recognized to be a common male sexual dysfunction, which is characterized by short intra-vaginal ejaculation latency time (IELT), decreased ability to delay ejaculation and negative personal consequences.[1] It has been hypothesized that both psycho-social and biological factors implicate in the etiological mechanism of PE.[2] Ejaculation is regulated by multiple neural structures including peripheral nervous system and central nervous system.[3] The coordination between these structures can be achieved by the excitatory and inhibitory circuits originating from the brain.[4,5] Abnormalities of the central system are considered as the main causes of PE. Several central neural structures, such as the hypothalamus, particularly preoptic area, thalamus, amygdala and basal ganglia structures, have been found to play a central role in this process.[6,7] In the impaired inhibitory network, it had been found in lifelong PE patients by the previous fMRI study.[8] Therefore, brain regions involved in the excitatory and inhibitory control over ejaculation might be impaired in PE patients. In addition, ejaculation is considered as a reward behavior including extragenital responses and subjective pleasurable feelings during ejaculation, which is regulated by the cingulate and limbic areas in the brain.[9] The rewarding properties of ejaculation is triggered by sensory information related to ejaculation in response to sexual stimulation, which is also regulated by the brain.[10] Moreover, ejaculation is regarded as a goal-directed behavior, which is regulated by higher central neural structures, such as prefrontal and cingulate cortex.[7] Physiologically, the prefrontal and cingulate cortex can produce and facilitate goal-directed behaviors.[11] The coordinated activity between these regions can exert cognitive control to suppress distractors, which further contribute to focus on goal-oriented behavior relating to emotion and reward, as well as motor control.[12] However, the neurological mechanism of ejaculation at the cerebral level is still poorly understood.

Based on previous neuropharmacological and neuroimaging studies, there has been increasing evidence that PE is associated with enhanced excitability or sensitization in the central nervous system, as well as decreased descending ejaculatory inhibition in the brain.[8,13] The prefrontal, cingulate cortex, and thalamus are regarded as an important regions for the executive function, particularly goal-oriented behavior (such as the behavior intended to delay ejaculation with efforts) that requires the coordination of multiple brain regions, impulsivity including impulsive choice and behavior (such as the tendency to ejaculate immediately instead of delaying ejaculation) and response inhibition (the ability to restrain learned behaviors, such as acquired rapid ejaculatory behavior).[14–17] The dorsolateral prefrontal cortex has been hypothesized to modulate impulsive behavior by providing inhibitory inputs to subcortical structures (e.g., hypothalamus, thalamus, amygdala) that induce aggressive behavior.[18–20] The cingulate cortex has been hypothesized to play an important role in the self-monitoring and self-regulation processes, which is implicated in conflict monitoring during tasks about goal-directed behaviors.[21,22] The cingulate cortex also serves as a hub region in the top-down regulation circuit and mediates interactions between the dorsolateral prefrontal cortex (involved in cognitive control) and subcortical structures including thalamus (involved in reward processing).[23,24] Voxel-based morphometry (VBM) is a widely utilized and non-invasive neuroimaging technique that investigates regional morphological changes of grey (GM) and white matter (WM) in the whole brain.[25] It has been applied to explore the microstructural mechanism underlying regional brain areas in various neuropsychological disorders.[26] Therefore, it can be used to explore the corresponding grey matter volume (GMV) changes in the prefrontal–cingulate–thalamic areas of PE patients, which are considered as the important basis for functional abnormalities, and whether these structural deficits are associated with declined ejaculatory control.

In the study, we speculated that rapid ejaculatory behavior and decreased ability to delay ejaculation might be driven by decreased regulatory control of the dorsolateral prefrontal cortex over reward-related areas including thalamus. Therefore, the method of VBM was utilized to test the hypothesis that PE patients

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13547 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

might show impaired GMV in the brain, especially the prefrontal–cingulate–thalamic areas. We further tested whether the changed volumes of these regions were related to the declined ejaculatory control.

2 | MATERIALS AND METHODS

2.1 | Participants

The present study was approved by the ethics committee of Jiangsu Province Hospital of Chinese Medicine. Written informed consents were acquired from all subjects participating in this study. According to the calculation method on size of sample in the previous study, a total of 50 lifelong PE patients and 50 healthy controls (HCs) matched for age, handedness, and education level were recruited in this study.[27]

PE was diagnosed based on the International Society for Sexual Medicine (ISSM) guidelines[28] with PE diagnostic tool (PEDT) (Chinese version)[29] scores≥11 and intravaginal ejaculatory latency time (IELT) < 1 min. All participants including PE and HCs were Han Chinese, right-handed, aged between 20 and 45 years with at least 9 years of education. In addition, all participants including PE and HCs (PEDT scores of HCs ≤ 8, IELT of HCs > 3 min) had a stable sexual relationship with the same female partner for at least 6 months with normal sexual desire, normal erectile function (5-items of the international index of erectile function (IIEF-5) (Chinese version)[30] scores > 21) and regular weekly sexual intercourse (at least 2 times per week).

Subjects including PE and HCs who met any of the following exclusion criteria were excluded: (1) presence or history of any unstable health condition including acute illness or severe chronic medical condition; (2) presence or history of substance abuse/dependence; (3) history of loss of consciousness; (4) taking medications which might potentially affect male sexual function and brain within 2 weeks (drug wash-out period)[31,32]; (5) any contraindication for magnetic resonance imaging (MRI) scanning; (6) structural abnormalities detected by T1-weighted MRI.

2.2 | MRI data acquisition

Structural MRI data were acquired using a 3.0-T MRI scanner (Siemens, Germany). The parameters of T1-weighted images were as follows: repetition time = 1,900 ms; echo time = 2.48 ms; flip angle = 9°; field of view = 250 mm×250 mm; matrix size = 256 × 256; slice thickness = 1 mm; number of slices = 176, acquisition time = 4 min 18 s.

2.3 | Voxel-based morphometry analysis

Structural MRI data were processed using the software of Data Processing Assistant for rs-fMRI advanced edition (DPARSF) in MATLAB software.[33] The preprocessing steps were as follows: (1) converting DICOM raw data to NIfTI format; (2) reorienting T1-weighted images; (3) the whole-brain T1-weighted images were segmented into GM, WM, and cerebrospinal fluid (CSF) using New Segment+DARTAL setting in DPARSF; (4) GM segments were normalized to the group-specific template by averaging the structural images of all subjects in the group and then transformed to the Montreal Neurological Institute (MNI) standard space; (5) to preserve the volume of tissue after warping, voxel values in the normalized GM images were modulated by the Jacobian determinants derived from the normalization; (6) the modulated GM images were smoothed using a 4 mm full-width half-maximum (FWHM) Gaussian filter. The detailed descriptions of participants and MRI data acquisition, VBM analysis, and statistical analysis were presented in Figure 1.

2.4 | Statistical analysis

The age, education, IIEF-5, and PEDT scores were compared between groups using two sample *t*-tests by the Statistical Package for the Social Sciences version 23.0 (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant. Two sample *t*-tests were performed to compare the differences of GMV between groups using the statistical parametric mapping software package (SPM8, http://www.fil. ion.ucl.ac.uk/spm) in Matlab 2009a. The significant differences were set at $P < 0.001$ (minimum cluster size was 12 voxels, which was corrected by the AlphaSim program). In addition, corrections between GMV of impaired brain regions and PEDT scores were examined in the patient group. Moreover, receiver operating characteristic (ROC) analysis was conducted separately for GMV of each brain region that shows differences between groups and the combined model. The area under the curve (AUC) was calculated to assess the diagnostic accuracy of these measures.

3 | RESULTS

3.1 | Differences of demographic and clinical characteristics between groups

There were no differences in the age, educational level, and IIEF-5 scores between PE patients and HCs. Higher PEDT scores were found in the patient group when compared with HCs (Table 1).

3.2 | Brain regions showed altered grey matter volume in premature ejaculation patients

Compared with HCs, PE patients revealed decreased GMV in the right dorsolateral superior frontal gyrus and left thalamus, and increased GMV in the left middle cingulate gyrus (Table 2 and Figure 2).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13547 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

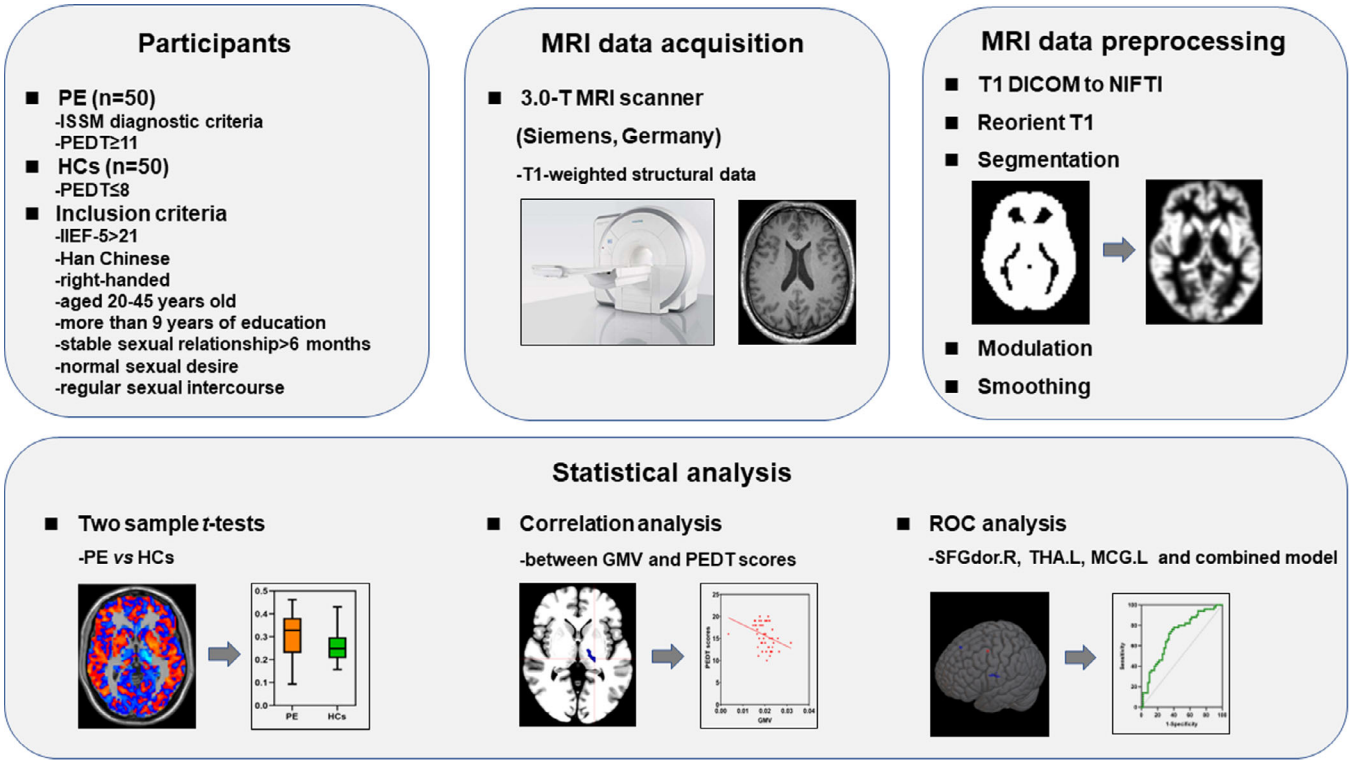


FIGURE 1 Schematic overview of participants and MRI data acquisition, VBM analysis and statistical analysis in this study. PE, premature ejaculation; HCs, healthy controls; ISSM, International Society for Sexual Medicine; PEDT, premature ejaculation diagnostic tool; IIEF-5, 5-Items of the International Index of Erectile Function; MRI, magnetic resonance imaging; GMV, grey matter volume; SFGdor.R, right dorsolateral superior frontal gyrus; THA.L, left thalamus; MCG.L, left middle cingulate gyrus; ROC, receiver operating characteristic; VBM, voxel-based morphometry.

TABLE 1 Demographic and clinical characteristics of participants.

Characteristics	PE (n = 50)	HCs (n = 50)	*t*	*P*
Age (years)	29.76 ± 6.19	31.98 ± 7.13	−1.66	0.10
Educational level (years)	13.50 ± 2.87	13.96 ± 1.59	−0.99	0.32
IIEF-5 (scores)	22.98 ± 1.02	22.70 ± 0.71	1.60	0.11
PEDT (scores)	15.74 ± 2.85	3.36 ± 1.77	26.10	<0.01

Abbreviations: HCs, healthy controls; IIEF-5, 5-items of the international index of erectile function; PE, premature ejaculation; PEDT, premature ejaculation diagnostic tool. *P*-values were obtained by two sample *t*-tests. $P < 0.05$ was considered to be statistically significant difference.

3.3 | Relationships between grey matter volume of impaired brain regions and PE diagnostic tool PE diagnostic tool scores in premature ejaculation patients

Significant negative associations were found between GMV of the left thalamus and PEDT scores in the patient group ($r = -0.35$; $P = 0.01$) (Figure 3).

3.4 | The value of grey matter volume for discriminating premature ejaculation patients from healthy controls

ROC analysis demonstrated that GMV of the right dorsolateral superior frontal gyrus (AUC = 0.71, $P < 0.01$, sensitivity = 60%, specificity = 78%), GMV of the left thalamus (AUC = 0.72, $P < 0.01$, sensitivity = 92%, specificity = 46%), GMV of the left middle cingulate gyrus (AUC = 0.69, $P < 0.01$, sensitivity = 50%, specificity = 90%), and the combined model (AUC = 0.84, $P < 0.01$, sensitivity = 78%, specificity = 80%) all had the ability to distinguish PE patients from HCs (Figure 4).

4 | DISCUSSION

In the present study, we used VBM analysis to evaluate structural changes of GMV in PE patients and their relationships with clinical characteristics. PE patients demonstrated decreased GMV in the right dorsolateral superior frontal gyrus and left thalamus, and increased GMV in the left middle cingulate gyrus when compared with HCs.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13547 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Brain regions showed altered grey matter volume in premature ejaculation patients.

Brain regions	Peak MNI coordinates			Clusters	Peak *T* values
	x	*y*	*z*		
Right dorsolateral superior frontal gyrus	18	30	46	13	−4.30
Left thalamus	−22	−22	0	47	−4.33
Left middle cingulate gyrus	−12	−8	46	12	4.02

Abbreviations: MNI, Montreal Neurological Institute; *x*, *y*, and *z*: the coordinates of peak voxel of each cluster in the MNI space.
The significant differences were set at $P < 0.001$ (minimum cluster size was 12 voxels, which was corrected by the AlphaSim program in REST software).

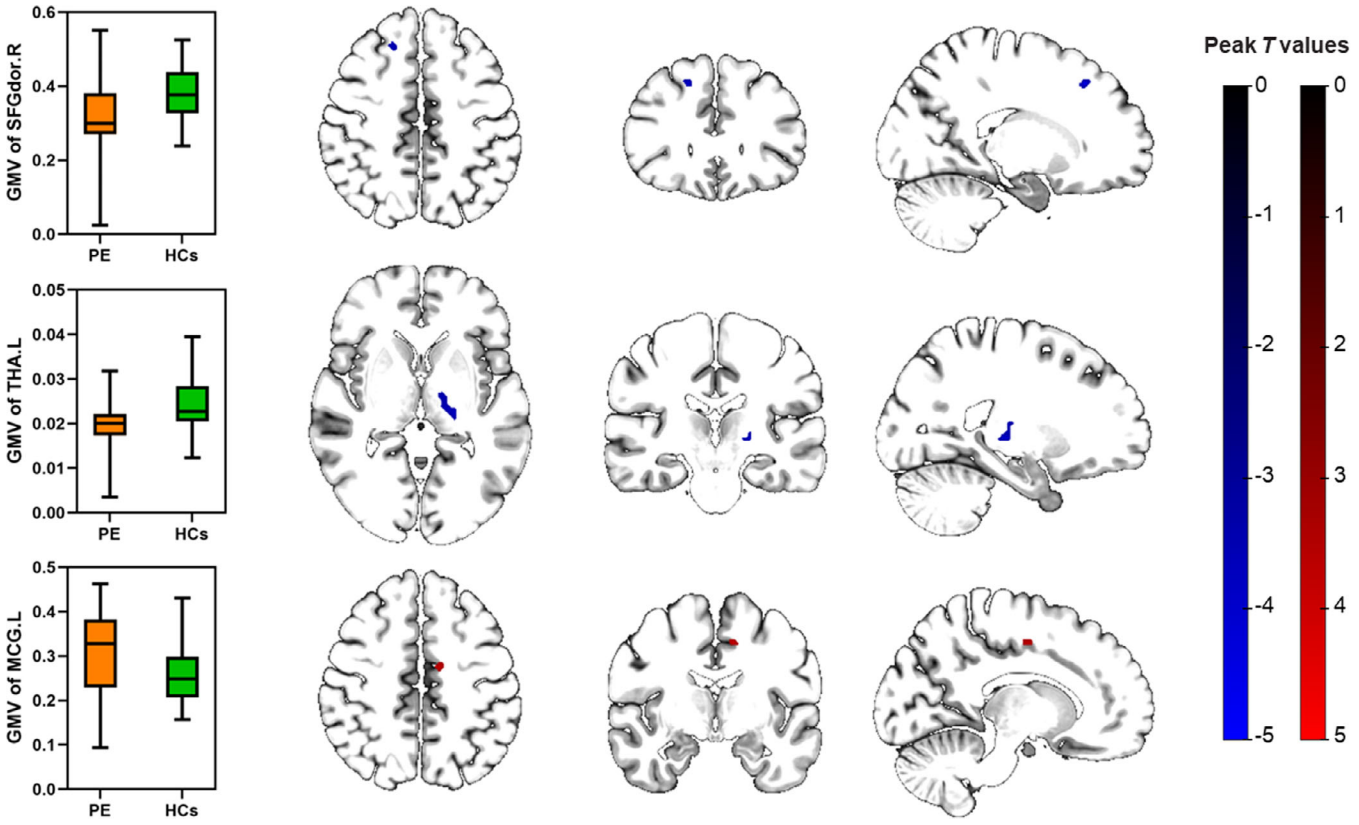


FIGURE 2 Brain regions showed altered grey matter volume in premature ejaculation patients. PE, premature ejaculation; HCs, healthy controls; GMV, grey matter volume; SFGdor.R, right dorsolateral superior frontal gyrus; THA.L, left thalamus; MCG.L, left middle cingulate gyrus.

In addition, negative relationships were detected between GMV of the left thalamus and PEDT scores of patients. Moreover, GMV of all these three brain regions had the ability to distinguish PE patients from HCs. GMV of the left thalamus had high sensitivity (92%), but low specificity (46%); conversely, GMV of the left middle cingulate gyrus had low sensitivity (50%), but high specificity (90%) for distinguishing PE patients from HCs. The combined model had both high sensitivity (78%) and high specificity (80%) for the diagnosis of PE.

The approaching behaviors toward the reward can be triggered by the rewarding properties of external stimulus, which is mediated by the motivational reward (wanting) and hedonic reward (liking) system in the brain.[34] The dorsolateral prefrontal cortex (i.e., superior frontal gyrus) is considered as a vital area in behavioral self-regulation through the high-order cognitive function including executive function, such as inhibitory control and reward processing, which are essential for regulating behavior.[35,36] The prefrontal cortex is the primary brain region for integrating internal and external sensory information including reward-related information from the thalamus, which affect reward-related behavior.[37–39] Abnormalities in the cognitive-control areas (e.g., dorsolateral prefrontal cortex) can lead to impaired top-down regulation of goal-direct behaviors in the presence of conflict, which further cause abnormal executive function associated with decreased ability to suppress automatic responses, disturbed control of behavior and decision-making.[40–42] Previous neuroimaging studies revealed abnormalities of GM in regions of the prefrontal cortex, which were associated with the impaired function of

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13547 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

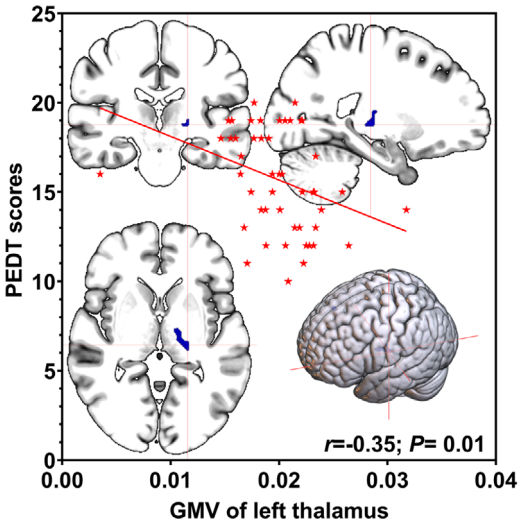


FIGURE 3 Relationships between GMV of impaired brain regions and PEDT scores in PE patients. GMV, grey matter volume; PEDT, premature ejaculation diagnostic tool.

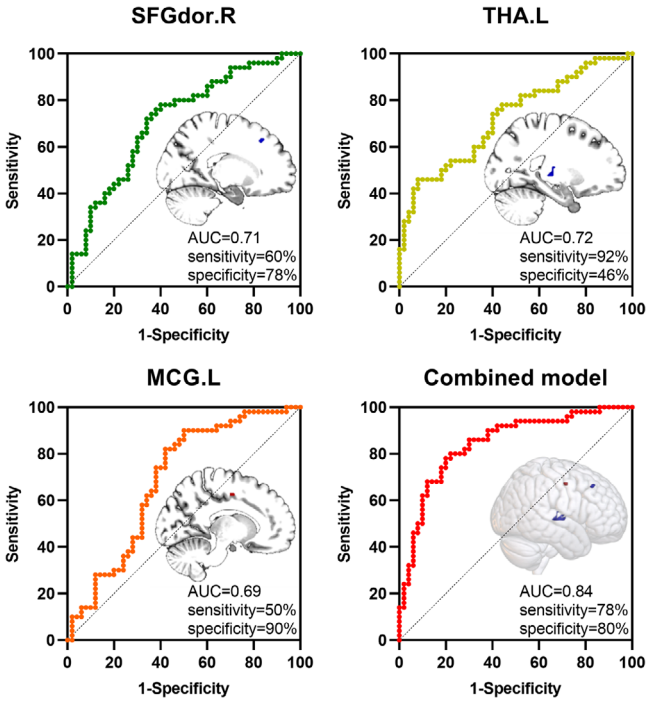


FIGURE 4 Receiver operating characteristic analysis of GMV for discriminating PE patients from HCs. SFGdor.R, right dorsolateral superior frontal gyrus; THA.L, left thalamus; MCG.L, left middle cingulate gyrus; AUC, area under the curve.

reward-processing and self-regulation.[43–45] In this study, PE patients showed decreased GMV in the right dorsolateral superior frontal gyrus, which is an important region located in the top-down regulating system involved in the cognitive control. This abnormality might decrease the ability of PE patients to suppress automatic responses (e.g., ejaculatory impulse) and patients might fail frequently in prolonging ejaculation latency due to the declined ejaculatory control ability and the increased urgent desire to achieve orgasm.

Cognitive control of behavior is supported by a top-down circuit composed of both the prefrontal cortex and subcortical structures including thalamus.[46–48] The thalamus is found to be a key mediator in the thalamo-cortical circuits, which involve in the top-down control of rewarding stimulus-motivated behavior via information updating.[49,50] The thalamus is also considered as the input modulator of the brain, where external sensory stimuli enter the brain, and then sensory information is transmitted to the cortex, especially prefrontal cortex for higher-level processing.[51,52] Diminished prefrontal modulation of thalamus (hypoactive top-down functional or structural connectivity) can down-regulate the activity and the structure of the thalamus.[53,54] In addition, the thalamus receiving ejaculation-related sensory information from the spinal cord and therefore it also plays an important role in controlling ejaculatory behavior by the top-down regulating system between brain and spinal cord.[3,5] The impaired structure or function of the thalamus can result in imbalanced facilitation and inhibition for controlling behavior, which is associated with impulsive choice and behavior.[55,56] Neuroimaging studies have revealed the dorsolateral prefrontal cortex and thalamus played a key role in controlling impulsive behavior and both functional and structural abnormalities in these regions were found to be related to aggressive behavior.[55,57] Therefore, decreased GMV in the left thalamus might lead PE patients more tendency to ejaculate immediately by the increased level of sexual arousal to evoke ejaculation due to the deficiency of information updating from thalamus, as well as decreased inhibitory control (top-down) over spinal center of ejaculation.

The middle cingulate gyrus is located in the midsection of the cingulate gyrus, which is critical for behavior control, such as performance monitoring, error detection, conflict monitoring, response selection, and working memory.[22,58] It is also considered as a central hub region for mediating brain regions involved in voluntary (top-down) behavioral conditioning and brain areas implicated in automatic (bottom-up) behavioral conditioning.[24,59] To optimize response selection and behavior, the middle cingulate gyrus is often recruited to adjust the control for better adaptive behavior based on the feedback from the top-down control in the prefrontal cortex and the down-top responses of the integration of stimulus features in the thalamus.[60–62] Therefore, the middle cingulate gyrus often exhibits compensatory increases in activation and morphology when individuals are in difficult situations or error is encountered.[61,63] In the study, we speculated that greater demand for the cognitive control was needed for PE patients to reorganize their ejaculator behavior when they were in the condition of rapid ejaculation, which caused compensatory increased GMV in the left middle cingulate gyrus. However, increased GMV of this region could not up-regulate the GMV of the dorsolateral superior frontal gyrus and thalamus, which were associated with the weakness in response inhibition circuitry underlying the maintenance of PE.

However, several limitations should be considered in this study when interpreting the findings. First, the sample size of the current study is not large enough to generalize these results, which also increased the likelihood of type II error. Second, these findings should

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13547 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

be verified by multimodality imaging methods including functional and structural MRI. Third, the changes of GMV associated with the course of PE and therapeutic intervention could not be detected in this study owing to the cross-sectional design of this study. Finally, other characteristics of PE patients, such as smoking condition, obesity, body mass index (BMI), frequency of sexual intercourse and some others, might be also associated with the development of PE. Therefore, more details about these characteristics of patients should be presented and their relationships with PE should be explored by regression analyses in further studies.

5 | CONCLUSION

In summary, the VBM analysis showed decreased GMV in the right dorsolateral superior frontal gyrus and left thalamus, and increased GMV in the left middle cingulate gyrus of lifelong PE patients in this study. These findings implied that aberrant GMV in the prefrontal–cingulate–thalamic areas might be involved in the pathological mechanism underlying PE. Decreased GMV in the dorsolateral prefrontal cortex and thalamus might be implicated in the development of PE while increased GMV in the middle cingulate gyrus might be the compensatory mechanism underlying the maintenance of PE.

AUTHOR CONTRIBUTIONS

Jianhuai Chen, Songzhan Gao, Xianfeng Yang, Yichun Guan, and Jie Yang designed the experiments. Jianhuai Chen, Songzhan Gao, and Jie Yang contributed to clinical data collection and assessment. Jianhuai Chen, Songzhan Gao, Jia Liu, Rusheng Liu, and Jie Yang analyzed the results. Jianhuai Chen, Songzhan Gao, Jia Liu, Rusheng Liu, and Jie Yang wrote the paper. All authors approved the final paper.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Henan Medical Science and Technology Research Program Project, Project No.: 2022-304; Jiangsu Traditional Chinese Medicine Science and Technology Development Plan Project, Project No.: MS2022023; Excellent Young Doctor Training Program of Jiangsu Province Hospital of Chinese Medicine, Project No.: 2023QB0126; Special Project of Innovation and Development Fund of Jiangsu Province Hospital of Chinese Medicine, Project No.: Y2021CX24; Jiangsu Province Graduate Research and Practice Innovation Program Project-School Assisted General Project, Project No.: No. SJCX23_0804; General project of Natural Science Foundation of Xinjiang Uygur Autonomous Region, Project No.: 2022D01A23

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Jianhuai Chen https://orcid.org/0009-0008-9786-2585

REFERENCES

1. Althof SE, McMahon CG, Waldinger MD, et al. An update of the International Society of Sexual Medicine's guidelines for the diagnosis and treatment of premature ejaculation (PE). *J Sexual Med*. 2014;11(6):1392-1422. doi:10.1111/jsm.12504 Epub 2014/05/23. PubMed PMID: 24848686
2. El-Hamd MA, Saleh R, Majzoub A. Premature ejaculation: an update on definition and pathophysiology. *Asian J Androl*. 2019;21(5):425-432. doi:10.4103/aja.aja_122_18 Epub 2019/03/13. PubMed PMID: 30860082; PubMed Central PMCID: PMCPMC6732885
3. Giuliano F. Neurophysiology of erection and ejaculation. *J Sexual Med*. 2011;8(Suppl 4):310-315. doi:10.1111/j.1743-6109.2011.02450.x Epub 2011/10/14. PubMed PMID: 21967393
4. Peeters M, Giuliano F. Central neurophysiology and dopaminergic control of ejaculation. *Neurosci Biobehav Rev*. 2008;32(3):438-453. doi:10.1016/j.neubiorev.2007.07.013 Epub 2007/10/09. PubMed PMID: 17919726
5. Coolen LM, Allard J, Truitt WA, McKenna KE. Central regulation of ejaculation. *Physiol Behav*. 2004;83(2):203-215. doi:10.1016/j.physbeh.2004.08.023 Epub 2004/10/19. PubMed PMID: 15488540
6. Veening JG, Coolen LM. Neural mechanisms of sexual behavior in the male rat: emphasis on ejaculation-related circuits. *Pharmacol Biochem Behav*. 2014;121:170-183. doi:10.1016/j.pbb.2013.12.017 Epub 2013/12/26. PubMed PMID: 24368305
7. Holstege G, Georgiadis JR, Paans AM, Meiners LC, van der Graaf FH, Reinders AA. Brain activation during human male ejaculation. *J Neurosci*. 2003;23(27):9185-9193. doi:10.1523/jneurosci.23-27-09185.2003 Epub 2003/10/10. PubMed PMID: 14534252; PubMed Central PMCID: PMCPMC6740826
8. Yang X, Gao M, Zhang L, et al. Central neural correlates during inhibitory control in lifelong premature ejaculation patients. *Frontiers in Human Neuroscience*. 2018;12:206. doi:10.3389/fnhum.2018.00206 Epub 2018/06/07. PubMed PMID: 29872385; PubMed Central PMCID: PMCPMC5972200
9. Holstege G, Georgiadis JR. The emotional brain: neural correlates of cat sexual behavior and human male ejaculation. *Prog Brain Res*. 2004;143:39-45. doi:10.1016/s0079-6123(03)43004-5 Epub 2003/12/05. PubMed PMID: 14653149
10. Tenk CM, Wilson H, Zhang Q, Pitchers KK, Coolen LM. Sexual reward in male rats: effects of sexual experience on conditioned place preferences associated with ejaculation and intromissions. *Horm Behav*. 2009;55(1):93-97. doi:10.1016/j.yhbeh.2008.08.012 Epub 2008/10/07. PubMed PMID: 18835271; PubMed Central PMCID: PMCPMC2659494
11. Sachuriga, Nishimaru H, Takamura Y, et al. Neuronal representation of locomotion during motivated behavior in the mouse anterior cingulate cortex. *Front Syst Neurosci*. 2021;15:655110. doi:10.3389/fnsys.2021.655110 Epub 2021/05/18. PubMed PMID: 33994964; PubMed Central PMCID: PMCPMC8116624
12. Niendam TA, Laird AR, Ray KL, Dean YM, Glahn DC, Carter CS. Meta-analytic evidence for a superordinate cognitive control network subserving diverse executive functions. *Cogn Affect Behav Neurosci*. 2012;12(2):241-268. doi:10.3758/s13415-011-0083-5 Epub 2012/01/28. PubMed PMID: 22282036; PubMed Central PMCID: PMCPMC3660731
13. Yusof F, Sidi H, Das S, Midin M, Kumar J, Hatta MH. Understanding the pathophysiology of premature ejaculation: bridging the link between pharmacological and psychological interventions. *Curr Drug Targets*. 2018;19(8):856-864. doi:10.2174/1389450117666161215161108 Epub 2016/12/21. PubMed PMID: 27993112
14. Wood JN, Grafman J. Human prefrontal cortex: processing and representational perspectives. *Nat Rev Neurosci*. 2003;4(2):139-147. doi:10.1038/nrn1033 Epub 2003/02/04. PubMed PMID: 12563285
15. Bubb EJ, Metzler-Baddeley C, Aggleton JP. The cingulum bundle: anatomy, function, and dysfunction. *Neurosci Biobehav Rev*.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13547 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2018;92:104-127. doi:10.1016/j.neubiorev.2018.05.008 Epub 2018/05/14. PubMed PMID: 29753752; PubMed Central PMCID: PMCPMC6090091
16. Friedman NP, Robbins TW. The role of prefrontal cortex in cognitive control and executive function. *Neuropsychopharmacology*. 2022;47(1):72-89. doi:10.1038/s41386-021-01132-0 Epub 2021/08/20. PubMed PMID: 34408280; PubMed Central PMCID: PMCPMC8617292
17. Ma J, du Hoffmann J, Kindel M, Beas BS, Chudasama Y, Penzo MA. Divergent projections of the paraventricular nucleus of the thalamus mediate the selection of passive and active defensive behaviors. *Nat Neurosci*. 2021;24(10):1429-1440. doi:10.1038/s41593-021-00912-7 Epub 2021/08/21. PubMed PMID: 34413514; PubMed Central PMCID: PMCPMC8484052
18. Wang W, Worhunsky PD, Zhang S, Le TM, Potenza MN, Li CR. Response inhibition and fronto-striatal-thalamic circuit dysfunction in cocaine addiction. *Drug Alcohol Depend*. 2018;192:137-145. doi:10.1016/j.drugalcdep.2018.07.037 Epub 2018/09/25. PubMed PMID: 30248560; PubMed Central PMCID: PMCPMC6200592
19. Gan G, Zilverstand A, Parvaz MA, et al. Habenula-prefrontal resting-state connectivity in reactive aggressive men—a pilot study. *Neuropharmacology*. 2019;156:107396. doi:10.1016/j.neuropharm.2018.10.025 Epub 2018/10/27. PubMed PMID: 30366001; PubMed Central PMCID: PMCPMC6478575
20. Ueltzhöffer K, Herpertz SC, Krauch M, Schmahl C, Bertsch K. Whole-brain functional connectivity during script-driven aggression in borderline personality disorder. *Prog Neuropsychopharmacol Biol Psychiatry*. 2019;93:46-54. doi:10.1016/j.pnpbp.2019.03.004 Epub 2019/03/20. PubMed PMID: 30885789
21. Yang J, Tian X, Wei D, et al. Macro and micro structures in the dorsal anterior cingulate cortex contribute to individual differences in self-monitoring. *Brain Imaging Behav*. 2016;10(2):477-485. doi:10.1007/s11682-015-9398-0 Epub 2015/05/11. PubMed PMID: 25958159
22. Parvaz MA, Maloney T, Moeller SJ, et al. Multimodal evidence of regional midcingulate gray matter volume underlying conflict monitoring. *NeuroImage Clin*. 2014;5:10-18. doi:10.1016/j.nicl.2014.05.011 Epub 2014/06/12. PubMed PMID: 24918068; PubMed Central PMCID: PMCPMC4050316
23. Boschin EA, Brkic MM, Simons JS, Buckley MJ. Distinct roles for the anterior cingulate and dorsolateral prefrontal cortices during conflict between abstract rules. *Cereb Cortex*. 2017;27(1):34-45. doi:10.1093/cercor/bhw350 Epub 2017/04/04. PubMed PMID: 28365775; PubMed Central PMCID: PMCPMC5939207
24. White MG, Panicker M, Mu C, et al. Anterior cingulate cortex input to the claustrum is required for top-down action control. *Cell Rep*. 2018;22(1):84-95. 10.1016/j.celrep.2017.12.023 Epub 2018/01/04. PubMed PMID: 29298436; PubMed Central PMCID: PMCPMC5779631
25. Ashburner J, Friston KJ. Voxel-based morphometry—the methods. *Neuroimage*. 2000;11(6 Pt 1):805-821. doi:10.1006/nimg.2000.0582 Epub 2000/06/22. PubMed PMID: 10860804
26. Pezzoli S, Sánchez-Valle R, Solanes A, et al. Neuroanatomical and cognitive correlates of visual hallucinations in Parkinson's disease and dementia with Lewy bodies: voxel-based morphometry and neuropsychological meta-analysis. *Neurosci Biobehav Rev*. 2021;128:367-382. doi:10.1016/j.neubiorev.2021.06.030 Epub 2021/06/26. PubMed PMID: 34171324
27. Desmond JE, Glover GH. Estimating sample size in functional MRI (fMRI) neuroimaging studies: statistical power analyses. *J Neurosci Methods*. 2002;118(2):115-128. doi:10.1016/s0165-0270(02)00121-8 Epub 2002/09/03. PubMed PMID: 12204303
28. Althof SE, McMahon CG, Waldinger MD, et al. An update of the International Society of Sexual Medicine's Guidelines for the diagnosis and treatment of premature ejaculation (PE). *Sex Med*. 2014;2(2):60-90. doi:10.1002/sm2.28 Epub 2014/10/31. PubMed PMID: 25356302; PubMed Central PMCID: PMCPMC4184677
29. Symonds T, Perelman MA, Althof S, et al. Development and validation of a premature ejaculation diagnostic tool. *Eur Urol*. 2007;52(2):565-573. doi:10.1016/j.eururo.2007.01.028 Epub 2007/02/06. PubMed PMID: 17275165
30. Rosen RC, Cappelleri JC, Smith MD, Lipsky J, Peña BM. Development and evaluation of an abridged, 5-item version of the International Index of Erectile Function (IIEF-5) as a diagnostic tool for erectile dysfunction. *Int J Impot Res*. 1999;11(6):319-326. doi:10.1038/sj.ijir.3900472 Epub 2000/01/19. PubMed PMID: 10637462
31. Mathers MJ, Klotz T, Roth S, Lümmen G, Sommer F. Safety and efficacy of vardenafil versus sertraline in the treatment of premature ejaculation: a randomised, prospective and crossover study. *Andrologia*. 2009;41(3):169-175. doi:10.1111/j.1439-0272.2008.00910.x Epub 2009/04/30. PubMed PMID: 19400851
32. Alghobary M, El-Bayoumy Y, Mostafa Y, Mahmoud E-HM, Amr M. Evaluation of tramadol on demand vs. daily paroxetine as a long-term treatment of lifelong premature ejaculation. *J Sex Med*. 2010;7(8):2860-2867. doi:10.1111/j.1743-6109.2010.01789.x Epub 2010/04/07. PubMed PMID: 20367773
33. Chao-Gan Y, Yu-Feng Z. DPARSF: a MATLAB toolbox for "Pipeline" data analysis of resting-state fMRI. *Front Syst Neurosci*. 2010;4:13. doi:10.3389/fnsys.2010.00013 Epub 2010/06/26. PubMed PMID: 20577591; PubMed Central PMCID: PMCPMC2889691
34. Morales I, Berridge KC. 'Liking' and 'wanting' in eating and food reward: brain mechanisms and clinical implications. *Physiol Behav*. 2020;227:113152. doi:10.1016/j.physbeh.2020.113152 Epub 2020/08/28. PubMed PMID: 32846152; PubMed Central PMCID: PMCPMC7655589
35. Bigliassi M, Filho E. Functional significance of the dorsolateral prefrontal cortex during exhaustive exercise. *Biol Psychol*. 2022;175:108442. doi:10.1016/j.biopsycho.2022.108442 Epub 2022/10/16. PubMed PMID: 36243197
36. Kane MJ, Engle RW. The role of prefrontal cortex in working-memory capacity, executive attention, and general fluid intelligence: an individual-differences perspective. *Psychon Bull Rev*. 2002;9(4):637-671. doi:10.3758/bf03196323 Epub 2003/03/05. PubMed PMID: 12613671
37. Staudinger MR, Erk S, Walter H. Dorsolateral prefrontal cortex modulates striatal reward encoding during reappraisal of reward anticipation. *Cereb Cortex*. 2011;21(11):2578-2588. doi:10.1093/cercor/bhr041 Epub 2011/04/05. PubMed PMID: 21459835
38. Haber SN, Knutson B. The reward circuit: linking primate anatomy and human imaging. *Neuropsychopharmacology*. 2010;35(1):4-26. doi:10.1038/npp.2009.129 Epub 2009/10/09. PubMed PMID: 19812543; PubMed Central PMCID: PMCPMC3055449
39. Otis JM, Zhu M, Namboodiri VMK, et al. Paraventricular thalamus projection neurons integrate cortical and hypothalamic signals for cue-reward processing. *Neuron*. 2019;103(3):423-431.e4. doi:10.1016/j.neuron.2019.05.018 Epub 2019/06/15. PubMed PMID: 31196673; PubMed Central PMCID: PMCPMC6773659
40. Lipton ML, Gulko E, Zimmerman ME, et al. Diffusion-tensor imaging implicates prefrontal axonal injury in executive function impairment following very mild traumatic brain injury. *Radiology*. 2009;252(3):816-824. doi:10.1148/radiol.2523081584 Epub 2009/07/02. PubMed PMID: 19567646
41. Arnsten AF, Rubia K. Neurobiological circuits regulating attention, cognitive control, motivation, and emotion: disruptions in neurodevelopmental psychiatric disorders. *J Am Acad Child Adolesc Psychiatry*. 2012;51(4):356-367. doi:10.1016/j.jaac.2012.01.008 Epub 2012/03/28. PubMed PMID: 22449642
42. Silton RL, Heller W, Towers DN, et al. The time course of activity in dorsolateral prefrontal cortex and anterior cingulate cortex during top-down attentional control. *Neuroimage*. 2010;50(3):1292-

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13547 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

1302. doi:10.1016/j.neuroimage.2009.12.061 Epub 2009/12/29. PubMed PMID: 20035885; PubMed Central PMCID: PMCPMC599-8337

43. Martinez-Horta S, Sampedro F, Pagonabarraga J, et al. Non-demented Parkinson's disease patients with apathy show decreased grey matter volume in key executive and reward-related nodes. *Brain Imaging Behav*. 2017;11(5):1334-1342. doi:10.1007/s11682-016-9607-5 Epub 2016/10/13. PubMed PMID: 27730477
44. Smolker HR, Depue BE, Reineberg AE, Orr JM, Banich MT. Individual differences in regional prefrontal gray matter morphometry and fractional anisotropy are associated with different constructs of executive function. *Brain Struct Funct*. 2015;220(3):1291-1306. doi:10.1007/s00429-014-0723-y Epub 2014/02/25. PubMed PMID: 24562372; PubMed Central PMCID: PMCPMC4320016
45. Cristofori I, Zhong W, Chau A, Solomon J, Krueger F, Grafman J. White and gray matter contributions to executive function recovery after traumatic brain injury. *Neurology*. 2015;84(14):1394-1401. doi:10.1212/wnl.0000000000001446 Epub 2015/03/10. PubMed PMID: 25746558; PubMed Central PMCID: PMCPMC4395-886
46. Radtke-Schuller S, Town SM, Yin P, et al. Dorsal prefrontal and premotor cortex of the ferret as defined by distinctive patterns of thalamo-cortical projections. *Brain Struct Funct*. 2020;225(5):1643-1667. doi:10.1007/s00429-020-02086-7 Epub 2020/05/28. PubMed PMID: 32458050; PubMed Central PMCID: PMCPMC728 6872
47. Ferguson BR, Gao WJ. Thalamic control of cognition and social behavior via regulation of gamma-aminobutyric acidergic signaling and excitation/inhibition balance in the medial prefrontal cortex. *Biol Psychiatry*. 2018;83(8):657-669. doi:10.1016/j.biopsych.2017.11.033 Epub 2018/01/27. PubMed PMID: 29373121; PubMed Central PMCID: PMCPMC5862785
48. Bruinsma B, Pattij T, Mansvelder HD. *eNeuro*. 2022;9(6):1-15. doi:10.1523/eneuro.0254-22.2022 Epub 2022/10/15. PubMed PMID: 36241421; PubMed Central PMCID: PMCPMC9636992
49. Herrero MT, Barcia C, Navarro JM. Functional anatomy of thalamus and basal ganglia. *Child Nervous Syst*. 2002;18(8):386-404. doi:10.1007/s00381-002-0604-1 Epub 2002/08/23. PubMed PMID: 12192499
50. Crosson B. The role of cortico–thalamo–cortical circuits in language: recurrent circuits revisited. *Neuropsychol Rev*. 2021;31(3):516-533. doi:10.1007/s11065-019-09421-8 Epub 2019/11/24. PubMed PMID: 31758291; PubMed Central PMCID: PMCPMC8418594
51. Sherman SM. Thalamus plays a central role in ongoing cortical functioning. *Nat Neurosci*. 2016;19(4):533-541. doi:10.1038/nn.4269 Epub 2016/03/30. PubMed PMID: 27021938
52. Parnaudeau S, Bolkan SS, Kellendonk C. The mediodorsal thalamus: an essential partner of the prefrontal cortex for cognition. *Biol Psychiatry*. 2018;83(8):648-656. doi:10.1016/j.biopsych.2017.11.008 Epub 2017/12/26. PubMed PMID: 29275841; PubMed Central PMCID: PMCPMC5862748
53. Huang AS, Rogers BP, Woodward ND. Disrupted modulation of thalamus activation and thalamocortical connectivity during dual task performance in schizophrenia. *Schizophr Res*. 2019;210:270-277. doi:10.1016/j.schres.2018.12.022 Epub 2019/01/12. PubMed PMID: 30630706; PubMed Central PMCID: PMCPMC6612476
54. Cao XH, Wang DH, Bai J, Zhou SC, Zhou YD. Prefrontal modulation of tactile responses in the ventrobasal thalamus of rats. *Neurosci Lett*. 2008;435(2):152-157. doi:10.1016/j.neulet.2008.02.030 Epub 2008/03/19. PubMed PMID: 18346849
55. Kose S, Steinberg JL, Moeller FG, et al. Neural correlates of impulsive aggressive behavior in subjects with a history of alcohol dependence. *Behav Neurosci*. 2015;129(2):183-196. doi:10.1037/bne0000038 Epub 2015/02/11. PubMed PMID: 25664566; PubMed Central PMCID: PMCPMC4777896
56. Cheng J, Ma X, Li C, et al. Diet-induced inflammation in the anterior paraventricular thalamus induces compulsive sucrose-seeking. *Nat Neurosci*. 2022;25(8):1009-1013. doi:10.1038/s41593-022-01129-y Epub 2022/08/02. PubMed PMID: 35915173
57. Jin C, Zhang T, Cai C, et al. Abnormal prefrontal cortex resting state functional connectivity and severity of internet gaming disorder. *Brain Imaging Behav*. 2016;10(3):719-729. doi:10.1007/s11682-015-9439-8 Epub 2015/08/28. PubMed PMID: 26311395
58. Brockett AT, Roesch MR. Anterior cingulate cortex and adaptive control of brain and behavior. *Int Rev Neurobiol*. 2021;158:283-309. doi:10.1016/bs.irn.2020.11.013 Epub 2021/04/01. PubMed PMID: 33785148
59. Huda R, Sipe GO, Breton-Provencher V, et al. Distinct prefrontal top-down circuits differentially modulate sensorimotor behavior. *Nat Commun*. 2020;11(1):6007. doi:10.1038/s41467-020-19772-z Epub 2020/11/28. PubMed PMID: 33243980; PubMed Central PMCID: PMCPMC7691329
60. Zheng C, Huang Y, Bo B, Wei L, Liang Z, Wang Z. Projection from the anterior cingulate cortex to the lateral part of mediodorsal thalamus modulates vicarious freezing behavior. *Neurosci Bull*. 2020;36(3):217-229. 10.1007/s12264-019-00427-z Epub 2019/09/19. PubMed PMID: 31531804; PubMed Central PMCID: PMCPMC7056816
61. Porter BS, Hillman KL, Bilkey DK. Anterior cingulate cortex encoding of effortful behavior. *J Neurophysiol*. 2019;121(2):701-714. doi:10.1152/jn.00654.2018 Epub 2019/01/10. PubMed PMID: 30625016
62. Gehring WJ, Knight RT. Prefrontal–cingulate interactions in action monitoring. *Nat Neurosci*. 2000;3(5):516-520. doi:10.1038/74899 Epub 2000/04/19. PubMed PMID: 10769394
63. Minzenberg MJ, Lesh T, Niendam T, et al. Conflict-related anterior cingulate functional connectivity is associated with past suicidal ideation and behavior in recent-onset schizophrenia. *J Psychiatr Res*. 2015;65:95-101. doi:10.1016/j.jpsychires.2015.04.002 Epub 2015/04/22. PubMed PMID: 25891474

How to cite this article: Gao S, Chen J, Liu J, et al. Decreased grey matter volume in dorsolateral prefrontal cortex and thalamus accompanied by compensatory increases in middle cingulate gyrus of premature ejaculation patients. *Andrology*. 2024;12:841–849. https://doi.org/10.1111/andr.13547

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13547 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 24 March 2023 | Revised: 15 August 2023 | Accepted: 30 September 2023

DOI: 10.1111/andr.13544

ANDROLOGY

WILEY

ORIGINAL ARTICLE

Inflammatory markers and androstenedione modify the effect of serum testosterone on obesity among men: Findings from a Chinese population

Changsheng Huan[1] | Mian Wang[1] | Yu Song[1] | Zexin Jia[1] | Dandan Wei[2] | Lulu Wang[2] | Qingqing Xu[2] | Juan Wang[2] | Mengzhen Zhao[2] | Jintian Geng[2] | Jiayu Shi[2] | Cuicui Ma[2] | Zhenxing Mao[2] | Chongjian Wang[2] | Wenqian Huo[1]

[1]Department of Occupational and Environmental Health Sciences, College of Public Health, Zhengzhou University, Zhengzhou, Henan, P. R. China

[2]Department of Epidemiology and Biostatistics, College of Public Health, Zhengzhou University, Zhengzhou, Henan, P. R. China

Correspondence
Wenqian Huo, Department of Occupational and Environmental Health Sciences, College of Public Health, Zhengzhou University, 100 Kexue Avenue, Zhengzhou, Henan 450001, P. R. China.
Email: huowenqian@zzu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 42177415, 21806146; Postdoctoral Science Foundation of China, Grant/Award Numbers: 2020T130604, 2021M702934; Science and Technique Foundation of Henan Province, Grant/Award Number: 212102310074; Scientific and Technological Innovation of Colleges and Universities in Henan Province Talent Support Programme, Grant/Award Number: 22HASTIT044; Young Backbone Teachers Program of Colleges and Universities in Henan Province, Grant/Award Number: 2021GGJS015; Excellent Youth Development Foundation of Zhengzhou University, Grant/Award Number: 2021ZDGGJS057

Abstract

Background: Few studies are available on the relationship of androstenedione with inflammation and obesity and the effect of androstenedione and inflammation on the association between testosterone and obesity. This study intended to examine the mediation effect of inflammatory markers on the association of testosterone with obesity and the moderation effect of androstenedione on the association of testosterone with inflammation and obesity in Chinese rural men.

Materials and methods: This cross-sectional research enrolled 2536 male rural inhabitants from the Henan Rural Cohort study. The serum concentrations of testosterone and androstenedione were determined by liquid chromatography–tandem mass spectrometry. Linear and logistic regression were used to examine the relationships between testosterone, inflammatory markers, and obesity. Mediation and moderation analyses were carried out to evaluate the potential effects of inflammatory markers on the relationship between testosterone and obesity, as well as androstenedione on the relationships of testosterone with inflammation and obesity.

Results: After adjusting for confounding factors, the results showed that testosterone and androstenedione were negatively related to obesity, and inflammatory markers were positively associated with obesity. Besides, testosterone and androstenedione were negatively associated with inflammatory markers. Mediation analysis showed that white blood cell, neutrophil, monocyte, and high-sensitivity C-reactive protein had mediating effects on the association between testosterone and obesity. The most vital mediator was high-sensitivity C-reactive protein, and its proportion of the effect was 11.02% (defined by waist circumference), 11.15% (defined by waist-to-hip ratio), 12.92% (defined by waist-to-height ratio), and full mediating effect (defined by body mass index). Moreover, androstenedione played negative moderation effects on the associations of testosterone with inflammation and obesity.

Changsheng Huan and Mian Wang contributed equally to this work.

© 2023 American Society of Andrology and European Academy of Andrology.

Conclusion: Inflammatory markers and androstenedione were first found to have modifying effects on the association of testosterone with obesity. Higher levels of testosterone and androstenedione could reduce the inflammation level and risk of obesity, indicating their potential roles in the prevention and treatment of chronic diseases.

KEYWORDS
androstenedione, inflammatory marker, mediation effect, moderation effect, obesity, testosterone

1 | INTRODUCTION

Obesity, a significant public health issue, is linked to an increased prevalence of chronic diseases.[1–3] According to the World Health Organization, the rate of obesity has tripled since 1975, and an estimated 50% of adults over the age of 18 are overweight/obese (WHO, 2018).[4] Despite significant efforts in China to eradicate severe obesity, the prevalence of obesity continues to rise, particularly in rural areas with few resources.[5] The number of overweight and obese individuals continues to rise, and by 2030, more than half of the people worldwide are expected to be overweight or obese.[6] Anthropometric measurements such as waist circumference (WC), waist-to-hip ratio (WHR), waist-to-height ratio (WHtR), and body mass index (BMI) are indicators of obesity and body fat distribution.[7] In addition, obesity rates vary by obesity indicator, and different obesity indicators have various meanings.[8,9] Therefore, when studying obesity, it is worthwhile to use various indicators to evaluate it.

Androgens, including testosterone and androstenedione, are synthesized in the gonads (testes and ovaries).[10] Research shows that testosterone acts as a steroid hormone that regulates prostate function, bone density, and muscle mass.[11] Both in vivo and in vitro research have revealed that testosterone plays an active role in all aspects of adipose tissue metabolism.[12,13] The enzyme aromatase can convert testosterone to estradiol, and then leads to preferential deposition of visceral fat.[12,14] Besides, significant associations between testosterone and obesity have been found in epidemiological studies.[15–17] Several clinical trials have also found that testosterone therapy is an effective treatment for obesity.[18,19] Androstenedione which is reversibly metabolized to testosterone by 17β-hydroxysteroid dehydrogenase is a precursor of androgen.[20] Only a few studies have explored the association between androstenedione and obesity.[21,22] Of note, testosterone and androstenedione are present together in the organism; however, no research has investigated whether there is a combination effect or modifying effect of androstenedione on testosterone-associated obesity.

Inflammation is involved in the pathophysiology of many chronic diseases, including obesity, diabetes, cardiovascular disease, and metabolic syndrome.[23] White blood cells (WBC) are important immune cells in the human body and are closely related to the body's inflammatory response.[24] Neutrophils (NEUT) are actively involved in inflammatory response and may have damaging effect on body tissues.[25] Lymphocytes (LYMPH) are directly involved in immune regulation.[26] Monocytes (MONO) are the core of innate immunity and are implicated in a variety of inflammatory diseases.[27] In addition, C-reactive protein (CRP) is an important marker for identifying inflammatory response as well as a key component of the innate immune response.[28] According to some studies, serum testosterone levels in men are negatively related to WBC count and granulocyte count.[29,30] An experimental animal study in mice showed that MONO decreased significantly with increasing testosterone concentrations.[31] Long-term testosterone treatment has been reported to result in a decrease in circulating LYMPH numbers.[32] Moreover, it was previously explored that serum testosterone levels were inversely related to CRP.[33,34] A study found a negative association between androstenedione and inflammatory markers (CRP).[35] However, it is unclear whether natural endogenous testosterone and androstenedione are associated with reducing aforementioned inflammatory markers. Additionally, published studies have suggested that inflammatory markers are positively related to obesity defined by various anthropometric indicators.[36,37] However, few studies have explored the role of inflammatory markers on the association between testosterone and obesity.

The mixed effect of serum testosterone and androstenedione on obesity is unknown and whether inflammation mediates the association of testosterone and androstenedione with obesity has not yet been fully elucidated. Therefore, the present study aimed to investigate the combined effects of androstenedione and testosterone on obesity and whether there is a mediating role for inflammatory markers in the association between testosterone and obesity.

2 | MATERIALS AND METHODS

2.1 | Study population

The data for this cross-sectional research came from the Henan Rural Cohort Study, which is presented elsewhere.[38] The current study enrolled 2808 male participants from Suiping County in Henan Province, China. Participants having missing values for either obesity or serum testosterone and androstenedione ($n = 228$) were eliminated from the study. We further excluded participants who had extreme

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by meryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

values or missing values for inflammatory markers ($n = 42$), leaving 2538 male participants. Finally, 2536 male individuals had complete information on the factors of interest, prompting them to be included in the current study. The Zhengzhou University Life Science Ethics Committee (code: [2015] MEC [S128]) approved the protocol. All subjects gave their informed consent.

2.2 | Data collection

The study participants' age, gender, educational attainment, per capita monthly income, marital status, high-fat diet, vegetable and fruit intake, physical activity, smoking and alcohol status, and family history of diseases were all acquired using a face-to-face questionnaire. There were three levels of educational attainment: never attended school, primary school, and junior secondary and above. The per capita monthly income was divided into three parts (<500, 500∼, or ≥1000 RMB). Marital status was divided into two categories: married/cohabiting or widowed/single/divorced. Based on the dietary guidelines, a high-fat diet was defined as consuming more than 75 g of meat per day. According to Chinese dietary guidelines,[39] adequate vegetable and fruit intake was defined as consuming more than 500 g of vegetables and fruits per day on average. Physical activity was determined to be low, middle, or high using the International Physical Activity Questionnaire.[40] Smoking and alcohol status was classified as current or non-current. Having at least one diseased parent, grandparent, or sibling was deemed a family history of disease.

Height, weight, WC, and hip circumference were measured as previously explained.[38]

2.3 | Outcome assessment

Obesity has previously been defined by WC, WHR, WHtR, and BMI.[41] BMI was computed from height and weight (kg/m^2). WHR was computed by the ratio of WC (cm) to hip circumference (cm), and WHtR was computed by the ratio of WC (cm) to height (cm).

Male individuals with a WC ≥90 cm, a WHR ≥90%, or a WHtR ≥50% were defined as obese individuals; otherwise, they were defined as non-obese individuals.[41] Obesity was conceptualized as maintaining a BMI higher than 28 kg/m^2.[42]

2.4 | Laboratory measurements

Venous blood sample was taken after fasting for more than 8 h at night. Fasting venous blood was gathered into a 10.0 mL ethylenediaminetetraacetic acid potassium anticoagulant tube and a 5.0 mL vacuum tube without anticoagulation. Part of the blood samples with anticoagulation were used for routine blood tests. Inflammatory cells (WBC, NEUT, LYMPH, MONO) were measured by automatic biochemical analyzer (XS-500i, SYSMEX). Blood samples without anticoagulation were centrifuged for 10 min at a relative centrifugal force of 2000 *g* and room temperature, and then a portion of the serum samples was transferred to the clinical laboratory center for further analysis. Immunoturbidimetry was used to examine the concentrations of high-sensitivity C-reactive protein (hsCRP) in the blood (Cobas c501, Roche).

Serum samples as described above were stored at −80°C. A liquid chromatography–tandem mass spectrometer (LC–MS/MS; Waters XEVO TQ-S system) was used to measure serum testosterone and androstenedione. To monitor the instrument's stability, blank and quality control samples were taken every 12 samples. At a signal-to-noise of 3, the limit of detection (LOD) was computed. Values less than the detection limit (0.04 g/L) or undetected were substituted for half of LOD. In Table S1, several parameters of experimental detection for testosterone and androstenedione, such as coefficients of variation (CVs), were tabulated. The measurement unit of testosterone and androstenedione is ng/mL.

2.5 | Statistical analysis

Normally and non-normally distribution continuous and categorical variables were represented as mean ± SD, medians (interquartile ranges), and frequencies (percentages), respectively. Chi-square tests (categorical variables), Wilcoxon rank-sum tests (continuous variables, skewed distribution), and Student's *t*-test (continuous variables, normal distribution) were used to assess the population's basic characteristics.

Because of their non-normal distributions, testosterone, androstenedione, WBC, NEUT, MONO, LYMPH, and hsCRP were natural log-transformed before analysis and given the designations ln-testosterone, ln-androstenedione, ln-WBC, ln-NEUT, ln-MONO, ln-LYMPH, and ln-hsCRP.

Serum concentrations of testosterone and androstenedione for participants were classified into tertiles, with the first tertile (T1) being the lowest and the referent group. The relationships of serum testosterone, androstenedione, and inflammatory marker levels with obesity were determined using logistic regression. Then, to examine the association across rising tertiles, trend tests were conducted by entering categorical data as continuous variables.

Linear regression was applied to investigate the relationships of testosterone and androstenedione levels with inflammatory markers (WBC, NEUT, LYMPH, MONO, and hsCRP). Model 1 was unadjusted; model 2 was adjusted for age, educational attainment, per capita monthly income, marital status, family history of hypertension, hyperlipidemia, and diabetes, smoking and drinking status, high-fat diet, physical activity, and fruit and vegetable intake. These covariates were chosen as confounders based on their significant differences between obese and non-obese populations (Table 1) and their potential impact on obesity.[5]

Furthermore, because age, per capita monthly income, marital status, smoking status, drinking status, high-fat diet, vegetable and fruit intake, and physical activity have effects on the chance of presenting obesity,[5] we further performed sensitivity analyses to

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Characteristics of the study participants.

Variable	Non-obesity ($N = 1031$)	Obesity ($N = 1505$)	*p*-Value
Age (years), mean ± SD	58.29 ± 11.36	57.16 ± 10.50	0.01
Educational attainment, *n* (%)			<0.01
Never attended school	148 (14.35)	146 (9.70)	
Primary school	289 (28.03)	353 (23.46)	
Junior secondary and above	594 (57.62)	1006 (66.84)	
Per capita monthly income, *n* (%)			<0.01
<500 RMB	382 (37.05)	459 (30.50)	
500~ RMB	298 (28.90)	416 (27.64)	
1000~ RMB	351 (34.05)	630 (41.86)	
Marital status, *n* (%)			<0.01
Married/cohabiting	881 (85.45)	1377 (91.50)	
Widowed/single/divorced/separation	150 (14.55)	128 (8.50)	
Fam HTN, *n* (%)	127 (12.32)	276 (18.34)	<0.01
Fam HD, *n* (%)	28 (2.72)	62 (4.12)	0.06
Fam TD, *n* (%)	19 (1.84)	33 (2.19)	0.54
Current smoker, *n* (%)	506 (49.08)	694 (46.11)	0.14
Current drinker, *n* (%)	279 (27.06)	576 (38.27)	<0.01
High-fat diet, *n* (%)	287 (27.84)	506 (33.62)	<0.01
Adequate vegetable and fruit intake, *n* (%)	679 (65.86)	1004 (66.71)	0.66
Physical activity, *n* (%)			<0.01
Low	248 (24.05)	433 (28.77)	
Mediate	380 (36.86)	568 (37.74)	
High	403 (39.09)	504 (33.49)	
WBC (mg/L), median (IQR)	5.80 (1.90)	6.20 (2.00)	<0.01
NEUT (mg/L), median (IQR)	3.22 (1.49)	3.45 (1.37)	<0.01
LYMPH (mg/L), median (IQR)	1.93 (0.87)	2.02 (0.85)	<0.01
MONO (mg/L), median (IQR)	0.36 (0.15)	0.38 (0.15)	<0.01
hsCRP (mg/L), median (IQR)	0.84 (1.03)	1.27 (1.59)	<0.01
Testosterone (ng/mL), median (IQR)	3.38 (2.79)	2.54 (2.25)	<0.01
Androstenedione (ng/mL), median (IQR)	0.72 (0.50)	0.66 (0.52)	<0.01

Abbreviations: Fam HD, family history of hyperlipidemia; Fam HTN, family history of hypertension; Fam TD, family history of diabetes; hsCRP, high-sensitivity C-reactive protein; IQR, interquartile range; LYMPH, lymphocytes; MONO, monocytes; NEUT, neutrophils; WBC, white blood cells.

assess the robustness of the studies by taking into account age (<60 or ≥60 years), per capita monthly income (<500, 500–999, or ≥1000 RMB), marital status (married or single), current smoker (yes or no), current drinker (yes or no), high-fat diet (yes or no), adequate vegetable and fruit intake (yes or no), and physical activity (low, mediate or high) in model 2 but not adjusting for these covariates.

A mediation analysis was used to investigate whether inflammatory markers had an impact on the association of serum testosterone with obesity. As previously stated,[43,44] the mediating variable required to be a continuous variable. A mediation variable exists when it satisfies the following conditions: (1) the independent variable has a significant effect on the change in the mediation variable; (2) the mediation variable has a significant effect on the change in the dependent variable; and (3) the relationship between the independent variable and the dependent variable is significantly reduced when both the effect of the independent variable on the mediation variable and the effect of the mediation variable on the dependent variable are controlled. We used PROCESS for SPSS to perform a mediation analysis on model 2 with 5000 bootstrap resamples. The mediator effect proportion was calculated as (indirect effect/total effect) × 100%.[45]

We used PROCESS for SPSS to evaluate the moderation effects of androstenedione. Statistically, moderating variables can be found by testing the significance of the interaction term between the moderating variable and the independent variable (moderating variable × independent variable) on the dependent variable. Additionally,

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon, Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

to examine conditional indirect effects, androstenedione levels were expressed as an SD below and above the mean. Moreover, the conditional effects were plotted to present the significant adjustments in order to visualize the moderation effects of androstenedione.

All data were analyzed using SPSS 22.0 and R 3.5.3. Statistical significance was defined as a two-tailed p values <0.05.

3 | RESULTS

3.1 | Basic characteristics

Table 1 summarizes the study participants' basic characteristics of the 2536 male participants according to whether they were obese or not. Obesity was defined as participants having either of the abnormal measures (WC or WHR or WHtR or BMI). Overall, a total of 1505 subjects were diagnosed with obesity.

In general, compared with the subjects without obesity, individuals with obesity had the following characteristics: higher educational attainment, per capita monthly income, married/cohabiting, family history of hypertension, current drinker, high-fat diet, higher levels of inflammatory markers (WBC, NEUT, LYMPH, MONO, and hsCRP), and lower levels of physical activity and serum hormones (testosterone and androstenedione).

3.2 | Associations of serum testosterone and androstenedione with obesity defined by four parameters

Table 2 illustrates the relationships of ln-testosterone and ln-androstenedione with four obesity parameters in tertiles and continuity. According to the findings, serum testosterone and androstenedione levels were protective factors against obesity.

After adjusting for covariates in model 2, a one-unit increase in ln-testosterone was linked to a 9% (95% confidence interval [CI]: 0.87–0.95), 8% (95% CI: 0.88–0.96), 8% (95% CI: 0.88–0.97), and 7% (95% CI: 0.87–0.99) lower risk of WC, WHR, WHtR, and BMI, respectively. In addition, compared with the first tertile, the third tertile was associated with a 68% (95% CI: 0.25–0.41), 62% (95% CI: 0.31–0.47), 61% (95% CI: 0.32–0.47), and 78% (95% CI: 0.14–0.35) lower risk of WC, WHR, WHtR, and BMI. Among the model 2, a one-unit increase in ln-androstenedione was associated with a 9% (95% CI: 0.85–0.98), 10% (95% CI: 0.84–0.96), and 12% (95% CI: 0.82–0.94) lower risk of WC, WHR, and WHtR, respectively.

3.3 | Sensitivity analyses

The results of sensitivity analyses are presented in Table S2. The relationships between testosterone and obesity were not different according to age (<60 or ≥60 years), per capita monthly income (<500, 500–999, or ≥1000 RMB), marital status (married or single), current smoker (yes or no), current drinker (yes or no), high-fat diet (yes or no), adequate vegetable and fruit intake (yes or no), and physical activity (low, moderate, or high).

3.4 | Associations of serum testosterone with inflammatory markers

Table 3 presents the findings of the linear regression of the associations of ln-testosterone and ln-androstenedione with inflammatory markers. After controlling for confounding variables, a one-unit increase in ln-testosterone was related to 0.038 (95% CI: −0.074, −0.002), 0.030 (95% CI: −0.058, −0.001), 0.004 (95% CI: −0.006, −0.001), and 0.091 (95% CI: −0.141, −0.040) decreases in WBC, NEUT, MONO, and hsCRP levels, respectively. The changes (95% CI) in the third tertile (T3) of WBC, NEUT, MONO, and hsCRP were −0.447 (−0.610, −0.284), −0.389 (−0.518, −0.261), −0.022 (−0.034, −0.010), and −0.736 (−0.964, −0.507), respectively, when compared with the first tertile of serum testosterone. After adjusting for covariates in model 2, a one-unit increase in ln-androstenedione was related to a 0.093 (95% CI: −0.149, −0.038), 0.092 (95% CI: −0.136, −0.048), and 0.006 (95% CI: −0.010, −0.002) decrease in WBC, NEUT, and MONO levels, respectively.

3.5 | Mediation effect

Figure S1 shows the changes and 95% CI of obesity with each tertile increment of inflammatory markers. The results showed that inflammatory markers were risk factors for obesity by any obesity-related parameters. Figure S2 shows the conceptual model of mediation. After adjusting for confounding factors, Table 4 illustrates the mediation effect of inflammatory markers on the association of testosterone with obesity. WBC and NEUT played a mediating role in the obesity defined by WC, WHR, and WHtR, and the proportions of the mediation effect were 4.53%, 4.28%, and 6.65% and 3.60%, 3.27%, and 5.65%, respectively. The proportion of mediating effects showed no significant difference between WBC and NEUT. LYMPH had no significant mediating effect. The mediating roles of MONO and hsCRP were all significant in obesity defined by four parameters (WC, WHR, WHtR, and BMI) and the proportions of the effect were 5.46%, 5.18%, and 7.28%, respectively, full mediating effect, and 11.02%, 11.15%, and 12.92%, respectively, full mediating effect. Among all inflammatory markers, hsCRP had the highest mediating effect, followed by MONO.

3.6 | Moderation effect

Given the preceding findings, we proceeded with the moderation effects of androstenedione on inflammatory markers and obesity. Figure S3 illustrates the conceptual model for the moderation effect of androstenedione. As presented in Table S3, after adjusting for covariates, serum androstenedione was found to be a significant moderator

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Associations of testosterone and androstenedione levels with obesity.

	Logistic regression OR (95% CI)			
Variables	**Obesity$_{-WC}$**	**Obesity$_{-WHR}$**	**Obesity$_{-WHtR}$**	**Obesity$_{-BMI}$**
Testosterone				
Model 1				
Continuous	**0.89 (0.85, 0.93)**	**0.90 (0.87, 0.94)**	**0.91 (0.87, 0.95)**	**0.91 (0.85, 0.98)**
T1	1	1	1	1
T2	**0.70 (0.57, 0.86)**	**0.76 (0.63, 0.93)**	0.88 (0.72, 1.07)	**0.68 (0.50, 0.93)**
T3	**0.30 (0.24, 0.37)**	**0.37 (0.30, 0.45)**	**0.37 (0.30, 0.45)**	**0.20 (0.13, 0.31)**
p_{trend}	<0.001	<0.001	<0.001	<0.001
Model 2				
Continuous	**0.91 (0.87, 0.95)**	**0.92 (0.88, 0.96)**	**0.92 (0.88, 0.97)**	**0.93 (0.87, 0.99)**
T1	1	1	1	1
T2	**0.69 (0.56, 0.85)**	**0.76 (0.63, 0.93)**	0.88 (0.72, 1.08)	**0.64 (0.47, 0.88)**
T3	**0.32 (0.25, 0.41)**	**0.38 (0.31, 0.47)**	**0.39 (0.32, 0.47)**	**0.22 (0.14, 0.35)**
p_{trend}	<0.001	<0.001	<0.001	<0.001
Androstenedione				
Model 1				
Continuous	**0.88 (0.83, 0.95)**	**0.88 (0.83, 0.94)**	**0.87 (0.81, 0.93)**	0.92 (0.83, 1.03)
T1	1	1	1	1
T2	**0.77 (0.63, 0.96)**	0.85 (0.70, 1.03)	**0.79 (0.65, 0.95)**	0.97 (0.70, 1.36)
T3	**0.73 (0.59, 0.90)**	**0.70 (0.58, 0.85)**	**0.68 (0.56, 0.83)**	0.80 (0.57, 1.14)
p_{trend}	0.003	<0.001	<0.001	0.22
Model 2				
Continuous	**0.91 (0.85, 0.98)**	**0.90 (0.84, 0.96)**	**0.88 (0.82, 0.94)**	0.94 (0.84, 1.05)
T1	1	1	1	1
T2	**0.79 (0.63, 0.98)**	0.86 (0.71, 1.05)	**0.80 (0.65, 0.97)**	0.98 (0.69, 1.38)
T3	**0.70 (0.56, 0.88)**	**0.70 (0.58, 0.86)**	**0.68 (0.55, 0.83)**	0.72 (0.50, 1.03)
p_{trend}	0.002	<0.001	<0.001	0.08

Note: Model 1—no adjustment. Model 2—adjusted for age, educational level, per capita monthly income, marital status, family history of hypertension, family history of hyperlipidemia, family history of diabetes, smoking status, drinking status, high-fat diet, physical activity, and fruit and vegetable intake. Bold values signify $p < 0.05$.

Abbreviations: BMI, body mass index; CI, confidence interval; OR, odds ratio; T, tertile; WC, waist circumference; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio.

in the associations of testosterone with inflammatory markers (defined by WBC and NEUT) and showed negative moderation effects (β [95% CI]: −0.0569 [−0.0859, −0.0278] for WBC, −0.0690 [−0.0918, −0.0462] for NEUT). Table S4 shows the associations between testosterone and obesity (defined by WHR) for low and high levels of androstenedione. Androstenedione was discovered to be a significant moderator in the relationship between testosterone and obesity (defined by WHR). The moderation effect varied with the range of serum androstenedione levels and was strongest at higher androstenedione levels. Figure 1 depicts these moderation effects by plotting simple slopes, suggesting that testosterone was negatively linked to inflammation and obesity, and individuals who had high levels of testosterone and androstenedione were less prone to inflammation and obesity.

4 | DISCUSSION

As far as we know, there is no prior study to investigate the role of inflammatory markers and androstenedione in the association of serum testosterone with obesity in Chinese rural men. This current research found inverse associations of testosterone and androstenedione with obesity (defined by WC, WHR, and WHtR). Likewise, we found negative associations of testosterone and androstenedione with inflammatory markers (WBC, NEUT, and MONO), and inflammatory markers were positively related to obesity. Mediation analysis revealed that inflammatory markers (WBC, NEUT, MONO, and hsCRP) were important mediators of the association of testosterone with obesity, suggesting that inflammatory response had a pivotal role

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 Associations of testosterone and androstenedione levels with inflammatory markers levels.

Variables	Linear regression β coefficients (95% CI)				
	WBC	NEUT	LYMPH	MONO	hsCRP
Testosterone					
Model 1					
Continuous	**−0.041 (−0.077, −0.005)**	**−0.030 (−0.059, −0.002)**	−0.003 (−0.018, 0.011)	**−0.004 (−0.006, −0.001)**	**−0.080 (−0.130, −0.030)**
T1	0	0	0	0	0
T2	**−0.253 (−0.416, −0.090)**	**−0.224 (−0.353, −0.096)**	0.004 (−0.061, 0.070)	**−0.016 (−0.028, −0.004)**	**−0.648 (−0.875, −0.420)**
T3	**−0.462 (−0.626, −0.299)**	**−0.391 (−0.520, −0.263)**	−0.046 (−0.111, 0.020)	**−0.021 (−0.033, −0.010)**	**−0.661 (−0.888, −0.433)**
Model 2					
Continuous	**−0.038 (−0.074, −0.002)**	**−0.030 (−0.058, −0.001)**	−0.001 (−0.015, 0.014)	**−0.004 (−0.006, −0.001)**	**−0.091 (−0.141, −0.040)**
T1	0	0	0	0	0
T2	**−0.259 (−0.421, −0.097)**	**−0.226 (−0.354, −0.099)**	0.001 (−0.064, 0.066)	**−0.016 (−0.027, −0.004)**	**−0.652 (−0.880, −0.425)**
T3	**−0.447 (−0.610, −0.284)**	**−0.389 (−0.518, −0.261)**	−0.031 (−0.097, 0.034)	**−0.022 (−0.034, −0.010)**	**−0.736 (−0.964, −0.507)**
Androstenedione					
Model 1					
Continuous	**−0.081 (−0.136, −0.025)**	**−0.082 (−0.125, −0.038)**	0.006 (−0.016, 0.029)	**−0.006 (−0.010, −0.002)**	−0.068 (−0.145, 0.010)
T1	0	0	0	0	0
T2	**−0.448 (−0.611, −0.285)**	**−0.361 (−0.489, −0.233)**	−0.056 (−0.121, 0.010)	**−0.034 (−0.046, −0.022)**	**−0.288 (−0.518, −0.059)**
T3	**−0.225 (−0.388, −0.062)**	**−0.298 (−0.426, −0.170)**	**0.074 (0.008, 0.139)**	**−0.019 (−0.030, −0.007)**	**−0.266 (−0.495, −0.037)**
Model 2					
Continuous	**−0.093 (−0.149, −0.038)**	**−0.092 (−0.136, −0.048)**	0.005 (−0.017, 0.028)	**−0.006 (−0.010, −0.002)**	−0.077 (−0.156, 0.001)
T1	0	0	0	0	0
T2	**−0.461 (−0.624, −0.298)**	**−0.369 (−0.498, −0.241)**	−0.060 (−0.126, 0.005)	**−0.034 (−0.045, −0.022)**	**−0.308 (−0.538, −0.077)**
T3	**−0.305 (−0.469, −0.140)**	**−0.354 (−0.484, −0.225)**	0.054 (−0.012, 0.120)	**−0.019 (−0.031, −0.007)**	**−0.270 (−0.502, −0.037)**

Note: Model 1—no adjustment. Model 2—adjusted for age, educational level, per capita monthly income, marital status, family history of hypertension, family history of hyperlipidemia, family history of diabetes, smoking status, drinking status, high-fat diet, physical activity, and fruit and vegetable intake. Bold values signify $p < 0.05$.

Abbreviations: CI, confidence interval; hsCRP, high-sensitivity C-reactive protein; LYMPH, lymphocytes; MONO, monocytes; NEUT, neutrophils; T, tertile; WBC, white blood cells.

20477927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by myram mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 4 Mediation effect of inflammatory markers on the association between testosterone and obesity (β, 95% confidence interval).

Variable	Total effect	Direct effect	Indirect effect	PE
WBC				
WC	**−0.097 (−0.144, −0.051)**	**−0.094 (−0.141, −0.048)**	**−0.004 (−0.010, −0.001)**	4.53%
WHR	**−0.089 (−0.133, −0.045)**	**−0.086 (−0.130, −0.042)**	**−0.004 (−0.009, −0.001)**	4.28%
WHtR	**−0.080 (−0.124, −0.035)**	**−0.076 (−0.120, −0.031)**	**−0.005 (−0.012, −0.001)**	6.65%
BMI	**−0.074 (−0.146, −0.002)**	**−0.073 (−0.145, −0.001)**	−0.002 (−0.009, 0.001)	–
NEUT				
WC	**−0.097 (−0.144, −0.051)**	**−0.095 (−0.141, −0.048)**	**−0.004 (−0.009, −0.001)**	3.60%
WHR	**−0.089 (−0.133, −0.045)**	**−0.086 (−0.130, −0.043)**	**−0.003 (−0.008, −0.001)**	3.27%
WHtR	**−0.080 (−0.124, −0.035)**	**−0.076 (−0.121, −0.031)**	**−0.005 (−0.011, −0.001)**	5.65%
BMI	**−0.074 (−0.146, −0.002)**	**−0.073 (−0.145, −0.001)**	−0.001 (−0.006, 0.002)	–
LYMPH				
WC	**−0.097 (−0.144, −0.051)**	**−0.098 (−0.144, −0.051)**	−0.001 (−0.003, 0.003)	–
WHR	**−0.089 (−0.133, −0.045)**	**−0.089 (−0.133, −0.045)**	−0.001 (−0.003, 0.003)	–
WHtR	**−0.080 (−0.124, −0.035)**	**−0.080 (−0.125, −0.035)**	−0.001 (−0.003, 0.003)	–
BMI	**−0.074 (−0.146, −0.002)**	**−0.074 (−0.146, −0.002)**	−0.001 (−0.003, 0.002)	–
MONO				
WC	**−0.097 (−0.144, −0.051)**	**−0.093 (−0.139, −0.046)**	**−0.005 (−0.011, −0.002)**	5.46%
WHR	**−0.089 (−0.133, −0.045)**	**−0.085 (−0.129, −0.041)**	**−0.005 (−0.010, −0.002)**	5.18%
WHtR	**−0.080 (−0.124, −0.035)**	**−0.075 (−0.120, −0.030)**	**−0.006 (−0.012, −0.002)**	7.28%
BMI	**−0.074 (−0.146, −0.002)**	−0.070 (−0.142, 0.003)	**−0.004 (−0.011, −0.001)**	Full
hsCRP				
WC	**−0.097 (−0.144, −0.051)**	**−0.088 (−0.135, −0.041)**	**−0.011 (−0.019, −0.005)**	11.02%
WHR	**−0.089 (−0.133, −0.045)**	**−0.081 (−0.123, −0.037)**	**−0.010 (−0.019, −0.004)**	11.15%
WHtR	**−0.080 (−0.124, −0.035)**	**−0.072 (−0.117, −0.027)**	**−0.010 (−0.020, −0.004)**	12.92%
BMI	**−0.074 (−0.146, −0.002)**	−0.064 (−0.137, 0.009)	**−0.009 (−0.018, −0.004)**	Full

Note: Full—full mediation effect. PE—indirect effect/total effect. Bold values signify $p < 0.05$.
Abbreviations: BMI, body mass index; hsCRP, high-sensitivity C-reactive protein; LYMPH, lymphocytes; MONO, monocytes; NEUT, neutrophils; WBC, white blood cells; WC, waist circumference; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio.

in the relationship between testosterone and obesity. Furthermore, androstenedione reinforced the inverse relationships of testosterone with inflammation and obesity.

As the two main natural androgenic hormones in the body, many studies have investigated the relationship between testosterone and obesity, but few studies are about the androstenedione. Our findings support previous research showing that low levels of serum testosterone and androstenedione are linked to an increased risk of obesity.[12,13,15,16,21,22,46,47] An animal study showed that testosterone has an anti-obesity effect and that testosterone inhibits the expansion of visceral and subcutaneous fat.[12] In addition, a cross-sectional study showed that obesity parameters (WC, WHR, BMI) were significantly negatively associated with serum testosterone concentrations.[15] Androstenedione has been shown to be negatively associated with obesity in several studies.[21,22] Although the mechanisms of testosterone in different kinds of obesity need further study, existing evidence suggests that androgens could be key regulators of body fat distribution.[48] When sex steroid hormones activate the cAMP cascade, hormone-sensitive lipase is activated, resulting in lipolysis in adipose tissues.[46] Testosterone inhibits triglyceride uptake and lipoprotein lipase activity, resulting in faster triglyceride turnover in abdominal subcutaneous adipose tissue and less triglyceride turnover in femoral fat, as well as the mobilization of lipids from visceral fat depots.[49] It has been demonstrated that androgens can reduce the activity of stearoyl-CoA desaturase-1 and increase the activity of lipoprotein lipase in human adipocytes, thereby lowering blood lipids, regulating lipid metabolism, and maintaining lipid homeostasis.[50]

The primary sources of pro-inflammatory cytokines are NEUT, LYMPH, and other WBC.[51] CRP is considered a representative biomarker of systemic inflammation.[52] Some previous studies have reported that inflammation involving pro-inflammatory cytokines promoted the development of obesity,[53] and serum CRP was positively related to obesity,[54] which is in line with our results. The two epidemiological studies mentioned above are prospective cohort studies and Mendelian randomization studies, which can better demonstrate the causal relation. The relationship between

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

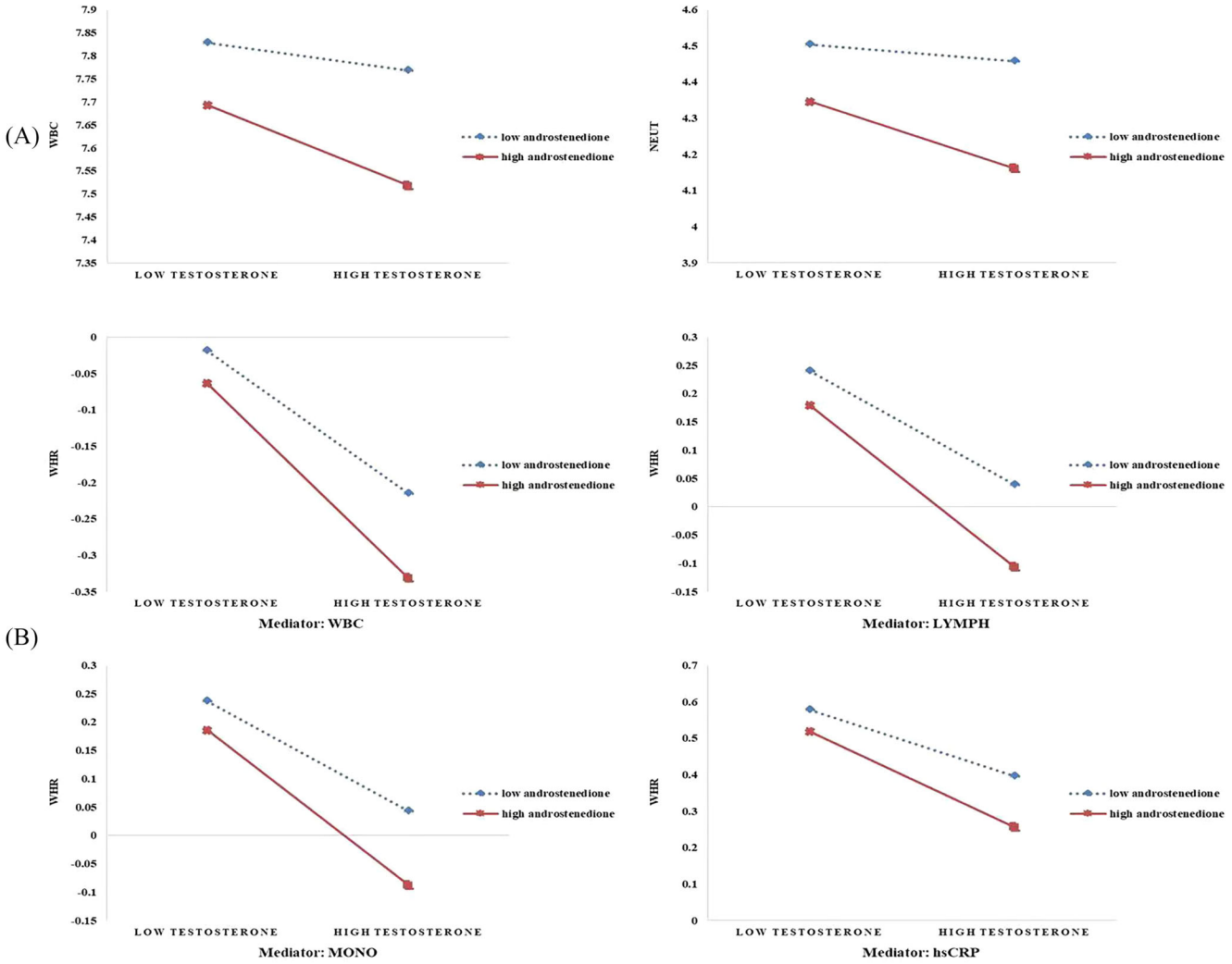


FIGURE 1 (A) Associations between testosterone and inflammatory markers for low and high levels of androstenedione. The model included the following covariates: age, educational level, per capita monthly income, marital status, family history of hypertension, family history of hyperlipidemia, family history of diabetes, smoking status, drinking status, high-fat diet, physical activity, and fruit and vegetable intake. (♦) Low level; (■) high level. (B) The direct effect between testosterone and obesity for low and high levels of androstenedione. The model included the following covariates: age, educational level, per capita monthly income, marital status, family history of hypertension, family history of hyperlipidemia, family history of diabetes, smoking status, drinking status, high-fat diet, physical activity, and fruit and vegetable intake. (♦) Low level; (■) high level.

inflammatory markers and obesity may be explained by the following facts. First, there is an emerging body of evidence that suggests that WBC secrete pro-inflammatory cytokines.[51] Moreover, a previous study has shown that inflammatory responses involving pro-inflammatory cytokines can contribute to the development of obesity.[53] Therefore, it is possible that WBC lead to an increase in body fat mass by secreting pro-inflammatory factors. Second, by regulating lipid metabolism and facilitating fat synthesis, CRP plays a crucial impact on the pathogenesis of obesity metabolic disorders.[55,56] Third, CRP is the major leptin-binding protein and interacts with leptin receptors, thus interfering with the action of leptin and causing a lack of response to leptin in the body, which in turn leads to obesity.[57] More studies are needed to further illuminate and validate the mechanisms between inflammation and obesity.

As far as we know, it is the first investigation to prove that the relationship between testosterone and obesity is mediated by inflammatory markers. A previous study of 1490 US men investigated associations between androgen activity and inflammatory markers.[30] The study found that testosterone was significantly associated with decreased WBC count (95% CI: −0.37, −0.14) and granulocyte count (95% CI: −0.29, −0.13). It has previously been shown that serum testosterone levels are negatively associated with hsCRP.[33] In addition, a study has reported a negative correlation between androstenedione and the levels of inflammatory markers (interleukin-6 [IL-6], hsCRP).[35] Our findings support the results of these prior studies. In this study, we proposed a hypothesis of a mediating role of inflammatory markers in testosterone-associated obesity. The results showed that there was a difference in the proportion of mediating effect between inflammatory

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

cells and hsCRP. Although hsCRP has the strongest mediating effect among these indicators, inflammatory cells are easier to obtain than hsCRP as a routine blood detection indicator, so they are more valuable in application. Testosterone inhibits the expression and release of pro-inflammatory cytokines via androgen receptors and non-classical surface receptors,[58–60] and pro-inflammatory cytokines are closely linked to the inflammatory response of the body.[61] Based on the above mechanism, it is plausible that high concentration of testosterone would lower the level of systemic inflammation, which leads to obesity.

Our findings demonstrated that testosterone was negatively associated with inflammatory markers and obesity and that the associations would be strengthened by androstenedione. It is well known that androstenedione and testosterone are both androgens. In addition, androstenedione is the primary precursor to testosterone, so androstenedione can play a role by converting to testosterone. So, consistent with our results, high androstenedione levels increase testosterone levels and therefore increase the inhibitory effect of testosterone on inflammatory levels and increase the protective effect against obesity. However, the biological role of androstenedione itself and the mechanism by which testosterone and androstenedione are associated with inflammation remain unknown. This could be a direction for the future research.

The strength of our study is a relatively large sample size consisting of 2536 male individuals. Furthermore, a mediation analysis, was performed to evaluate direct and indirect causal effects between testosterone, obesity, and inflammatory markers. Moreover, to our knowledge, no prior studies have investigated the moderation effects of androstenedione on testosterone-associated inflammation and obesity in a population-based study. Finally, LC–MS/MS was used to accurately measure testosterone concentration. Meanwhile, the current research has a few inadequacies. First, because of the cross-sectional design, this research may not be able to establish a causal relationship among testosterone, inflammatory markers, and obesity. Second, we only estimated the effect of testosterone and androstenedione, so other hormone levels may have been overlooked. Third, the study was limited by the lack of data on more inflammatory markers in addition to WBC and hsCRP, such as IL-4 or IL-6. Finally, because our participants are from Henan rural areas in China, we may be bounded in our ability to apply these findings to other countries and regions. Despite these drawbacks, this large-scale population study can shed light on the critical role of inflammatory markers and androstenedione in testosterone and obesity.

5 | CONCLUSION

We first found that androstenedione has an enhanced effect on testosterone-associated inflammation and obesity, and the inflammatory markers mediate the association of testosterone with obesity in the current study. This suggests that inflammation and androstenedione may influence the link between testosterone and obesity. More research is required to verify our findings and clarify the underlying mechanism.

ACKNOWLEDGMENTS

We would like to thank the study participants for their contributions. We also want to thank the research team for their help with data collection and laboratory measurements. This research was supported by the National Natural Science Foundation of China (grant nos. 42177415 and 21806146), the Postdoctoral Science Foundation of China (grant nos. 2020T130604 and 2021M702934), the Science and Technique Foundation of Henan Province (grant no. 212102310074), the Scientific and Technological Innovation of Colleges and Universities in Henan Province Talent Support Programme (grant no. 22HASTIT044), the Young Backbone Teachers Program of Colleges and Universities in Henan Province (grant no. 2021GGJS015), and the Excellent Youth Development Foundation of Zhengzhou University (grant no. 2021ZDGGJS057).

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Wenqian Huo https://orcid.org/0000-0002-7898-093X

REFERENCES

1. Del Ry S, Cabiati M, Salvadori C, et al. Transcriptional alterations of ET-1 axis and DNA damage in lung tissue of a rat obesity model. *DNA Cell Biol*. 2015;34(3):170-177.
2. Wu D, Gao B, Li M, et al. Hydrogen sulfide mitigates kidney injury in high fat diet-induced obese mice. *Oxid Med Cell Longev*. 2016;2016:2715718.
3. Sohouli MH, Sharifi-Zahabi E, Lari A, Fatahi S, Shidfar F. The impact of low advanced glycation end products diet on obesity and related hormones: a systematic review and meta-analysis. *Sci Rep*. 2020;10(1):22194.
4. Al-Serri A, Al-Bustan SA, Al-Sabah SK, Annice BG, Alnaqeeb MA, Mojiminiyi OA. Association between the lipoprotein lipase rs1534649 gene polymorphism in intron one with body mass index and high density lipoprotein-cholesterol. *Saudi J Biol Sci*. 2021;28(8):4717-4722.
5. Liu X, Wu W, Mao Z, et al. Prevalence and influencing factors of overweight and obesity in a Chinese rural population: the Henan Rural Cohort Study. *Sci Rep*. 2018;8(1):13101.
6. Jeffery E, Church CD, Holtrup B, Colman L, Rodeheffer MS. Rapid depot-specific activation of adipocyte precursor cells at the onset of obesity. *Nat Cell Biol*. 2015;17(4):376-385.
7. Woldegebriel AG, Fenta KA, Aregay AB, et al. Effectiveness of anthropometric measurements for identifying diabetes and prediabetes among civil servants in a regional city of northern Ethiopia: a cross-sectional study. *J Nutr Metab*. 2020;2020:8425912.
8. Fu W, Cao S, Liu B, et al. Association of general and central adiposity with blood pressure among Chinese adults: results from the China National Stroke Prevention Project. *J Hypertens*. 2018;36(12):2406-2413.
9. Bragg F, Tang K, Guo Y, et al. Associations of general and central adiposity with incident diabetes in Chinese men and women. *Diabetes Care*. 2018;41(3):494-502.
10. Schipf S, Haring R, Friedrich N, et al. Low total testosterone is associated with increased risk of incident type 2 diabetes mellitus in men:

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

results from the Study of Health in Pomerania (SHIP). *Aging Male*. 2011;14(3):168-175.
11. Chiang JM, Kaysen GA, Segal M, Chertow GM, Delgado C, Johansen KL. Low testosterone is associated with frailty, muscle wasting and physical dysfunction among men receiving hemodialysis: a longitudinal analysis. *Nephrol Dial Transplant*. 2019;34(5):802-810.
12. Sebo ZL, Rodeheffer MS. Testosterone metabolites differentially regulate obesogenesis and fat distribution. *Mol Metab*. 2021;44:101141.
13. Lima TFN, Frech FS, Blachman-Braun R, Rakitina E, Patel P, Ramasamy R. Association of aging and obesity with decreased 17-hydroxyprogesterone, a serum biomarker of intratesticular testosterone. *Int J Impot Res*. 2022;34(1):44-49.
14. Zhang C, Gilardi G, Di Nardo G. Depicting the proton relay network in human aromatase: new insights into the role of the alcohol-acid pair. *Protein Sci*. 2022;31(9):e4389.
15. Rotter I, Rył A, Grzesiak K, et al. Cross-sectional inverse associations of obesity and fat accumulation indicators with testosterone in non-diabetic aging men. *Int J Environ Res Public Health*. 2018;15(6):1207.
16. Saad F, Doros G, Haider KS, Haider A. Differential effects of 11 years of long-term injectable testosterone undecanoate therapy on anthropometric and metabolic parameters in hypogonadal men with normal weight, overweight and obesity in comparison with untreated controls: real-world data from a controlled registry study. *Int J Obes (Lond)*. 2020;44(6):1264-1278.
17. Ng Tang Fui M, Hoermann R, Cheung AS, Gianatti EJ, Zajac JD, Grossmann M. Obesity and age as dominant correlates of low testosterone in men irrespective of diabetes status. *Andrology*. 2013;1(6):906-912.
18. Ng Tang Fui M, Hoermann R, Grossmann M. Effect of testosterone treatment on adipokines and gut hormones in obese men on a hypocaloric diet. *J Endocr Soc*. 2017;1(4):302-312.
19. Høst C, Bojesen A, Erlandsen M, et al. A placebo-controlled randomized study with testosterone in Klinefelter syndrome: beneficial effects on body composition. *Endocr Connect*. 2019;8(9):1250-1261.
20. Colldén H, Landin A, Wallenius V, et al. The gut microbiota is a major regulator of androgen metabolism in intestinal contents. *Am J Physiol Endocrinol Metab*. 2019;317(6):E1182-E1192.
21. Tchernof A, Labrie F, Bélanger A, Després JP. Obesity and metabolic complications: contribution of dehydroepiandrosterone and other steroid hormones. *J Endocrinol*. 1996;150 (suppl):S155-S164.
22. Khaw KT, Barrett-Connor E. Lower endogenous androgens predict central adiposity in men. *Ann Epidemiol*. 1992;2(5):675-682.
23. Kim HY, Lee J, Kim J. Association between dietary inflammatory index and metabolic syndrome in the general korean population. *Nutrients*. 2018;10(5):648.
24. Kabashima K, Honda T, Ginhoux F, Egawa G. The immunological anatomy of the skin. *Nat Rev Immunol*. 2019;19(1):19-30.
25. Boyko AA, Azhikina TL, Streltsova MA, Sapozhnikov AM, Kovalenko EI. HSP70 in human polymorphonuclear and mononuclear leukocytes: comparison of the protein content and transcriptional activity of HSPA genes. *Cell Stress Chaperones*. 2017;22(1):67-76.
26. d'Engremont C, Vernerey D, Pointet AL, et al. Additive value of pre-operative and one-month post-operative lymphocyte count for death-risk stratification in patients with resectable pancreatic cancer: a multicentric study. *BMC Cancer*. 2016;16(1):823.
27. Timmons GA, O'Siorain JR, Kennedy OD, Curtis AM, Early JO. Innate rhythms: clocks at the center of monocyte and macrophage function. *Front Immunol*. 2020;11:1743.
28. Moutachakkir M, Lamrani Hanchi A, Baraou A, Boukhira A, Chellak S. Immunoanalytical characteristics of C-reactive protein and high sensitivity C-reactive protein. *Ann Biol Clin (Paris)*. 2017;75(2):225-229.
29. Brand JS, van der Schouw YT, Dowsett M, et al. SHBG and differential white blood cell count in middle-aged and older men. *Maturitas*. 2012;71(3):274-278.
30. Schooling CM. Androgen activity and markers of inflammation among men in NHANES III. *Am J Hum Biol*. 2013;25(5):622-628.
31. Nunn CL, Lindenfors P, Pursall ER, Rolff J. On sexual dimorphism in immune function. *Philos Trans R Soc Lond B Biol Sci*. 2009;364(1513):61-69.
32. Koçar IH, Yesilova Z, Ozata M, Turan M, Sengül A, Ozdemir I. The effect of testosterone replacement treatment on immunological features of patients with Klinefelter's syndrome. *Clin Exp Immunol*. 2000;121(3):448-452.
33. Tang YJ, Lee WJ, Chen YT, Liu PH, Lee MC, Sheu WH. Serum testosterone level and related metabolic factors in men over 70 years old. *J Endocrinol Invest*. 2007;30(6):451-458.
34. Tsilidis KK, Rohrmann S, McGlynn KA, et al. Association between endogenous sex steroid hormones and inflammatory biomarkers in US men. *Andrology*. 2013;1(6):919-928.
35. Sesmilo G, Miller KK, Hayden D, Klibanski A. Inflammatory cardiovascular risk markers in women with hypopituitarism. *J Clin Endocrinol Metab*. 2001;86(12):5774-5781.
36. Dixon JB, O'Brien PE. Obesity and the white blood cell count: changes with sustained weight loss. *Obes Surg*. 2006;16(3):251-257.
37. Kawada T, Otsuka T, Endo T, Kon Y. The metabolic syndrome, smoking, inflammatory markers and obesity. *Int J Cardiol*. 2011;151(3):367-368. Author reply 373-374.
38. Liu X, Mao Z, Li Y, et al. Cohort profile: the Henan Rural Cohort: a prospective study of chronic non-communicable diseases. *Int J Epidemiol*. 2019;48(6):1756-1756j.
39. Mi YJ, Zhang B, Wang HJ, et al. Prevalence and secular trends in obesity among Chinese adults, 1991–2011. *Am J Prev Med*. 2015;49(5):661-669.
40. Tu R, Li Y, Shen L, et al. The prevalence and influencing factors of physical activity and sedentary behaviour in the rural population in China: the Henan Rural Cohort Study. *BMJ Open*. 2019;9(9):e029590.
41. Liu X, Tu R, Qiao D, et al. Association between long-term exposure to ambient air pollution and obesity in a Chinese rural population: the Henan Rural Cohort Study. *Environ Pollut*. 2020;260:114077.
42. Zhou BF. Effect of body mass index on all-cause mortality and incidence of cardiovascular diseases–report for meta-analysis of prospective studies open optimal cut-off points of body mass index in Chinese adults. *Biomed Environ Sci*. 2002;15(3):245-252.
43. Zhang L, Li Y, Wang C, et al. Early menarche is associated with an increased risk of type 2 diabetes in rural Chinese women and is partially mediated by BMI: the Henan Rural Cohort Study. *Menopause*. 2019;26(11):1265-1271.
44. Valeri L, Vanderweele TJ. Mediation analysis allowing for exposure-mediator interactions and causal interpretation: theoretical assumptions and implementation with SAS and SPSS macros. *Psychol Methods*. 2013;18(2):137-150.
45. Yang BY, Markevych I, Bloom MS, et al. Community greenness, blood pressure, and hypertension in urban dwellers: the 33 Communities Chinese Health Study. *Environ Int*. 2019;126:727-734.
46. Saad F, Aversa A, Isidori AM, Gooren LJ. Testosterone as potential effective therapy in treatment of obesity in men with testosterone deficiency: a review. *Curr Diabetes Rev*. 2012;8(2):131-143.
47. Saad F, Yassin A, Doros G, Haider A. Effects of long-term treatment with testosterone on weight and waist size in 411 hypogonadal men with obesity classes I-III: observational data from two registry studies. *Int J Obes*. 2016;40(1):162-170.
48. Tchernof A, Brochu D, Maltais-Payette I, et al. Androgens and the regulation of adiposity and body fat distribution in humans. *Compr Physiol*. 2018;8(4):1253-1290.
49. Traish AM, Abdou R, Kypreos KE. Androgen deficiency and atherosclerosis: the lipid link. *Vascul Pharmacol*. 2009;51(5-6):303-313.
50. Zerradi M, Dereumetz J, Boulet MM, Tchernof A. Androgens, body fat distribution and adipogenesis. *Curr Obes Rep*. 2014;3(4):396-403.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

51. Mikirova N, Casciari J, Rogers A, Taylor P. Effect of high-dose intravenous vitamin C on inflammation in cancer patients. *J Transl Med*. 2012;10:189.
52. Wu Y, Potempa LA, El Kebir D, Filep JG. C-reactive protein and inflammation: conformational changes affect function. *Biol Chem*. 2015;396(11):1181-1197.
53. Perrin EM, O'Shea TM, Skinner AC, et al. Elevations of inflammatory proteins in neonatal blood are associated with obesity and overweight among 2-year-old children born extremely premature. *Pediatr Res*. 2018;83(6):1110-1119.
54. Bochud M, Marquant F, Marques-Vidal PM, et al. Association between C-reactive protein and adiposity in women. *J Clin Endocrinol Metab*. 2009;94(10):3969-3977.
55. Nieto-Vazquez I, Fernández-Veledo S, Krämer DK, Vila-Bedmar R, Garcia-Guerra L, Lorenzo M. Insulin resistance associated to obesity: the link TNF-alpha. *Arch Physiol Biochem*. 2008;114(3):183-194.
56. Kovacs A, Green F, Hansson LO, et al. A novel common single nucleotide polymorphism in the promoter region of the C-reactive protein gene associated with the plasma concentration of C-reactive protein. *Atherosclerosis*. 2005;178(1):193-198.
57. Sudhakar M, Silambanan S, Chandran AS, Prabhakaran AA, Ramakrishnan R. C-reactive protein (CRP) and leptin receptor in obesity: binding of monomeric CRP to leptin receptor. *Front Immunol*. 2018;9:1167.
58. Liu L, Benten WP, Wang L, et al. Modulation of *Leishmania donovani* infection and cell viability by testosterone in bone marrow-derived macrophages: signaling via surface binding sites. *Steroids*. 2005;70(9):604-614.
59. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biol Reprod*. 2008;78(3):432-437.
60. Malkin CJ, Pugh PJ, Jones RD, Kapoor D, Channer KS, Jones TH. The effect of testosterone replacement on endogenous inflammatory cytokines and lipid profiles in hypogonadal men. *J Clin Endocrinol Metab*. 2004;89(7):3313-3318.
61. Hlapčić I, Belamarić D, Bosnar M, Kifer D, Vukić Dugac A, Rumora L. Combination of systemic inflammatory biomarkers in assessment of chronic obstructive pulmonary disease: diagnostic performance and identification of networks and clusters. *Diagnostics*. 2020;10(12):1029.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Huan C, Wang M, Song Y, et al. Inflammatory markers and androstenedione modify the effect of serum testosterone on obesity among men: Findings from a Chinese population. *Andrology*. 2024;12:850–861. https://doi.org/10.1111/andr.13544

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13544 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 7 March 2023 | Revised: 30 August 2023 | Accepted: 3 September 2023

DOI: 10.1111/andr.13531

ANDROLOGY

WILEY

ORIGINAL ARTICLE

Tip-microVapour Fast Freezing: A novel easy method for cryopreserving severe oligozoospermic samples

Sara Dabizzi[1,2] | Costanza Calamai[1,2] | Selene Degl'Innocenti[1] | Luca Boni[3] | Linda Vignozzi[1,2] | Mario Maggi[2,4] | Elisabetta Baldi[1,5] | Monica Muratori[2]

[1]Unit of Andrology, Women's Endocrinology and Gender Incongruence, Centre for the Prevention, Diagnosis and Treatment of Infertility, Azienda Ospedaliera Universitaria Careggi Hospital, Florence, Italy

[2]Department of Experimental and Clinical Biomedical Sciences 'Mario Serio', University of Florence, Florence, Italy

[3]Unit of Clinical Epidemiology, IRCCS Ospedale Policlinico San Martino, Genova, Italy

[4]Unit of Endocrinology, Azienda Ospedaliera Universitaria Careggi Hospital, Florence, Italy

[5]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

Correspondence
Sara Dabizzi, Unit of Andrology, Women's Endocrinology and Gender Incongruence, Centre for the Prevention, Diagnosis and Treatment of Infertility, Azienda Ospedaliera Universitaria Careggi Hospital, Florence, Italy. Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy.
Email: dabizzis@aou-careggi.toscana.it; sara.dabizzi@stud.unifi.it

Funding information
Fondazione Ente Cassa di Risparmio di Firenze, Grant/Award Number: ECFR 2017; Andrological Sciences Onlus

Abstract

Background: Sperm cryopreservation is an important procedure for oligozoospermic subjects at risk of azoospermia and after surgical recovery of spermatozoa in non-obstructive azoospermic men. Conventional procedures for sperm cryopreservation might be, however, not suitable for samples with a very low sperm number.

Objectives: In this pilot study, we investigated the recoveries of sperm motility and viability in severe oligozoospermic subjects ($n = 39$) after cryopreservation with a tip-microVapour Fast Freezing, a procedure previously developed by our group for men with good semen quality. Sperm DNA fragmentation was also evaluated in a second group of oligozoospermic samples ($n = 16$).

Materials and methods: We used a Vapour Fast Freezing procedure using 10 μL tips as carrier, and Test Yolk Buffer as freezing medium (tip-microVapour Fast Freezing). In a subset of samples ($n = 22$), we compared recovery of motility and viability as obtained with tip-microVapour Fast Freezing and with a Vapour Fast Freezing procedure using 500 μL straws. Sperm DNA fragmentation was evaluated by the sperm chromatin dispersion test.

Results: We found a recovery rate (median [interquartile range]) of 0.29 (0.13–0.41) for progressive motility, 0.30 (0.21–0.52) for total motility and 0.48 (0.29–0.60) for viability. Interestingly, we observed that samples with the poorest motility were apparently less damaged by freezing/thawing. In a subset of samples ($n = 22$), we directly compared values of viability, progressive motility and total motility by freezing/thawing with tip-microVapour Fast Freezing and Vapour Fast Freezing conducted with 500 μL straws. We found much better values of all sperm parameters in samples after freezing/thawing with tip-microVapour Fast Freezing than with Vapour Fast Freezing in 500 μL straws: that is, progressive motility: 7.00 (3.00–8.50)% versus 2.00 (0.00–4.25)%, $p < 0.001$; total motility: 12.00 (8.00–16.25)% versus 6.50 (1.00–9.25)%, $p < 0.001$; viability: 29.75 (23.75–45.25) versus 22.50 (13.75–28.13), $p < 0.001$, respectively. In the second group of oligozoospermic samples, we found that tip-microVapour Fast Freezing produced lower levels of sperm DNA fragmentation than straws (33.00 [19.75–36.00]% vs. 36.00 [22.75–41.87]%, $p < 0.001$).

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2023 The Authors. *Andrology* published by Wiley Periodicals LLC on behalf of American Society of *Andrology* and European Academy of *Andrology*.

Discussion and conclusion: Tip-microVapour Fast Freezing appears to be a very promising method to cryopreserve semen samples from severe oligozoospermic patients.

KEYWORDS
carriers, motility, oligozoospermia, sperm cryopreservation, Vapour Fast Freezing, viability

1 | INTRODUCTION

One in every seven couples experiences fertility problems in the western societies, with a male factor responsible for up to 50% of the cases. Men with severe oligozoospermia or azoospermia account for 3.1% and 4.3% of infertile subjects,[1] respectively, and such percentages are expected to increase over time because of the declining trend in sperm count/concentration recently reported.[2,3] The introduction of intracytoplasmic sperm injection (ICSI) gave the chance of parenthood to couples with severe male factor infertility by allowing the treatment of men with severe oligozoospermia and even those with azoospermia. In men with severe oligozoospermia, an extended search of spermatozoa in semen can be pursued, making possible the recovery of a sufficient number of spermatozoa for oocyte injection. However, these patients are at major risk to become azoospermic than men with mild or moderate oligozoospermia.[4] For azoospermic subjects, percutaneous or microsurgical aspiration from epididymis or surgical retrieval from testis of spermatozoa are available,[5] including those mainly used in case of non-obstructive azoospermia (NOA): testicular sperm aspiration (TESA) and testicular sperm extraction (TESE). These approaches have highly variable sperm recovery rates (RRs): in obstructive azoospermia (OA), 45%–97% for epididymal sperm aspiration[6] and near 100% for TESA and TESE[7,8]; in NOA, 36%–64% for TESE and even lower for TESA.[5,6] Such a scenario poses the possibility that no spermatozoon are found on the day of oocyte pick-up. This situation might provoke ICSI cycle cancellation and request for repetition of surgical sperm recovery, increasing the risk of medical complications other than the psychological and economic burden for the couple.

In this scenario, sperm cryopreservation can be of help for oligozoospermic subjects at risk of azoospermia. In addition, in men undergoing surgical recovery of spermatozoa, it can reduce the number and consequences of repeated surgical interventions and assure that spermatozoa are always available for the ongoing ICSI cycle.

Sperm cryopreservation procedures conventionally consist of adding cryoprotectants, freezing with gradual temperature decrease and finally storing semen samples in liquid nitrogen. These procedures are used for sperm banking in subjects presenting with middle/fine semen quality but appear not suitable for samples with very low sperm number, as they present several drawbacks. First, conventional procedures use relatively large carriers and sample volumes which endanger sperm retrieval after thawing. Second, conventional procedures lead to a high reduction in sperm viability and motility and to sperm loss because of washing step of thawed samples.[9] This issue can be balanced in samples with mild/good quality but represents a serious drawback with samples with very low sperm numbers. Another relevant sperm parameter is the integrity of DNA as it is well known that freezing/thawing provokes an increase in sperm DNA fragmentation (sDF).[10] The latter, in turn, endangers the outcomes of assisted reproductive techniques, including ICSI,[11,12] when frozen samples are used. To overcome some of the drawbacks of conventional cryopreservation procedures, several biological or non-biological carriers have been tested,[6] but the optimal carrier for cryopreservation of a low number of spermatozoa is still sought.

Our group recently tested two procedures of Vapour Fast Freezing (VFF) to cryopreserve spermatozoa in low semen volumes (microVFF) and found that such procedures better maintain sperm viability/motility or DNA quality, depending on the carrier used, with respect to conventional VFF. In particular, using 10 μL tips (tip-microVFF), thawed samples showed percentages of motility and viability similar to a conventional VFF procedure but a lower percentage of sDF.[9] We also found that washing/centrifuging steps after sample thawing provoked a relevant fraction of damage to motile and viable spermatozoa.[9] In this study, we aimed at investigating the recovery of sperm motility and viability in subjects presenting with extremely low sperm number after cryopreservation with a tip-microVFF and skipping the washing/centrifuging steps in thawed samples.

2 | MATERIALS AND METHODS

2.1 | Population and study design

In this pilot study, semen samples were collected from 39 patients with severe oligozoospermia, afferent to the Semen Cryopreservation and Andrology Laboratory of Careggi Hospital, from December 2019 to August 2021, to undergo semen cryopreservation because they were affected by oncological disease (testicular cancer, $n = 7$; lymphoma, $n = 2$; glioma, $n = 1$) or oligozoospermia ($n = 29$). All these subjects were offered sperm banking by a standard procedure used in the laboratory since 1992,[13] that is, a VFF method using 500 μL high security straws (Cryo Bio System) as carrier and Test Yolk Buffer (TYB, containing 20% egg yolk and 12% glycerol as cryoprotectants) (Fujifilm, Irvine Scientific) as freezing medium (from here on indicated as conventional procedure). In all 39 subjects, with remaining semen samples from cryopreservation with conventional procedure, we also used a

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13531 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

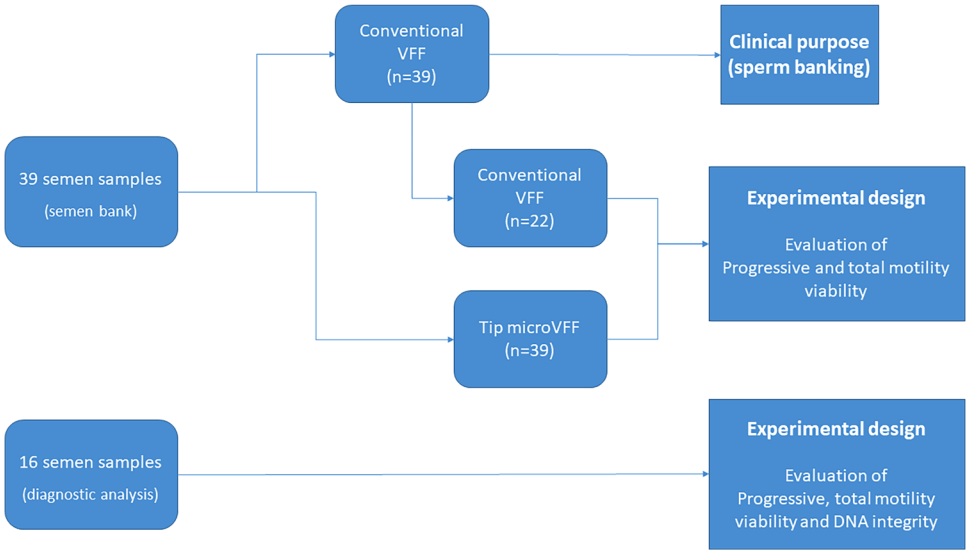


FIGURE 1 Scheme of the study design. VFF, Vapour Fast Freezing.

TABLE 1 Population characteristics ($n = 39$).

Age (year)	Abstinence (day)	Volume (mL)	pH	Sperm concentration (million/mL)	Sperm count (million/ejaculate)
33.54 ± 9.18	4.00 (3.00–5.00)	3.30 ± 1.99	7.60 (7.40–7.80)	1.20 (0.48–2.10)	2.85 (1.14–8.06)

Note: Values are mean ± SD or median (interquartile range).

new method, that is, a VFF using TYB as the freezing medium but 10 μL polypropylene tips as carrier (tip-microVFF).[9] Finally, when the remaining semen volume was at least 150 μL ($n = 22$), an additional straw was prepared in order to compare recovery of motility and viability of conventional procedure to that of tip-microVFF (Figure 1). The population characteristics are reported in Table 1. To compare tip-microVFF and conventional procedure in terms of sperm DNA integrity after thawing, we recruited further 16 patients with oligozoospermia (sperm concentration <15 million/mL) among men undergoing routine semen analysis, resulting in a group with higher sperm concentration and number than the group with severe oligozoospermia (Tables S1 and 1). Table S1 also reports the values for age, abstinence and the other semen parameters as found in fresh samples in this second group. All the recruited subjects signed a written informed consent form. The study was approved by the Ethical Committee of AOU Careggi (protocol no. 15554_bio).

2.2 | Semen analysis

Semen samples were collected after sexual abstinence for 2–7 days (Table 1). After 30 min for liquefaction, semen analysis was conducted following the WHO 2010 guidelines[14] and consisted of determination of: (i) sperm number and concentration, (ii) progressive and total sperm motility, and (iii) sperm viability. Briefly, sperm concentration was determined by using a Neubauer improved cell counting chamber and examining all nine grids, as suggested by WHO manual 2010, in case of very low sperm number[14]; sperm concentration was then multiplied by semen volume to obtain sperm number/ejaculate. Motility was determined, using light microscopy at 40× magnification on fresh and thawed samples by scoring progressive ($a + b$), non-progressive (c) and immobile spermatozoa (d) in 200 cells, in different fields. Total motility ($a + b + c$) was scored as progressive + non-progressive motility. Sperm viability was evaluated by using eosin test: the sample was mixed with an eosin solution (1:2), spread on a slide and examined by light microscopy, scoring stained and non-stained spermatozoa.

The RR of sperm parameters was calculated by the ratio: post-thaw value/fresh sample value. For RR of sperm concentration, we used the fresh value at freezing time after the addition of freezing medium.

2.3 | Sperm cryopreservation

TYB was slowly added (1:1) to semen samples; hence, the samples were aspirated into the straws, which were subsequently sealed on both sides (conventional procedure). For tip-microVFF, samples were aspired by a micropipette into the tips and then carefully inserted one by one into a cryovial for storage. In both procedures, carriers were first cooled in liquid nitrogen (LN_2) vapour for 8 min by placing them in a floating support at 5 cm above the surface of LN_2 (cooling rate of −15.6°C/min). Hence, the carriers were immersed in LN_2.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13531 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.4 | Thawing

After removal from storage, tips were thawed by carefully opening the cryovial still immersed in LN_2, but attention was given to avoid entry of LN_2 into the vial. Hence, tips were placed vertically on a sterile Petri dish until thawing (few seconds). Samples cryopreserved with conventional procedure were thawed by placing them at 37°C for 15 min. Then, we evaluated motility and viability. In the comparison between tip-microVFF and conventional procedure, in the first 13 samples, we also evaluated post-thawed sperm concentration. All tested parameters were evaluated in duplicate.

2.5 | Sperm chromatin dispersion assay

sDF was detected with Halosperm kit (Halotech DNA) by following manufacturer's instructions with slight modifications. Briefly, 50,000 spermatozoa were added to 1% low melting point agarose and layered on pre-coated agarose slides. Slides were then covered with a coverslip until solidification (4°C). Then, samples were treated with the acid denaturation solution and then with the lysing solution (both provided by the kit). Hence, slides were dehydrated with 70% ethanol and then with 100% ethanol. Staining was conducted with eosin (15 min at RT) and thiazine (15 min at RT) solutions. After drying, slides were scored for halos by bright field microscopy by counting at least 200 spermatozoa. sDF was expressed as the percentage of spermatozoa without or with small halo on total spermatozoa.[9]

2.6 | Statistical analyses

Data were analysed with Statistical Package for the Social Sciences (SPSS 25) for Windows (SPSS, Inc.). The Kolmogorov–Smirnov test was used to check the normal distribution of the variables and data are expressed as the mean ± SD or median (interquartile range), accordingly. We used the Wilcoxon signed rank test to compare: (i) post-thawing values to the fresh ones and (ii) after-thawing sperm parameters obtained with conventional procedure and tip-microVFF. Correlation analysis between the fresh–thawed difference and fresh values was evaluated by calculating the Spearman's correlation coefficient (r). To evaluate the regression to the mean and mathematical coupling effects, we applied the Oldham test.[15] The comparison between conventional procedure and tip-microVFF was sized considering progressive motility as primary endpoint.

2.7 | Sample size calculation

Preliminary experiments of comparison between conventional and tip-microVFF indicated that paired differences showed a mean ± SD of 3.89 ± 4.73 and a normal distribution. Hence, assuming a power of 0.90 and an alpha error of 0.05, the number of subjects to be recruited resulted to be 19, as calculated by a two-sided Wilcoxon signed rank test for quantitative, non-parametric and paired data. Analysis of sample size was computed using PASS software (PASS 2022, v22.0.2, NCSS).

3 | RESULTS

The results of tip-microVFF in the 39 oligozoospermic samples are reported in Table 2. As shown, tip-microVFF recovered nearly all frozen spermatozoa (concentration RR = 0.85), whereas, as expected, both progressive and total motility were highly decreased (RR = 0.29 and 0.30, respectively). Sperm viability was less affected by freezing/thawing, showing an RR of 0.48. Interestingly, when we plotted the difference between fresh and thawed values against fresh values, we observed a sharp direct correlation for progressive ($r = 0.95$, $p < 0.001$, Figure 2A) and total motility ($r = 0.87$, $p < 0.001$, Figure 2B) and viability ($r = 0.43$, $p < 0.01$, Figure S1). Therefore, these results might indicate that the better the value of the aforementioned parameters, in fresh samples, the more severe was their deterioration during cryopreservation. We also investigated whether such relationships were merely because of a regression to the mean and/or mathematical coupling effect, by analysing data with Oldham test.[15] The results showed no evidence of relationship between differences in fresh–thawed and fresh values for sperm vitality (Pearson's correlation coefficient: 0.08; 95% confidence limits: −0.24, 0.39; $p = 0.609$), at variance with both progressive (Pearson's correlation coefficient: −0.88; 95% confidence limits: −0.93, −0.77; $p < 0.001$) and total motility (Pearson's correlation coefficient: −0.69; 95% confidence limits: −0.83, −0.48; $p < 0.001$).

In a subset of 22 semen samples, we compared motility and viability after conventional procedure and tip-microVFF. The results are reported in Figure 3, showing that the new method better recovered both progressive and total motility than conventional procedure (Figure 3A,B). This result is also present when evaluating viability (Figure 3C). Consistently, using tip-microVFF, RR for progressive (RR = 0.29 [0.13–0.41] vs. 0.14 [0.00–0.21], $p < 0.001$) and total motility (RR = 0.30 [0.21–0. 52] vs. 0.19 [0.03–0.29], $p < 0.001$) were higher than those of the conventional procedure. Similar results were found for viability (RR = 0.49 [0.29–0.60] vs. 0.29 [0.18–0.41], $p < 0.001$). Regarding sperm concentration ($n = 13$), the two procedures showed similar recoveries, with a ratio value between after and before cryopreservation of 0.91 [0.68–1.00] for microVFF and 0.87 [0.74–1.00] for conventional procedure ($p = 0.374$). To assess whether baseline semen alteration could affect the comparison between the two cryopreservation methods, we grouped subjects according to baseline semen quality. These analyses, however, confirmed better RRs with tip-microVFF than straws in subjects both below and above the median values of baseline progressive motility, sperm count and viability (data not shown).

To verify whether tip-microVFF protected sperm DNA integrity better than 500 μL straws, we recruited further 16 patients with oligozoospermia among subjects undergoing routine semen analysis. Figure 4 reports the results of this comparison showing the expected increase in sDF after thawing but a lower level of sperm DNA damage

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13531 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Recovery of sperm concentration, progressive and total motility and viability after cryopreservation with tip-microVapour Fast Freezing (VFF) ($n = 39$).

	Sperm concentration (million/mL)	Progressive motility (%)	Total motility (%)	Viability (%)
Fresh	0.90 (0.50–2.00)	15.00 (7.00–29.00)	30.00 (17.00–40.00)	75.00 (58.50–80.00)
Thawed	0.80 (0.43–1.50)	4.00 (2.00–8.00)	11.00 (4.00–15.00)	29.5 (20–40.50)
p-Value[a]	<0.001	<0.001	<0.001	<0.001
Recovery	0.85 (0.73–1.00)	0.29 (0.13–0.41)	0.30 (0.22–0.52)	0.48 (0.29–0.60)

Note: Data are median (interquartile range).
[a]Wilcoxon signed rank test.

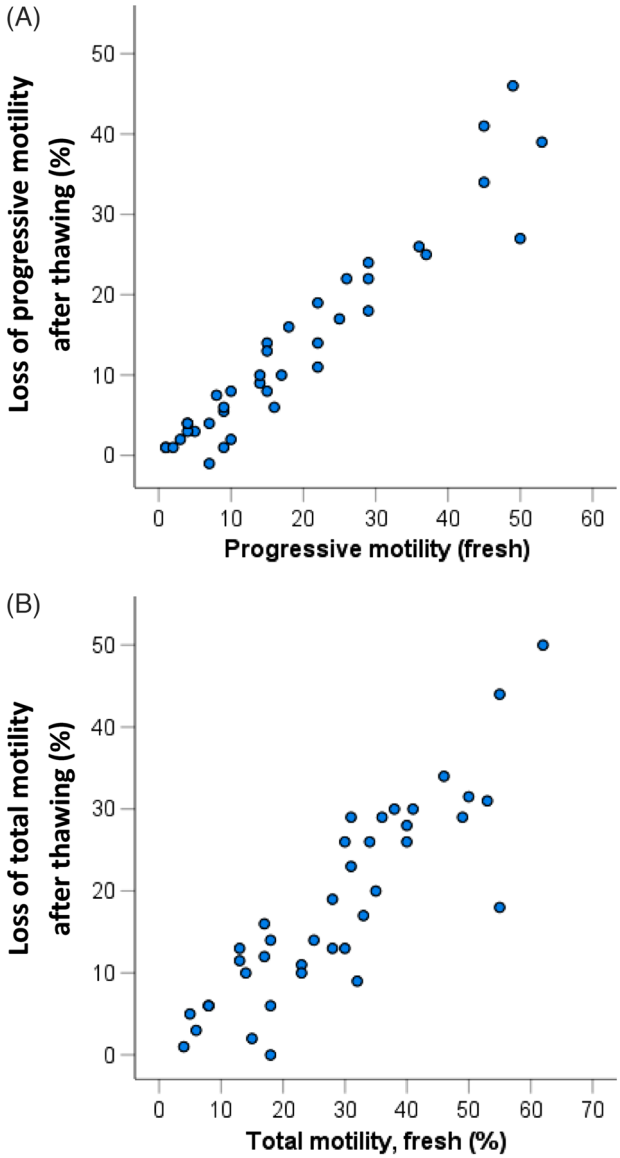


FIGURE 2 Dispersion plots reporting differences in fresh–thawed values (loss) against basal progressive (A) and total motility (B).

in samples cryopreserved with tips than straws. The results of motility and viability in these 16 patients confirmed a better recovery of progressive motility by tips (4.50 [2.00–10.00]%) than straws (4.00 [2.00–8.00]%, $p < 0.05$). A trend towards higher recoveries, albeit not statistically significant, was also observed for total motility (9.00 [3.25–15.75]% vs. 7.50 [3.50–9.75]%, tips vs. straws, respectively) and viability (34.50 [22.50–40.00]% vs. 27.00 [24.00–39.00]%, tips vs. straws, respectively).

4 | DISCUSSION

In this pilot study, we show that the use of tip-microVFF to cryopreserve semen samples with very low sperm counts guarantees a better recovery of both motility parameters and viability of spermatozoa than conventional procedure. Interestingly, the new method appears to preserve sperm motility, especially when poor values are present in fresh samples; hence, there is a pressing need for small-volume cryopreservation procedure.

This project stems from a previous study by our group, aimed at comparing the effect of freezing with microVFF and that of the conventional VFF in 500 μL straws in samples of patients with normal sperm count and motility.[9] That study found that microVFF by using 10 μL tips yielded similar recovery of motility and viability but lower sperm DNA damage than conventional VFF in 500 μL straws.[9] The ultimate scope of current research was to develop a new method to cryopreserve very low semen volumes, in order to extend the service of sperm banking to patients with low sperm counts. Because it is well known that recovery of sperm motility and viability highly depends on baseline semen quality,[13,16] in the current study, we challenged tip-microVFF using samples from severely oligozoospermic patients, and in a subset of samples, we compared results with those found using 500 μL straws. Surprisingly, we found that the use of tips yielded very good after-thawing values of both motility and viability, resulting even better than those previously published in 2013 by our group in a similar group of patients (i.e., oligozoospermic ones) using the same procedure but with 500 μL straws.[13] Indeed, Degl'Innocenti et al.[13] showed that the median value of recovery approximated 0% for motility parameters and 20% for viability ($n = 219$), versus 4% and 11% for progressive and total motility, respectively, and 29.5% for viability observed in the 39 recruited men of this study. The better recovery of motility and viability obtained with 10 μL tips than 500 μL straws was also confirmed by the direct comparison done in this study in 22 samples (Figure 3), which also showed that after-thawing motility and viability with straws are similar to those reported in 2013.[13]

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13531 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

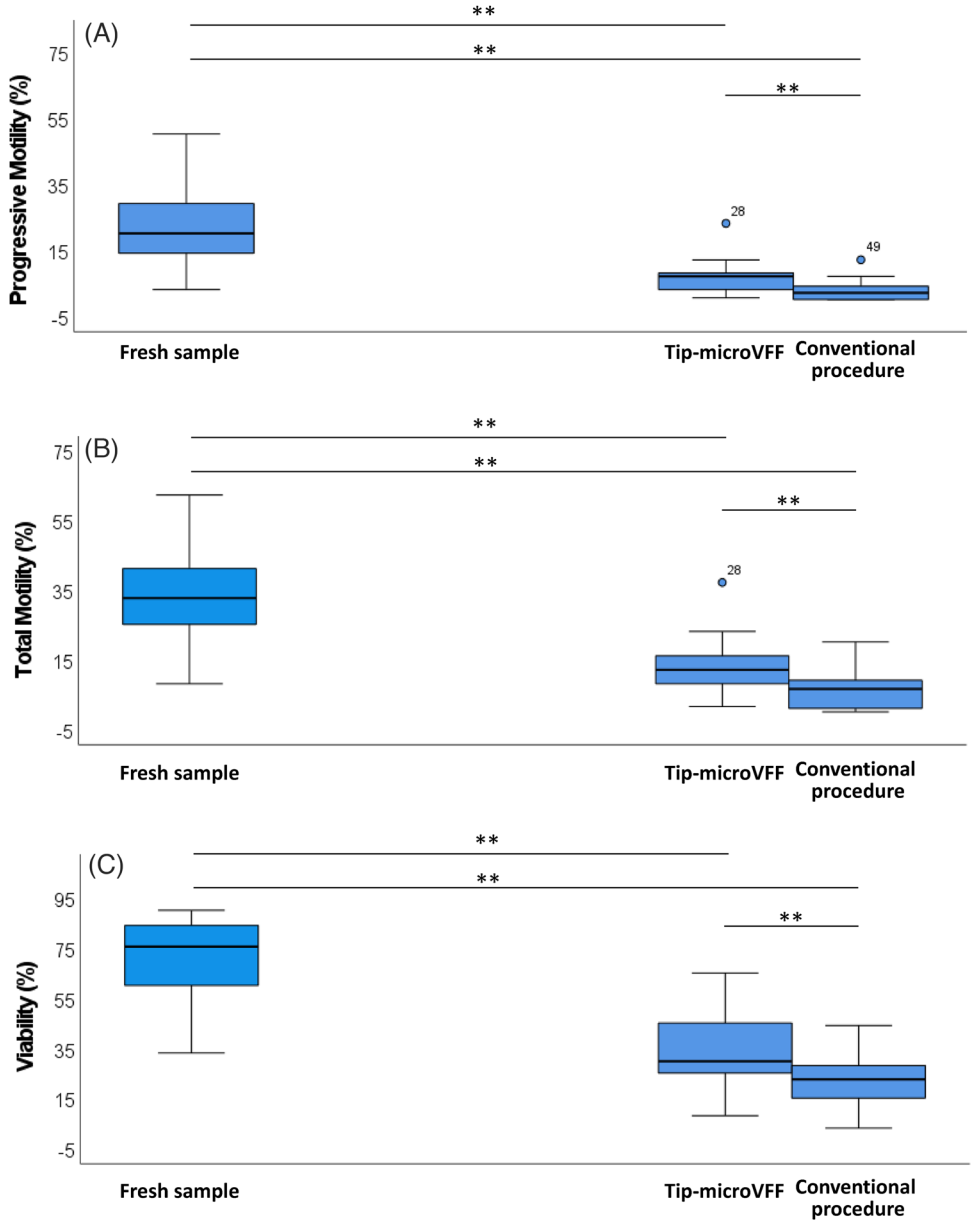


FIGURE 3 Box graphs reporting median values (interquartile range) of progressive (A) and total (B) motility and viability (C), as found before and after cryopreservation with conventional procedure and tip-microVapour Fast Freezing (VFF). $^{**}p < 0.001$; Wilcoxon signed rank test.

As mentioned, tip-microVFF resulted safer than conventional procedure in terms of sperm DNA integrity in semen samples with good quality.[9] To confirm this finding, because of the very low availability of sperm counts in severely oligozoospermic subjects, we recruited a second group of subjects with moderate oligozoospermia, where we compared tip-microVFF and conventional procedure in terms of damage to DNA. We found that straws yielded higher post thawing values of sDF than tips, although the latter remained over the 30% threshold.[17,18] These results further underline the importance of the carrier for protection of semen samples from cryodamage in oligozoospermic subjects, as already indicated by others.[19] As mentioned, when the comparison between tips and straws was conducted in samples with good semen quality, tips and straws yielded very similar results,[9] contrary to what was reported in this study with oligozoospermic samples. One possible explanation for this result might rely on the fact that in current study, we skipped the washing/centrifugation step after thawing samples. The method to thaw samples is very important in terms of induced damage by different cryopreservation procedures. For instance, we recently showed that the additional damage induced by vitrification with respect to VFF was nearly blunted when motility and viability were immediately evaluated after thawing, skipping the centrifugation for sample washing.[9] An alternative intriguing explanation of the different results obtained in oligozoospermic subjects versus normozoospermic ones might rely on intrinsic characteristics of the samples. Indeed, we found a sharp relationship between both progressive and total motility in fresh samples and their loss during cryopreservation, indicating that the lower is the value before and the lower is the damage after thawing. As we excluded that such relationship was

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13531 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

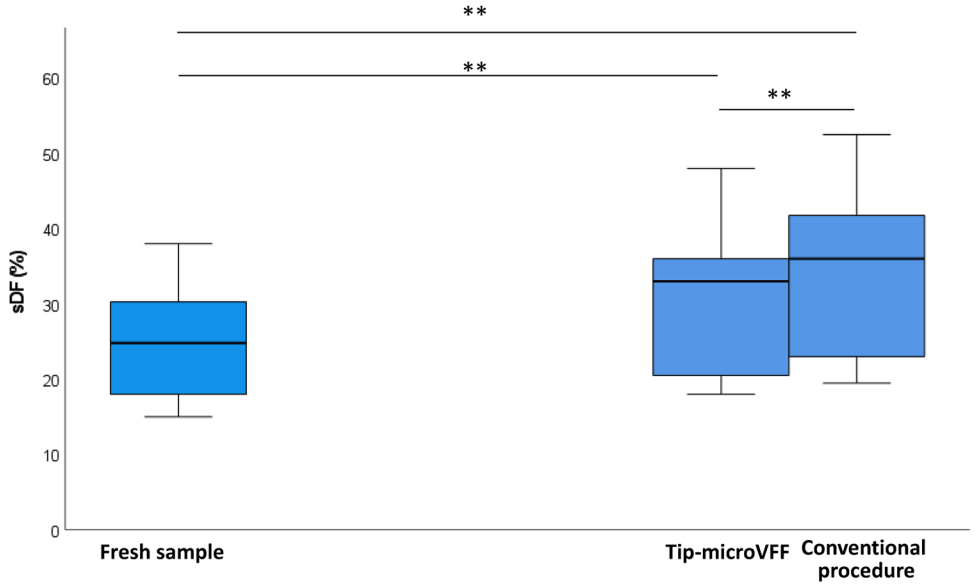


FIGURE 4 Box graphs reporting median values (interquartile range) of sperm DNA fragmentation (sDF), as found before and after cryopreservation with conventional procedure and tip-microVapour Fast Freezing (VFF). $^{**}p < 0.001$; Wilcoxon signed rank test.

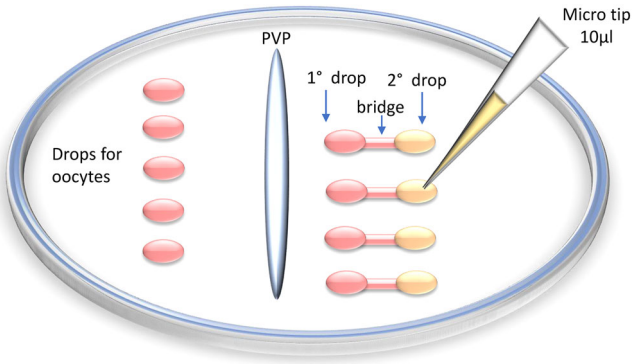


FIGURE 5 A cartoon depicting the suggested use of tip-microVapour Fast Freezing (VFF) thawed samples in the assisted reproductive technique cycle. PVP, polyvinylpyrrolidone.

due to mathematical coupling and/or regression to the mean effects, we can conclude that the worst samples may be somehow more protected by injuries to sperm motility by freezing/thawing with respect to good samples.

As mentioned, in this study, sample washing after thawing was skipped in tip-microVFF, in order to limit sperm loss as already reported with very small semen volumes.[20–22] This aspect guarantees a shortened time for handling samples and avoids noxious effects of centrifugation.[9] On the other hand, skipping washing step might be detrimental for semen samples, as cryoprotectant compounds could be toxic to cells and tissues.[23] The strength point of the study is the idea to overcome this limitation by preparing an ICSI plate with some 20 μL drops of sperm medium, linking them in pair with a thin medium bridge using a pipette (Figure 5) and covering with mineral oil until use. After thawing as described in M&M, tip can be discharged in a drop, allowing spermatozoa to swim across the medium bridge for washing and selection.

Beside the ease of handling sample, the new method is cheap, as it is time-saving and does not require special equipment and material. In addition, it is safe, as tips are stored in closed vials thus preventing viral cross-contamination. Hence, tip-microVFF appears to overcome most drawbacks previously reported with other carriers.[24–28]

This study has two major limitations. Firstly, although better recovery of motility, viability and DNA integrity was observed (Ref.[9] and present study), tips were not tested for biocompatibility at temperature of liquid nitrogen. Secondly, up to now, we have no data on reproductive outcomes with spermatozoa frozen in tips.

In conclusion, this study presents an easy, cheap and simple new method to cryopreserve semen from severely oligozoospermic subjects. In addition, tip-microVFF yields very good values of recovery of both sperm motility and viability. Indeed, such values result much better than those found with 500 μL straws observed in this and in a previous study by our group[13] conducted with similar subjects. Hence, tip-microVFF appears to be very promising new method to cryopreserve semen samples with very low sperm numbers, such as those of severely oligozoospermic subjects.

AUTHOR CONTRIBUTIONS

Sara Dabizzi conceived and designed the study, performed cryopreservation procedures and participated in writing the manuscript. Selene Degl'Innocenti and Costanza Calamai performed cryopreservation procedures. Costanza Calamai also performed sperm DNA fragmentation determination. Luca Boni performed Oldham test. Mario Maggi and Linda Vignozzi prompted the group to challenge the conventional VFF procedure and critically discussed the results. Monica Muratori performed statistical analyses and drafted the manuscript. All authors critically reviewed the manuscript and gave their approval.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13531 by meryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

ACKNOWLEDGMENTS

We thank Salvatore Zimmitti, who calculated the sample size of comparison between tip-microVFF and conventional procedure. The study was supported by Fondazione Ente Cassa di Risparmio di Firenze (ECFR 2017) and Andrological Sciences Onlus.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Sara Dabizzi https://orcid.org/0009-0005-2904-7245
Linda Vignozzi https://orcid.org/0000-0003-0907-0630

REFERENCES

1. Irvine DS. Epidemiology and aetiology of male infertility. *Hum Reprod*. 1998;13:33-44.
2. Levine H, Jørgensen N, Martino-Andrade A, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. *Hum Reprod Update*. 2017;23:646-659.
3. Levine H, Jørgensen N, Martino-Andrade A, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis of samples collected globally in the 20th and 21st centuries. *Hum Reprod Update*. 2023;29:157-176.
4. Bak CW, Song SH, Yoon TK, Lim JJ, Shin TE, Sung S. Natural course of idiopathic oligozoospermia: comparison of mild, moderate and severe forms. *Int J Urol*. 2010;17:937-943.
5. Ishikawa T. Surgical recovery of sperm in non-obstructive azoospermia. *Asian J Androl*. 2012;14:109-115.
6. AbdelHafez F, Bedaiwy M, El-Nashar SA, Sabanegh E, Desai N. Techniques for cryopreservation of individual or small numbers of human spermatozoa: a systematic review. *Hum Reprod Update*. 2009;15:153-164.
7. Thornhill JA, Fanning DM, Davis NF, Ward F, Shamoun O, Brinsden P. Testicular sperm extraction and intracytoplasmic sperm injection: outcomes in a specialist fertility centre. *Ir Med J*. 2015;108:263-265.
8. Hu Y, Cao S, Wu S, Zhao J, Arhin SK, Shan D. Comparison of the effects of different testicular sperm extraction methods on the embryonic development of azoospermic men in intracytoplasmic sperm injection (ICSI) cycles: a retrospective cohort study. *Biomed Res Int*. 2021;2021:5515247.
9. Arciero V, Ammar O, Maggi M, Vignozzi L, Muratori M, Dabizzi S. Vapour fast freezing with low semen volumes can highly improve motility and viability or DNA quality of cryopreserved human spermatozoa. *Andrology*. 2022;10:1123-1133.
10. Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. *Hum Reprod Update*. 2015;21:209-227.
11. Cissen M, Wely MV, Scholten I, et al. Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: a systematic review and meta-analysis. *PLoS One*. 2016;11:e0165125.
12. Simon L, Zini A, Dyachenko A, Ciampi A, Carrell DT. A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. *Asian J Androl*. 2017;19:80-90.
13. Degl'Innocenti S, Filimberti E, Magini A, et al. Semen cryopreservation for men banking for oligospermia, cancers, and other pathologies: prediction of post-thaw outcome using basal semen quality. *Fertil Steril*. 2013;100:1555-1563.
14. WHO. *World Health Organization Laboratory Manual for the Examination and Processing of Human Semen*. Geneva: WHO Press; 2010.
15. Tu YK, Gilthorpe MS. Revisiting the relation between change and initial value: a review and evaluation. *Stat Med*. 2007;26:443-457.
16. Stanic P, Tandara M, Sonicki Z, Simunic V, Radakovic B, Suchanek E. Comparison of protective media and freezing techniques for cryopreservation of human semen. *Eur J Obstet Gynecol Reprod Biol*. 2000;91:65-70.
17. Ni W, Xiao S, Qiu X, et al. Effect of sperm DNA fragmentation on clinical outcome of frozen-thawed embryo transfer and on blastocyst formation. *PLoS One*. 2014;9:e94956.
18. Le MT, Nguyen TAT, Nguyen HTT, et al. Does sperm DNA fragmentation correlate with semen parameters? *Reprod Med Biol*. 2019;18:390-396.
19. Ziarati N, Topraggaleh TR, Rahimizadeh P, et al. Micro-quantity straw as a carrier for cryopreservation of oligozoospermic semen samples: effects of storage times and cryoprotectant. *Cryobiology*. 2019;86:65-70.
20. Jensen CF, Ohl DA, Parker WR, et al. Optimizing human semen cryopreservation by reducing test vial volume and repetitive test vial sampling. *Fertil Steril*. 2015;103:640-646.
21. Berkovitz A, Miller N, Silberman M, Belenky M, Itsykson P. A novel solution for freezing small numbers of spermatozoa using a sperm vitrification device. *Hum Reprod*. 2018;33:1975-1983.
22. Sereni E, Bonu MA, Fava L, et al. Freezing spermatozoa obtained by testicular fine needle aspiration: a new technique. *Reprod Biomed Online*. 2008;16:89-95.
23. Best BP. Cryoprotectant toxicity: facts, issues, and questions. *Rejuvenation Res*. 2015;18:422-436.
24. AbdelHafez F, Bedaiwy M, El-Nashar SA, Sabanegh E, Desai N. Techniques for cryopreservation of individual or small numbers of human spermatozoa: a systematic review. *Hum Reprod*. 2008;15:153-164.
25. Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H. Simple vitrification for small numbers of human spermatozoa. *Reprod Biomed Online*. 2012;24:301-307.
26. Peng QP, Cao SF, Lyu QF, et al. A novel method for cryopreservation of individual human spermatozoa. *In Vitro Cell Dev Biol Anim*. 2011;47:565-572.
27. Stein A, Shufaro Y, Hadar S, Fisch B, Pinkas H. Successful use of the Cryolock device for cryopreservation of scarce human ejaculate and testicular spermatozoa. *Andrology*. 2015;3:220-224.
28. Sun J, Chen W, Zhou L, et al. Successful delivery derived from cryopreserved rare human spermatozoa with novel cryopiece. *Andrology*. 2017;5:832-837.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Dabizzi S, Calamai C, Degl'Innocenti S, et al. Tip-microVapour Fast Freezing: A novel easy method for cryopreserving severe oligozoospermic samples. *Andrology*. 2024;12:862–869. https://doi.org/10.1111/andr.13531

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13531 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 17 July 2023 | Revised: 19 September 2023 | Accepted: 20 September 2023

DOI: 10.1111/andr.13539

ANDROLOGY WILEY

ORIGINAL ARTICLE

The relationship between sperm nuclear DNA fragmentation, mitochondrial DNA fragmentation, and copy number in normal and abnormal human ejaculates

Ana Tímermans[1,2] | Fátima Otero[1,2] | Manuel Garrido[3] | Jaime Gosálvez[4] | Stephen Johnston[5,6] | José Luis Fernández[1,2]

[1]INIBIC-Complexo Hospitalario Universitario A Coruña (CHUAC), Spain

[2]Laboratory of Molecular Genetics and Radiobiology, Centro Oncológico de Galicia, Doctor Camilo Veiras, Spain

[3]Complexo Hospitalario Universitario A Coruña (CHUAC), Clinical Analysis Service, Spain

[4]Genetics Unit, Facultad de Biología, Universidad Autónoma de Madrid, Spain

[5]School of Environment, The University of Queensland, Gatton, Australia

[6]School of Veterinary Science, The University of Queensland, Gatton, Gatton, Australia

Correspondence
Dr. José Luis Fernández, Genetics Unit, Complexo Hospitalario Universitario a Coruña (CHUAC), Xubias, 84, 15006-A Coruña, Spain.
Email: Jose.Luis.Fernandez.Garcia@sergas.es; joseluis.fernandez@cog.es

Abstract

Background: While it is common to clinically evaluate sperm nuclear DNA fragmentation, less attention has been given to sperm mitochondrial DNA. Recently, a digital PCR assay has allowed accurate estimation of the proportion of fragmented mtDNA molecules and relative copy number.

Objectives: To determine the correlation of classical sperm parameters, average mtDNA copies per spermatozoon and the level of mtDNA fragmentation (SDF-mtDNA) to that of nuclear DNA fragmentation (SDF-nDNA), measured as the proportion of global, single-strand DNA (SDF-SSBs) and double-strand DNA breaks (SDF-DSBs). To determine whether the level of nuclear and mitochondrial DNA fragmentation and/or copy number can differentiate normozoospermic from non-normozoospermic samples.

Materials and methods: Ejaculates from 29 normozoospermic and 43 non-normozoospermic were evaluated. SDF was determined using the sperm chromatin dispersion assay. mtDNA copy number and SDF-mtDNA were analyzed using digital PCR assays.

Results: Relative mtDNA copy increased as sperm concentration or motility decreased, or abnormal morphology increased. Unlike SDF-mtDNA, mtDNA copy number was not correlated with SDF-nDNA. SDF-mtDNA increased as the concentration or proportion of non-vital sperm increased; the higher the mtDNA copy number, the lower the level of fragmentation. Non-normozoospermic samples showed double the level of SDF-nDNA compared to normozoospermic (median 25.00 vs. 13.67). mtDNA copy number per spermatozoon was 3× higher in non-normozoospermic ejaculates (median 16.06 vs. 4.99). Although logistic regression revealed SDF-Global and mtDNA copy number as independent risk factors for non-normozoospermia, when SDF-Global and mtDNA copy number were combined, ROC curve analysis resulted in an even stronger discriminatory ability for predicting the probability of non-normozoospermia (AUC = 0.85, 95% CI 0.76–0.94, $p < 0.001$).

© 2023 American Society of Andrology and European Academy of Andrology.

Conclusion: High-quality ejaculates show lower nuclear SDF and retain less mtDNA copies, with approximately half of them fragmented, so that the absolute number of non-fragmented mtDNA molecules per spermatozoon is extremely low.

KEYWORDS
mitochondrial DNA copy number, sperm chromatin dispersion test, sperm DNA fragmentation, sperm mtDNA damage

1 | INTRODUCTION

The human spermatozoan is a highly specialized cell, designed to contain, protect, and deliver the male DNA to the oocyte. Maintenance of the integrity of sperm DNA is an obligatory requirement for achieving a successful pregnancy and healthy offspring.[1–5] Even in a normal ejaculate, a certain proportion of spermatozoa show fragmented DNA. This proportion may be increased in males with low sperm quality, infertility, and those with pathologies such as varicocele, infections, cancer, or exposed to certain drugs or toxic agents.[1–6]

The mechanisms that result in sperm DNA fragmentation (SDF), have been shown to be a consequence of an apoptotic-like process,[7] impaired chromatin remodeling during spermiogenesis,[8] or due to attack by reactive oxygen species (ROS).[9] DNA breakages in the deoxyribose-phosphate backbone may be distributed throughout the molecule, affecting only one of the strands at a specific position (single-strand DNA breaks: SSBs) or both strands at a similar or close location (double-strand DNA breaks: DSBs).[10,11]

The current techniques for determining SDF, include the sperm chromatin structure assay (SCSA), the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay, the alkaline single-cell electrophoresis (alkaline comet) assay or the Sperm Chromatin Dispersion (SCD) test. A meta-analysis concluded that all these assays have a good sensitivity but possibly the SCSA and SCD could be less predictive of assisted reproduction outcome.[12] Nevertheless, recent reports indicate a good predictive value of the SCD test.[4,13] These assays do not distinguish if the DNA fragmentation of an individual sperm cell is only due to SSBs (SDF-SSBs) or DSBs (SDF-DSBs).[3,4,14] The presence and differentiation of DSBs, may however, be detected using the non-denaturing comet assay,[15] or a more recently reported variant of the SCD test (DSBs-SCD), where DNA fragments spread through passive diffusion.[16] The subtraction of SDF-DSBs value from that of global SDF obtained with the standard SCD test can thereby provide an estimate of the SSBs' sub-population.[16]

Sperm mitochondria form tight helices around the mid-piece of the flagellum to constitute the mitochondrial sheath and have particular features different than those of somatic cells, contributing to sperm motility, hyperactivation, capacitation, acrosome reaction, and fertilization.[17–22] While SDF is typically evaluated in nuclear DNA (nDNA), there also exists small 16,569 bp circular DNA copies present in human sperm mitochondria (mtDNA).[22] Some reports have estimated the average copy number of mtDNA per individual human spermatozoon, using real-time quantitative PCR normalized to a nuclear gene as control for relative quantitation.[23–26] The use of digital PCR (dPCR) of particular mtDNA sequences has allowed a direct and absolute estimation of the mtDNA copies.[27,28] In dPCR, the entire DNA sample is not processed in a single PCR, but it is distributed in thousands of individual and parallel end-point PCR reactions.[29]

Unlike the estimation of copy number, the evaluation of the breakage level of the human sperm mtDNA remains essentially unexplored due to the technical inability of current methods to distinguish between intact and fragmented mtDNA copies.[30] Recently, we have designed a dPCR assay that allows an accurate and reliable estimation of the proportion of fragmented mtDNA copies.[31] The assay amplifies two small target sequences, within ND1 and ND6, distantly located in the circular molecule, in independent microchambers running parallel end-point PCR reactions. Each target is detected by hybridization with hydrolysis-TaqMan probes labeled with different fluorochromes (HEX and FAM). A microchamber containing one mtDNA molecule without fragmentation will present both signals co-localized; that is, if HEX or FAM appears located alone in a different microchamber, then this corresponds to a fragmented mtDNA molecule, containing at least, two DSBs.[31]

In this study, the average number of mtDNA copies per sperm cell was determined and complemented with their corresponding fragmentation level obtained using our dPCR assay and may provide a more accurate estimation of potentially intact mtDNA molecules transmitted by the sperm to the oocyte. The relationship of mtDNA copies and fragmented mtDNA was also correlated with classical sperm quality parameters (concentration, motility, morphology, and vitality), as well as with the presence of SDF in nuclear DNA, assessed as either global SDF, or compared to the subpopulation with SSBs only or with DSBs.

2 | MATERIALS AND METHODS

2.1 | Semen samples

The study was performed using neat semen samples from 72 males attending the andrology laboratory from the Complexo Hospitalario Universitario A Coruña (CHUAC). All participants provided informed consent and the study was approved by the Institutional Review Board (reference number 2022/117). Individuals who required semen analysis and men from couples undergoing assisted reproduction techniques were included. Individuals with heavy alcohol and/or drug use in the previous 3 months, with a history of recent illness, fever, or exposure

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

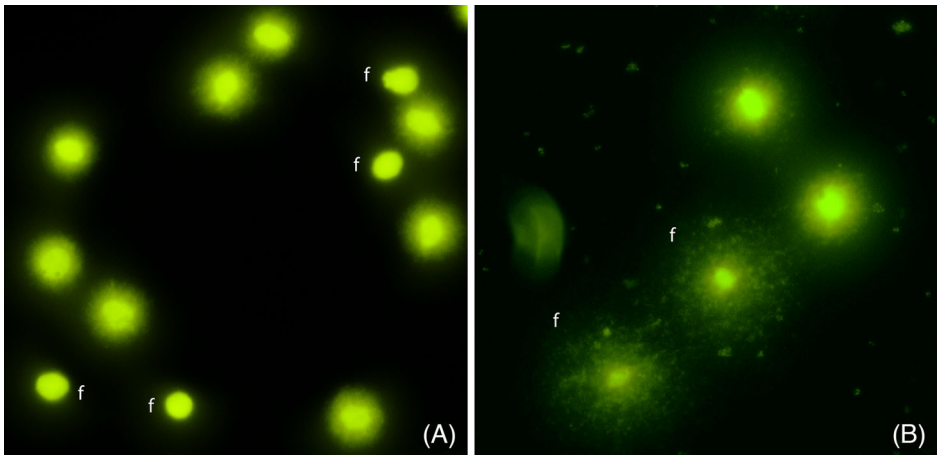


FIGURE 1 The sperm chromatin dispersion (SCD) assay for the detection of nuclear global-sperm DNA fragmentation (A) and DNA fragmentation containing DNA double-strand breaks (SDF-DSBs) (B). (a) Sperm with DNA fragmentation corresponded to those nucleoids with small halos. (B) Sperm with SDF-DSBs corresponded to those nucleoids with large dispersed halos. (f: fragmented).

to chemotherapy, radiotherapy, pesticides, heavy metals, or with positive serology for HIV, hepatitis B or C, syphilis, cytomegalovirus, *Chlamydia* and gonococcus, were excluded. The age of the patients was 37.4 ± 5.5 (mean ± sd) years. Concentration and motility were determined using CASA (Sperm Class Analyzer; Microptic SL, Barcelona, Spain). Morphology and vitality were evaluated under the microscope after Papanicolau and eosin–nigrosin staining, respectively.

Semen samples were categorized according to the WHO manual (5th edition, 2010)[32] as normozoospermic ($n = 29$;) or with abnormal semen parameters ($n = 43$; 12 asthenozoospermic, 2 teratozoospermic,14 asthenoteratozoospermic, 3 oligozoospermic, 4 oligoasthenozoospermic, 8 oligoasthenoteratozoospermic). The age of normozoospermic and non-normozoospermic individuals was 36.7 ± 4.8 and 37.8 ± 5.9 years, respectively. The fifth percentile of the fertile population was established as the cut-off point; concentration: 15 million/mL; total sperm number: 39 million; total motility: 40%; progressive motility: 32%; abnormal forms: 4%, and vitalilty: 58%.

2.2 | Sperm chromatin dispersion test

The SCD test was performed using the HalospermG2 kit (Halotech DNA SL, Madrid, Spain), accordingly to the manufacturer's recommendations.[14] In brief, an aliquot of each semen sample was diluted to 5−10 million spermatozoa mL^{-1}, mixed with molten agarose and pipetted onto a slide and covered with a coverslip. The slide was then placed onto a cold plate in the refrigerator (4°C) for 5 min to allow the agarose to form a microgel in which the sperm cells were embedded for further processing. The coverslip was subsequently removed, and the slide immediately covered with drops of HCl solution for 7 min at room temperature (22°C). The slide was then incubated with the lysing solution for 25 min. After washing with distilled water, the slide was dehydrated in increasing ethanol baths (70%–90%–100%), air-dried and stained with SYBR Gold and observed under the epifluorescence microscope. Sperm without DNA fragmentation showed large haloes of dispersed DNA loops, whereas those with fragmented DNA show no halo or a small halo (see Figure 1A).

2.3 | DSBs-SCD assay

For the DSBs-SCD assay, spermatozoa were processed in a similar manner to the standard SCD test but with modifications.[16] Spermatozoa in the microgel were only incubated with a specifically adapted lysing solution for 2 min (Halotech DNA SL, Madrid, Spain). Slides were washed, dehydrated, and stained as per the standard SCD assay. For the DSBs-SCD assay, spermatozoa without DSBs showed compact haloes of chromatin loops from the central core, whereas spermatozoa with fragmented DNA associated with DSBs exhibited large haloes of diffused "spotty" DNA fragments (Figure 1B). Rarely, extreme DNA fragmentation and mobilization away from the nucleoid made the corresponding halo very "faint" so that only a residual core of the nucleoid remained detectable.

2.4 | Isolation of sperm DNA

For isolation of sperm DNA, 150 μL of semen sample was mixed with 300 μL of PBS and centrifuged at 4000 rpm for 10 min. After the pellet was resuspended in 100 μL of PBS, 100 μL of extraction buffer (20 mM Tris-HCl pH 8; 20 mM Ethylenediaminetetraacetic acid (EDTA); 200 mM NaCl; 4% Sodium Dodecyl Sulfate (SDS) and 1 μL of DTT 1 M (final concentration 5 mM) were added. This mixture was incubated in the dark at room temperature for 60 min. Automated DNA extraction was performed with the QIAamp DNA Blood Mini Kit in QIAcube Connect (QIAgen) according to the manufacturer's instructions. Total DNA concentration and quality were estimated using Thermo Scientific NanoDrop One (Thermo Fisher Scientific, Madrid, Spain).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.5 | mtDNA copy number per spermatozoon

The average copy number of mtDNA per individual spermatozoon was estimated using dPCR for concurrent amplification of a target within the mtDNA gene (ND6) and another target in a single-copy nuclear gene (RPP30) as a reference for relative quantitation, differentially labeled. Given the haploid nature of sperm, each RPP30 copy estimated by the dPCR assay must correspond to an individual spermatozoon present in the sample.

dPCR was performed using the microwell-on-chip system (Thermo Fisher Scientific). For this procedure, 1 ng of isolated DNA in 2 μL was mixed with 7.5 μL of master mix (Thermo Fisher Scientific), 0.75 μL of primer solution and TaqMan hydrolysis probe for target RPP30, HEX-labeled (ddPCR Gene Expression Assay RPP30, human, Bio-Rad), 0.75 μL of primer solution and TaqMan probe for target MT-ND6, FAM-labeled (ddPCR Gene Expression Assay MT-ND6, human, Bio-Rad) and 4 μL of water. This final mixture volume (15 μL) was then evenly distributed on a dPCR chip. dPCR was performed with a QuantStudio 3D Digital PCR System (Thermo Fisher Scientific). The PCR conditions were set at one cycle of 96°C, 10 min; 39 cycles of hybridization-extension at 55°C, 1 min and denaturation at 98°C, 30 s, and a final cycle of 60°C, 2 min.

Chip analysis was carried out by QuantStudio 3D AnalysisSuite software. This analysis allowed us to determine the number of copies of ND6 (mtDNA) and RPP30 (nuclear single-copy gene) present. The ratio between the two genes provided an estimate of the average number of mtDNA copies present in each spermatozoon from the sample.

2.6 | mtDNA fragmentation level

In parallel to the determination of mtDNA copy number per spermatozoon, mtDNA fragmentation was estimated by a different dPCR performed under the same technical conditions. The only difference was that the primer for the RPP30 nuclear gene was replaced by a primer to amplify a target within another mitochondrial gene, MT-ND1.[31] In brief, in our design, Target Sequence 1 was located within the ND1 mitochondrial gene, comprising bases 3,629 to 3,775 (146 pb); as indicated. Target Sequence 2 was found within the ND6 mitochondrial gene, comprising bases 14,250 to 14,382 (132 pb) (ddPCR Gene Expression Assay MT-ND1 and MT-ND6, human, Bio-Rad). The two targets were connected by two segments: the longer of 10,475 bp, and the shorter of 5,816 bp. Since the two DNA segments between the two targets were very long, if DSBs were present, they will be located almost exclusively in these regions.

Amplification of Target 1 was detected by hybridization with a TaqMan probe labeled with HEX, while amplification of Target 2 was detected with another probe labeled with FAM. Since mtDNA is circular, the joint amplification of both targets, that is, the colocalization of HEX + FAM in a microchamber, will correspond to a non-fragmented molecule. If the molecule has at least two DSBs, one in segment X and another in segment Y, each target will be located in a different fragment, so they can be separated and distributed in different microchambers ("break-apart"). When the PCR reaction takes place, there will be a chamber with a single HEX signal and another chamber with the FAM signal (Figure 2). As with all quantitative studies with dPCR,

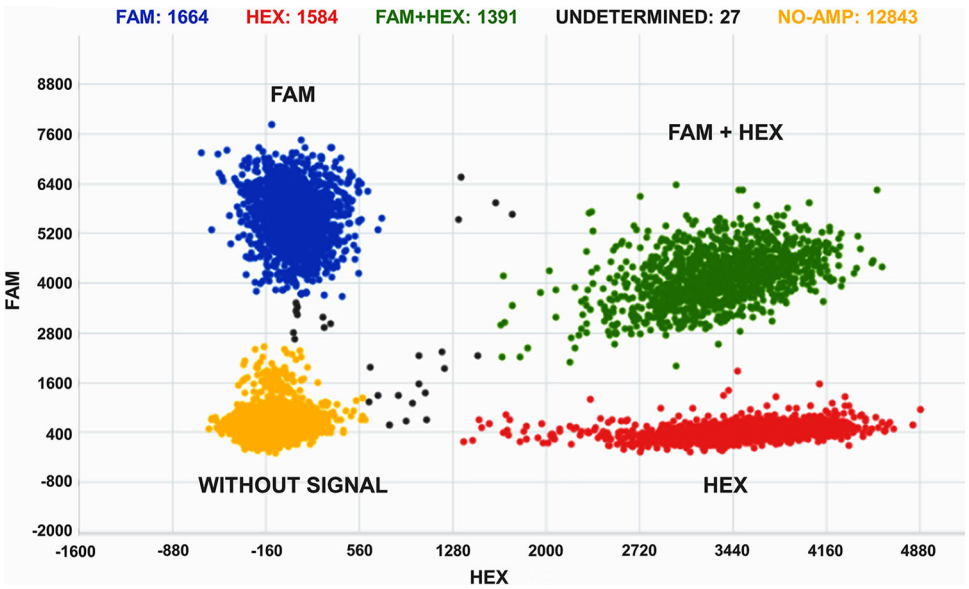


FIGURE 2 Graphic representation of the distribution of the number of microwells of dPCR chips, without and with amplification signals of the mtDNA targets in a sperm sample. The *X*-axis corresponds to the HEX signal intensity (signal from amplification of the target within the ND1 gen) and the *Y*-axis to the FAM signal intensity (signal from amplification of the target within the ND6 gen). Upper right: microwells with colocalized FAM + HEX signals (green dots), corresponded to non-fragmented mtDNA copies. Upper left: microwells with only a FAM signal (blue dots). Bottom right: microwells with only a HEX signal (red dots). The software indicated that these correspond to 60.68% fragmented mtDNA molecules. Bottom left: microwells without signal (yellow dots).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Descriptive statistics of the parameters of semen samples.

	N	Mean	SD	Median	Minimun	Maximum
Concentration (million/mL)	72	62.52	60.92	36.25	1.30	303.00
Total sperm n° (million)	72	191.70	185.72	115.10	6.50	706.80
Total motility (%)	72	38.86	19.74	36.50	6.00	89.00
Progressive motility (%)	72	31.99	19.80	31.00	0.00	86.00
Abnormal forms (%)	72	93.46	4.10	94.00	80.00	99.00
Non-vital cells (%)	49	34.45	14.71	34.00	10.00	65.00
SDF-global nDNA (%)	72	26.02	16.39	22.84	4.33	78.33
SDF-DSBs nDNA (%)	72	10.86	11.09	7.67	0.67	66.67
SDF -SSBs nDNA (%)	72	15.16	10.19	12.67	1.67	37.33
Contribution DSBs (%)	72	41.00	20.70	37.14	4.11	85.11
Contribution SSBs (%)	72	59.00	20.70	62.86	14.90	95.89
mtDNA copy n°	72	16.33	18.71	9.24	1.17	107.91
SDF-mtDNA (%)	72	53.29	13.33	52.43	31.61	100

Abbreviations: DSBs, double-strand DNA breaks; SD, standard deviation; SSBs, single-strand DNA breaks.

this assay must be performed ensuring that an adequate proportion of microchambers are without signal, to avoid as far as possible the colocalization of both targets that were separated due to DSBs, but that may coincide in the same microchamber by random. Under these conditions, the Poisson distribution allows its estimation for subsequent correction.

2.7 | Statistical analysis

Three hundred spermatozoa were scored per SDF technique. All data were analyzed and graphs generated using the SPSS 26 software package for Windows (SPSS Inc., Chicago). Data were not normally distributed according to the Kolmogorov–Smirnov test so that Spearman's rho was employed to evaluate the correlation of distributions of SDF-Global, SDF-SSBs, SDF-DSBs, SDF-mtDNA, mtDNA copy number per spermatozoon, and seminal parameters. The distribution of SDF-SSBs and SDF-DSBs in the Global-SDF was evaluated with Chi-squared (χ^2) test. Regression analysis was used to analyze mathematical models of relationship between different parameters. Comparisons between samples with normal and abnormal semen parameters were performed using the Mann–Whitney *U*-test. Logistic regression and receiver operating characteristic curves (ROC) curves were constructed and used to investigate a model that allowed the prediction of the non-normozoospermia. Statistical significance was defined as $p < 0.05$.

3 | RESULTS

Descriptive statistics (mean ± SD) of the different parameters evaluated for the whole sample population are summarized in Table 1. Values of classical sperm parameters in normo- and non-normozoospermic samples are presented in Table S1.

3.1 | Sperm nuclear DNA

Table 2 reports the Spearman's rho correlation between SDF of nuclear DNA (nDNA), mtDNA copy number, and the DNA fragmentation of mtDNA (SDF-mtDNA). These resulted showed that the higher the level of the global SDF, the higher the spermatozoa with SSBs, and spermatozoa with DSBs in the sample (rho = 0.85 and 0.67, respectively; $p < 0.001$). Following logarithmic transformation of the data to obtain a normal distribution, linear regression was used to examine the relationship between SDF-SSBs and that of SDF-DSBs on global-SDF; global-SDF showed a coefficient of determination (r^2) value of 0.68 ($p < 0.001$) with a linear coefficient-slope of 0.68 when plotted against SDF-SSBs and an r^2 value of 0.52 ($p < 0.001$) with a linear coefficient of 0.54 for SDF-DSBs (Figure 3). These results suggest that sperm with SSBs contributed to a higher proportion of SDF-global sperm that those with DSBs. Significant correlations were also found between nuclear DNA damage and the classical seminal parameters, with the exception of sperm concentration (Table 3). Thus, the higher the level of SDF-Global, and/or SDF-SSBs and SDF-DSBs, the higher the frequency of non-motile sperm (total and progressive), sperm with abnormal morphology, and non-vital sperm.

3.2 | Sperm mitochondrial DNA

Table 2 shows that the higher the frequency of spermatozoa with fragmented global nuclear DNA, the higher the fraction of fragmented mtDNA molecules found in the sample (rho = 0.29; $p = 0.013$); a similar correlation was not found with SDF-DSBs but was evident with SDF-SSBs (rho = 0.35; $p = 0.003$; Table 2). Interestingly, while the copy number of mtDNA per sperm cell was not correlated with that of SDF of nuclear DNA, it was negatively correlated with the fragmentation level of mtDNA (rho = −0.51; $p < 0.001$) (Table 2); the data fitted signif-

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Correlations between SDF in nuclear DNA (nDNA), mtDNA copy number and SDF-mtDNA.

		SDF-global nDNA	SDF-DSBs nDNA	SDF-SSBs nDNA	mtDNA copy n°	SDF-mtDNA
SDF-global nDNA	*R*					
	p-Value					
SDF-DSBs nDNA	*R*	0.672**				
	p-Value	< 0.001				
SDF-SSBs nDNA	*R*	0.852**	0.284*			
	p-Value	< 0.001	0.016			
mtDNA copy n°	*R*	0.163	0.185	0.082		
	p-Value	0.172	0.121	0.496		
SDF-mtDNA	*R*	0.290*	0.034	0.348**	−0.512**	
	p-Value	0.013	0.777	0.003	< 0.001	

Abbreviations: DSBs, double-strand DNA breaks; SSBs, single-strand DNA breaks.
*$p < 0.05$.
**$p < 0.01$.

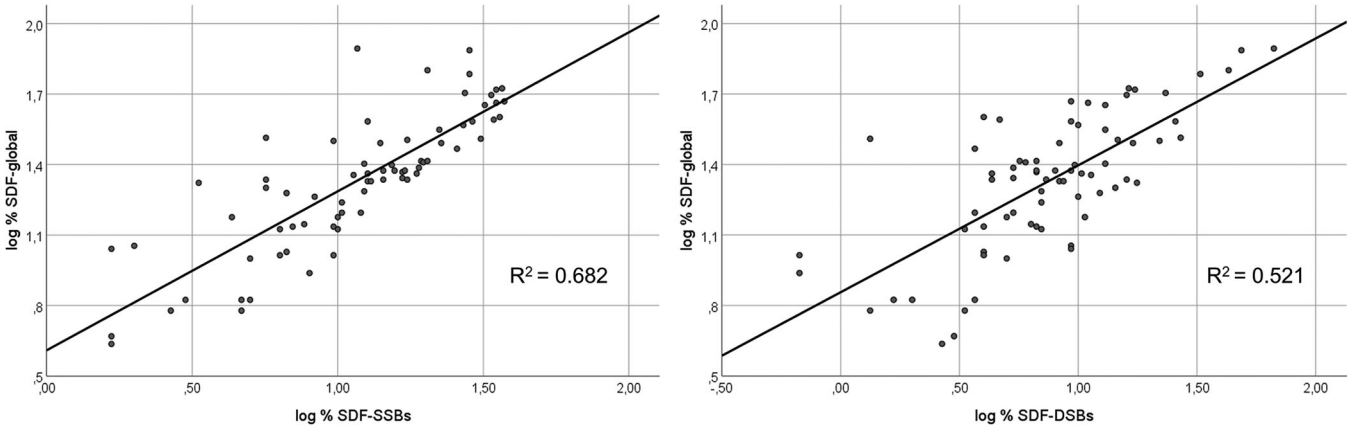


FIGURE 3 Linear regression to evaluate the influence of SDF-SSBs (left) and of SDF-DSBs (right) with respect to global-SDF.

TABLE 3 Correlations between classical sperm parameters, SDF in nuclear DNA (nDNA), mtDNA copy number, and mtDNA fragmentation.

		SDF-global nDNA	SDF-DSBs nDNA	SDF-SSBs nDNA	mtDNA copy n	SDF-mtDNA
Concentration	*r*	−0.196	−0.140	−0.134	**−0.805**	**0.440**
	p-Value	0.099	0.241	0.260	**< 0.001**	**< 0.001**
Total sperm n°	*r*	−0.205	−0.130	−0.141	**−0.815**	**0.488**
	p-Value	0.083	0.278	0.239	**< 0.001**	**< 0.001**
Total motility	*r*	**−0.541**	**−0.392**	**−0.454**	**−0.397**	−0.056
	p-Value	**< 0.001**	**0.001**	**< 0.001**	**0.001**	0.639
Progressive motility	*r*	**−0.546**	**−0.412**	**−0.443**	**−0.356**	−0.049
	p-Value	**< 0.001**	**< 0.001**	**< 0.001**	**0.002**	0.680
Abnormal forms	*r*	**0.481**	**0.279**	**0.477**	**0.328**	0.109
	p-Value	**< 0.001**	**0.018**	**< 0.001**	**0.005**	0.363
Non-vital cells	*r*	**0.653**	**0.557**	**0.406**	−0.182	**0.451**
	p-Value	**< 0.001**	**< 0.001**	**0.004**	0.212	**0.001**

Abbreviations: DSBs, double-strand DNA breaks; SSBs, single-strand DNA breaks.
Bold values correspond to significant correlations.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

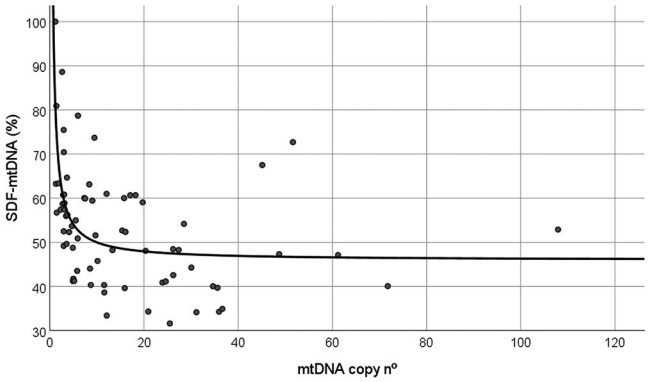


FIGURE 4 The regression curve of the influence of the number of mtDNA copies per sperm (*X*-axis) in the proportion of fragmented sperm mtDNA molecules (SDF-mtDNA; *Y*-axis). The data were consistent an inverse regression model; the higher the mtDNA copies, the fewer the proportion of fragmented ones.

icantly to an inverse regression model ($y = 45.91 + (40.75/x)$; $r^2 = 0.33$; $p < 0.001$), suggesting that the SDF-mtDNA fraction decreased very rapidly at first and then more slowly as the number of mtDNA copies increased (Figure 4).

Regarding the classical sperm parameters, SDF-mtDNA correlated positively only with concentration, total sperm number and with non-vital sperm (Table 3). The mtDNA copy number significantly correlated negatively with concentration, motility, either total or progressive, and positively with abnormal morphology (Table 3). An inverse regression model revealed a substantial influence of concentration on the variation of the number of mtDNA copies ($y = 8.24 + (151.96/x)$; $r^2 = 0.65$; $p < 0.001$).

3.3 | A comparison of non-normozoospermic and normozoospermic ejaculates

With respect to the fragmentation of nuclear DNA, non-normozoospermic samples ($n = 43$; Table 4) showed nearly double global SDF average values than normozoospermic samples ($n = 29$) (median: 25.0 vs. 13.7; Mann–Whitney *U*-test, $p < 0.001$). However, the differences were mostly dependent on SDF-SSBs (median: 16.7 vs. 9.7; $p < 0.001$). While SDF-DSBs were also significantly increased in non-normozoospermic, the difference was lower (median: 9.3 vs. 6.0; $p = 0.004$). The relative contribution of SDF-SSBs and SDF-DSBs to the SDF-Global sperm population was not found to be significant between non-normozoospermic and normozoospermic ($p = 0.487$), with the SDF-SSBs population representing 66.0% and 60.5% of the median, respectively.

However, when mtDNA was analyzed, non-normozoospermic samples showed three times more copies per sperm than normozoospermic (median: 16.1 vs. 5.0; $p = 0.001$). The dPCR assay for estimation of SDF-mtDNA also revealed that approximately half of these mtDNA copies were fragmented on average; this proportion was very similar in both groups (median: 52.4 vs. 53.7; $p = 0.570$).

When SDF-Global, SDF-DSBs, mtDNA copy number, and SDF-mtDNA were included in a multivariate analysis, using binary logistic regression, only SDF-Global and mtDNA copy number per spermatozoon remained as significant independent risk factors associated with non-normozoospermia (Figure 5; SDF-Global: OR 1.09, 95% CI 1.03–1.14, $p = 0.002$; mtDNA copy number: OR 1.10, 95% CI 1.03–1.18, $p = 0.003$). When SDF-Global and mtDNA copy number were combined, ROC curve analyses resulted in even stronger discriminatory ability for predicting the probability of a non-normozoospermic ejaculate (Area under the curve (AUC) = 0.85, 95% CI 0.76–0.94, $p < 0.001$), when compared to SDF-Global and mtDNA copy number, separately (Figure 5). The highest discriminant level of the combined factors, which corresponded to 72.10% sensitivity and 89.7% specificity, was obtained when the multivariant model results in a probability value ≥ 0.64.

Most of the non-normozoospermic samples showed mixed categories of oligozoospermia, and/or teratozoospermia and/or asthenozoospermia, so that there were not sufficient samples to make a confident comparison with normozoospermic. Nevertheless, a preliminary approach was attempted with those categories with a small but acceptable number (see Table S2). Asthenozoospermic samples ($n = 12$) showed near double levels of SDF-global (median: 25.5; $p = 0.015$), but mtDNA copy number and SDF-mtDNA were not found significantly different to those from normozoospermic samples. Asthenoteratozoospermic ($n = 14$) showed 2.3 times increase of the median of SDF-Global (median: 31.5; $p = 0.001$); in this category, mtDNA copies per sperm doubled that of normozoospermic (median: 11.8; $p = 0.01$), whereas their fragmentation level was not different (median: 55.7). Finally, oligoasthenoteratozoospermic ($n = 8$), that is, the most abnormal of the samples, also showed a median value 2.3 times higher for SDF-global levels (median: 31.3; $p = 0.004$) compared to normozoospermic samples. Moreover, this cohort also showed the highest level of mtDNA copies per sperm, 8 times higher (median: 40.9; $p < 0.001$) than normozoospermic samples. Again, no differences were found with respect to the fragmentation level of mtDNA (median: 47.8) between oligoasthenoteratozoospermic and normozoospermic samples.

4 | DISCUSSION

During spermatogenesis, there is a considerable depletion of mtDNA, so that a mature human spermatozoon typically only contains around ten copies, but ranging from 0 to 226, as estimated by qPCR.[23,33,34] This phenomenon is peculiar to sperm cells, since mtDNA depletion may be pathological if present in somatic cells. Moreover, other studies have pointed out that good quality-normozoospermic samples tend to have sperm with less mtDNA copy number than poorer quality samples, similar to what was found in the current study; a finding that also suggests that increased mtDNA copy number could serve as a potential biomarker for spermatogenic dysfunction.[24,26,35,36]

Estimation of mtDNA copy number in stallion sperm has been shown to vary depending on the mitochondrial target sequence

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 4 Comparison between normozoospermic and non-normozoospermic samples, regarding SDF in nuclear DNA (nDNA), relative contribution of SDF-DSBs and of SDF-SSBs to SDF-global nDNA (%), mtDNA copy number and SDF-mtDNA.

		N	Mean	SD	Median	Minimum	Maximum
SDF-global nDNA	**Normo**	29	17.56	12.51	13.67	4.33	61.00
	Non-normo	43	31.73	16.35	25.00*	10.00	78.33
SDF-DSBs nDNA	**Normo**	29	7.72	7.52	6.00	0.67	32.67
	Non-normo	43	12.98	12.60	9.33*	1.33	66.67
SDF-SSBs nDNA	**Normo**	29	9.84	6.64	9.67	1.67	28.33
	Non-normo	43	18.75	10.64	16.67*	2.00	37.33
Contribution DSBs	**Normo**	29	42.09	19.49	39.42	6.49	84.82
	Non-normo	43	40.26	21.67	33.80	4.11	85.11
Contribution SSBs	**Normo**	29	57.92	19.48	60.54	15.18	93.61
	Non-normo	43	59.74	21.67	66.20	14.90	95.89
mtDNA copy n°	**Normo**	29	7.60	7.06	4.99	1.17	28.47
	Non-normo	43	22.21	21.69	16.06*	1.27	107.91
SDF-mtDNA	**Normo**	29	54.42	13.78	53.68	31.61	100
	Non-normo	43	52.53	13.13	52.36	34.15	88.61

Abbreviations: Normo, normozoospermia; Non-normo, non-normozoospermia; DSBs, double-strand DNA breaks; SD, standard deviation; SSBs, single-strand DNA breaks.

*$p < 0.05$ indication for significant differences between Normo and Non-normo.

amplificated by qPCR, so it has been suggested that this phenomenon could be a result of fragmentation of the mitochondrial genome during spermatogenesis.[35] Nevertheless, unlike nuclear DNA, sperm mtDNA fragmentation has typically not been routinely evaluated given the technical difficulty to distinguish fragmented and intact copies.[30] The majority of the reports concerning sperm mtDNA integrity have been related to current known deletions or point mutations, estimated by PCR and/or sequencing. These alterations have been found more frequent in poor quality sperm samples.[33,36–38]

Song and Lewis[33] evaluated sperm mtDNA fragmentation and deletions using a long PCR assay for half of the mtDNA genome (8.7 kb). This procedure was based on the capacity of many DNA lesions, and obviously DSBs, to block or impede the progression of the DNA polymerase. The technique revealed that abnormal sperm samples had lower amplification levels than normozoospermic samples, showing a decrease in mtDNA integrity. Although the long PCR target may increase the possibility of finding a blocking DNA lesion, it also resulted in a significant processivity problem of the DNA polymerase, with low efficiency and low sensitivity and reproducibility.[39] Moreover, quantification using this procedure was semiquantitative.

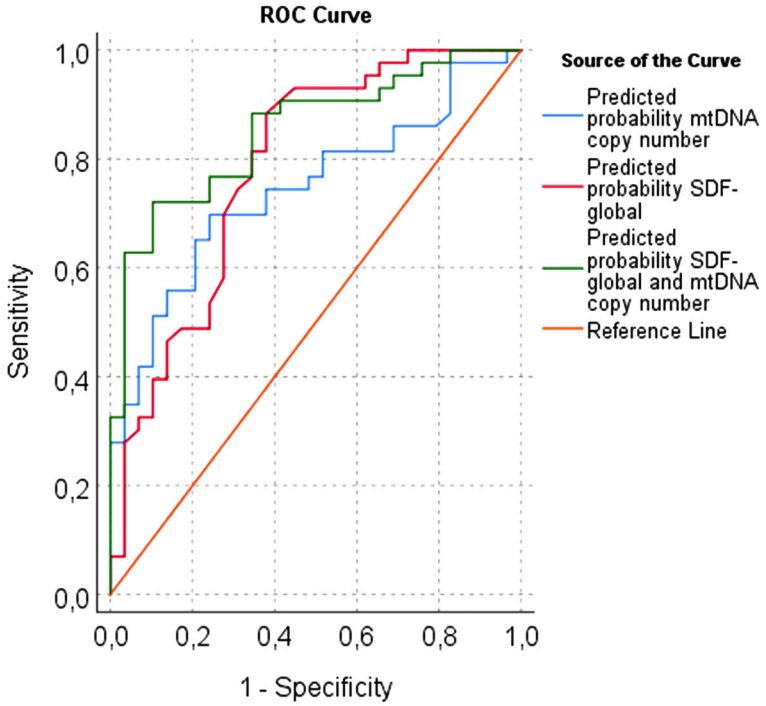
An opposite result was reported in a recent study performed by next generation sequencing.[40] First, dPCR, confirmed a lower mtDNA copy number in good quality spermatozoa. Second, high-throughput sequencing revealed that mtDNA from good quality sperm showed a much higher frequency of large deletions and duplications, these being heterogeneous and random, so they could be potentially related to DSBs, presumably generated by mtDNA degradation.[40] Nevertheless, the described next generation sequencing procedure required an initial step, where the mitochondrial genome was amplified in two large segments of 8397 and 8289 bp. As previously indicated, if DSBs were present in these segments, they would not allow the amplification by DNA polymerase, so the resulting native fragments would not be detected.

Our dPCR methodology, which is based on an adapted "break-apart" approach, has been specifically designed to quantify accurately the proportion of fragmented mtDNA copies. Our dPCR mtDNA fragmentation assay revealed that sperm from normozoospermic samples, not only contained very few mtDNA copies, but also that an average of half of them appeared fragmented. For comparison, the same specific dPCR procedure revealed that mtDNA from human peripheral blood cells ($n = 10$ samples) showed a fragmented fraction of only 7.97 % ± 0.39% (mean ± sd).[31]

Although the normozoospermic samples in the current study showed three times lower mtDNA copy number than the non-normozoospermic samples, SDF-mtDNA was not found significantly different between these groups. However, when all the samples irrespective of categorization were examined, mtDNA fragmentation clearly increased as mtDNA copy number decreased. This finding may mean that good-quality sperm samples, which have less mtDNA copy number, express most of these copies as degraded, so that the absolute number of remaining complete circular mtDNA copies in sperm must be quite rare. mtDNA fragmentation also increased significantly with high sperm concentration, whereas correspondingly, mtDNA copies decreased as sperm concentration increased.

A complete and perfect spermatogenesis process should theoretically at least result in a high-quality mature sperm with a lower amount of mtDNA molecules and with most of these remaining copies being fragmented. This is likely to be a dynamically-progressive process, so

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License



Area Under the ROC Curve

Test Result Variable(s)	Area	CI 95%		p-value
		Lower Bound	Upper Bound	
Predicted probability SDF-global	0.78	0.67	0.90	<0.001
Predicted probability mtDNA copy	0.74	0.63	0.86	<0.001
Predicted probability SDF-global and mtDNA copy	0.85	0.76	0.94	<0.001

FIGURE 5 Receiver operating characteristic (ROC) curves showing the discriminatory power of nuclear global sperm DNA fragmentation (global-SDF) and average mtDNA copy number per spermatozoon, either isolated or combined, to distinguish non-normozoospermic from normozoospermic samples. CI: confidence interval.

that a high frequency of mtDNA fragmentation would be evidence of effective mtDNA degradation and elimination. Conversely, a high relative amount of mtDNA, with a low frequency of fragmentation would be indicative of poor sperm quality, and a consequence of inefficient or defective spermatogenesis; in fact, the high copy number could be due to an impaired or slower degradation mtDNA process and reflect a spermatozoon of less maturity.

While the evolutionary reason of elimination of paternal mtDNA from the sperm cell is still equivocal, it is well established that human mtDNA shows a strict matrilineal inheritance,[41,42] although some exceptions have been reported.[43] It has generally been implied that mtDNA molecules from sperm cell must be eliminated in the oocyte after fertilization,[44,45] but perhaps this removal would be greatly facilitated if the male mtDNA copies are reduced in number prior to fertilization, or at least made susceptible to degradation through extensive fragmentation.[46]

We might suppose that good sperm quality means less nuclear SDF and less mtDNA copy number with the remaining mtDNA being more fragmented. However, the results of the current study revealed a positive correlation between mtDNA fragmentation and global-nuclear DNA fragmentation. Global-SDF determined by the alkaline-denaturant comet assay was also found correlated with the number and size of mtDNA deletions within a 8.7 kb segment amplified by long PCR.[47] Given the preceding discussion, it would seem counter-intuitive that more nuclear SDF be associated with more mtDNA fragmentation. In the current study, non-vital dead sperm and mtDNA fragmentation levels showed a positive correlation, so this phenomenon could be associated with the number of dead sperm cells, with these dead or dying cells contributing to a co-incident increase in both nuclear and mitochondrial DNA fragmentation.

If we focus on the DNA breakage type, SDF-mtDNA, in spite of being a consequence of DSBs, correlated more strongly with nuclear

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by meryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

SSBs. This association is somewhat difficult to explain and should be interpreted with caution, since nuclear and mtDNA fragmentation are estimated using different techniques. Nevertheless, it must be recognized that mtDNA nucleoids are less protected by associated proteins than nuclear DNA, so that SSBs produced by nucleases or ROS could be relatively more frequent in mtDNA than in nuclear DNA. In addition, the accumulation of SSBs could easily and rapidly result in the coincidence of SSBs in adjacent but opposite sites in both strands leading to DSBs.[16]

Unlike SDF-mtDNA, there was no significant correlation between nuclear-SDF and mtDNA copy number, further confirming the results of Faja et al.[24] In that report, SDF had been analyzed by the TUNEL assay. Our logistic regression identified SDF-Global and mtDNA copy number per spermatozoon as independent risk factors associated with non-normozoospermia. A similar conclusion was achieved by Shi et al., where SDF was evaluated using the SCSA.[48] A high predictive accuracy for consecutive diagnoses of clinical infertility has previously been described for mtDNA copy number (AUC: 0.91).[38] Our ROC curve analysis established strong potential discriminatory power of the two combined factors to distinguish non-normozoospermic samples.

The results from the analysis of the whole sperm samples studied here suggest that besides nuclear SDF, mtDNA copy number, and possibly mtDNA fragmentation are related to sperm quality and may serve as potential biomarkers for spermatogenic dysfunction. Expanding the study to larger cohorts would provide further detail about the relevance of mtDNA fragmentation in sperm quality and male fertility.

AUTHOR CONTRIBUTIONS

Experimental design: JLF, JG. *Experimental performing*: AT, FO. *Data analysis*: SJ, AT, FO, JG. *Manuscript writing*: JLF, JG, SJ.

CONFLICT OF INTEREST STATEMENT

JG and JLF are consultants of Halotech DNA SL.

DATA AVAILABILITY STATEMENT

Data are available upon request.

ORCID

Stephen Johnston https://orcid.org/0000-0002-0290-5458
José Luis Fernández https://orcid.org/0000-0002-2985-7169

REFERENCES

1. Lewis SEM, Aitken RJ. DNA damage to spermatozoa has impact on fertilization and pregnancy. *Cell Tissue Res*. 2005;322:33-41.
2. Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. *Fertil Steril*. 2014;102:998-1005.
3. Zini A, Agarwal A, eds. *A Clinician's Guide to Sperm DNA and Chromatin Damage*. Springer; 2018.
4. Esteves SC, Zini A, Coward RM, et al. Sperm DNA fragmentation testing: summary evidence and clinical practice recommendations. *Andrologia*. 2021;53:e13874.
5. Marinaro JA, Schlegel PN. Sperm DNA damage and its relevance in fertility treatment: a review of recent literature and current practice guidelines. *Int J Mol Sci*. 2023;24:1446. doi:10.3390/ijms24021446
6. Evenson DP, Wixon R. Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA). *Toxicol Appl Pharmacol*. 2005;207:532-537.
7. Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online*. 2003;7:428-432.
8. Leduc F, Maquennehan V, Nkoma GB, Boissonneault G. DNA damage response during chromatin remodeling in elongating spermatids of mice. *Biol Reprod*. 2008;78:324-332.
9. Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. *Reprod Fertil Dev*. 2016;28:1-10.
10. von Sonntag C. *The Chemical Basis of Radiation Biology*. Taylor and Francis Ltd; 1987.
11. Cortés-Gutiérrez EI, López-Fernández C, Fernandez JL, et al. Interpreting sperm DNA damage in a diverse range of mammalian sperm by means of the two-tailed comet assay. *Front Genet*. 2014;5:404.
12. Cissen M, Mv Wely, Scholten I, et al. Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: a systematic review and meta-analysis. *PLoS ONE*. 2016;11(11):e0165125. doi:10.1371/journal.pone.0165125
13. Chua SC, Yovich SJ, Hinchliffe PM, Yovich JL. The sperm DNA fragmentation assay with SDF level less than 15% provides a useful prediction for clinical pregnancy and live birth for women aged under 40 years. *J Pers Med*. 2023;13(7):1079. doi:10.3390/jpm13071079
14. Fernández JL, Muriel L, Goyanes V, et al. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril*. 2005;84:833-842.
15. Dunkenberger L, Reiss K, Del Valle L. Comet assay for the detection of single and double-strand DNA breaks. *Methods Mol Biol*. 2022;2422:263-269.
16. Tímermans A, Vázquez R, Otero F, et al. DNA fragmentation of human spermatozoa: simple assessment of single-and double-strand DNA breaks and their respective dynamic behavioral response. *Andrology*. 2020;8:1287-1303.
17. Moraes CR, Meyers S. The sperm mitochondrion: organelle of many functions. *Anim Reprod Sci*. 2018;194:71-80. doi:10.1016/j.anireprosci.2018.03.024
18. Durairajanayagam D, Singh D, Agarwal A, Henkel R. Causes and consequences of sperm mitochondrial dysfunction. *Andrologia*. 2021;53:e13666. doi:10.1111/and.13666. Epub 2020 Jun 8
19. Boguenet M, Bouet PE, Spiers A, Reynier P, May-Panloup P. Mitochondria: their role in spermatozoa and in male infertility. *Hum Reprod Update*. 2021;27:697-719. doi:10.1093/humupd/dmab001
20. Wang X, Yin L, Wen Y, Yuan S. Mitochondrial regulation during male germ cell development. *Cell Mol Life Sci*. 2022;79:91. doi:10.1007/s00018-022-04134-3
21. Tesarik J, Mendoza-Tesarik R. Mitochondria in human fertility and infertility. *Int J Mol Sci*. 2023;24:8950. doi:10.3390/ijms24108950
22. Taanman JW. The mitochondrial genome: structure, transcription, translation and replication. *Biochem Biophys Acta Bioenerg*. 1999;1410:103-123.
23. May-Panloup P, Chrétien MF, Savagner F, et al. Increased sperm mitochondrial DNA content in male infertility. *Hum Reprod*. 2003;18:550-556. doi:10.1093/humrep/deg096
24. Faja F, Carlini T, Coltrinari G, et al. Human sperm motility: a molecular study of mitochondrial DNA, mitochondrial transcription factor A gene and DNA fragmentation. *Mol Biol Rep*. 2019;46:4113-4121. doi:10.1007/s11033-019-04861-0
25. Wu H, Huffman AM, Whitcomb BW, et al. Sperm mitochondrial DNA measures and semen parameters among men undergoing fertility

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

treatment. *Reprod Biomed Online*. 2019;38:66-75. doi:10.1016/j.rbmo.2018.10.004
26. Tiegs AW, Tao X, Landis J, et al. Sperm mitochondrial DNA copy number is not a predictor of intracytoplasmic sperm injection (ICSI) cycle outcomes. *Reprod Sci*. 2020;27:1350-1356. doi:10.1007/s43032-020-00163-0
27. Belmonte FR, Martin JL, Frescura K, et al. Digital PCR methods improve detection sensitivity and measurement precision of low abundance mtDNA deletions. *Sci Rep*. 2016;6:25186.
28. Li B, Kaushik S, Kalinowski P, et al. Droplet digital PCR shows the D-Loop to be an error prone locus for mitochondrial DNA copy number determination. *Sci Rep*. 2018;8:11392.
29. Quan P-L, Sauzade M, dPCR BrouzesE. A technology review. *Sensors*. 2018;18:1271.
30. Malik AN, Czajka A. Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction? *Mitochondrion*. 2013;13:481-492.
31. Mosquera A, Guillén R, Otero F, et al. Design of a digital-PCR assay to quantify fragmented human mitochondrial DNA. *Environ Mol Mutagen*. 2021;62(6):1-10. doi:10.1002/em.22449
32. WHO. World Health Organisation (WHO) laboratory manual for the examination and processing of human semen. 5th ed. WHO; 2010: 271 p.
33. Song GY, Lewis V. Mitochondrial DNA integrity and copy number in sperm from infertile men. *Fertil Steril*. 2008;90:2238-2244.
34. Amaral A, Ramalho-Santos J, St John JC. The expression of polymerase gamma and mitochondrial transcription factor A and the regulation of mitochondrial DNA content in mature human sperm. *Hum Reprod*. 2007;22:1585-1596. doi:10.1093/humrep/dem030. Epub 2007 Mar 5.
35. Darr CR, Moraes LE, Connon RE, et al. The relationship between mitochondrial DNA copy number and stallion sperm function. *Theriogenology*. 2017;94:94-99. doi:10.1016/j.theriogenology.2017.02.015. Epub 2017 Feb 21.
36. Zhang G, Wang Z, Ling X, et al. Mitochondrial biomarkers reflect semen quality: results from the MARCHS study in Chongqing, China. *PLoS One*. 2016;11:e0168823. [PubMed: 28006017].
37. Ambulkar PS, Waghmare JE, Chaudhari AR, et al. Large scale 7436-bp deletions in human sperm mitochondrial DNA with spermatozoa dysfunction and male infertility. *J Clin Diagn Res*. 2016;10. GC09–GC12. [PubMed: 28050401].
38. Wu H, Huffman AM, Whitcomb BW, et al. Sperm mitochondrial DNA measures and semen parameters among men undergoing fertility treatment. *Reprod Biomed Online*. 2019;38:66-75. doi:10.1016/j.rbmo.2018.10.004. Epub 2018 Nov 16.
39. Shokolenko I, LeDoux S, Wilson G, Alexeyev M. Mitochondrial DNA damage, repair, degradation and experimental approaches to studying these phenomena. In: Storici F, ed. DNA repair—on the pathways to fixing DNA damage and errors. *InTechOpen*. 2011:339-356.
40. Boguenet M, Desquiret-Dumas V, Goudenège D, et al. Mitochondrial DNA content reduction in the most fertile spermatozoa is accompanied by increased mitochondrial DNA rearrangement. *Hum Reprod*. 2022;37:669-679. doi:10.1093/humrep/deac024
41. Giles RE, Blanc H, Cann HM, Wallace DC. Maternal inheritance of human mitochondrial DNA. *Proc Natl Acad Sci USA*. 1980;77:6715-6719.
42. Cummins JM. Fertilization and elimination of the paternal mitochondrial genome. *Hum Reprod*. 2000;15:92-101. Suppl. S2.
43. Luo S, Valencia CA, Zhang J, et al. Biparental inheritance of mitochondrial DNA in humans. *Proc Natl Acad Sci*. 2018;115:13039-13044. doi:10.1073/pnas.1810946115. USA. Epub 2018 Nov 26.
44. Song WH, Yi YJ, Sutovsky M, Meyers S, Sutovsky P. Autophagy and ubiquitin-proteasome system contribute to sperm mitophagy after mammalian fertilization. *Proc Natl Acad Sci*. 2016;113:E5261-E5270. doi:10.1073/pnas.1605844113. USA. Epub 2016 Aug 22.
45. Nishimura Y, Yoshinari T, Naruse K, et al. Active digestion of sperm mitochondrial DNA in single living sperm revealed by optical tweezers. *Proc Natl Acad Sci*. 2006;103:1357-1382. doi:10.1073/pnas.0506911103
46. DeLuca SZ, O'Farrell PH. Barriers to male transmission of mitochondrial DNA in sperm development. *Dev Cell*. 2012;22:660-668. doi:10.1016/j.devcel.2011.12.021
47. O'Connell M, McClure N, Powell LA, Steele EK, Lewis SE. Differences in mitochondrial and nuclear DNA status of high-density and low-density sperm fractions after density centrifugation preparation. *Fertil Steril*. 2003;79(Suppl 1>):754-762. doi:10.1016/s0015-0282(02)04827-6
48. Shi WH, Ye MJ, Qin NX, et al. Associations of sperm mtDNA copy number, DNA fragmentation index, and reactive oxygen species with clinical outcomes in ART treatments. *Front Endocrinol (Lausanne)*. 2022;13:849534. doi:10.3389/fendo.2022.849534

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Tímermans A, Otero F, Garrido M, Gosálvez J, Johnston S, Fernández JL. The relationship between sperm nuclear DNA fragmentation, mitochondrial DNA fragmentation, and copy number in normal and abnormal human ejaculates. *Andrology*. 2024;12:870–880. https://doi.org/10.1111/andr.13539

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13539 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 22 June 2023 | Revised: 16 September 2023 | Accepted: 26 September 2023

DOI: 10.1111/andr.13541

ANDROLOGY WILEY

ORIGINAL ARTICLE

Differential sperm histone retention in normozoospermic ejaculates of infertile men negatively affects sperm functional competence and embryo quality

Riddhi Kirit Pandya[1] | Ameya Jijo[1] | Aswathi Cheredath[1] | Shubhashree Uppangala[2] | Sujith Raj Salian[1] | Vani R Lakshmi[3] | Pratap Kumar[4] | Guruprasad Kalthur[5] | Sanjay Gupta[6] | Satish Kumar Adiga[1]

[1]Centre of Excellence in Clinical Embryology, Department of Reproductive Science, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India

[2]Division of Reproductive Genetics, Department of Reproductive Science, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India

[3]Department of Data Science, Prasanna School of Public Health, Manipal Academy of Higher Education, Manipal, India

[4]Department of Reproductive Medicine and Surgery, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India

[5]Division of Reproductive Biology, Department of Reproductive Science, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India

[6]KS313, Epigenetics and Chromatin Biology Group, Gupta Lab, Cancer Research Institute, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Navi Mumbai, India

Correspondence
Satish Kumar Adiga, Centre of Excellence in Clinical Embryology, Department of Reproductive Science, Kasturba Medical College, Manipal Academy of Higher Education, Manipal 576104, India.
Email: satish.adiga@manipal.edu

Sanjay Gupta, KS313, Epigenetics and Chromatin Biology Group, Gupta Lab, Cancer Research Institute, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Navi Mumbai, India.
Email: sgupta@actrec.gov.in

Funding information
Indian Council of Medical Research, Grant/Award Number: ICMR # 5/10/FR/8/2014-RCH

Abstract

Background: The unique epigenetic architecture that sperm cells acquire during spermiogenesis by retaining <15% of either canonical or variant histone proteins in their genome is essential for normal embryogenesis. Whilst heterogeneous levels of retained histones are found in morphologically normal spermatozoa, their effect on reproductive outcomes is not fully understood.

Methods: Processed spermatozoa ($n = 62$) were tested for DNA integrity by sperm chromatin dispersion assay, and retained histones were extracted and subjected to dot-blot analysis. The impact of retained histone modifications in normozoospermic patients on sperm functional characteristics, embryo quality, metabolic signature in embryo spent culture medium and pregnancy outcome was studied.

Results: Dot-blot analysis showed heterogeneous levels of retained histones in the genome of normozoospermic ejaculates. Post-wash sperm yield was affected by an increase in H3K27Me3 and H4K20Me3 levels in the sperm chromatin ($p < 0.05$). Also, spermatozoa with higher histone H3 retention had increased DNA damage ($p < 0.05$). Spermatozoa from these cohorts, when injected into donor oocytes, correlated to a significant decrease in the fertilisation rate with an increase in sperm histone H3 ($p < 0.05$) and H3K27Me3 ($p < 0.01$). An increase in histone H3 negatively affected embryo quality ($p < 0.01$) and clinical pregnancy outcome post-embryo transfer ($p < 0.05$). On the other hand, spent culture medium metabolites assessed by high-resolution (800 MHz)

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2023 The Authors. *Andrology* published by Wiley Periodicals LLC on behalf of American Society of *Andrology* and European Academy of *Andrology*.

nuclear magnetic resonance showed an increased intensity of the amino acid methionine in the non-pregnant group than in the pregnant group ($p < 0.05$) and a negative correlation with sperm histone H3 in the pregnant group ($p < 0.05$).

Discussion and conclusion: Histone retention in spermatozoa can be one of the factors behind the development of idiopathic male infertility. Such spermatozoa may influence embryonic behaviour and thereby affect the success rate of assisted reproductive technology procedures. These results, although descriptive in nature, warrant further research to address the underlying mechanisms behind these clinically important observations.

KEYWORDS
assisted reproductive technology, infertility, metabolomics, pregnancy outcome, retained histone post-translational modifications, sperm epigenetics

1 | INTRODUCTION

Classically, the contribution of spermatozoa to embryogenesis was limited to the transfer of paternal DNA[1,2] and centrioles[3] and as a signal to start the metabolic activity of the oocytes.[4] However, recent literature involving high-throughput technologies indicates that their contribution is much more central than previously thought.[5–7] Several reports have demonstrated that the male gamete imparts various epigenetic marks, RNA and protein molecules to the resultant zygotes.[7–9] These factors might have a vital role in embryo development and future health of the progeny.[5,10–12]

During spermiogenesis, human spermatozoa attain a unique epigenetic architecture by replacing nucleosome-bound DNA with protamines and retaining <15% of either canonical or variant histones in the genome.[13–15] The retained nucleosomes in mature spermatozoa exhibit specific post-translational modifications (PTMs), such as acetylation and methylation majorly on H3 and H4 histones. These heritable marks are highly enriched in the imprinted gene regions (microRNA and HOX gene clusters), promoters and regulatory regions of various developmentally important genes.[8,16–18] The epigenetic memory delivered by spermatozoa during fertilisation may aid in the epigenetic reprogramming of zygotes and the development of early embryos by regulating their gene expression patterns.[19–21]

Earlier reports have demonstrated that an abnormal histone-to-protamine ratio in infertile men leads to altered semen parameters, fertilisation failure and low pregnancy outcomes.[22–24] In addition, the differential profiles of sperm DNA methylation were detected on imprinted and developmental genes located at retained histones in oligozoospermic patients.[25,26] Furthermore, patients with astheno and teratozoospermic conditions had differential epigenetic signatures.[27] A recent report also demonstrated that normozoospermic patients have differential epigenetic signatures making a sub-cohort of unexplained infertility,[28] where changes in the sperm histone retained epigenome were associated with fertilisation failure, poor blastocyst development and development of imprinted disorders in the progeny.[28–31]

Heterogeneous levels of histone PTMs in mature normal human spermatozoa have been reported.[15,18,32,33] The enrichment of gene activation marks (H3K4Me3 and H4K12Ac) and repression marks (H3K9Me3, H3K27Me3 and H4K20Me3) were observed at the developmental promoter region and certain imprinted genes in the spermatozoa.[8,26,33] Also, these histone modifications are transmitted to embryos during fertilisation.[15,18,21] However, the influence of altered retained histone PTMs of the paternal genome on embryogenesis has not been elucidated. Importantly, concerns associated with increased imprinting disorders among those born through assisted reproductive technology (ART)[34,35] raise the need to study the sperm epigenetic signature and its impact on embryos. Therefore, the current study looked at the retained histones and their PTMs in the processed sperm fraction of men undergoing ART treatment and their impact on sperm functional characteristics and embryo development.

2 | MATERIALS AND METHODS

2.1 | Study subjects

Samples obtained from patients ($n = 62$) visiting the university infertility clinic for their treatment were included in the study after obtaining ethical approval from the Institutional Ethics Committee (IEC 464/2017 and 568/2020) and written informed consent from all the patients. Ejaculates were evaluated according to World Health Organization criteria.[36] Based on semen analysis reports, only normozoospermic subjects were included. These patients did not have varicocele, urogenital infections, hypogonadotropic hypogonadism, obstruction or agenesis of the seminal ducts. The patients' sperm characteristics are described in Table 1. The study outline is depicted in Figure 1. Briefly, processed sperm samples ($n = 62$) were used to

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

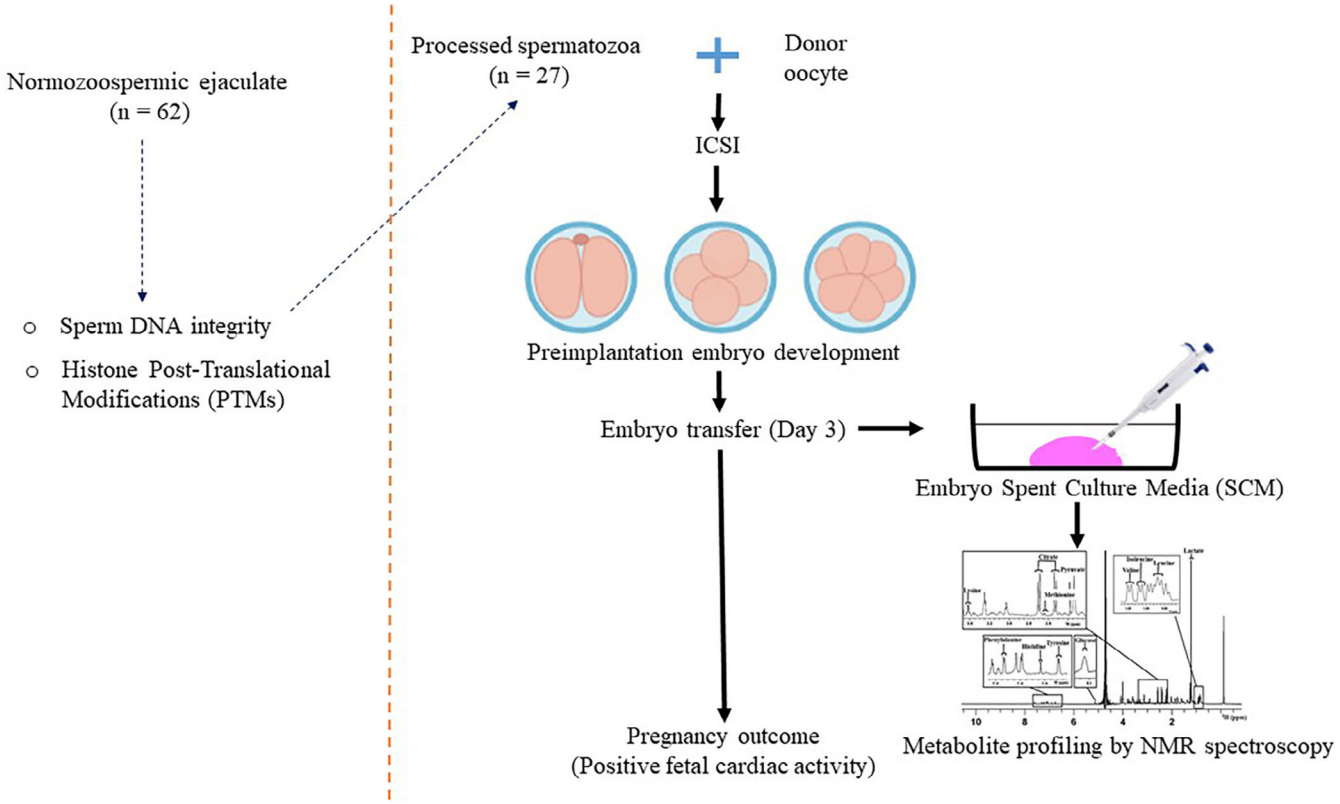


FIGURE 1 Schematic outline of the study: experimental outline to determine the influence of retained sperm epigenetic signature on pre-implantation embryos. NMR, nuclear magnetic resonance.

TABLE 1 Sperm characteristics in neat ejaculate and processed fraction.

	Median (Q_1 –Q_3)
Neat ejaculate ($n = 62$)	
Male age (years)	36.00 (34.00–39.75)
Semen volume (mL)	2.50 (1.87–3.63)
Sperm concentration (10^6/mL)	62.50 (46.50–80.75)
Total sperm number (10^6/ejaculate)	157.00 (95.25–204.75)
Total motility (%)	70.00 (59.25–76.75)
Processed fraction ($n = 62$)	
Sperm concentration (10^6/mL)	27.66 (20.51–44.13)
DNA damage (%)	9.00 (2.00–23.25)

extract retained histones using the acid extraction method followed by dot-blot analysis. Furthermore, part of the sample was also subjected to a sperm chromatin dispersion (SCD) test to understand its sperm chromatin integrity. To understand the association between histone PTMs and sperm characteristics, a correlation analysis was performed. Out of 62 patients, 27 underwent Intracytoplasmic Sperm Injection (ICSI) cycles using donor oocytes. All the recipient females ($n = 27$; partner of the male patients) had a history of poor ovarian reserve as evidenced by low Anti-Mullerian Hormone <0.5 ng/mL in addition to previous in vitro fertilisation failure because of poor ovarian response ($n = 8$) and advanced maternal age ($n = 2$). The processed sperm samples of these patients were used to understand the impact of sperm histone PTMs on embryo developmental competence and reproductive outcomes in pregnant ($n = 17$) and non-pregnant groups ($n = 10$). Post-embryo transfer spent culture medium was collected from both the groups and analysed for the levels of metabolites using nuclear magnetic resonance (NMR) spectroscopy and associated with sperm histone PTMs to determine the relationship between them.

2.2 | Semen sample and sperm preparation

After semen analysis, ejaculates were subjected to density gradient separation as described earlier.[37] Briefly, samples were overlaid on the gradient solutions (80% and 40%) (^{V}GRAD, Cat. No. V-GRD83_100P, Vitromed GmbH) and centrifuged at 350 *g* for 20 min. The pellet was resuspended in pre-warmed V-HEPES plus media (Cat. No. V-HTF-P1, Vitromed GmbH) and washed by centrifuging at 300 *g* for 8 min. The pellet was resuspended in pre-warmed V-ONESTEP (Cat. No. V-OSM-20, Vitromed GmbH) and washed again by centrifuging at 200 *g* for 8 min. After wash, the sperm concentration was calculated and 0.1 million spermatozoa were used to determine sperm chromatin integrity. The remaining fraction was stored in phosphate buffered saline (PBS) at −80°C for histone PTM analysis.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.3 | Sperm chromatin integrity assay

On processed sperm samples, an SCD assay was carried out as described by Fernández et al.[38] with a few minor modifications. A mixture of 0.1 million spermatozoa and 1% low melting agarose kept at 37°C was layered on slides pre-coated with 0.65% normal melting agarose and allowed to solidify. The slides were immersed in denaturation solution (0.08 N HCl), lysis solution 1 (0.4 m Tris, 20 mM Dithiothreitol (DTT), 1% SDS, 50mM Ethylenediamine tetraacetic acid) and lysis solution 2 (0.4 M Tris, 2 M NaCl). The slides were then air-dried after being neutralised in Tris buffer (0.4 M Tris) and serially dehydrated in alcohol. Sperm chromatin integrity was assessed under a fluorescent microscope (Imager-A1; Zeiss) after staining the cells with ethidium bromide (7 μg/mL). The large halo spermatozoa (without any DNA damage or normal) produce halos with a thickness equal to or greater than the length of the minor diameter of the core, whereas small halo spermatozoa produce halos with a thickness equal to or smaller than one-third diameter of the minor diameter of the core.[38,39] The percentage of spermatozoa with damaged DNA was calculated by counting spermatozoa with a small + no halo, and the percentage of spermatozoa with severely damaged DNA was calculated by counting spermatozoa with no halo.

2.4 | Histone extraction

Acid extraction of sperm histone was carried out as described previously with minor modifications.[33,40] Approximately 2 million spermatozoa were washed twice with PBS at 4000 *g* for 5 min at 4°C. Pellet was resuspended in hypotonic lysis buffer (10 mM Tris–Cl pH 8.0, 0.1 mM KCl, 1.5 mM $MgCl_2$, 100 mM DTT) along with a protease inhibitor and vortexed thoroughly. After incubation for 1 h at 4°C, their nuclei were pelleted by centrifuging at 10,000 *g* for 10 min at 4°C, and 0.4 N of H_2SO_4 solution was added. The tubes were vortexed with intermittent incubation on ice for 2 h. The solution was centrifuged at 16,000 *g* for 10 min at 4°C and the supernatant was transferred to a fresh tube. The proteins were allowed to precipitate by adding 4 volumes of chilled acetone and stored at −20°C overnight. The next day, they were pelleted down and washed once with chilled acidified acetone (0.05 M HCl in 100% acetone) and twice with chilled 100% acetone at 16,000 *g* for 10 min at 4°C. The pellet was vacuum dried and dissolved in 0.1% β-mercaptoethanol and stored at −20°C.

2.5 | Dot-blot analysis

Total protein extracted from 2 million spermatozoa was slotted on a polyvinylidene difluoride membrane using a vacuum and proteins were visualised. The membrane was incubated in 5% Bovine serum albumin in Tris-buffered saline with 0.1% Tween (TBST) for 1 h and probed with histone H3, histone H4 and histone PTMs such as H3K4Me3, H4K12Ac, H3K9Me3, H3K27Me3 and H4K20Me3 antibodies overnight at 4°C. The membrane was washed three times in TBST and incubated with a secondary antibody at room temperature for 1 h. Blots were washed using three washes and developed using Clarity Max western ECL substrate (Cat. No. 1705062, Bio-Rad). Antibodies used and dilutions are given in Table S1. The intensity of the signal for all the modifications for each patient was calculated by densitometric analysis using ImageJ software. The relative expression of each modification was expressed in fold change by normalising the signal intensity to the total protein spotted on the membrane (Figure S1).

2.6 | Fertilisation and embryo grading

Controlled ovarian hyperstimulation of the donors was performed by a standard antagonist protocol. Follicular aspiration was performed and retrieved oocyte cumulus complexes were washed and then incubated in V-ONESTEP medium media (Cat. No. V-OSM-20, Vitromed GmbH) at 37°C in 6% CO_2 and 5% O_2 in an incubator. An average of 10-11 donor MII oocytes per couple (n = 27) were inseminated with the spermatozoa by ICSI and cultured individually in V-ONESTEP culture media. After 16–18 h of insemination, oocytes were assessed for fertilisation. On day 3, fertilised embryos were graded as per the ESHRE consensus,[41] and per patient (n = 2), embryos were transferred to the recipient females (partner of the male patients). Grade I and II embryos were considered good- and average-quality embryos, respectively, whereas Grade III embryos were considered poor-quality embryos.

2.7 | Biochemical and chemical pregnancy outcomes

Fourteen days after embryo transfer, a blood test was performed to determine the level of human chorionic gonadotropin (β-hCG). β-hCG levels above 100 mIU/mL were considered positive for biochemical pregnancy. After 4 weeks, ultrasonography was performed to determine the presence of a gestational sac or foetal cardiac activity to confirm clinical pregnancy.

2.8 | Collection of embryo spent culture media, NMR sample preparation and data analysis

The spent culture medium samples and media controls were collected post-embryo transfer, snap-frozen and stored at −80°C for the NMR analysis. The samples were prepared as described in Cheredath et al.[42] Briefly, 25 μL of embryo spent culture media samples were thawed for 10 min at room temperature. The samples were prepared by dilution with sodium salt of 2,2,3,3 tetradeutero 3-(trimethyl silyl) propionate (TSP) as a standard reference compound and transferred to 1.7 mm

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

NMR tubes for profiling the spectra. All spectra were acquired on a Bruker AVANCE NMR spectrometer operating at a ^{1}H resonance frequency of 800 MHz equipped with a 1.7 mm cryo-probe at 298 K. The NMR experiments were recorded using Carr–Purcell–Meiboom–Gill sequence 'cpmgpr1d' pulse program available in the Bruker library. Data were analysed using the Bruker TOPSPIN 3.6.2 software. The peak integrals were measured with respect to the corresponding integral of the TSP signal.

2.9 | Statistical analysis

All the data are presented as the mean and SD, median or interquartile range (Q_1–Q_3) after checking the normality assumption with the help of the Shapiro–Wilk test. Subsequently, an age-adjusted Pearson's correlation analysis was performed using Jamovi (version 2.2.5; RRID:SCR_016142).[43] The strength of the correlation was represented by the value of the correlation coefficient (r value). To compare male age, semen parameters, DNA damage, female and donor age, the number of retrieved oocytes, endometrial thickness and the number of embryos transferred between pregnant and non-pregnant groups, two samples independent t-tests or Mann–Whitney U-test were performed after appropriate assumption checks. Female age, male age and sperm DNA damage-adjusted analysis of covariance (ANCOVA) were performed between the levels of sperm histone PTMs and clinical pregnancy. To determine the levels of methionine in media control and spent culture medium (SCM) of the pregnant and non-pregnant groups, the Kruskal–Wallis test was performed. All graphs were plotted using GraphPad Prism 8 (GraphPad Prism software). The level of significance was set at 5% for the entire study.

3 | RESULTS

3.1 | Sperm histone PTMs and post-wash sperm characteristics

Sperm characteristics analysed in neat and processed samples are summarised in Table 1. To determine the association between processed sperm characteristics, retained core histones and histone PTM levels, age-adjusted Pearson's correlation was performed as our data demonstrated age-related changes in sperm characteristics such as total motility ($r = -0.305$, $p < 0.05$), progressive motility ($r = -0.313$, $p < 0.01$) and tail defects ($r = 0.341$, $p < 0.01$) in the neat ejaculate (Table S2). Correlation analysis demonstrated a weak negative correlation between sperm concentration and H3K27Me3 ($r = -0.27$, $p < 0.05$) and H4K20Me3 ($r = -0.285$, $p < 0.05$). Although the percentage of spermatozoa with DNA damage (% small halo + % no halo) did not show any correlation with core histones and PTMs, the percentage of spermatozoa with severe damage (% no halo) had a weak positive correlation with H3 histone ($r = 0.26$, $p < 0.05$) (Table 2).

3.2 | Core histone (H3) and its modification (H3K27Me3) demonstrated a relationship between fertilisation and embryo quality

Sperm histone modifications play an important role during fertilisation and embryo development. Therefore, the age-adjusted correlation analysis was carried out between retained sperm histone levels, their PTM levels, and embryological outcomes such as fertilisation rate and embryo quality on day 3 of the development. Table 3 represents the embryological outcomes. Among the histones and modifications considered in this study, histone H3 ($r = -0.397$, $p < 0.05$) and H3K27Me3 ($r = -0.503$, $p < 0.01$) showed a moderate negative correlation with the fertilisation rate (Table 4). However, no correlation was obtained between sperm histone PTMs and cleavage rate. Interestingly, histone H3 showed a moderate negative correlation with the percentage of good-quality embryos on day 3 of development ($r = -0.568$, $p < 0.01$).

3.3 | Pregnancy outcome is associated with core histone H3 in spermatozoa

As we found a negative association of retained histone PTMs present in spermatozoa with fertilisation rate and embryo quality, the association between sperm histone PTMs and pregnancy outcome was assessed. Out of 27 infertile couples from the donor oocyte program, 17 patients conceived successfully, resulting in a 62.9% clinical pregnancy rate. Here, the patients were categorised into two groups. Patients who had positive foetal cardiac activity post-embryo transfer were considered pregnant ($n = 17$) and those with negative foetal cardiac activity were considered non-pregnant ($n = 10$). The factors influencing pregnancy outcomes such as the age of the male patients, semen characteristics, sperm DNA damage, donor age, recipient age, oocytes used, embryos transferred and endometrial thickness, were comparable between the groups, as presented in Table S3. The mean levels of sperm histone and their PTMs were compared between the pregnant and non-pregnant groups by applying ANCOVA adjusting for the influence of the female recipients' age, male age and sperm DNA damage. It was found that the pregnant group had a significantly low level of histone H3 in sperm fraction used in ICSI than the non-pregnant group (Figure 2). However, other histone modification levels did not show any significant difference between the groups (Table S4).

3.4 | Sperm histone H3 is associated with altered metabolite levels in SCM of preimplantation embryos

As we found that the high histone H3 levels might influence pregnancy outcomes, we explored whether sperm histones and their modifications have any role in the embryo metabolomic signature. Hence, SCM was profiled using high-sensitivity enhanced 800 MHz NMR spectroscopy. A total of 12 metabolites consisting of eight amino acids and four carbohydrates were identified by 1D ^{1}H NMR spectroscopy in

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Correlation analysis between sperm histone post-translational modifications and processed sperm parameters ($n = 62$).

	Histone 3	H3K4Me3	H3K9Me3	H3K27Me3	Histone 4	H4K12Ac	H4K20Me3
Sperm concentration (10^6/mL)	0.058	−0.095	−0.125	−0.270*	0.154	−0.153	−0.285*
DNA damage (%)	0.075	0.060	0.075	0.139	0.052	−0.168	0.120
Severe DNA damage (%)	0.260*	0.021	0.143	−0.007	0.086	−0.249	0.058

Note: Pearson's correlation coefficient (r value) is presented in the table (adjusted for men's age). DNA damage includes small halo + no halo (%). Severe DNA damage includes no halo (%).
*Significant at alpha = 5%.

TABLE 3 Pre-implantation embryo developmental outcomes ($n = 27$).

	Mean ± SD
Fertilisation rate (%)	73.94 ± 16.08
Cleavage rate (%)	96.50 ± 6.50
Good-quality embryos (%) (day 3) (Grade I embryos)	38.66 ± 28.74

the medium control (blank) and SCM of embryos in the pregnant and non-pregnant categories (Table S5). The methionine intensity in SCM of the embryos that resulted in pregnancy was significantly reduced when compared to embryos that did not lead to pregnancy. ($p < 0.05$, Figure 3A). Further correlation analysis demonstrated that levels of methionine in SCM of the pregnant group showed a moderate negative correlation with sperm histone H3 levels ($r = -0.648$, $p < 0.05$) (Figure 3B and Table S6).

4 | DISCUSSION

In this study, heterogeneous levels of retained histones in spermatozoa demonstrated a significant association with sperm DNA fragmentation, fertilisation, embryo quality, and pregnancy outcome suggesting that histone retention in the spermatozoa from normozoospermic ejaculate might influence embryonic behaviour and pregnancy outcome.

Heterogeneous levels of histone PTMs in the chromatin of normal human spermatozoa have been reported.[15,32,44,45] Sperm chromatin enriched with histone PTMs such as H3K4Me2, H3K36Me3 and H4S1ph is indicative of poor sperm quality.[46–48] In line with this, our findings demonstrated the heterogenous levels of retained histones and their PTMs in processed sperm fractions of normozoospermic ejaculates. Also, a negative correlation was observed between post-wash sperm concentration and heterochromatin marks, H3K27Me3 and H4K20Me3, indicating that increased histone retention in chromatin could affect the post-wash sperm yield. Histone methylation marks such as H3K9Me3, H3K27Me3 and H4K20Me3 are present in the heterochromatin region and are crucial for the progression of normal spermatogenesis.[49,50] Any alterations during this process can lead to poor sperm competence and decreased fertility.[15,51]

It has been shown that an altered histone-to-protamine ratio, or more specifically, increased histone retention in sperm chromatin affects male fertility by decreasing sperm concentration, and motility, increasing DNA damage and poor embryo outcome.[22,23,26,32,52] We observed a positive association between histone H3 and spermatozoa with high DNA damage. Nuclear compaction by the incorporation of protamines protects the genetic and epigenetic integrity of sperm chromatin from external stress.[53,54] Therefore, sperm chromatin with increased histone retention reduces the chromatin structure stability, thereby rendering it more vulnerable to DNA fragmentation.[22,55] Our findings and reports mentioned above imply that alteration in the histone-to-protamine ratio can hinder the epigenetic stability of sperm chromatin, thereby impairing sperm competence.

Genes important for embryonic development are marked by specific histone PTMs in spermatozoa.[8,56] It has been shown that retained histone marks, H3K9Me3 and H4K20Me3 of the spermatozoa are transmitted to the embryos and demarcate the heterochromatin region in the zygote; furthermore, they act as a template for copying epigenetic information through maternal machinery.[57] Therefore, to understand the influence of normozoospermic ejaculates with heterogenous paternal histone PTMs on fertilisation and preimplantation embryo developmental competence, we selected sperm samples used for the donor oocyte ICSI program from the above normozoospermic cohort. The use of donor oocytes likely minimised the influence of female factors and thus enabled the study to assess the contribution of paternal epigenome to embryogenesis. Sperm histone H3

TABLE 4 Correlation analysis between sperm histone post-translational modifications and embryo developmental competence ($n = 27$).

	Histone H3	H3K4Me3	H3K9Me3	H3K27Me3	Histone H4	H4K12Ac	H4K20Me3
Fertilisation rate (%)	−0.397*	−0.316	−0.305	−0.503**	0.111	0.033	−0.337
Cleavage rate (%)	−0.136	−0.138	−0.332	−0.275	−0.025	0.126	0.255
Good-quality embryos (%) (day 3)	−0.568**	0.072	−0.067	−0.114	0.014	−0.218	0.119
Poor-quality embryos (%) (day 3)	0.365	−0.115	−0.038	0.085	−0.039	0.103	−0.039

Note: Pearson's correlation coefficient (r value) is presented in the table (adjusted for men's age).
*Significant at alpha = 5%; * Significant at alpha = 1%.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

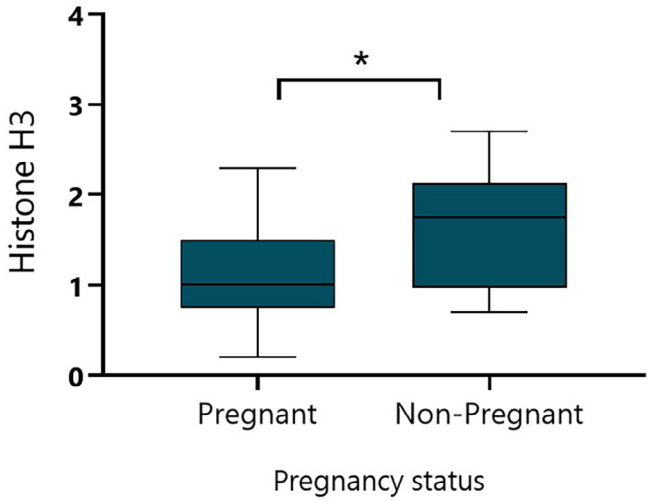


FIGURE 2 Box-and-whisker plot showing the level of histone H3 in the spermatozoa of male partners in pregnant ($n = 17$) and non-pregnant groups ($n = 10$). The data were analysed by unpaired student's *t*-test and represented as mean ± SD. Asterisk (*) represents significance at $p < 0.05$ (adjusted for the female recipient's age, male age and sperm DNA damage).

and H3K27Me3 showed a negative association with the fertilisation rate. The enrichment of H4K12Ac in the sperm nucleus and the zygote following fertilisation has been demonstrated and is associated with genes expressed at the four-cell stage to blastocyst.[20] Whereas experimentally induced abnormal histone retention during spermiogenesis showed differential expression of genes in the two-cell stage to blastocyst,[58] suggesting abnormal retention of histones during spermiogenesis leading to altered gene expression in early embryos.[49,59,60] These studies support our findings that altered levels of paternally retained histones and their PTMs in normozoospermic individuals may have an impact on the fertilisation process. Interestingly, a negative correlation between histone H3 and good-quality embryos was observed, indicating that an increase in histone retention can affect the development of human preimplantation embryos. Earlier studies have demonstrated that the cleavage rate and quality of the embryos are adversely affected because of increase in histone retention in sperm chromatin.[61] However, embryo production was not affected.[62] Furthermore, poor-quality blastocysts were observed in normozoospermic spermatozoa with abnormal histone CpG island methylation profiles, suggesting that alteration in the epigenetic signature on the paternal genome is associated with unexplained male infertility and poor blastocyst development.[29]

We noticed unsuccessful pregnancies in couples with a significantly increased level of histone H3 in spermatozoa. When normozoospermic spermatozoa were subjected to dot-blot analysis, the non-pregnant group had higher levels of histones than the pregnant group, demonstrating the impact on pregnancy outcome.[10] The clinical pregnancy rate was lower in spermatozoa with a lower protamine 1:protamine 2 ratio in the chromatin.[23,24] Together, these findings support the fact that levels of retained histones in spermatozoa and their enrichment at developmental genes are essential for fertilisation and early embryo development. Since the establishment of a successful pregnancy is multifactorial, our argument suggesting the association between retained histones and clinical pregnancy may be overstated. However, there could be a link between histone levels in spermatozoa and pregnancy outcome.

Previous studies have shown that the uptake of metabolites from culture media can be a predictive tool for embryo viability and implantation potential.[62–65] Data from our laboratory have shown that sperm DNA integrity negatively affects embryo metabolism.[65,66] In the current study, we observed that the intensity of methionine was significantly lower in the SCM of embryos in successful pregnancy groups than in those of failed pregnancy. Methionine is a key element in the 1-C metabolism cycle that provides methyl moieties to S-adenosyl methionine, which is required for DNA and histone methylation.[67,68] Also, methionine is a precursor to the methyl donors required for protein synthesis during embryonic development.[69] Methionine in embryo culture media is essential because the methylation levels on embryo genomes are highly maintained after fertilisation and start to decline after the four-cell stage.[70,71] Abnormal methionine metabolism

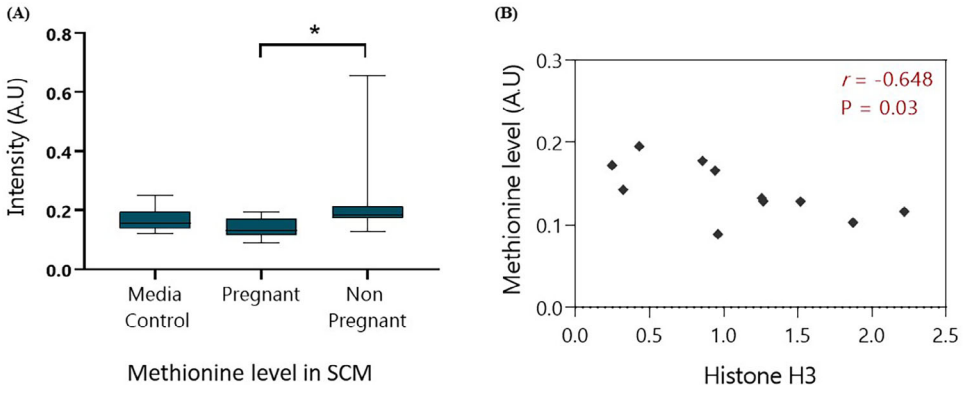


FIGURE 3 (A) Levels of methionine in spent embryo culture media obtained from media control ($n = 13$), pregnant ($n = 11$) and non-pregnant ($n = 7$) groups are represented by a box-and-whisker plot. The data were analysed by the Kruskal–Wallis test. Asterisk (*) represents significance at $p < 0.05$. (B) Scatterplot depicting the association between levels of sperm histone H3 and methionine obtained from spent embryo culture media of the pregnant group ($n = 11$). '*r*' represents Pearson's correlation coefficient. Data are significant at $p < 0.05$. SCM, spent culture medium.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

affects the transition of the morula to the blastocyst because of hypomethylation of DNA, which changes the expression of developmental genes, indicating a critical role for methionine metabolism during pre-implantation embryo development.[72,73] However, at this juncture, it is not possible to claim whether a similar phenomenon exists in our study as we did not use SCM beyond day 3 of development. On the other hand, we noticed that the level of methionine in SCM of the successful pregnancy group was found to be negatively correlated with the sperm histone H3 levels. According to earlier reports, differential levels of amino acids in spent embryo media were observed in pregnant and non-pregnant groups.[74–76] Our results are in line with previous studies, where methionine levels in the SCM of embryos might regulate embryo development, which is also associated with sperm histone H3. A molecular understanding of the significance of methionine metabolism in preserving H3K4Me3 levels in the chromatin during early embryogenesis has been provided by expression experiments in human embryonic stem cells.[77,78] However, we could not identify the molecular or biochemical mechanisms to link methionine metabolism and sperm histone H3 levels in the current study.

Various intrinsic and extrinsic factors, such as altered lifestyle, dietary exposure, nutritional status (malnutrition or obesity) or environmental toxicants (smoking, alcoholism and other environmental toxins) can affect the sperm epigenome.[79–82] Our findings provide support for existing research that morphologically normal spermatozoa may contain altered epigenetic signatures that could potentially be a contributing factor to idiopathic/unexplained infertility.[15,18,32,33] By minimising the influence of the female factor using donor oocytes, the current study demonstrates that the heterogeneous amounts of retained sperm histones in normozoospermic individuals can affect fertilisation and embryo quality, thereby impacting the pregnancy outcome. Importantly, altered epigenetic signatures in spermatozoa may be passed on to the next generation, potentially impacting the health of the offspring.[81,83–85] Patients with abnormal semen characteristics were not included because the sperm numbers obtained after the ICSI procedure were not adequate to perform epigenetic analysis. Due to restrictions on using human embryos for research, we were unable to determine the underlying molecular mechanism responsible for the retained histone H3 and H3K27Me3-mediated alterations during fertilisation, development of the embryos, and changes in the levels of methionine in SCM.

5 | CONCLUSION

Heterogenous levels of retained histones in spermatozoa can be one of the factors underlying the aetiology of idiopathic male infertility. Such spermatozoa may influence fertilisation, the quality of the embryos, and embryo metabolism, thereby affecting the assisted reproductive technology outcome. These results, although descriptive in nature, warrant further research to address the underlying mechanisms behind these clinically important observations.

AUTHOR CONTRIBUTIONS

Satish Kumar Adiga conceived and designed the experiments. Riddhi Kirit Pandya, Ameya Jijo, Aswathi Cheredath and Sujith Raj Salian performed the experiments and was involved in the acquisition of data. Riddhi Kirit Pandya, Vani Lakshmi R. and Shubhashree Uppangala analysed and interpreted the data. Satish Kumar Adiga, Riddhi Kirit Pandya and Sanjay Gupta wrote the manuscript. Guruprasad Kalthur, Sanjay Gupta and Pratap Kumar revised the manuscript critically for important intellectual content. Riddhi Kirit Pandya is the guarantor of this work and as such, has full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have given final approval for publication.

ACKNOWLEDGEMENTS

The facilities provided by the NMR Research Centre at the Indian Institute of Science, Bengaluru, and the Advanced Centre for Treatment, Research, and Education in Cancer, Tata Memorial Centre, Navi Mumbai, India, are gratefully acknowledged. Aswathi Cheredath and Ameya Jijo acknowledge the Dr. TMA Pai Doctoral Fellowship from the Manipal Academy of Higher Education. This work was supported by research grants from the Indian Council of Medical Research (ICMR # 5/10/FR/8/2014-RCH).

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data related to this article are available in both the article and the online supporting information.

ORCID

Guruprasad Kalthur https://orcid.org/0000-0002-4554-2917
Satish Kumar Adiga https://orcid.org/0000-0002-2897-4697

REFERENCES

1. Sutton WS. The chromosomes in heredity. *Biol Bull*. 1903(4):231-251.
2. Baccetti B, Afzelius BA. The biology of the sperm cell. *Monogr Dev Biol*. 1976(10):1-254.
3. Sathananthan AH, Kola I, Osborne J, et al. Centrioles at the beginning of human development. *Proc Natl Acad Sci U S A*. 1991;88(11):4806-4810.
4. Saunders CM, Larman MG, Parrington J, et al. PLC zeta: a sperm-specific trigger of Ca (2+) oscillations in eggs and embryo development. *Development*. 2002;129(15):3533-3544.
5. Castillo J, Estanyol JM, Ballescá JL, Oliva R. Human sperm chromatin epigenetic potential: genomics, proteomics, and male infertility. *Asian J Androl*. 2015;17(4):601-609.
6. Castillo J, Bogle OA, Jodar M, et al. Proteomic changes in human sperm during sequential in vitro capacitation and acrosome reaction. *Front Cell Dev Biol*. 2019;7:295.
7. Siklenka K, Erkek S, Godmann M, et al. Disruption of histone methylation in developing sperm impairs offspring health transgenerationally. *Science*. 2015;350(6261):aab2006.
8. Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. *Nature*. 2009;460(7254):473-478.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

9. Gross N, Strillacci MG, Peñagaricano F, Khatib H. Characterization and functional roles of paternal RNAs in 2-4 cell bovine embryos. *Sci Rep*. 2019;9(1):20347.
10. Azpiazu R, Amaral A, Castillo J, et al. High-throughput sperm differential proteomics suggests that epigenetic alterations contribute to failed assisted reproduction. *Hum Reprod*. 2014;29(6):1225-1237.
11. Chen Q, Yan M, Cao Z, et al. Sperm tsRNAs contribute to the intergenerational inheritance of an acquired metabolic disorder. *Science*. 2016;351(6271):397-400.
12. Dimofski P, Meyre D, Dreumont N, Leininger-Muller B. Consequences of paternal nutrition on offspring health and disease. *Nutrients*. 2021;13(8):2818.
13. Miller D, Brinkworth M, Iles D. Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction*. 2010;139(2):287-301.
14. Schagdarsurengin U, Paradowska A, Steger K. Analysing the sperm epigenome: roles in early embryogenesis and assisted reproduction. *Nat Rev Urol*. 2012;9(11):609-619.
15. Schon SB, Luense LJ, Wang X, et al. Histone modification signatures in human sperm distinguish clinical abnormalities. *J Assist Reprod Genet*. 2019;36(2):267-275.
16. Arpanahi A, Brinkworth M, Iles D, et al. Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. *Genome Res*. 2009;19(8):1338-1349.
17. Brykczynska U, Hisano M, Erkek S, et al. Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nat Struct Mol Biol*. 2010;17(6):679-687.
18. Vieweg M, Dvorakova-Hortova K, Dudkova B, et al. Methylation analysis of histone H4K12ac-associated promoters in sperm of healthy donors and subfertile patients. *Clin Epigenetics*. 2015;7(1):31.
19. van der Heijden GW, Ramos L, Baart EB, et al. Sperm-derived histones contribute to zygotic chromatin in humans. *BMC Dev Biol*. 2008;8:34.
20. Paradowska AS, Miller D, Spiess AN, et al. Genome wide identification of promoter binding sites for H4K12ac in human sperm and its relevance for early embryonic development. *Epigenetics*. 2012;7(9):1057-1070.
21. van de Werken C, van der Heijden GW, Eleveld C, et al. Paternal heterochromatin formation in human embryos is H3K9/HP1 directed and primed by sperm-derived histone modifications. *Nat Commun*. 2014;5:5868.
22. Aoki VW, Liu L, Carrell DT. Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. *Hum Reprod*. 2005;20(5):1298-1306.
23. Aoki VW, Liu L, Jones KP, et al. Sperm protamine 1/protamine 2 ratios are related to in vitro fertilization pregnancy rates and predictive of fertilization ability. *Fertil Steril*. 2006;86(5):1408-1415.
24. de Mateo S, Gázquez C, Guimerà M, et al. Protamine 2 precursors (pre-P2), protamine 1 to protamine 2 ratio (P1/P2), and assisted reproduction outcome. *Fertil Steril*. 2009;91(3):715-722.
25. Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT. Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertil Steril*. 2010;94(5):1728-1733.
26. Hammoud SS, Nix DA, Hammoud AO, Gibson M, Cairns BR, Carrell DT. Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. *Hum Reprod*. 2011;26(9):2558-2569.
27. Jenkins TG, Aston KI, Hotaling JM, Shamsi MB, Simon L, Carrell DT. Teratozoospermia and asthenozoospermia are associated with specific epigenetic signatures. *Andrology*. 2016;4(5):843-849.
28. Denomme MM, McCallie BR, Parks JC, Schoolcraft WB, Katz-Jaffe MG. Alterations in the sperm histone-retained epigenome are associated with unexplained male factor infertility and poor blastocyst development in donor oocyte IVF cycles. *Hum Reprod*. 2017;32(12):2443-2455.
29. White CR, Denomme MM, Tekpetey FR, Feyles V, Power SG, Mann MR. High frequency of imprinted methylation errors in human preimplantation embryos. *Sci Rep*. 2015;5:17311.
30. Stuppia L, Franzago M, Ballerini P, Gatta V, Antonucci I. Epigenetics and male reproduction: the consequences of paternal lifestyle on fertility, embryo development, and children lifetime health. *Clin Epigenetics*. 2015;7:120.
31. Esteves SC, Roque M, Bedoschi G, Haahr T, Humaidan P. Intracytoplasmic sperm injection for male infertility and consequences for offspring. *Nat Rev Urol*. 2018;15(9):535-562.
32. La Spina FA, Romanato M, Brugo-Olmedo S, et al. Heterogeneous distribution of histone methylation in mature human sperm. *J Assist Reprod Genet*. 2014;31(1):45-49.
33. Luense LJ, Wang X, Schon SB, et al. Comprehensive analysis of histone post-translational modifications in mouse and human male germ cells. *Epigenet Chromatin*. 2016;9:24.
34. Lazaraviciute G, Kauser M, Bhattacharya S, Haggarty P, Bhattacharya S. A systematic review and meta-analysis of DNA methylation levels and imprinting disorders in children conceived by IVF/ICSI compared with children conceived spontaneously. *Hum Reprod Update*. 2014;20(6):840-852.
35. Cortessis VK, Azadian M, Buxbaum J, et al. Comprehensive meta-analysis reveals association between multiple imprinting disorders and conception by assisted reproductive technology. *J Assist Reprod Genet*. 2018;35(6):943-952.
36. World Health Organization. WHO laboratory manual for the examination and processing of human semen. *World Health Organization*; 2021.
37. Meitei HY, Uppangala S, Sharan K, et al. A simple, centrifugation-free, sperm-sorting device eliminates the risks of centrifugation in the swim-up method while maintaining functional competence and DNA integrity of selected spermatozoa. *Reprod Sci*. 2021;28(1):134-143.
38. Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J Androl*. 2003;24(1):59-66.
39. Tandara M, Bajić A, Tandara L, et al. Sperm DNA integrity testing: big halo is a good predictor of embryo quality and pregnancy after conventional IVF. *Andrology*. 2014;2(5):678-686.
40. Shechter D, Dormann HL, Allis CD, Hake SB. Extraction, purification and analysis of histones. *Nat Protoc*. 2007;2(6):1445-1457.
41. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Hum Reprod*. 2011;26(6):1270-1283.
42. Cheredath A, Uppangala S, Asampille G, et al. Duration of dry and humidified incubation of single-step embryo culture medium and oxygen tension during sham culture do not alter medium composition. *F1000Res*. 2022;11:242.
43. The Jamovi Project 2021 (Jamovi version 2.2). Computer software. Accessed 17 Mar 2022. RRID:SCR_016142.
44. Krejčí J, Stixová L, Pagáčová E, et al. Post-translational modifications of histones in human sperm. *J Cell Biochem*. 2015;116(10):2195-2209.
45. Yu B, Zhou H, Liu M, et al. Epigenetic alterations in density selected human spermatozoa for assisted reproduction. *PLoS One*. 2015;10(12):e0145585.
46. Godmann M, Auger V, Ferraroni-Aguiar V, et al. Dynamic regulation of histone H3 methylation at lysine 4 in mammalian spermatogenesis. *Biol Reprod*. 2007;77(5):754-764.
47. Štiavnická M, García-Álvarez O, Ulčová-Gallová Z, et al. H3K4me2 accompanies chromatin immaturity in human spermatozoa: an epigenetic marker for sperm quality assessment. *Syst Biol Reprod Med*. 2020;66(1):3-11.
48. Zhang B, Zheng H, Huang B, et al. Allelic reprogramming of the histone modification H3K4me3 in early mammalian development. *Nature*. 2016;537(7621):553-557.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

49. Boissonnas CC, Jouannet P, Jammes H. Epigenetic disorders and male subfertility. *Fertil Steril*. 2013;99(3):624-631.
50. Mozzetta C, Boyarchuk E, Pontis J, Ait-Si-Ali S. Sound of silence: the properties and functions of repressive Lys methyltransferases. *Nat Rev Mol Cell Biol*. 2015;16(8):499-513.
51. Peters AH, Mermoud JE, O'Carroll D, et al. Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. *Nat Genet*. 2002;30(1):77-80.
52. Carrell DT, Liu L. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. *J Androl*. 2001;22(4):604-610.
53. Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod*. 1991;44(4):569-574.
54. Zini A, Kamal KM, Phang D. Free thiols in human spermatozoa: correlation with sperm DNA integrity. *Urology*. 2001;58(1):80-84.
55. Zini A, Zhang X, San Gabriel M. Sperm nuclear histone H2B: correlation with sperm DNA denaturation and DNA stainability. *Asian J Androl*. 2008;10(6):865-871.
56. Wu J, Xu J, Liu B, et al. Chromatin analysis in human early development reveals epigenetic transition during ZGA. *Nature*. 2018;557(7704):256-260.
57. van der Werken C, van der Heijden GW, Eleveld C, et al. Paternal heterochromatin formation in human embryos is H3K9/HP1 directed and primed by sperm-derived histone modifications. *Nat Commun*. 2014;5:5868.
58. Ihara M, Meyer-Ficca ML, Leu NA, et al. Paternal poly (ADP-ribose) metabolism modulates retention of inheritable sperm histones and early embryonic gene expression. *PLoS Genet*. 2014;10(5):e1004317.
59. van der Heijden GW, Derijck AA, Ramos L, Giele M, van der Vlag J, de Boer P. Transmission of modified nucleosomes from the mouse male germline to the zygote and subsequent remodeling of paternal chromatin. *Dev Biol*. 2006;298(2):458-469.
60. Liu X, Wang C, Liu W, et al. Distinct features of H3K4me3 and H3K27me3 chromatin domains in pre-implantation embryos. *Nature*. 2016;537(7621):558-562.
61. Fournier C, Labrune E, Lornage J, et al. The impact of histones linked to sperm chromatin on embryo development and ART outcome. *Andrology*. 2018;6(3):436-445.
62. Tsuribe PM, Lima Neto JF, de Assis Golim M, de Paula Freitas Dell'Aqua C, Issa JP, Gobbo CAM. Assessment of sperm DNA in patients submitted the assisted reproduction technology procedures. *JBRA Assist Reprod*. 2016;20(1):17-22.
63. Seli E, Vergouw CG, Morita H, et al. Noninvasive metabolomic profiling as an adjunct to morphology for noninvasive embryo assessment in women undergoing single embryo transfer. *Fertil Steril*. 2010;94(2):535-542.
64. Pudakalakatti SM, Uppangala S, D'Souza F, et al. NMR studies of preimplantation embryo metabolism in human assisted reproductive techniques: a new biomarker for assessment of embryo implantation potential. *NMR Biomed*. 2013;26(1):20-27.
65. D'Souza F, Uppangala S, Asampille G, et al. Spent embryo culture medium metabolites are related to the in vitro attachment ability of blastocysts. *Sci Rep*. 2018;8(1):17025.
66. Uppangala S, Pudakalakatti S, D'souza F, et al. Influence of sperm DNA damage on human preimplantation embryo metabolism. *Reprod Biol*. 2016;16(3):234-241.
67. Tarahomi M, Vaz FM, van Straalen JP, et al. The composition of human preimplantation embryo culture media and their stability during storage and culture. *Hum Reprod*. 2019;34(8):1450-1461.
68. Leese HJ, McKeegan PJ, Sturmey RG. Amino acids and the early mammalian embryo: origin, fate, function and life-long legacy. *Int J Environ Res Public Health*. 2021;18(18):9874.
69. Drábková P, Andrlová L, Hampl R, Kanďár R. Amino acid metabolism in human embryos. *Physiol Res*. 2016;65(5):823-832.
70. Fulka H, Mrazek M, Tepla O. DNA methylation pattern in human zygotes and developing embryos. *Reproduction*. 2004;128(6):703-708.
71. Menezo Y, Clément P, Dale B. DNA methylation patterns in the early human embryo and the epigenetic/imprinting problems: a plea for a more careful approach to human assisted reproductive technology (ART). *Int J Mol Sci*. 2019;20(6):1342.
72. Bonilla L, Luchini D, Devillard E, Hansen PJ. Methionine requirements for the preimplantation bovine embryo. *J Reprod Dev*. 2010;56(5):527-532.
73. Ikeda S, Sugimoto M, Kume S. Importance of methionine metabolism in morula-to-blastocyst transition in bovine preimplantation embryos. *J Reprod Dev*. 2012;58(1):91-97.
74. Brison DR, Houghton FD, Falconer D, et al. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod*. 2004;19(10):2319-2324.
75. Houghton FD, Hawkhead JA, Humpherson PG, et al. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod*. 2002;17(4):999-1005.
76. Wallace M, Cottell E, Cullinane J, McAuliffe FM, Wingfield M, Brennan L. (1)H NMR based metabolic profiling of day 2 spent embryo media correlates with implantation potential. *Syst Biol Reprod Med*. 2014;60(1):58-63.
77. Steele W, Allegrucci C, Singh R, et al. Human embryonic stem cell methyl cycle enzyme expression: modelling epigenetic programming in assisted reproduction? *Reprod Biomed Online*. 2005;10(6):755-766.
78. van Winkle LJ, Ryznar R. One-carbon metabolism regulates embryonic stem cell fate through epigenetic DNA and histone modifications: implications for transgenerational metabolic disorders in adults. *Front Cell Dev Biol*. 2019;7:300.
79. Carone BR, Fauquier L, Habib N, et al. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell*. 2010;143(7):1084-1096.
80. Ng SF, Lin RC, Laybutt DR, Barres R, Owens JA, Morris MJ. Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature*. 2010;467(7318):963-966.
81. Schaefer S, Nadeau JH. The genetics of epigenetic inheritance: modes, molecules, and mechanisms. *Q Rev Biol*. 2015;90(4):381-415.
82. Wei J, Sun X, Chen Y, et al. Perinatal exposure to bisphenol A exacerbates nonalcoholic steatohepatitis-like phenotype in male rat offspring fed on a high-fat diet. *J Endocrinol*. 2014;222(3):313-325.
83. Champroux A, Cocquet J, Henry-Berger J, Drevet JR, Kocer A. A decade of exploring the mammalian sperm epigenome: paternal epigenetic and transgenerational inheritance. *Front Cell Dev Biol*. 2018;6:50.
84. Xu X, Miao Z, Sun M, Wan B. Epigenetic mechanisms of paternal stress in offspring development and diseases. *Int J Genomics*. 2021:6632719.
85. Fitz-James MH, Cavalli G. Molecular mechanisms of transgenerational epigenetic inheritance. *Nat Rev Genet*. 2022;23(6):325-341.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Pandya RK, Jijo A, Cheredath A, et al. Differential sperm histone retention in normozoospermic ejaculates of infertile men negatively affects sperm functional competence and embryo quality. *Andrology*. 2024;12:881–890. https://doi.org/10.1111/andr.13541

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13541 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 31 July 2023 | Revised: 21 September 2023 | Accepted: 5 October 2023

DOI: 10.1111/andr.13546

ANDROLOGY

WILEY

ORIGINAL ARTICLE

Testicular function after non-cytotoxic and immunotherapy drug treatment

David J. Handelsman[1,2] | Amanda Idan[1] | Sue Sleiman[1] | Fey Bacha[1] | Georgina V. Long[3] | Alexander M. Menzies[3] | Tejnei Vaishnav[4] | Noosha Litkouhi[4] | Xanthie Volckmar[4] | William Ledger[4,5] | Antoinette Anazodo[4,5,6]

[1]Andrology Department and Clinical Andrology Laboratory, Concord Hospital, Sydney, New South Wales, Australia

[2]ANZAC Research Institute, University of Sydney, Sydney, New South Wales, Australia

[3]Melanoma Institute of Australia, University of Sydney, Royal North Shore & Mater Hospitals, Sydney, New South Wales, Australia

[4]School of Women's and Childrens Health, Royal Hospital for Women, Sydney, New South Wales, Australia

[5]University of New South Wales, Sydney, New South Wales, Australia

[6]Sydney Children's Hospital, Sydney, New South Wales, Australia

Correspondence
David J. Handelsman, ANZAC Research Institute, University of Sydney, Sydney, 2139 NSW, Australia.
Email: djh@sydney.edu.au

Funding information
NHMRC Investigator Grants, University of Sydney; Nicholas and Hele Moore and Melanoma Institute of Australia

Abstract

Background: The effects of novel non-cytotoxic and immunotherapy drugs for cancer treatment on human testicular function have not been studied systematically.

Objectives: The present study aimed to characterize effects of non-cytotoxic and immunotherapy drugs in patients with cancers who had not been previously treated with gonadotoxic chemo- or radiotherapy.

Materials and methods: This study involved 34 men, not previously treated with gonadotoxic regimens, in a mixed longitudinal (Cohort 1: 19 men about to start and approximately 1 year on non-cytotoxic and immunotherapy treatment) and cross-sectional (Cohort 2: 15 men already on non-cytotoxic and immunotherapy treatment) study using data modeling to estimate within-person time-course changes in testicular exocrine and endocrine functions. Cohort 1 provided 45 paired semen and blood samples (34 prior to and nine during treatment) and Cohort 2 provided 45 sets of samples (15 pre-treatment, 30 on treatment), including six men in Cohort 2 who had pre-treatment spermatozoa cryostorage prior to the study. Men on non-cytotoxic and immunotherapy treatment had undergone a median of 33.5 months long-term treatment.

Results: Spermatozoa output and concentration were reduced by about 50%, with corresponding increases in serum follicle-stimulating hormone and decreases in serum inhibin B. Serum testosterone, luteinizing hormone, and sex hormone-binding globulin were unaffected by non-cytotoxic and immunotherapy treatment.

Conclusion: Within limits of the present study of sample size and duration of on-non-cytotoxic and immunotherapy treatment, non-cytotoxic and immunotherapy drugs have a modest effects on testicular exocrine function (sperm production) or its hormonal correlates (follicle-stimulating hormone, inhibin B), with minimal impact on testicular endocrine (testosterone, luteinizing hormone) function.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2023 Commonwealth of Australia. *Andrology* published by Wiley Periodicals LLC on behalf of American Society of *Andrology* and European Academy of *Andrology*.

KEYWORDS
AMH, cancer, chronic myeloid leukemia, enzyme inhibitors, FSH, immune checkpoint inhibitors, inhibin B, melanoma, non-cytotoxic drugs, spermatozoa, testis, testosterone

1 | INTRODUCTION

Over decades, prognosis for long-term remission or cure of cancers has improved due to therapeutic advances in conventional treatment based on chemotherapy and/or radiotherapy.[1] For the increasing numbers of long-term survivors, quality of life is a pivotal concern, with reproductive function and fertility of prominent concern for younger men. However, both conventional treatment modalities are gonadotoxic creating severe, and often irreversible, spermatogenic damage, leading to male infertility and/or hypogonadism.[2] Gonadotoxic spermatogenic damage reflects the exquisite sensitivity of the intensely replicating germinal epithelium to cytotoxic drugs and irradiation, a sensitivity even greater than the often dose-limiting bone marrow toxicity of cancer treatments. Impaired reproductive function and fertility have serious adverse effects on survivor's quality of life, creating psychological distress in current and future relationships.[3–6]

Hence, fertility preservation procedures aiming to preserve or protect reproductive potential arising from gonadotoxic therapy have developed as fertility insurance to minimize the impact of treatment on a patient's future fertility.[7,8] The original, most widely available fertility preservation procedure is sperm cryostorage, a long standard clinical practice recommended by most international guidance documents on comprehensive cancer care,[9,10] and the forerunner of the new oncofertility discipline.[11] Over decades, well-established spermatozoa banking programs have been available for men with cancer or non-cancer disorders where medical gonadotoxic treatment involves risk of temporary or permanent male infertility.[12–14]

An important contemporary advance in cancer treatment has been the advent of non-cytotoxic regimens including immunotherapy using monoclonal antibody checkpoint inhibitors,[15] as well as small molecule enzyme inhibitors.[16] Conventional cancer treatment aims to directly kill any replicating cells, albeit indiscriminately, leading to off-target effects damaging rapidly proliferating epithelia such as the germinal epithelium as well as the often dose-liming bone marrow toxicity and bystander damage to spermatogenesis. Such treatment regimens are provided in discrete treatment periods of limited duration, leaving residual gonadotoxic damage in its long-term wake. In contrast, newer non-cytotoxic agents have a different molecular mechanism of action, with selective immunological or biochemical targeting to malignant cells, but sparing the intensely replicative reproductive and bone marrow epithelia. Furthermore, such non-cytotoxic treatments are deployed differently, intended as ongoing treatment that could turn malignant disease into a chronic, treatable disease like diabetes, epilepsy, hypertension, heart failure or HIV infection. Such new non-cytotoxic and immunotherapy (NCIT) drugs have been successfully applied to improving prognosis for several cancers, notably melanoma, lung cancer, lymphoma, and leukemia with wider application anticipated.[17]

Currently, there are limited clinical data on NCIT drug effects on human reproductive function. A comprehensive review of 32 new non-cytotoxic oncology drugs marketed between 2014 and 2018 by the US Federal Drug Administration or the Australian Therapeutic Goods Administration identified no human studies of reproductive effects for any of these drugs.[18] Furthermore, pre-clinical data were very limited, with nine drugs showing impaired male fertility and three showing impaired male and female fertility. As a result, it remains prudent for men to undertake sperm cryostorage prior to NCIT treatments. Nevertheless, as the impact of these newer NCIT drugs on male fertility remains largely unknown, we aimed to characterize the impact of novel NCIT drugs on male reproductive function in patients not previously treated with gonadotoxic drugs or irradiation (Figure 1).

2 | METHODS

2.1 | Study design

This observational study had a mixed longitudinal and cross-sectional design with two parallel cohorts of men with cancer or non-cancer diseases who had planned, or had undergone, treatment with NCIT drugs. Participants were recruited from two centers, the Andrology Department, Concord Hospital and the Royal Hospital for Women, both in Sydney, NSW, Australia.

Cohort 1 involved longitudinal investigation of men scheduled to be treated with NCIT drugs who had not been treated with cytotoxic drugs or radiotherapy. They were studied before and on long-term NCIT drugs at about 12 months after starting treatment. Participants were usually referred for sperm cryostorage as part of standard care. Men who had stored spermatozoa before their treatment were already involved in routine follow-up to determine ongoing need for sperm cryostorage.[14] Cohort 2 included men who were already on NCIT drug treatment who were referred to participate in this observational study. Some men in Cohort 2 had previously cryostored spermatozoa prior to entering this study and those data if available were used.

The planned sample size was 25 men in each cohort. Formal pre-study sample size projections were not feasible due to the lack of suitable prior data on NCIT drug effects. Consequently, the sample size was based on what would be expected to be significant spermatogenic damage on conventional gonadotoxic treatments. The study, including the written informed consent form, was approved by the Sydney Local Health District Human Ethics Committee (Concord Hospital).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13546 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

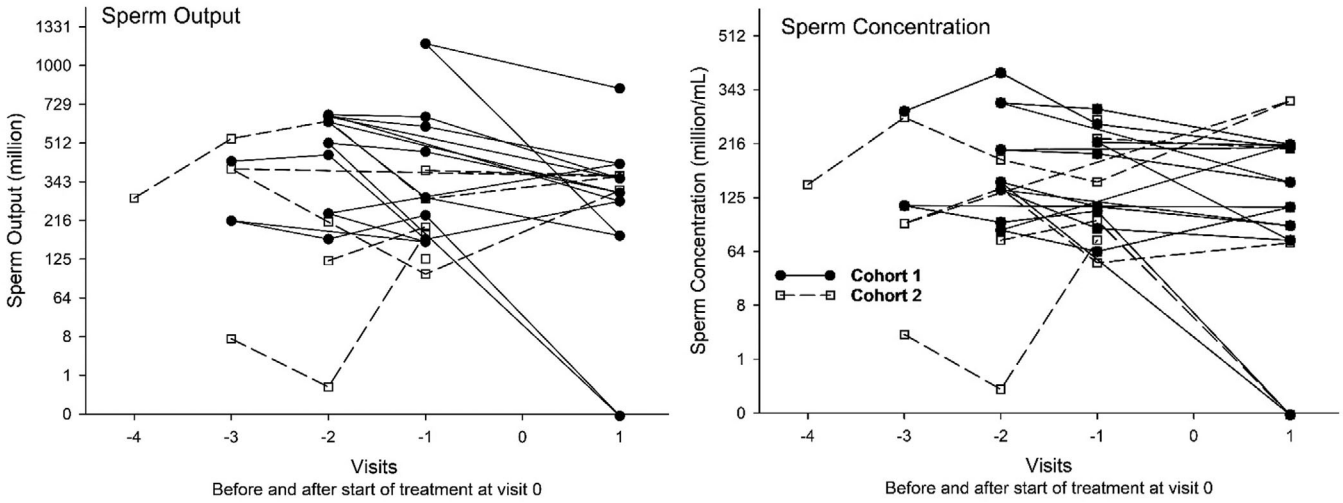


FIGURE 1 Plots of sperm output (million spermatozoa per ejaculate, left panel) and sperm concentration (million spermatozoa per milliliter ejaculate, right panel) of study participants who had multiple pre-treatment and on-treatment semen analyses due to their use of sperm cryostorage either coinciding with recruitment to the study for non-cytotoxic and immunotherapy (NCIT) treatment (Cohort 1) or having been completed before starting NCIT treatment (Cohort 2). The results for each man are plotted as connected symbols and lines with 15 men in Cohort 1 (filled circles, straight lines) and six men in Cohort 2 (open squares, dashed lines). The *y* axis is on a cube-root scale, which optimizes normalization of sperm distributions, and the *x* axis represents sequential visits before and after the start of NCIT treatments, with the start of treatment indicated as the 0 visit and uneven time intervals between visits. Prior to start of treatment, men were having spermatozoa cryostored, and the visits were usually within 1–2 weeks. After treatment, the men in Cohort 1 were scheduled to be studied at 12 months after start of treatment. However, the time on treatment for Cohort 2 was by definition longer and more variable, as they only entered the study after the start of NCIT treatment. For further details see text.

2.2 | Study procedures

The inclusion criteria were (i) aged between 18 and 65 years, (ii) diagnosis of cancer or non-cancer disease requiring treatment with NCIT drugs (immunotherapy checkpoint or enzyme inhibitors or analogous non-cytotoxic drugs), and (iii) provide written informed consent to participate in the research including undergoing additional tests and providing blood and semen samples. Exclusion criteria were (a) having received chemotherapy or pelvic radiotherapy prior to current treatment, and (b) major psychiatric disease or psychological condition that in the investigator's opinion, limited participant's understanding of, and compliance with, study requirements. After providing written informed consent, men completed a standardized medical and reproductive history, underwent physical examination (including testicular size), and provided blood and semen samples. At entry, men provided information on their reproductive, marital and fertility status, smoking, and alcohol consumption, as well as age, height, and weight. Their underlying diseases were classified as melanoma, hematological, or other, with treatments classified as antibody-based and/or enzyme inhibitor. At subsequent visits, the men again provided semen and blood samples.

2.3 | Semen analyses and sperm cryostorage

Semen analysis was conducted according to the WHO Semen Analysis Manual[19] in a NATA-accredited Andrology laboratory, providing semen analysis and sperm cryostorage, and consistent with the journal's guidelines for semen analysis.[20] In addition to routine semen analysis (abstinence interval, semen volume, sperm concentration and output, sperm motility and morphology), sperm DNA fragmentation was measured by Halosperm G2, an improved commercial version of the Halosperm sperm chromatin dispersion test.[21]

2.4 | Reproductive hormone assays

As far as possible, blood samples were obtained non-fasting in the mornings, but collections during sperm cryostorage were opportunistic and considered at random times. Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and sex hormone-binding globulin (SHBG) were measured as they were drawn in routine hospital pathology laboratory using commercial (Roche) immunoassays, subject to routine internal and external quality control programs. Sera were also stored frozen for analysis in a single batch by liquid chromatography-mass spectrometry (serum testosterone, dihydrotestosterone, dehydroepiandrosterone, estradiol, estrone, 3α androstanediol, and 3β androstanediol),[22] as well as serum inhibin B and AntiMullerian Hormone (AMH) by ELISA immunoassays (ANSH laboratories, Banksia Scientific) performed according to the manufacturer's instruction.

2.5 | Data analysis

The primary study endpoints were the impact of NCIT drug treatment on sperm output and concentration in gonadotoxic-naïve men with

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13546 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

cancer. Secondary endpoint included semen volume, sperm motility and morphology, serum testosterone, LH, FSH, SHBG, and testicular volume.

The impact of NCIT drugs on the primary outcomes (sperm output, concentration) was analyzed by mixed model analysis for repeated measures using an autoregressive covariance structure with Fisher scoring and Newton–Raphson maximization (NCSS 2022 Statistical Software, NCSS). This method fits an intercept for each individual with the slope of the regression based exclusively on the sperm parameters from that individual. This optimizes the integrated analysis of longitudinal and cross-sectional data with unequal numbers of samples and spacing of endpoints, and circumvents the bias or inefficiency of reducing the data to individuals or samples alone. Covariate adjustments were performed for continuous (age, height, weight, body mass index, body surface area) or categorical (type of NCIT drug [antibody, enzyme inhibitor], alcohol, smoking, fertility status) variables.

Descriptive summary of variables was reported as mean and standard error of the mean (for gaussian data) or median with interquartile range (for non-gaussian data). Normality of data distribution was evaluated by the Shapiro–Wilks test together with the Box–Cox transformation to identify optimal power transformations to normalize non-gaussian distributions prior to parametric analyses. Sperm concentration and output were right-skewed and cube-root transformed[23] with serum testosterone, FSH, and DNA fragmentation log transformed and serum SHBG cube-root transformed prior to analysis. After analysis, transformed variables were backtransformed for ease of interpretation. Each semen analysis was classified as pre-treatment or on NCIT treatment.

3 | RESULTS

3.1 | Patients (Table 1)

The 34 participants were divided into 19 men in Cohort 1 providing 45 paired semen and blood samples (34 prior to and nine during treatment), and another 15 in Cohort 2 providing 45 sets of samples (15 pre-treatment, 30 on treatment), which included six men in Cohort 2 also having had pre-treatment sperm cryostorage prior to the study. Men on NCIT treatment had undergone a median of 33.5 (IQR: 8–55) months of treatment.

Overall, most were married or de facto (22/33), but only a minority had prior paternity (10/35, including one successful sperm donor prior to illness), and none had prior infertility. Two men on treatment had produced a pregnancy in a partner. Most were non-smokers (never = 25, current = 5, past = 3), and reported light alcohol consumption (never, light, or social drinking = 30, heavy or binge = 3), and none had cryptorchidism.

Their underlying diseases were melanoma ($n = 16$), hematological (chronic myeloid leukemia = 10, lymphoma = 1), and other ($n = 7$; multiple sclerosis = 2, liver cancer = 1, medullary thyroid cancer = 1, lung cancer = 1, sarcoma = 1, systemic lupus nephropathy = 1). Treatments were antibody-based ($n = 21$), small molecule enzyme inhibitor ($n = 14$) or both ($n = 3$) in the following therapeutic classes: monoclonal antibody (rituximab, belimumab, pembrolizumab), checkpoint inhibitor (nivolumab), immunomodulator (natalizumab), kinase inhibitors (imatinib, vandetanib, dasatenib, nilotinib, erlotinib, levatinib, dabrafenib, trametinib, encorafinib, binimetanib), ALK kinase inhibitor (alectanib), or BCR-ABL tyrosinase kinase inhibitor (asciminib).

3.2 | Sperm output and function

By linear mixed model for repeated measures, sperm output ($p < 0.001$) and concentration ($p = 0.005$) were significantly lower on treatment compared with pre-treatment when adjusted for semen volume (see also Table 2 and Figure 1). For either sperm outcome, none of the covariables (age, height, weight, body mass index, body surface area, marital status, fertility status, number of children, alcohol, smoking, antibody treatment, enzyme inhibitor treatment, or the combination of treatments) had significant influence on that difference (data not shown). One man with melanoma had normal sperm output (median 133 million) and serum FSH (7.3 IU/L) at entry, but after 18 months of treatment with both antibody and enzyme inhibitors was azoospermic, with marked elevation of serum FSH (22.5 IU/L) but no other known gonadotoxin exposure.

Semen volume was not different according to NCIT treatment, but was significantly lower in men who were single versus married, de facto or separated (2.8 vs. 3.6 mL, $p = 0.043$) or with proven versus unknown fertility (3.1 vs. 4.7 mL, $p = 0.014$), but not influenced by any other covariables. Sperm motility and morphology and DNA fragmentation were not significantly different according to NCIT treatment or any covariates.

3.3 | Serum reproductive hormones

NCIT treatment was associated with increased serum FSH (6.5 vs. 2.6 IU/L, $p = 0.005$) and decreased serum Inhibin B (218 vs. 293 pg/mL, $p = 0.020$). Serum testosterone, LH, and SHBG did not show any difference according to NCIT treatment, with or without adjustment for all covariates (excluding sperm output or concentration, data not shown).

4 | DISCUSSION

The present study demonstrates that among men not previously exposed to therapeutic gonadotoxicity, NCIT drug treatments reduce sperm output by about 50% after a median of over 30 months on treatment. The reduction in sperm production was associated with increased serum FSH and decreased serum inhibin B, expected effects of any drug having adverse impact on spermatogenesis.[24,25] One man treated with both antibody and enzyme inhibitor drugs for 18 months displayed severe reduction of sperm production, becoming azoospermic with highly elevated serum FSH and undetectable inhibin B consistent with severe spermatogenic damage. No specific

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13546 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Baseline descriptive data of male participants.

Variable	Number	Mean ± SEM	Median [IQR]
Age (years)	34	31.9 ± 1.2	32 [26–38]
Height (cm)	32	180 ± 1.5	181 [173–185]
Weight (kg)	32	87.0 ± 3.1	84.0 [74.0–96.0]
BMI (kg/m^2)	32	26.8 ± 0.9	26.0 [23.9–30.3]
BSA (m^2)	32	2.09 ± 0.04	2.05 [1.93–2.20]
Mean testis volume (mL)	32	25.6 ± 1	25 [22–29]
Semen volume (mL)	34	3.5 ± 0.3	3.6 [2.2–4.3]
Sperm density (million per mL)	34	82 ± 12	57 [21–136]
Total sperm output (million)	34	295 ± 47	203 [76–478]
Sperm motility (%)	34	30 ± 4	39 [0.6–49]
Sperm morphology (%)	25	4.9 ± 0.8	4.0 [2.0–7.5]
Sperm DNA fragmentation (%)	17	19 ± 5	11 [8.5–22]
Serum testosterone[a] (ng/mL)	21	5.0 ± 0.4	5.0 [3.3–6.3]
Serum LH (IU/L)	23	5.6 ± 0.6	4.5 [3.6–6.7]
Serum FSH (IU/L)	23	4.0 ± 0.5	2.9 [2.2–5.5]
Serum SHBG (nmol/L)	22	38 ± 4	32 [25–46]
Serum inhibin B (pg/mL)	21	270 ± 20	242 [215–326]
Serum AMH (ng/mL)	21	10.4 ± 1.0	7.8 [7.4–14.9]
Serum testosterone[b] (ng/mL)	21	6.2 ± 0.5	6.4 [4.0–7.5]
Serum DHT (ng/mL)	21	0.51 ± 0.04	0.51 [0.38–0.66]
Serum DHEA (ng/mL)	21	4.8 ± 0.6	5.2 [2.4–5.9]
Serum estradiol (pg/mL)	21	46 ± 7	42 [26–63]
Serum estrone (pg/mL)	21	33 ± 4	29 [19–39]
Serum 3α androstanediol (ng/mL)	21	0.47 ± 0.08	0.60 [0.07–0.80]
Serum 3β androstanediol (ng/mL)	21	0.82 ± 0.02	0.81 [0.75–0.88]

Note: Data are expressed as mean ± standard error of the mean (SEM) and as median and interquartile range (IQR) [Q1–Q3].
Abbreviations: AMH, AntiMullerian Hormone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBD, sex hormone-binding globulin.
[a]Measured by testosterone immunoassay as samples became available.
[b]Measured batchwise at end of study by high-pressure liquid chromatography mass spectrometry.

cause was identified, but autoimmune orchitis triggered by checkpoint inhibitor treatment[26,27] was not excluded, although the lack of testicular pain and swelling made it unlikely. The changes in sperm production could not be attributed statistically to effects of antibody or enzyme inhibitor treatments alone, or their combination. The potential contribution from autoimmune hypophysitis[28] contributing to partial gonadotrophin deficiency, representing a potentially reversible mechanism for the reduction in sperm output, could not be excluded; however, none of the men in this study had clinical evidence of hypophysitis, which, while a well-recognized, is often temporary and/or reversible, complication of immunotherapy treatments,[29] remains rare with a prevalence of about 1.5%.[29,30] The lack of significant change in serum testosterone, LH, and SHBG indicates that androgen deficiency is unlikely because of NCIT treatments. Overall, however, these effects on sperm production and its related hormones (FSH, Inhibin B) appear substantially less than expected for conventional gonadotoxic cancer treatments involving chemo- and/or radiotherapy. For men in whom NCIT drugs are therapeutically successful, it is likely they may continue to be used indefinitely, so further research into longer term effects is still needed. Conversely, despite the relatively mild testicular impact of NCIT drugs, if they are used in combined regimens with gonadotoxic therapies, the net effect on testicular function and fertility is likely to be more severe.

These data are consistent with limited prior finding that men on similar non-cytotoxic drug treatments are usually still capable of fathering children,[31–33] although quantitative estimates of male fertility, such as time to pregnancy are not available.[34–36] The median sperm output and concentration in men on NCIT treatment (89 million and 48 million/mL, respectively) are still well above lower 95% confidence limits (20 million and 9 million/mL, respectively) for unselected men in the community[37] or for young Australian men.[38] Furthermore, they exceed the threshold for sperm concentration (8 million/mL) required to achieve paternity in gonadotropin-treated infertile men with hypogonadotropic hypogonadism,[39] a group that has fertile female partners and no known defects in sperm function. These considerations support and extend previous findings on the high likelihood of paternity among

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13546 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Effects of non-cytotoxic and immunotherapy treatment.

Variable	Pre-treatment	On treatment	*p*
Number of men	17	14	
Number of samples	52	40	
Semen volume (mL)	3.2 ± 0.2	3.6 ± 0.2	0.24
Sperm density (million per mL)	**65 [48–86]**	**28 [17–43]**	**0.002**
Total sperm output (million)	**202 [151–264]**	**77 [48–117]**	**0.002**
Sperm motility (%)	34 ± 3	31 ± 4	0.67
Sperm morphology (%)	6.2 ± 0.6	5.1 ± 0.6	0.24
Sperm DNA fragmentation (%)	11 [8–46]	11 [9–17]	0.65
Serum testosterone[a] (ng/mL)	5.0 ± 0.4	5.3 ± 0.4	0.72
Serum LH (IU/L)	5.2 ± 0.5	5.9 ± 0.6	0.41
Serum FSH (IU/L)	**3.5 ± 0.4**	**6.6 ± 0.9**	**0.009**
Serum SHBG (nmol/L)	36.2 ± 4.2	37.4 ± 3.7	0.83
Serum inhibin B (pg/mL)	**281 ± 23**	**209 ± 21**	**0.026**
Serum AMH (ng/mL)	10.7 ± 1.1	11.6 ± 1.7	0.65
Serum testosterone[b] (ng/mL)	6.3 ± 0.5	6.1 ± 0.5	0.81
Serum DHT (ng/mL)	0.53 ± 0.04	0.52 ± 0.05	0.79
Serum DHEA (ng/mL)	5.1 ± 0.7	3.9 ± 0.6	0.19
Serum estradiol (pg/mL)	43 ± 7	44 ± 7	0.99
Serum estrone (pg/mL)	33 ± 5	29 ± 4	0.49
Serum 3α androstanediol (ng/mL)	0.47 ± 0.08	0.53 ± 0.08	0.56
Serum 3β androstanediol (ng/mL)	0.84 ± 0.02	0.77 ± 0.02	0.053

Note: Descriptive data are tabulated as mean ± SEM if gaussian, or median and interquartile range (IQR) [Q1–Q3] if non-gaussian. *p*-Values from unpaired *t*-test or Mann–Whitney rank test (if non-gaussian) with **Bold data** to indicate statistically significant difference. Semen variables are adjusted by covariance analysis for semen volume as a surrogate for abstinence interval.

Abbreviations: AMH, AntiMullerian Hormone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBD, sex hormone-binding globulin.

[a]Measured by testosterone immunoassay as samples became available.

[b]Measured batchwise at end of study by high-pressure liquid chromatography mass spectrometry.

men treated with NCIT drugs. However, this is subject to the reservation that spermatogenic damage may occur on combination or more intense NCIT regimens, so that men embarking on NCIT drug treatments, when the ultimate treatment regimen(s) may not be predictable, should continue to be advised on both pre-treatment sperm cryostorage as well as adequate contraception, both options intended to ensure timely future paternity.

The present study extends previous reports on male reproductive function on NCIT drug treatments. A post-mortem study of men with metastatic melanoma reported that six of seven men treated with immunotherapy, as well as two of six men not treated with immunotherapy, had severe spermatogenic damage.[40] The lack of statistically significant differences reflects both the small sample as well as prior knowledge that terminal illness and its treatments are major, non-specific determinants of post-mortem testicular damage.[41] Consequently, post-mortem testicular damage may not reflect pre-mortem testicular function or sperm output. Checkpoint inhibitor treatment-related orchitis may also damage sperm output,[26,27] although we observed no history or evidence of orchitis in our study. A cross-sectional study of 25 men treated for a median 20 months with immune checkpoint inhibitors for melanoma reported that semen analysis was qualitatively normal in 18/22, including four of 22 on treatment, as well as one of five men having abnormal pre-treatment semen analysis.[33] No quantitative data on sperm output or function nor any reproductive hormone analyses were reported.

More data are available for effects of imatinib on paternity[31,32] with limited congruent data on paternity data among men treated with second-generation tyrosine kinase inhibitors (nilotinib, dasatinib, bosutinib).[32] The significant increase in serum FSH in this study is consistent with the moderate decline in sperm production, noting one man treated most intensively with both types of NCIT drugs, becoming azoospermic with a high serum FSH. Other than our finding of a significant increase in serum FSH, the present findings are consistent with a study of 48 Chinese men with CML in which serum testosterone, LH, FSH, and estradiol by immunoassay did not differ from 50 outpatient control or 10 infertile men[31]; however, sperm output was not reported.[31] Similarly, the inverse correlation between serum SHBG and BSA in this study is consistent with the known effects of larger body size in obesity with lower serum SHBG.[42]

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13546 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Strengths of this study were its longitudinal component with analysis by a data modeling technique that focused on within-person time-course changes, notably before versus on treatment, that was robust to irregular sampling times and missing data. The relatively limited duration of observation on NCIT treatment (median 33.5 months) is long enough to observe gonadotoxic effects on spermatogenesis, which has a cycle time of 3 months. The main limitations of this study are its relatively small sample size and the lack of data on sexual function. Further prospective comprehensive studies of the systematic impact of non-cytotoxic drugs on male reproductive function are needed.

Overall, we conclude that these non-cytotoxic and immunotherapy drugs mostly have little or no effects on testicular exocrine function and none on testicular endocrine function. Only in the most heavily treated individuals—also those with the most advanced underlying disease—the effects on sperm output may be due to the non-specific effects of underlying disease and its severity rather than the non-cytotoxic and immunotherapy drug therapies. Further larger and longer studies including disease-specific measures of underlying disease severity would be valuable.

AUTHOR CONTRIBUTIONS

David J. Handelsman: conception, design, data acquisition and analysis, and drafting manuscript. Amanda Idan, Sue Sleiman, and Fey Bacha: data acquisition and reviewing manuscript. Georgina V. Long and Alexander M. Menzies: design, data acquisition, and reviewing manuscript. Tejnei Vaishnav, Noosha Litkouhi, and Xanthie Volckmar: data acquisition and reviewing manuscript. William Ledger: design and reviewing manuscript. Antoinette Anazodo: conception, design, data acquisition, and reviewing manuscript.

ACKNOWLEDGMENTS

There was no funding for this project. David J. Handelsman, Alexander M. Menzies, and Georgina V. Long were supported by NHMRC Investigator Grants, University of Sydney. Alexander M. Menzies was also supported by Nicholas and Hele Moore and Melanoma Institute of Australia.

Open access publishing facilitated by The University of Sydney, as part of the Wiley - The University of Sydney agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST STATEMENT

Georgina V. Long is consultant advisor for Agenus, Amgen, Array Biopharma, AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Evaxion, Hexal AG (Sandoz Company), Highlight Therapeutics S.L., Innovent Biologics USA, Merck Sharpe & Dohme, Novartis, OncoSec, PHMR Ltd, Pierre Fabre, Provectus, Qbiotics, and Regeneron. Alexander M. Menzies is a consultant advisor for BMS, MSD, Novartis, Roche, Pierre-Fabre, and Qbiotics. The remaining authors have no relevant disclosures for this study.

DATA AVAILABILITY STATEMENT

The data from this study are not publicly available and are subject to privacy restrictions. Summary data may be provided on reasonable request to the corresponding author.

ORCID

David J. Handelsman https://orcid.org/0000-0002-4200-7476
Antoinette Anazodo https://orcid.org/0000-0002-5495-6062

REFERENCES

1. Shapiro CL. Cancer survivorship. *N Engl J Med*. 2018;379:2438-2450.
2. Meistrich ML. Effects of chemotherapy and radiotherapy on spermatogenesis in humans. *Fertil Steril*. 2013;100:1180-1186.
3. Dohle GR. Male infertility in cancer patients: review of the literature. *Int J Urol*. 2010;17:327-331.
4. Goldfarb S, Mulhall J, Nelson C, Kelvin J, Dickler M, Carter J. Sexual and reproductive health in cancer survivors. *Semin Oncol*. 2013;40:726-744.
5. Logan S, Anazodo A. The psychological importance of fertility preservation counseling and support for cancer patients. *Acta Obstet Gynecol Scand*. 2019;98:583-597.
6. Schover LR. Sexual quality of life in men and women after cancer. *Climacteric*. 2019;22:553-557.
7. Chiba K, Fujisawa M. Fertility preservation in men with cancer. *Reprod Med Biol*. 2014;13:177-184.
8. Brannigan RE, Fantus RJ, Halpern JA. Fertility preservation in men: a contemporary overview and a look toward emerging technologies. *Fertil Steril*. 2021;115:1126-1139.
9. Oktay K, Harvey BE, Partridge AH, et al. Fertility preservation in patients with cancer: ASCO clinical practice guideline update. *J Clin Oncol*. 2018;36:1994-2001.
10. Mulder RL, Font-Gonzalez A, Green DM, et al. Fertility preservation for male patients with childhood, adolescent, and young adult cancer: recommendations from the PanCareLIFE Consortium and the International Late Effects of Childhood Cancer Guideline Harmonization Group. *Lancet Oncol*. 2021;22:e57-e67.
11. Woodruff TK. The emergence of a new interdiscipline: oncofertility. *Cancer Treat Res*. 2007;138:3-11.
12. Rozati H, Handley T, Jayasena CN. Process and pitfalls of sperm cryopreservation. *J Clin Med*. 2017;6:89.
13. Barak S. Fertility preservation in male patients with cancer. *Best Pract Res Clin Obstet Gynaecol*. 2019;55:59-66.
14. Shankara-Narayana N, Di Pierro I, Fennell C, et al. Sperm cryopreservation prior to gonadotoxic treatment: experience of a single academic centre over 4 decades. *Hum Reprod*. 2019;34:795-803.
15. Carlino MS, Larkin J, Long GV. Immune checkpoint inhibitors in melanoma. *Lancet*. 2021;398:1002-1014.
16. Huang L, Jiang S, Shi Y. Tyrosine kinase inhibitors for solid tumors in the past 20 years (2001–2020). *J Hematol Oncol*. 2020;13:143.
17. Pan C, Liu H, Robins E, et al. Next-generation immuno-oncology agents: current momentum shifts in cancer immunotherapy. *J Hematol Oncol*. 2020;13:29.
18. Volckmar X, Vallejo M, Bertoldo MJ, et al. Oncofertility information available for recently approved novel non cytotoxic and immunotherapy oncology drugs. *Clin Pharmacol Ther*. 2022;111:382-390.
19. World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. World Health Organization; 2021.
20. Bjorndahl L, Barratt CL, Mortimer D, Jouannet P. 'How to count sperm properly': checklist for acceptability of studies based on human semen analysis. *Hum Reprod*. 2016;31:227-232.
21. Fernandez JL, Muriel L, Goyanes V, et al. Halosperm is an easy, available, and cost-effective alternative for determining sperm DNA fragmentation. *Fertil Steril*. 2005;84:860.
22. Harwood DT, Handelsman DJ. Development and validation of a sensitive liquid chromatography-tandem mass spectrometry assay to simultaneously measure androgens and estrogens in serum without derivatization. *Clin Chim Acta*. 2009;409:78-84.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13546 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

23. Handelsman DJ. Optimal power transformations for analysis of sperm concentration and other semen variables. *J Androl*. 2002;23:629-634.
24. Jensen TK, Andersson AM, Hjollund NH, et al. Inhibin B as a serum marker of spermatogenesis: correlation to differences in sperm concentration and follicle-stimulating hormone levels. A study of 349 Danish men. *J Clin Endocrinol Metab*. 1997;82:4059-4063.
25. van Beek RD, Smit M, van den Heuvel-Eibrink MM, et al. Inhibin B is superior to FSH as a serum marker for spermatogenesis in men treated for Hodgkin's lymphoma with chemotherapy during childhood. *Hum Reprod*. 2007;22:3215-3222.
26. Brunet-Possenti F, Opsomer MA, Gomez L, Ouzaid I, Descamps V. Immune checkpoint inhibitors-related orchitis. *Ann Oncol*. 2017;28:906-907.
27. Quach HT, Robbins CJ, Balko JM, et al. Severe epididymo-orchitis and encephalitis complicating anti-PD-1 therapy. *Oncologist*. 2019;24:872-876.
28. Özdemir BC. Immune checkpoint inhibitor-related hypogonadism and infertility: a neglected issue in immuno-oncology. *J Immunother Cancer*. 2021;9:e002220.
29. Chang LS, Barroso-Sousa R, Tolaney SM, Hodi FS, Kaiser UB, Min L. Endocrine toxicity of cancer immunotherapy targeting immune checkpoints. *Endocr Rev*. 2019;40:17-65.
30. Barroso-Sousa R, Barry WT, Garrido-Castro AC, et al. Incidence of endocrine dysfunction following the use of different immune checkpoint inhibitor regimens: a systematic review and meta-analysis. *JAMA Oncol*. 2018;4:173-182.
31. Chang X, Zhou L, Chen X, et al. Impact of imatinib on the fertility of male patients with chronic myelogenous leukaemia in the chronic phase. *Target Oncol*. 2017;12:827-832.
32. Rambhatla A, Strug MR, De Paredes JG, Cordoba Munoz MI, Thakur M. Fertility considerations in targeted biologic therapy with tyrosine kinase inhibitors: a review. *J Assist Reprod Genet*. 2021;38(8):1897-1908.
33. Salzmann M, Tosev G, Heck M, et al. Male fertility during and after immune checkpoint inhibitor therapy: a cross-sectional pilot study. *Eur J Cancer*. 2021;152:41-48.
34. Joffe M. Time to pregnancy: a measure of reproductive function in either sex. Asclepios Project. *Occup Environ Med*. 1997;54:289-295.
35. Joffe M, Key J, Best N, Keiding N, Scheike T, Jensen TK. Studying time to pregnancy by use of a retrospective design. *Am J Epidemiol*. 2005;162:115-124.
36. Romero Herrera JA, Bang AK, Priskorn L, Izarzugaza JMG, Brunak S, Jørgensen N. Semen quality and waiting time to pregnancy explored using association mining. *Andrology*. 2021;9:577-587.
37. Cooper TG, Noonan E, von Eckardstein S, et al. World Health Organization reference values for human semen characteristics. *Hum Reprod Update*. 2010;16:231-245.
38. Hart RJ, Doherty DA, McLachlan RI, et al. Testicular function in a birth cohort of young men. *Hum Reprod*. 2015;30:2713-2724.
39. Liu PY, Baker HW, Jayadev V, Zacharin M, Conway AJ, Handelsman DJ. Induction of spermatogenesis and fertility during gonadotropin treatment of gonadotropin-deficient infertile men: predictors of fertility outcome. *J Clin Endocrinol Metab*. 2009;94:801-808.
40. Scovell JM, Benz K, Samarska I, et al. Association of impaired spermatogenesis with the use of immune checkpoint inhibitors in patients with metastatic melanoma. *JAMA Oncol*. 2020;6:1297-1299.
41. Handelsman DJ, Staraj S. Testicular size: the effects of aging, malnutrition, and illness. *J Androl*. 1985;6:144-151.
42. Cooper LA, Page ST, Amory JK, Anawalt BD, Matsumoto AM. The association of obesity with sex hormone-binding globulin is stronger than the association with ageing–implications for the interpretation of total testosterone measurements. *Clin Endocrinol (Oxf)*. 2015;83:828-833.

How to cite this article: Handelsman DJ, Idan A, Sleiman S, et al. Testicular function after non-cytotoxic and immunotherapy drug treatment. *Andrology*. 2024;12:891–898. https://doi.org/10.1111/andr.13546

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13546 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 15 March 2023 | Revised: 15 August 2023 | Accepted: 10 September 2023

DOI: 10.1111/andr.13537

ANDROLOGY WILEY

ORIGINAL ARTICLE

Differential roles of cyclooxygenase enzymes in the regulation of murine juvenile undifferentiated spermatogonia

Amy Tran-Guzman | **Amina Khan** | **Martine Culty**

Department of Pharmacology and Pharmaceutical Sciences, Alfred E. Mann School of Pharmacy and Pharmaceutical Sciences, University of Southern California, Los Angeles, California, USA

Correspondence
Martine Culty, AE Mann School of Pharmacy and Pharmaceutical Sciences; University of Southern California; Los Angeles, CA 90089–9121, USA.
Email: culty@usc.edu

Funding information
USC Alfred E. Mann School of Pharmacy and Pharmaceutical Sciences

Abstract

Background: Acetaminophen and ibuprofen are widely administered to babies due to their presumed safety as over-the-counter drugs. However, no reports exist on the effects of cyclooxygenase inhibitors on undifferentiated spermatogonia and spermatogonial stem cells. Infancy represents a critical period for spermatogonial stem cell formation and disrupting spermatogonial stem cells or their precursors may be associated with infertility and testicular cancer formation.

Objectives: The goal of this study was to examine the molecular and functional impact of cyclooxygenase inhibition and silencing on early steps of undifferentiated spermatogonia (u spg) and spermatogonial stem cell development, to assess the potential reproductive risk of pharmaceutical cyclooxygenase inhibitors.

Methods: The effects of cyclooxygenase inhibition were assessed using the mouse C18-4 undifferentiated juvenile spermatogonial cell line model, previously shown to include cells with spermatogonial stem cell features, by measuring prostaglandins, cell proliferation, and differentiation, using cyclooxygenase 1- and cyclooxygenase 2-selective inhibitors NS398, celecoxib, and FR122047, acetaminophen, and ibuprofen. Cyclooxygenase 1 gene silencing was achieved using a stable short-hairpin RNA approach and clone selection, then assessing gene and protein expression in RNA sequencing, quantitative real-time polymerase chain reaction, and immunofluorescence studies.

Results: Cyclooxygenase 2 inhibitors NS398 and celecoxib, as well as acetaminophen, but not ibuprofen, dose-dependently decreased retinoic acid-induced expression of the spg differentiation gene *Stra8*, while NS398 decreased the spg differentiation marker Kit, suggesting that cyclooxygenase 2 is positively associated with spg differentiation. In contrast, short-hairpin RNA-based cyclooxygenase 1 silencing in C18-4 cells altered cellular morphology and upregulated *Stra8* and *Kit*, implying that cyclooxygenase 1 prevented spg differentiation. Furthermore, RNA sequencing analysis of cyclooxygenase 1 knockdown cells indicated the activation of several signaling pathways including the TGFb, Wnt, and Notch pathways, compared to control C18-4

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2023 The Authors. *Andrology* published by Wiley Periodicals LLC on behalf of American Society of *Andrology* and European Academy of *Andrology*.

cells. Notch pathway genes were upregulated by selective cyclooxygenase inhibitors, acetaminophen and ibuprofen.

Conclusion: We report that cyclooxygenase 1 and 2 differentially regulate undifferentiated spermatogonia/spermatogonial stem cell differentiation. Cyclooxygenases regulate Notch3 expression, with the Notch pathway targeted by PGD2. These data suggest an interaction between the eicosanoid and Notch signaling pathways that may be critical for the development of spermatogonial stem cells and subsequent spermatogenesis, cautioning about using cyclooxygenase inhibitors in infants.

KEYWORDS
analgesics, C18-4 spermatogonial cell line, cyclooxygenases, eicosanoid pathway, NSAIDs, prostaglandins

1 | INTRODUCTION

Acetaminophen (ACE) and ibuprofen (IBU) are widely administered to babies due to their presumed safety as over-the-counter drugs.[1,2] Despite their prevalent use, studies have suggested that treating infants with cyclooxygenase (Cox) inhibitors could jeopardize their long-term reproductive functions.[3–5] However, no reports exist on the effects of Cox inhibitors on spermatogonial stem cells (SSCs) required for sperm formation. Infancy represents a critical period for SSC formation and disrupting SSCs or their precursors may be associated with infertility and testicular germ cell tumor formation.

Testicular cancer and infertility are part of a series of disorders believed to have a common developmental origin and are known collectively as testicular dysgenesis syndrome (TDS).[6] TDS can develop during fetal life or within a few months after birth, during which a pool of pluripotent cells, known as gonocytes, differentiate to form a reservoir of SSCs to ensure the life-long production of sperm. Molecular signals dictate the balance between differentiation and self-renewal during the different stages of pre-meiotic spermatogenesis, ensuring that there is a constant pool of SSCs that can be induced to differentiate into mature sperm throughout a male's life.[7] Steps of differentiation and meiosis in spermatocytes and spermatids comprise the remainder of spermatogenesis. Improper germ cell development can result in carcinoma in situ, the pathological precursor of testicular cancer which has origins in primordial germ cells or gonocytes that have failed to differentiate into u-spgs. In addition to classical seminomas, another class of seminomas can arise from more differentiated postnatal germ cells such as the SSCs, known as spermatocytic seminomas (SS). While SS can initially form a benign tumor, it can result in life-threatening disease if allowed to progress to more serious sarcomas.[8]

Eicosanoids are fatty-acid-based signaling molecules that have effects ranging from the regulation of physiological systems such as pain perception and cell growth to specific roles in females during pregnancy and childbirth.[9,10] They are formed through the release of esterified arachidonic acid (AA) from membrane phospholipids by an enzyme called phospholipase A2 (Pla2) in response to inflammatory stimuli. Upon release, AA gets further oxidized by various enzymes, epoxygenases, lipoxygenases, and Coxs. Two Coxs exist in cells, Cox1, which is constitutively expressed, and Cox2, whose expression is inducible and tissue-specific. The binding of AA on the Cox active site of the Cox enzymes is responsible for its cyclization, forming prostaglandin (PG) H2. Due to the unstable nature of PGH2, it is quickly converted to lipid-based mediators PGs, prostacyclins, and thromboxanes.[11] Several isoforms of PG synthases, which have varying expression from tissue to tissue, isomerize PGH2 to various PGs that exert both autocrine and paracrine effects on nearby membrane receptors.

We previously reported that rat gonocytes and spgs express Cox2 and that gonocytes produce PGs at significant levels.[12] We also characterized in a mouse SSC model, the C18-4 cell line, expression of both Cox1 and Cox2 mRNA and protein, as well as other intermediates of the eicosanoid biosynthetic pathway including Pla2 and measurable levels of PGs -D2, E2, and F2a production.[13] In this study, we are continuing to interrogate the role of the eicosanoid pathway on male reproductive development using the C18-4 cell line as a relevant in vitro model, to elucidate the mechanism of action of Cox inhibitors on spg development that could underlie potentially toxic effects. Using several Cox1, Cox2, and non-selective pharmacological Cox inhibitors, we have identified unique roles played by Cox1 and Cox2 in regulating SSC differentiation and downstream molecular signaling pathways. The upregulation of Notch pathway genes and significant changes in cell morphology were observed in a Cox1 knockdown model of the C18-4 cell line, suggesting that eicosanoids may regulate cellular maintenance and differentiation.

2 | MATERIALS AND METHODS

2.1 | Cell line and treatments

The mouse type A spg C18-4 cell line, gifted by Marie-Claude Hofmann, was generated by LTAg-immortalization of spg from 6-day-old Balb/c mice (The University of Texas MD Anderson Cancer Center).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

The absence of mycoplasma contamination in the C18-4 cell line was confirmed (Figure S1). The C18-4 cell line was shown to include a subset of cells with characteristics representative of SSCs.[14] Cells were cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L d-Glucose, L-Glutamine, and 110 mg/L of sodium pyruvate (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich) and 1% Penicillin-Streptomycin (P/S Solution 100X (Corning) at 35°C, 7% CO_2. The cells were allowed to grow in 25 and 75 cm^2 Corning cell culture-treated flasks and passaged every other day.

Pharmacological Cox inhibitors NS398, celecoxib, and FR122047 were purchased from Sigma Aldrich and were dissolved in ultra-pure grade dimethyl sulfoxide (DMSO from VWR International) to obtain concentrations 5, 20, and 50 mg/ml, respectively. Treatment solutions were subsequently diluted in cell culture media to obtain 1, 10, and 100 μM final concentrations. ACE and IBU were purchased from Sigma Aldrich. The drugs were dissolved in 100% ethanol to obtain concentrations of 0.05 g/ml. Treatments were subsequently diluted in cell culture media to obtain 50 and 100 μM final concentrations. All-trans Retinoic Acid purchased from Sigma Aldrich was dissolved in ultra-pure grade dimethyl sulfoxide and diluted in culture medium to obtain a final concentration of 1 μM. Supplemental PGD2 (Cat No. 538909) was purchased from EMD Millipore Pore. The compound was dissolved in 100% EtOH to obtain a stock concentration of 1 mg/ml. Treatments were subsequently diluted in cell culture media to obtain 50 and 100 μM final concentrations. Stock solutions of the drugs were stored at −20°C.

2.2 | Cox1 short-hairpin RNA-mediated silencing

Cox1 silencing was achieved using short-hairpin RNA (shRNA)-based transfection in which plasmids containing several silencer sequences targeted to Cox1 were integrated into the C18-4 cellular genome. Control samples were treated with plasmids containing scrambled sequences. Cox1 shRNA plasmid for a mouse (sc-35097-SH) and control shRNA plasmid (sc-108060) were purchased from Santa Cruz Biotech. Cells were plated at 500,000 cells/well in 6-well plates and transfected with 0.5 μg plasmid and introduced into a 1.5 μL Lipofectamine 3000 mix (ThermoFisher Scientific) diluted in Opti-MEM (ThermoFisher Scientific). Positively transfected cells were selected with two weeks of 4 μg/ml puromycin treatment. We screened several candidate clones using different concentrations of plasmid DNA and Lipofectamine 3000, generating groups A-D pairs of C18-$4^{Scrambled}$ and C18-$4^{Cox1\text{-}KD1}$ transfected cells. Successful silencing of Cox1 was validated with immunoblot and quantitative real-time polymerase chain reaction (qPCR) assays, before isolating cells into 1 cell/well concentrations into 96 well plates to select for stably transfected clones. Cells were allowed to proliferate for several weeks to obtain enough cells for further downstream validation and analysis.

2.3 | Liquid chromatography-mass spectrometry of PGs

Liquid chromatography-mass spectrometry (LC-MS) of PGs was conducted according to the previously reported protocol.[12,13] In short, C18-4 cells were plated at 1,000,000 cells/well in 6-well Corning culture-treated microplates overnight in a culture medium consisting of DMEM supplemented with 10% charcoal stripped-FBS (CS-FBS) and 1% Penicillin-Streptomycin (CS-culture medium). Cells were treated with either medium containing DMSO/ethanol (used at the same final percentage as in samples treated with Cox inhibitors) as a control medium, or with Cox inhibitors diluted in a CS-culture medium. Aliquots of CS-culture medium without cells were collected and stored at −80°C to establish base line levels of PGs in LC-MS analysis. The conditioned media from control cells and cells treated with Cox inhibitors were collected and stored at −80°C until LC-MS analysis. Samples were analyzed using a Shimadzu Nexera ultra-high-performance LC (UHPLC) coupled with a Sciex TripleTOF quadrupole time of flight MS. PGs were detected using electrospray negative mode ionization followed by tandem MS fragmentation. Sciex Analyst v1.7 software was used for data acquisition. Sciex MultiQuant v3.02 software was used to select peak area measurements from selected product ions and to perform calibration curve regression analysis and sample quantifications. PG concentrations were normalized to total protein/well and protein was measured by BCA quantification as described below. Data for PGD2 represent the means ± SEM of two independent experiments, each conducted in duplicates. PGE2 was measured in 2 experiments, each with one control well, one well with 10 μM NS398, and another well with 100 μM NS398.

2.4 | Prostaglandin D2 enzyme-linked immunosorbent assay

For PGD2 enzyme-linked immunosorbent assay (ELISA) quantification, C18-4 cells were plated at 400,000 cells/well in 12-well Corning culture-treated plates and treated with Cox inhibitors diluted in a culture medium supplemented with CS-FBS. Control samples consisted of cells grown in a culture medium containing the same proportion of DMSO/ethanol as that present in treatments. After treatments, cell culture supernatants were collected and saved for PG analysis. Samples were stored at −80°C until use. PGD2 levels were measured using the Prostaglandin D2 ELISA Kit (Cayman Chemical) according to the manufacturer's instructions. ELISA standards detecting PGD2 at a range from 19.5 to 2500 pg/ml were prepared in a CS-culture medium without cells. The VICTOR X5 Multilabel Plate Reader (PerkinElmer, Inc.) was used to detect the OD measurement of Ellman's reagent at wavelength 405 nm. Data analysis was evaluated using the Cayman PGD2 computer spreadsheet, and $\%B/B_0$ from standards S1-S8 versus PGD2 concentration was plotted in Prism version 7.0 (GraphPad Software) software using a 4-parameter logistic fit. PGD2 concentrations

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

were normalized to total protein/well quantified with Bradford reagent according to the manufacturer's protocol (VWR Life Science). Cell layers were solubilized using 0.1 N NaOH and stored at −80°C for total protein quantification. All experiments were performed using a minimum of three independent experiments, with data normalized to the control medium. The mean ± SEM is shown.

2.5 | EdU proliferation assay

Cells were plated in 96-well Corning culture-treated black bottom microplates at 8–10,000 cells/well density and allowed to grow overnight in serum-free DMEM containing 1% P/S. The next day, cells were treated with a CS-culture medium containing ethanol/DMSO (control medium) or Cox inhibitors diluted in a CS-culture medium for 24 h. For the last 6 h of treatment, cells were incubated with 10 μM EdU (5-ethynyl-2′-deoxyuridine) as recommended by the manufacturer (Click-IT EdU HCS Assay, Invitrogen). Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde followed by a 0.1% Triton X-100 permeabilization surfactant. The Click-IT reaction cocktail was then added to each well and allowed to incubate in the dark for 30 min. Cells were washed and 100 μL of 1:2000 HCS NuclearMask was added to stain DNA for 30 min in the dark. The plates were imaged and quantified with the Cytation 5 Cell Imaging Multi-Mode Reader (Biotek).

2.6 | RNA extraction, complementary DNA synthesis, and qPCR

C18-4 cells were plated at 100—200,000 cells/well in 24 well plates and treated with a CS-culture medium containing ethanol/DMSO (control medium) or with Cox inhibitors diluted in CS-culture medium for 24 h. Cells were collected after treatment and stored in RNA lysis buffer and extracted based on manufacturer recommendations using the RNAqueous Micro Total RNA Isolation Kit (Invitrogen). Complementary DNA (cDNA) was synthesized from purified RNA using the PrimeScript RT Master Mix (Takara Bio) also according to the manufacturer's instructions. qPCR was performed using a LightCycler 480 with a SYBR Green PCR Master Mix kit (Roche Diagnostics). Primers were designed using Primer-BLAST from the NCBI-NIH gene database and are listed in Table 1.

qPCR cycling conditions were as follows: initial step at 95°C followed by 40 cycles at 95°C for 15 s, 60°C for 1 min. This was followed by both melting curves and cooling cycles. Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA, and the comparative threshold cycle (C_t) method was used to analyze the data. Glyceraldehyde 3-phosphate dehydrogenase was used for data normalization for C18-4 cells. Assays were performed in triplicates. All experiments were performed using a minimum of three independent sample preparations and the mean ± SEM is shown.

2.7 | Immunoblot analysis

Cells were plated at 1,000,000 cells/well in 6-well Corning culture-treated microplates overnight and treated as described above. Treated C18-4 cells were scraped from plates using chilled PBS (Santa Cruz Biotechnology), centrifuged, and collected. Cell pellets were stored at −80°C for western blot analysis.

Protein was extracted in 30 μL from cell pellets using RIPA lysis buffer (Santa Cruz Biotechnology) containing Pierce protease and phosphatase inhibitors (Thermo Fisher Scientific). Contents were vortexed to lyse cells and protein extracts were collected after centrifugation for 30 min at 4°C. Protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), following the manufacturer's instructions. Samples were calculated against the standard curve diluted in RIPA lysis buffer based on the working range of 20–2000 μg/mL bovine serum albumin. 16 μg/sample of protein were mixed at a 1:1 ratio with 2x Laemmli Sample Buffer + beta-mercaptoethanol (Bio-Rad Laboratories), and boiled for 10 min at 95°C to allow for protein denaturation. Samples were frozen at −20°C until western blotting. Western blotting was conducted according to the protocol previously reported.[13] Antibodies used are as follows: Cox1 (Cat No. 4841, Cell Signaling Technologies, RRID: AB_2084807), Cox2 (Cat No. 13314, Cell Signaling Technologies, RRID: AB_2798178), Notch3 (Cat No. ab23426, Abcam, RRID: AB_776841), Hes1 (Cat No. 11988, Cell Signaling Technologies, RRID: AB_2728766). Protein expression was either compared or normalized to alpha-tubulin (Cat No. T9026, Sigma Aldrich, RRID: AB_477593) or to total protein (TotalStain Q, Azure Biosystems).

2.8 | Immunofluorescence analysis

C18-4 cells were plated at 50,000 cells/well in 96 well Corning culture-treated black bottom microplates and were allowed to reach 80% confluency overnight. The next day, they were treated with a CS-culture medium containing ethanol/DMSO (control medium) or with Cox inhibitors diluted in CS-culture media for 24 h. After treatment, the samples were fixed with cold 4% paraformaldehyde in PBS (Santa Cruz Biotechnology) for 10 min at room temperature and washed with PBS. The cells were then incubated with 1% Triton X-100 (Promega) in PBS for 10 min at room temperature, washed, and incubated in 5% donkey serum (Sigma-Aldrich) in PBS for another 30 min. Cells were incubated with primary antibodies, either Cox1 1:300 (Cat #4841 Cell Signaling Technology, RRID: AB_2084807), Notch3 1:300 (Cat No. ab23426, Abcam, RRID: AB_776841), or c-Kit 1:100 (SC168, Santa Cruz Biotech, RRID: AB_631033) diluted in 5% donkey serum overnight at 4°C. On the next day, the wells were washed and corresponding Alexafluor anti-mouse or anti-rabbit secondary antibodies (Thermo Fisher) were added at 1:400 in 5% donkey serum for 30 min at room temperature. The cells were washed one last time, and 100 μL DAPI (D1306; Thermo Fisher) at 300 nM was added to the wells for 5 min. The Cytation 5 Cell Imaging Multi-Mode Reader (Biotek) was used to capture images and

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 List of primers used for quantitative real-time polymerase chain reaction (qPCR) analysis.

Gene	Forward Primer	Reverse Primer
Gapdh	AAGGTCATCCCAGAGCTGAA	CTGCTTCACCACCTTCTTGA
Cox1	CCTCTTTCCAGGAGCTCACA	TCGATGTCACCGTACAGCTC
Foxo1	CTTCAAGGATAAGGGCGACA	GACAGATTGTGGCGAATTGA
Gapdh	AAGGTCATCCCAGAGCTGAA	CTGCTTCACCACCTTCTTGA
Id4	CAGGGTGACAGCATTCTCTG	CCGGTGGCTTGTTTCTCTTA
Kit	AGCAAATGTCACAACAACCT	CCTCGTATTCAACAACCAAA
Mcam	CAAACTGGTGTGCGTCTTCTT	CTTTTCCTCTCCTGGCACAC
Stra8	GCCTCAAAGTGGCAGGTACTG	CTTATCCAGGCTTTCTTCCTGTTC
Jam1	CAAGGCAAGGGTTCGGTGTA	TAGGGAGCTGTGATCTGGCT
Mmp2	GCCCCCATGAAGCCTTGTTT	TAGCGGTCTCGGGACAGAAT
Tgbfr3	CCTCCGCAGTACAGACCAAG	AACCCTCCGAAACCAGGAAG
Tgbf2	TCCCCTCCGAAAATGCCATC	TGCTATCGATGTAGCGCTGG
Tgbf3	ATGACCCACGTCCCCTATCA	CAGACGGCCAGTTCATTGTG
Smad3	CAGCCATGTCGTCCATCCTG	CCATCCAGTGACCTGGGGAT
Smad9	CCTGAGCTCTGCCTCCTATG	ACACACTTGCTAGGCTGACC
Notch1	TGCCATATACAGGAGCCACG	ATTGGTGTTCTGGCAGGAGG
Notch3	AGGTGGTCACAGACTTGAATGA	GTGGGGTGAAGCCATCAGG
Hes1	CGGAATCCCCTGTCTACCTC	CTTGGAATGCCGGGAGCTAT
Hey1	CCACTGCAGTTAACTCCTCCT	CGCGTCAAAATAACCTTTCCCT

quantify protein expression levels after adjusting for background signal and normalizing data to nuclei counts by the Cytation 5 software. Briefly, data were obtained from three independent experiments conducted in triplicates, based on the analysis of 4 pictures per well taken at different locations of the field. Signal quantification was normalized to cell nuclei counts, by dividing the number of immunofluorescence (IF) signal-positive cells by the total number of cells in the sample. The total cell number was obtained by automatic counting of DAPI-labelled nuclei. A gate was set against the background, and cells were considered positive if they had an IF signal above background fluorescence. Note that 50,000 cells were screened per well for each protein marker.

2.9 | Total RNA sequencing

The total RNA of three samples representing different passages of Scrambled Controls and C18-4$^{Cox1\text{-}KD1}$ were collected as described in section 2.7, and they were sent to the USC Molecular Genomics Core lab for whole transcriptome RNA sequencing (RNAseq). RNA was extracted from cells using the RNAqueous Micro Total RNA Isolation Kit (Invitrogen) as previously described, and quality control was performed using Agilent BioAnalyzer 2100. The integrity of RNA used for RNAseq of C18-4$^{Cox1\text{-}KD1}$ was assessed by agarose gel electrophoresis. Only samples that passed quality checks with RIN > 8 were selected to move forward with RNAseq, resulting in two scrambled controls (SCR-1 and SCR-3) and 3 Cox1 knockdown samples (KD1, 2, and 3). cDNA libraries were prepared using Takara SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian (Takara Bio Inc.) following the manufacturer's protocol. Prepared libraries were sequenced on the Illumina Nextseq500 platform at a read length of 2 × 75 at 50 million paired reads.

Transcriptomic datasets of each sample were imported into Partek Flow software (Partek Inc.) as FASTQ files. Pre-alignment QA/QC was conducted, and bases were trimmed based on a quality score (Phred) of 20. Bases were trimmed on both ends for a minimum read length of 25 base pairs. The reminder reads were aligned to the mouse genome (mm10-GENCODE Genes-release M24) using the STAR aligner tool. Post-alignment QA/QC was conducted to confirm that over 70% of the reads were aligned to the reference genome. Gene counts were conducted, and a noise reduction filter was applied to exclude features in which the maximum value is less than equal to 10 (<10 counts/gene excluded). Upper quartile normalization and gene-specific analysis were conducted, resulting in a list of 28,123 differentially expressed genes (DEGs) between Scrambled controls and C18-4.$^{Cox1\text{-}KD1}$ Applying parameters of false discovery rate greater than or equal to 0.1 and fold changes of −2 and 2 narrowed down the gene list to 1,265 genes which were used for gene ontology and pathway analysis. The PANTHER Classification System was used to evaluate gene ontologies and relationships,[15,16] and the Qiagen Ingenuity Pathway Analysis Software (IPA; Qiagen) was used to evaluate functional pathways.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.10 | Statistical analysis

Statistical analysis was conducted by Student's t-test or one-way ANOVA with post-hoc multiple comparison analysis using Prism version 7.0 (GraphPad Software). Multiple comparison analyses were done using Tukey's (using statistical hypothesis testing) or the Benjamini, Krieger, and Yekutieli methods (controlling for false discovery rate). Statistical significance was considered at $p \leq 0.05$. Results are shown as the mean ± SEM. Significant difference in treatments compared to control samples: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

3 | RESULTS

3.1 | Cox inhibitors decrease PG production in the C18-4 cell line

Confirmatory studies were conducted to validate that PG production was inhibited with pharmacological Cox inhibitors as evidence that Cox enzymatic activity was blocked as expected. Cells were plated overnight and treated for 24 h with various concentrations of selective and non-selective Cox inhibitors in culture media supplemented with 10% CS-FBS, which contains significantly lower amounts of PGs than conventional FBS used for cell culture. NS398 and celecoxib are Cox2 selective inhibitors that target Cox2 at IC50 = 3.8 μM and at IC50 = 0.04 μM respectively.[17,18] FR122047 is a selective Cox1 inhibitor (IC50 = 28 nM), and ACE and IBU are non-selective Cox inhibitors (ace: IC50 = 26 μM for Cox2, 114 μM for Cox1, ibu: IC50 = 12 μM for Cox1, IC50 = 80 μM for Cox2).[19–22] Since the concentrations of PGD2 measured by ELISA and LC-MS were in the same range (20 ± 2 pg/mL with LC-MS and 50 ± 31 pg/mL by ELISA), we pooled the fold changes calculated from these experiments. PGD2 levels were decreased in a dose-dependent manner by NS398, with a 33% decrease at 10 μM and 45% decrease at 100 μM, while 10 μM of celecoxib showed a non-significant 27% decrease similar to that of NS398 at the same concentration, suggesting the contribution of Cox2 activity on PGD2 synthesis in C18-4 cells, that was prevented by blocking this enzyme (Figure 1A). On the other end, FR122047 had no effect (small not significant decrease at 10 μM) suggesting that Cox1 was not involved in PGD2 production (Figure 1A). Ace induced a significant 30% decrease at 50 μM, similar to the effects of Cox2-specific inhibitors at 10 μM, but it had no effect at 100 μM, whereas Ibu significantly inhibited PGD2 production at both doses, by 38% at 50 μM and 40% at 100 μM, suggesting that the effects of Ibu were driven by its inhibitory effect on Cox2. The levels of PGE2 were examined only in a few samples treated with medium (control samples), 10 and 100 μM NS398, measured by LC-MS. The means ± SD of PGE2 in cell supernatants of control samples were 396 ± 32 pg/mL, around 10-time higher than those of PGD2, as expected from our previous study on C18-4 cells.[13] Due to the limited number of samples analyzed, one could not perform statistical analysis. However, replicates had small variability that allowed raw data comparison. PGE2 levels from cells treated with 10 μM NS398 were slightly lower than those of control samples (358 ± 33 pg/mL), while PGE2 production by cells treated with 100 μM NS398 was 222 ± 2 pg/mL (44% lower than control levels). This effect was similar to that induced by the same concentration of NS398 on PGD2 production by C18-4 cells, suggesting that PGE2 was also formed via Cox2 activation.

3.2 | Effects of pharmacological Cox inhibitors on cell proliferation and differentiation

The effects of 24-h treatment with Cox inhibitors selectively targeting Cox1, Cox2, or with non-selective Cox inhibitors, on proliferation and differentiation were evaluated. Proliferation, measured by EdU incorporation, showed that 100 μM ACE treatment significantly increased proliferation by 20% of control levels, while 50 μM of ACE and both doses of IBU did not significantly alter cell proliferation (Figure 1B,C). The selective Cox2 inhibitor NS398 slightly increased cell proliferation (17% above control) at 10 μM, but 100 μM NS398 had no effect (Figure 1B,D). EDU incorporation did not show proliferation changes in cells treated with the selective Cox2 inhibitor celecoxib from 1 to 50 μM (Figure 1E). Similarly, the selective Cox1 inhibitor FR122047 did not alter cell proliferation at doses from 1 to 50 μM (Figure 1F). However, both celecoxib and FR122047 at 50 μM significantly reduced total cell counts by almost 50% (Figure 1G–H). Since cell proliferation measured by EdU incorporation was not altered by 50 μM of these Cox inhibitors (Figure 1E,F), the decreases in cell counts could be due to either a cytostatic or apoptotic effect of the inhibitors. Therefore, our subsequent experiments were limited to concentrations of 10–100 μM of NS398, and 1–10 μM of celecoxib and FR122047. We also chose to focus on concentrations of 50 and 100 μM of ACE/IBU, as these corresponded to blood levels of ACE and IBU derived from a clinical trial conducted on feverish children treated with a single dose of ACE and IBU.[12,22]

Taken together, the differential effects of the Cox inhibitors observed on PGD2 (Figure 1A) and cell proliferation (Figure 1B–F) suggest that the synthesis of PGD2 via Cox1 and Cox2 is not or minimally involved in C18-4 cell proliferation. However, the reduction of cell numbers by the highest dose tested (50 μM) of celecoxib and FR122047 indicates cytotoxicity, since proliferation was not affected at that dose (Figure 1E,F). Since PG levels were normalized to cell numbers, one might be able to determine whether the adverse effects of the two inhibitors on cell numbers are related to their effects on PGs at 50 μM in future experiments. However, the comparison of the inhibitory effects of 100 μM NS398 on PGD2 production, with its apparent lack of effect on cell numbers as seen in Figure 1E, suggests that PGD2 levels are not related to cell survival, implying that celecoxib and FR122047 might exert off-target cytotoxic effects.

Next, the ability of pharmacological Cox inhibitors ACE and IBU to alter the differentiation status of SSCs was evaluated by measuring the expression levels of gene markers of SSC, progenitor spgs, and differentiated spgs, in cells treated with medium (basal conditions) or

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

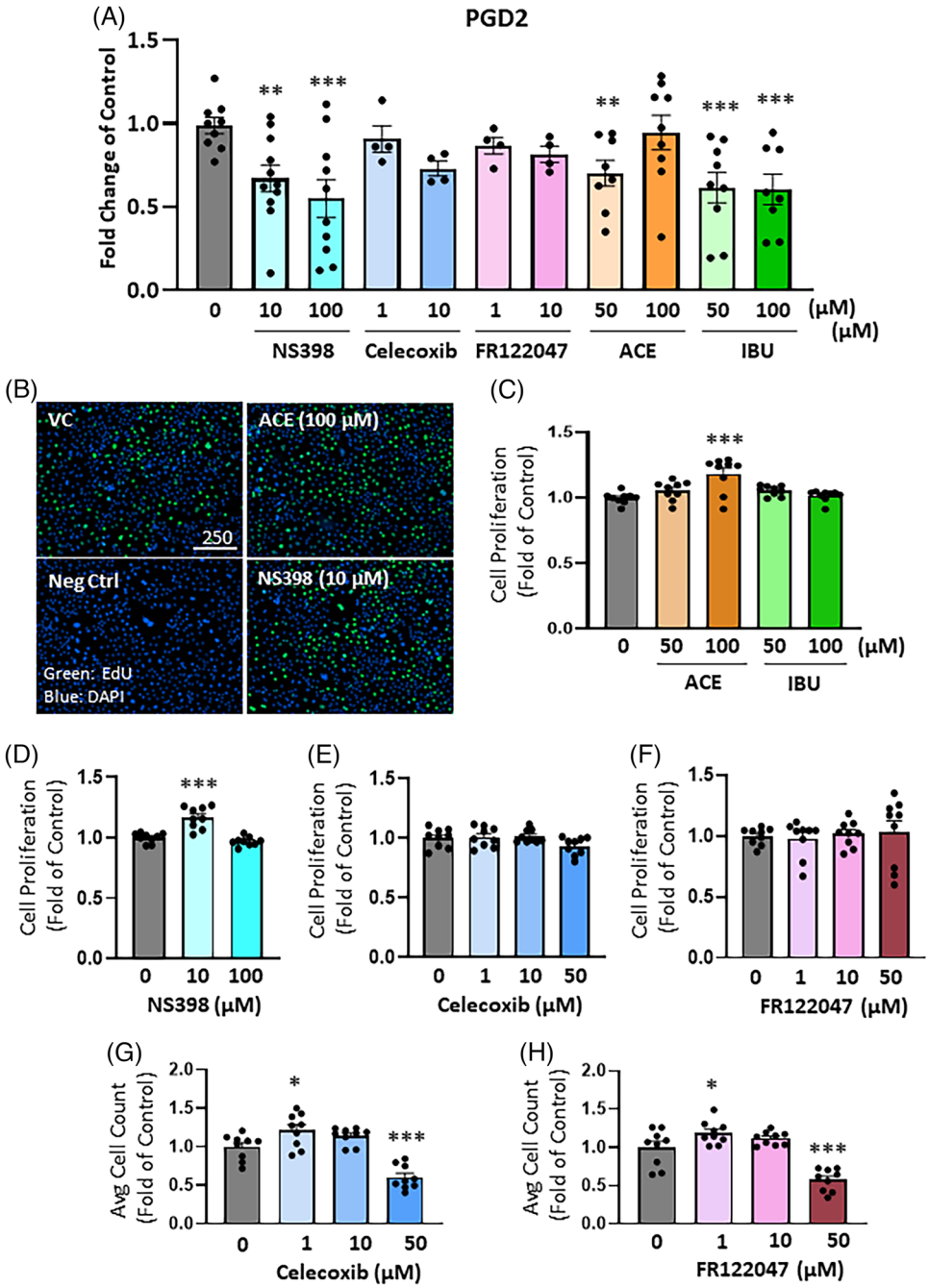


FIGURE 1 Effects of cyclooxygenase (Cox) inhibitors on prostaglandin and cell proliferation in C18-4 spermatogonia. (A) Prostaglandin D2 levels measured by enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-mass spectrometry (LC-MS) after 24 h treatment. Treatments were 10 and 100 μM NS398, 1 and 10 μM of celecoxib and FR122047, or 50 and 100 μM acetaminophen and ibuprofen. ELISA results were obtained from N = 3 independent experiments conducted in triplicates, while LC-MS was performed on N = 2 independent experiments conducted in duplicates. Since the ELISA data gave similar concentrations of PGD2 to those obtained by LC-MS, the calculated fold changes of all experiments were pooled. Data are reported as fold change/ control (indicated by a zero). (B–F). COXIB's effects on proliferation assessed by visualizing EDU incorporation into C18-4 cells. (B) Example of EDU labeling in cells treated with control medium, 10 μM NS398 and 100 μM acetaminophen. Green: EDU, Blue: DAPI. Scale in μm. (C-F) The percent of proliferating cells after 24 h treatment with medium or the indicated COXIBs was quantified as a percent of total cell numbers and expressed as fold change of control. (C) 50 and 100 μM acetaminophen and ibuprofen. (D) 10 and 100 μM NS398; (E) 1–50 μM celecoxib; (F) 1—50 μM FR122047. (G-H) Quantification of average cell number normalized to control medium (fold change of controls) with treatment of up to 50 μM celecoxib (F) and FR122047 (G). For all data, N = 3 independent experiments were conducted in triplicates. Significant difference relative to controls with one-way ANOVA test and multiple comparisons: * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

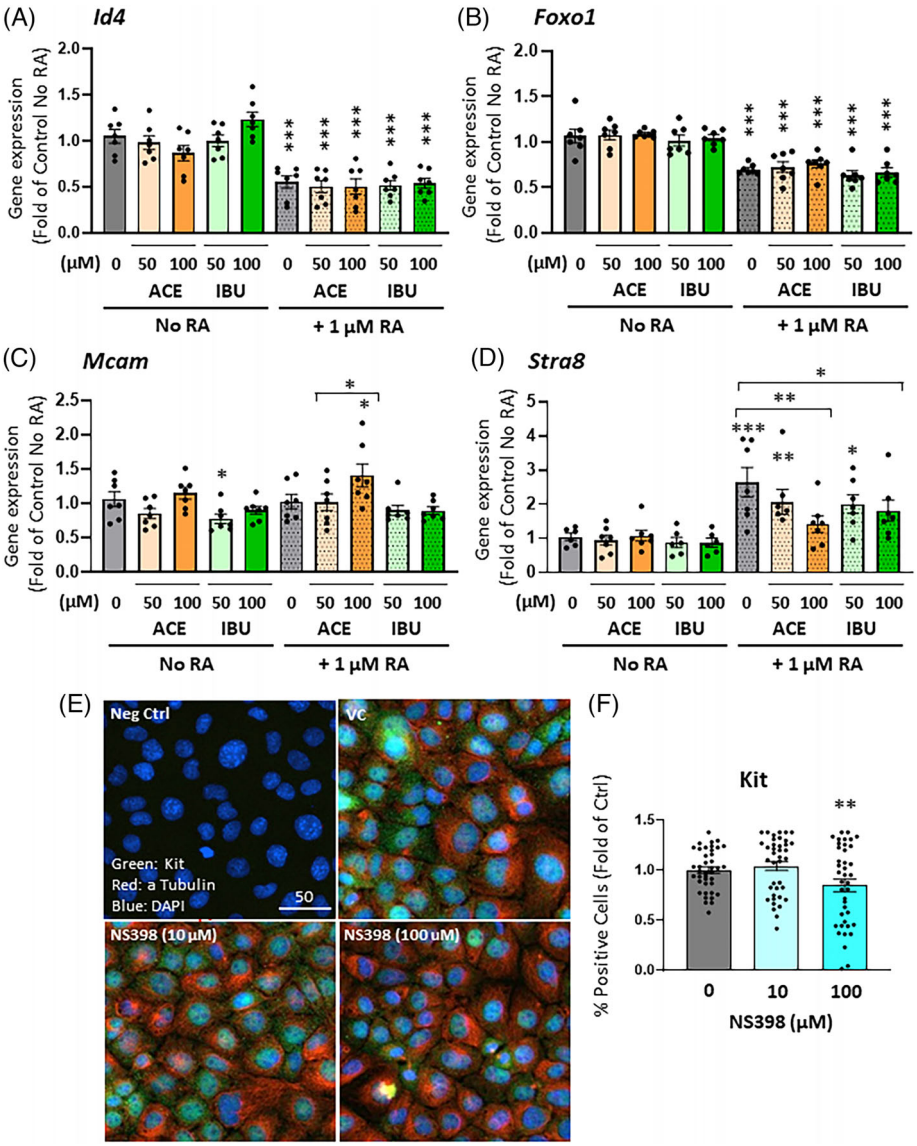


FIGURE 2 Effects of cyclooxygenase (Cox) inhibitors on cell differentiation. Effect on differentiation was examined by measuring genes (A–D) and protein (E, F) markers after 24 h treatment of C18-4 cells with acetaminophen and ibuprofen +/- 1 μM retinoic acid. mRNA expression of (A) *Id4*, (B) *Foxo1*, (C) *Mcam*, (D) *Stra8*. mRNA expression data were normalized to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). (E, F) Visualization and quantification of Kit protein expression after 24 h treatment with 10 and 100 μM NS398. Green: Kit, Red: alpha-tubulin, Blue: DAPI. Scale in μm. N = 3 independent experiments conducted in triplicates. Significant difference relative to controls with one-way ANOVA test and multiple comparisons: * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

1 μM retinoic acid (RA) used to induce SSC differentiation, alongside medium or the drugs. In the absence of RA, ACE at 50 or 100 μM had no effect on the expression of the classical SSC marker Inhibitor Of DNA Binding 4 (*Id4*), Forkhead Box O1 (*Foxo1*), a gene expressed in SSCs and progenitor spgs, the progenitor marker Melanoma Cell Adhesion Molecule (*Mcam*) or the differentiated spg marker Stimulated by RA 8 (*Stra8*) (Figure 2A–D). Similarly, 50 and 100 μM IBU did not affect *Id4*, *Foxo1*, *Mcam*, or *Stra8* expression in the absence of RA, but it induced a 22% decrease in *Mcam* at 50 μM. In contrast, treatment with 1 μM RA for 24 h reduced the gene expression of *Id4* and *Foxo1* (Figure 2A,B), while it induced the expression of (*Stra8*) (Figure 2D), as expected, but it did not affect *Mcam* levels (Figure 2C). Moreover, in the presence of RA, ACE at 100 μM significantly induced the expression of *Mcam* by 41% above basal levels, whereas it inhibited by 47% the RA-induced upregulation of *Stra8* (Figure 2C,D). IBU at 100 μM decreased the RA effect on *Stra8* expression by *32%*. Since ACE had stronger effects on cell differentiation and is known to have a greater inhibitory effect on Cox2 than Cox1, we examined the effect of the Cox2 selective inhibitor NS398 on Kit, another marker of spermatogonial differentiation. 24-h treatment with 100 μM NS398 decreased Kit protein expression (Figure 2E,F), suggesting that Cox2 may uniquely exert pro-differentiation effects.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

3.3 | Morphological and transcriptomic characterization of C18-4$^{Cox1\text{-}KD1}$

To further characterize the downstream effects of Cox inhibition on SSCs, we examined the role of Cox1 by stably knocking down its expression as described in the methods section. From the A-D pairs of C18-4^{Scrambled} and C18-4$^{Cox1\text{-}KD1}$ transfected cells, cells in the knockdown "B" condition were the most promising candidates for single-cell isolation (Figure 3A). Scrambled and Knockdown B cells were plated into 96 well plates at a density of a single cell per well and allowed to proliferate. Isolated clones, referred now as C18-4$^{Cox1\text{-}KD1}$, displayed significantly lower expression of Cox1 at both the protein and transcript levels, expressing approximately 30% of the levels present in Scrambled cells (Figure 3B–D).

We observed significant morphological differences in C18-4$^{Cox1\text{-}KD1}$ compared to scrambled control cells (Figure 3E). The cells adopted a more fibroblastic or mesenchymal phenotype, likely due to loss of integrity of adhesion between cells. Indeed, the epithelial adhesion marker junctional adhesion molecule 1 (*Jam-1*), was significantly downregulated in C18-4$^{Cox1\text{-}KD1}$ compared to its control (Figure 3F). *Mmp2*, matrix metalloproteinase 2, which encodes a protein involved in extracellular matrix remodeling, was also significantly downregulated (Figure 3G). Silencing of Cox1 also induced the expression of spermatogonial differentiation marker *Stra8* (Figure 3H), suggesting that Cox1 is important for maintaining cells at an undifferentiated state.

3.4 | Ontology and pathway analysis of DEGs in C18-4$^{Cox1\text{-}KD1}$ cells

To evaluate the signaling pathways altered by silencing Cox1, we characterized the C18-4$^{Cox1\text{-}KD1}$ cell transcriptome using total RNAseq (RNAseq). FASTQ files were imported into Partek Flow and the two control samples averaged approximately 12,422,840 total reads, whereas the knockdown samples averaged approximately 13,353,310 total reads. Number of reads removed in the control samples were ~1410 (0.011%) and in the knockdown samples ~2070 (0.015%). Cleaned reads were aligned to the mouse genome assembly, and counts were normalized. This resulted in 436 down- and 829 up-regulated genes in C18-4$^{Cox1\text{-}KD1}$ illustrated by the volcano plot (Figure S2). Overall, we identified 1265 DEGs using a false discovery rate of < 0.1 and fold change parameters of −2–2. Hierarchical clustering analysis of the three individual C18-4$^{Cox1\text{-}KD1}$ samples and two scrambled samples showed high similarity in DEGs between controls and between knockdown samples (Figure 4A).

The PANTHER classification system was employed for functional and gene ontology annotation of DEGs based on the gene list of 1265 genes compiled by Partek Flow. The top biological processes identified by PANTHER included cellular processes (557 genes), biological regulation (376 genes), metabolic processes (347 genes), response to stimulus (221 genes), and signaling (176 genes) (Figure S3A). As for the molecular function analysis, the greatest percentage of genes were annotated with binding (350 genes), followed by catalytic activity (242 genes), and molecular function regulation (163 genes) (Figure S3B). Although the transcriptome of C18-4 cells was very different between C18-4^{Scrambled} and C18-4$^{Cox1\text{-}KD1}$ transfected cells, "cell differentiation" was not identified among altered functional pathways, and genes related to spg differentiation were not in the DEGs list. This may be due to the fact that the Cox1 silencing studies were performed in basal conditions with small amounts of RA from FBS insufficient to observe effects on differentiation. It could also suggest that the reduction of Cox1 by RA contributes but is not sufficient to induce spg differentiation, a complex process known to involve several genes.

Note that, 995 genes were annotated as pathway-related genes, with 26 genes associated with the Wnt signaling pathway, 12 genes associated with the TGFb signaling pathway, and 4 genes associated with the Notch signaling pathway. The regulation of the epithelial-to-mesenchymal transition (EMT) pathway (192 genes), inhibition of matrix metalloproteases (39 genes), and inhibition of prostanoid synthesis (10 genes) were pathways identified as altered by Cox1 knockdown using IPA analysis (Figure S3C). Wnt, TGFb, and Notch signaling pathways that were identified as significantly differentially altered by PANTHER classification were also identified as top pathways altered via IPA. Interestingly, all three pathways were predicted to be involved in the regulation of EMT, one of the top canonical pathways activated in our data set (Figure S4). Top DEGs associated with EMT include several Wnt family members *Wnt11*, *Wnt5a*, *Wnt7a*, and *Wnt9a*, TGFb genes *Tgfb2*, *Tgfb3*, *and Smad3*, and Notch receptors *Notch1* and *Notch3* (Table 2). Next, qPCR was performed to verify RNAseq expression values. We chose to verify the genes that were most relevant to our model and that were amongst the most significantly altered. Amongst the genes identified with the major signaling pathways predicted to be altered, TGFb signaling comprised several genes significantly altered in RNAseq by more than 2-folds, including *Tgfb2*, *Tgfb3*, and *Tgfbr3* (Table 2; Table S1). *Tgfbr3* was significantly increased by over 2-fold in qPCR analysis, in agreement with the RNAseq data, while non-significant increases (~1.5-fold) were observed in *Tgfb2* and *Tgfb3* mRNA expression by qPCR (Figure 4B). *Smad3* and *Smad9*, genes that are also associated with TGFb signaling, were significantly increased when measured with qPCR consistent with RNAseq expression values (Figure 4B). Another major signaling pathway was the Notch signaling, in which *Notch1* and *Notch3* transcripts were significantly upregulated as well. Overall, qPCR data were consistent with what was being measured using RNAseq.

3.5 | Activation of Notch3 signaling pathway in C18-4$^{Cox1\text{-}KD1}$

Immunofluorescence analysis showed increased expression of Notch3 in C18-4$^{Cox1\text{-}KD1}$ compared to the scrambled controls (Figure 5A), confirming the induction of this signaling pathway when Cox1 expression is reduced. To further investigate the effect of Cox1 knockdown on Notch pathway expression, we evaluated protein levels of Notch3 and its downstream target, transcription factor Hes1 using western

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

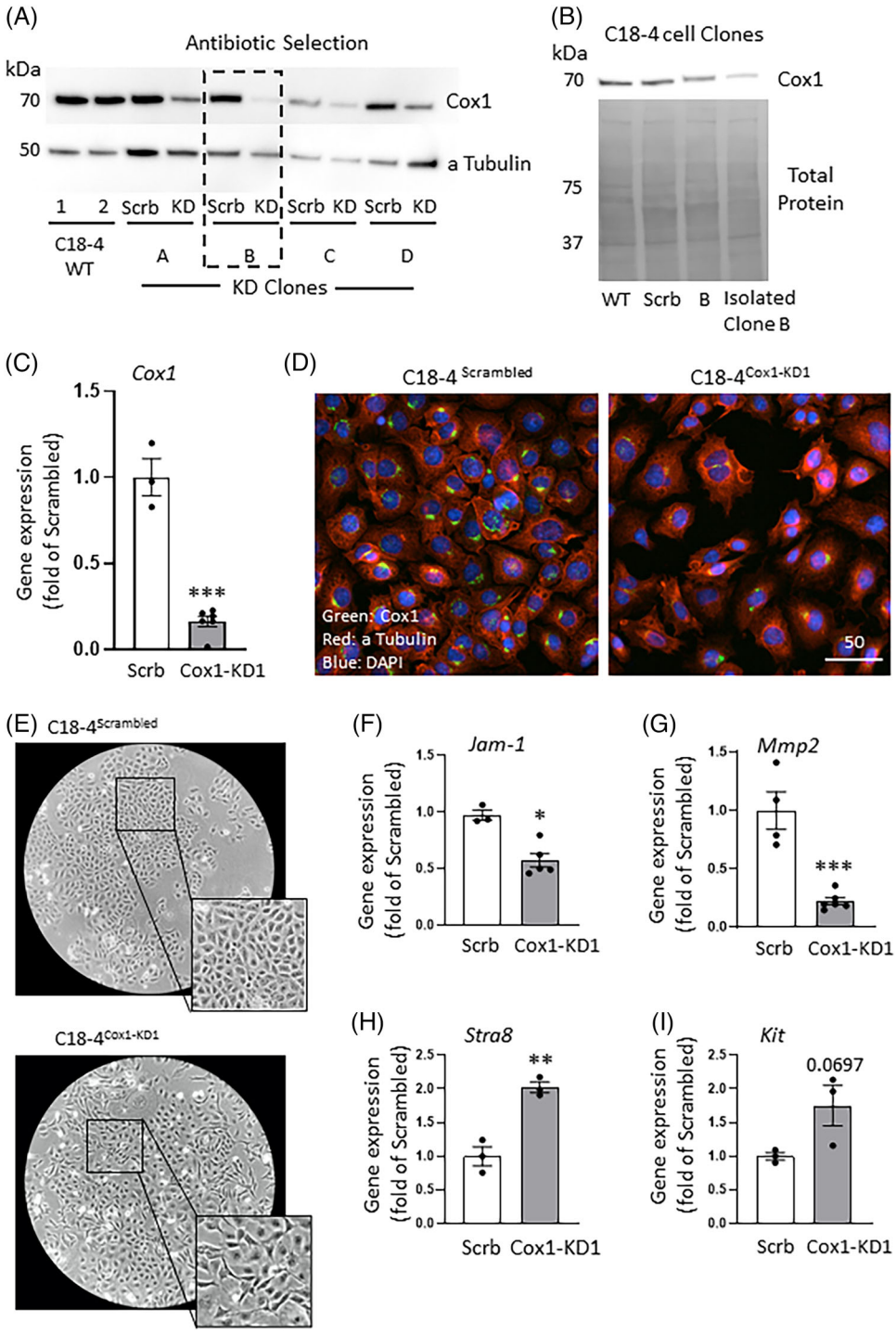


FIGURE 3 Characterization of C18-4$^{Cox1\text{-}KD1}$ cells and KD-associated morphological and gene changes. (A) Expression of cyclooxygenase (Cox)1 on populations of transfected cells with varying concentrations of shRNA and lipofectamine 3000 showing that knockdown "B" had the most downregulated expression of Cox1. (B) Cox1 protein expression on immunoblot, comparing wildtype cells (WT), scrambled cells (Scrb), knockdown "B" clone, and an isolated clonal population of C18-4$^{Cox1\text{-}KD1}$. C18-4$^{Cox1\text{-}KD1}$ Cox1 protein expression validated against the total protein. (C) Gene expression of Cox1 in C18-4$^{Cox1\text{-}KD1}$ and Scrambled controls, (D) Immunofluorescence staining of Cox1 in C18-4$^{Cox1\text{-}KD1}$ and Scrambled controls. Green: Cox1, red: alpha-tubulin, blue: DAPI. Scale in μm. (E) Brightfield visualization illustrating morphological differences between C18-4$^{Cox1\text{-}KD1}$ and Scrambled controls. (F) Differences in gene expression between C18-4$^{Cox1\text{-}KD1}$ and Scrambled controls of *Jam-1*, *Mmp2*, *Stra8*, and *Kit*. Results are presented as fold change of controls. mRNA expression data were normalized to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*); N = 3 independent experiments conducted in triplicates. Significant difference relative to controls with t-test: * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

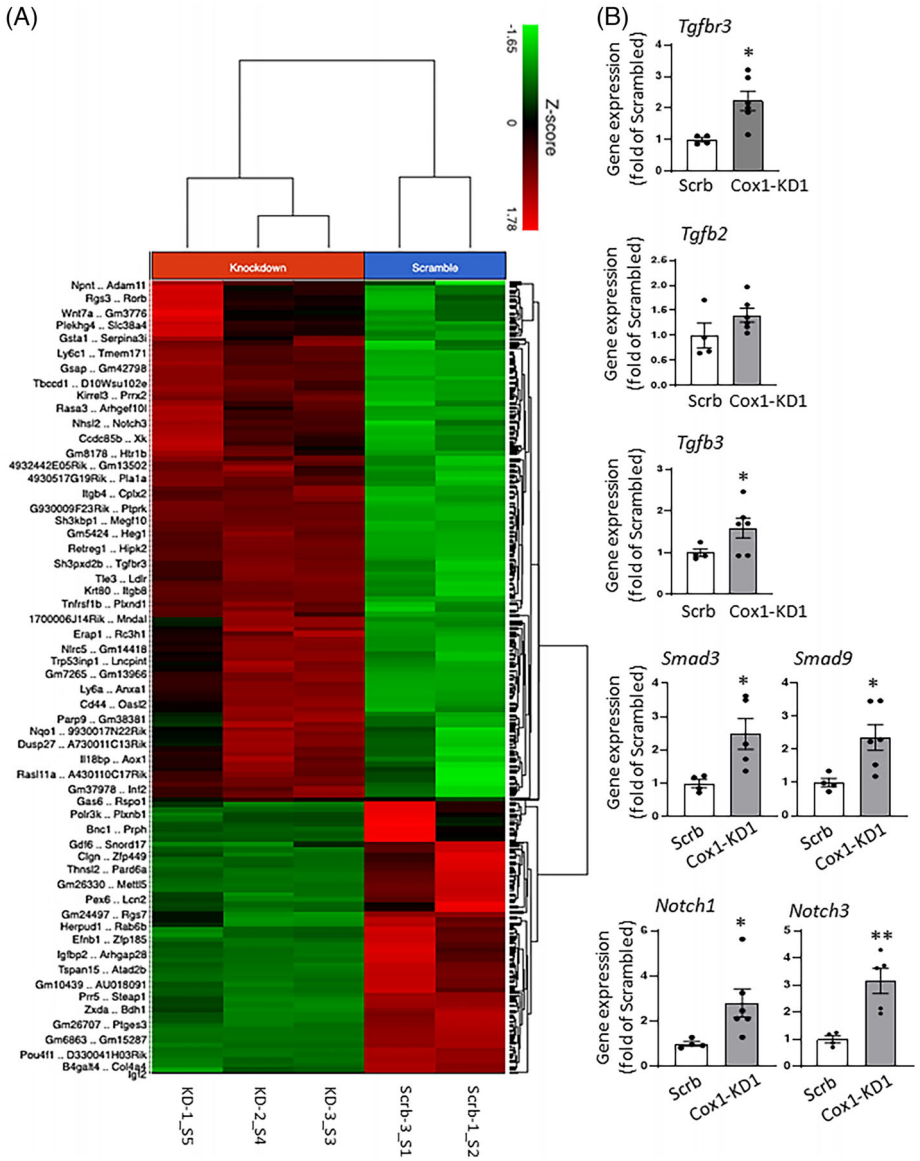


FIGURE 4 Ontology and pathway analysis of differentially expressed genes in C18-4$^{Cox1\text{-}KD1}$. RNA-seq analysis was performed on Scrambled and C18-4$^{Cox1\text{-}KD1}$ clones. (A) Hierarchical clustering of 1,265 differentially expressed genes (DEGs) between C18-4$^{Cox1\text{-}KD1}$ and Scrambled controls evaluated by total RNA sequencing and analyzed using Partek Flow (FDR < 0.1, Fold change −2–2). (B) qPCR analysis of TGFb and Notch pathway genes: *Tgfbr3*, *Tgfb2*, *Tgfb3*, *Smad3*, *Smad9*, *Notch1*, *Notch3*. mRNA expression data were normalized to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*); N = 3 independent experiments conducted in triplicates. Significant difference relative to controls with t-test: * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

blotting (Figure 5B). Compared to the scrambled controls, Notch3 receptor expression was upregulated in C18-4$^{Cox1\text{-}KD1}$ by approximately 50% (Figure 5C). While Hes1 was significantly increased in C18-4$^{Cox1\text{-}KD1}$ compared to the scrambled controls by western blot (Figure 5B,C), the percentage of Hes1-positive cells in C18-4$^{Cox1\text{-}KD1}$ showed a non-significant increasing trend compared to scrambled controls (Figure 5D). Hes1 immunolocalization was mainly diffuse in nuclei, but not different between scrambled controls and Cox1 KD cells. The alteration of Cox2 expression was consistent with what was observed using RNAseq and qPCR (Table S1).

3.6 | Differential effects of pharmacological Cox inhibitors on Notch pathway expression

To validate the effects of blocking eicosanoid synthesis on Notch signaling, wildtype C18-4 cells were treated for 24 h with pharmacological Cox inhibitors that target Cox enzymes selectively and non-selectively. Both drugs targeting Cox2 significantly induced the expression of *Notch3*- NS398 at 50 and 100 μM, and Celecoxib at 1 and 10 μM (Figure 6A,B). NS398 reduced *Notch1* expression at 50 μM but celecoxib had no effect on the expression of this gene. Celecoxib

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 List of differentially expressed genes involved in signaling pathways predicted to be altered by **cyclooxygenase (Cox)**1 silencing.

Gene name	Gene symbol	FDR	Fold change
Peroxisome proliferator-activated receptor gamma	*Pparg*	2.16E-05	32.7
Inhibitor of DNA binding 2	*Id2*	1.80E-03	6.185
Phosphoinositide-3-kinase regulatory subunit 5	*Pik3r5*	3.13E-04	5.868
Platelet-derived growth factor receptor beta	*Pdgfrb*	1.17E-03	4.673
Twist family bhlh transcription factor 2	*Twist2*	7.60E-04	4.627
Wnt family member 7A	*Wnt7a*	3.41E-02	3.653
Phosphoinositide-3-kinase regulatory subunit 1	*Pik3r1*	1.33E-03	3.488
Notch receptor 3	*Notch3*	1.26E-03	2.785
Transforming growth factor beta 2	*Tgfb2*	2.78E-03	2.761
Notch receptor 1	*Notch1*	1.84E-03	2.653
Jagged canonical Notch ligand 1	*Jag1*	7.95E-04	2.632
Fibroblast growth factor 2	*Fgf2*	9.14E-02	2.565
Fibroblast growth factor 5	*Fgf5*	2.04E-02	2.536
SMAD family member 3	*Smad3*	7.14E-04	2.308
Transforming growth factor beta 3	*Tgfb3*	4.72E-03	2.268
Phosphoinositide-3-kinase regulatory subunit 3	*Pik3r3*	2.15E-02	2.059
AKT serinethreonine kinase 3	*Akt3*	1.28E-03	−2.007
Epithelial splicing regulatory protein 2	*Esrp2*	2.56E-02	−2.171
Wnt family member 5A	*Wnt5a*	6.41E-03	−2.462
Par-6 family cell polarity regulator alpha	*Pard6a*	1.39E-02	−2.471
Wnt family member 9A	*Wnt9a*	5.83E-04	−2.8
Wnt family member 11	*Wnt11*	5.22E-04	−2.994
Matrix metallopeptidase 2	*Mmp2*	1.47E-04	−3.751
Fibroblast growth factor 22	*Fgf22*	8.97E-02	−4.516
Fibroblast growth factor 21	*Fgf21*	6.90E-04	−11.362

induced the expression of *Hes1* at both 1 and 10 μM, whereas only a non-significant increase was observed with NS398 treatment, which only reached significance at 50 μM ($p = 0.054$). Interestingly, 10 μM NS398 induced *Hey1* expression while the drug concentration had to reach 100 μM before a decrease in the expression of this gene was observed. On the other hand, celecoxib decreased *Hey1* expression at 1 μM, but the effect was not significant at 10 μM.

FR122047 was used in the same conditions as the Cox2 selective inhibitors to evaluate any differential effects between Cox1 and Cox2 selective inhibition. Similar to what was observed with the Cox2 selective inhibitors, FR122047 treatment over 24 h induced the gene expression of *Notch3* and *Hes1* at both doses of the drugs, 1 and 10 μM (Figure 6C). There was no effect of FR122047 on *Notch1* expression, and similarly to what was observed with 100 μM NS398, high dose FR122047 induced a significant decrease in *Hey1* expression.

Lastly, ACE and IBU were chosen as representative non-selective Cox inhibitors at doses of 50 and 100 μM, which were converted from human-relevant doses.[22] Both 100 μM ACE and IBU significantly upregulated *Notch3* expression, while only 100 μM IBU induced *Hes1* expression (Figure 6D). An increase was observed with 100 μM ACE on *Hes1* expression, though not significant. Interestingly, while not observed with the Cox selective inhibitors, 100 μM ACE induced the expression of *Notch1*, which indicates that ACE is capable of targeting both types of Notch receptors, perhaps due to independent effects outside Cox inhibition. 100 μM IBU was also able to induce *Hey1* expression, which is another effect not observed with the selective Cox inhibitors at high doses and may be attributed uniquely to this drug.

Evaluation of Notch3 protein expression using immunofluorescence staining illustrated similar effects, with NS398 and FR122047 increasing the percent of cells expressing Notch3, visible as bright green fluorescence spots in nuclei (Figure 6E). Quantification of positive cells showed significant increases with the high doses of 100 and 10 μM NS398 and FR122047, respectively (Figure 6F,G). Western blot validation showed similar results, however, induction of Notch3 expression was not observed with either dose of celecoxib (Figure 6H).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

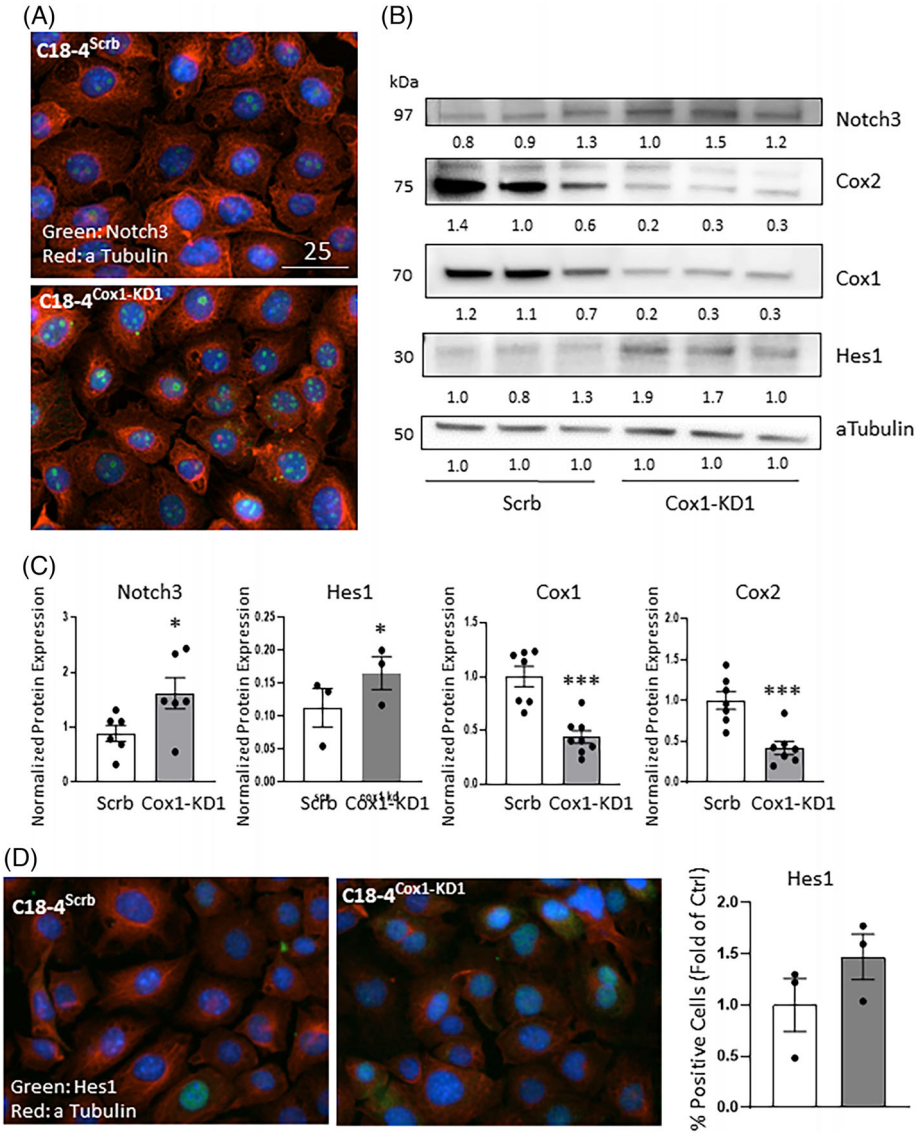


FIGURE 5 Activation of Notch3 signaling pathway in C18-4$^{Cox1\text{-}KD1.}$ (A) Immunofluorescence (IF) images of Notch3 expression in C18-4$^{Cox1\text{-}KD1}$ and Scrambled controls. Green: Notch3, red: alpha-tubulin, blue: DAPI. Scale in μm. (B) Immunoblots of Notch3, Hes1, cyclooxygenase (Cox)1, and Cox2 expression in C18-4$^{Cox1\text{-}KD1}$ and Scrambled controls are cropped at indicated bands, and protein expression, indicated by values under the bands, is normalized to alpha-tubulin. (C) Quantification of Notch3, Hes1, Cox1, and Cox2 protein expression on western blot normalized against the total protein. Results are presented as fold change of controls. (D) IF images of Hes1 expression in C18-4$^{Cox1\text{-}KD1}$ and Scrambled controls. Green: Hes1, red: alpha-tubulin, blue: DAPI. Hes1-positive cells were counted and the data show the percentage of Hes-positive cells as fold of control samples. N = 3 independent experiments. Significant difference relative to controls with t-test: * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

3.7 | PGD2 negatively regulates Notch signaling in C18-4 spermatogonial cells

We next wanted to explore whether PGs are involved in mediating the interaction between the eicosanoid and Notch signaling pathways and whether they are responsible for the activation of the Notch pathway observed with silencing Cox1. Wildtype C18-4 cells were treated for 24 h with 1, 10, and 50 μM of supplemental PGD2. PGD2 treatment resulted in the suppression of *Notch3* mRNA expression (Figure 7A), and a similar effect was observed with *Hes1* expression as suppression was also observed at the 10 and 50 μM doses (Figure 7C). Note that, 10 μM PGD2 downregulated *Notch1* and *Hey1* expression (Figure 7B,D), but at 50 μM, the opposite effect was observed. Such biphasic response is reminiscent of adaptative hormetic effects observed with substances acting on the hormonal system and chemical stressors.[23] However, this effect of PGD2 was found only for *Hey1* expression, suggesting that the higher dose of PGD2 had activated a unique signaling pathway regulating *Hey1* expression.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

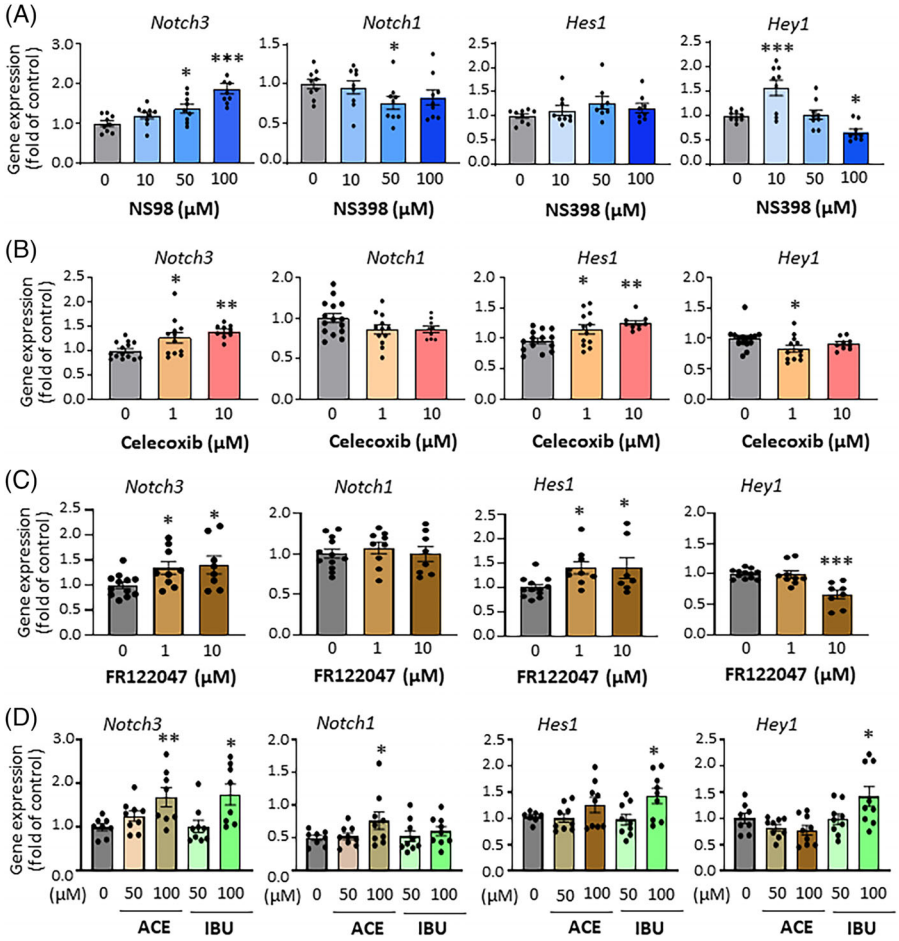


FIGURE 6 Differential effects of cyclooxygenase (Cox) inhibitors on Notch pathway expression. Effect of 24 h treatment of Cox inhibitors on expression of Notch pathway genes *Notch3*, *Notch1*, *Hes1*, and *Hey1* with (A) 10–100 μM NS398, (B) 1–10 μM celecoxib, (C) 1–10 μM FR122047, and (D) 50 and 100 μM acetaminophen and ibuprofen. Results are presented as fold change of control (0). mRNA expression data is normalized to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). (E) Immunofluorescence images of Notch3 expression after 24 h treatment with 100 μM NS398 and 10 μM FR122047 against their respective controls. Green: Notch3, red: alpha-tubulin, blue: DAPI. Scale in μm. Quantification of % positive cells is normalized to controls with (F) 10 and 100 μM NS398 and (G) 1 and 10 μM FR122047 24-h treatment. N = 3 independent experiments conducted in triplicates. Significant difference relative to control medium with one-way analysis of variance (ANOVA) test and multiple comparisons: * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). (H) Representative western blots are cropped to show Notch3 expression with the 24-h treatment of NS398, celecoxib, and FR122047. Expression was compared to total protein.

4 | DISCUSSION

The objective of this study was to explore the role of the eicosanoid pathway in spermatogonial development. We chose to target the Cox enzymes due to their properties as rate-limiting enzymes of the eicosanoid pathway and as targets of over-the-counter analgesics ACE and IBU. We first observed that concentrations of ACE and IBU equivalent to those measured in treated infants did not alter spg proliferation. However, the Cox1 selective inhibitor FR122047 and Cox2 selective inhibitor Celecoxib reduced cell numbers at the highest dose used, indicating some toxicity. Although cell numbers were not measured for the high 100 μM concentration of NS398, subsequent experiments using that dose did not show remarkable changes in cell numbers compared to controls (Figure 2E) suggesting that NS398 is less toxic for spermatogonia than Celecoxib and FR122047. This also suggests that Cox enzymes might play a role in spermatogonia survival and that two selective Cox2 inhibitors might not have the exact same effects, either due to different affinities for Cox2 or via unknown off-target effects.

As Cox1 is the more prevalent of the two enzymes, and being one that is constitutively expressed in all cell types,[24] we focused our efforts towards interrogating the role of Cox1. We believe that a greater understanding of its involvement in regulating spg development will also aid in elucidating potentially toxic properties of analgesic drugs that target the eicosanoid pathway. Observations from Cox1 knockdown studies were confirmed with pharmacological Cox inhibitors that target Cox1 and Cox2 both selectively and non-selectively. Our data indicate that blocking Cox enzymes either with shRNA-based silencing or pharmaceutical Cox inhibitors can alter the cellular properties of the SSCs, as modeled by the C18-4 cell line. Cox2

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

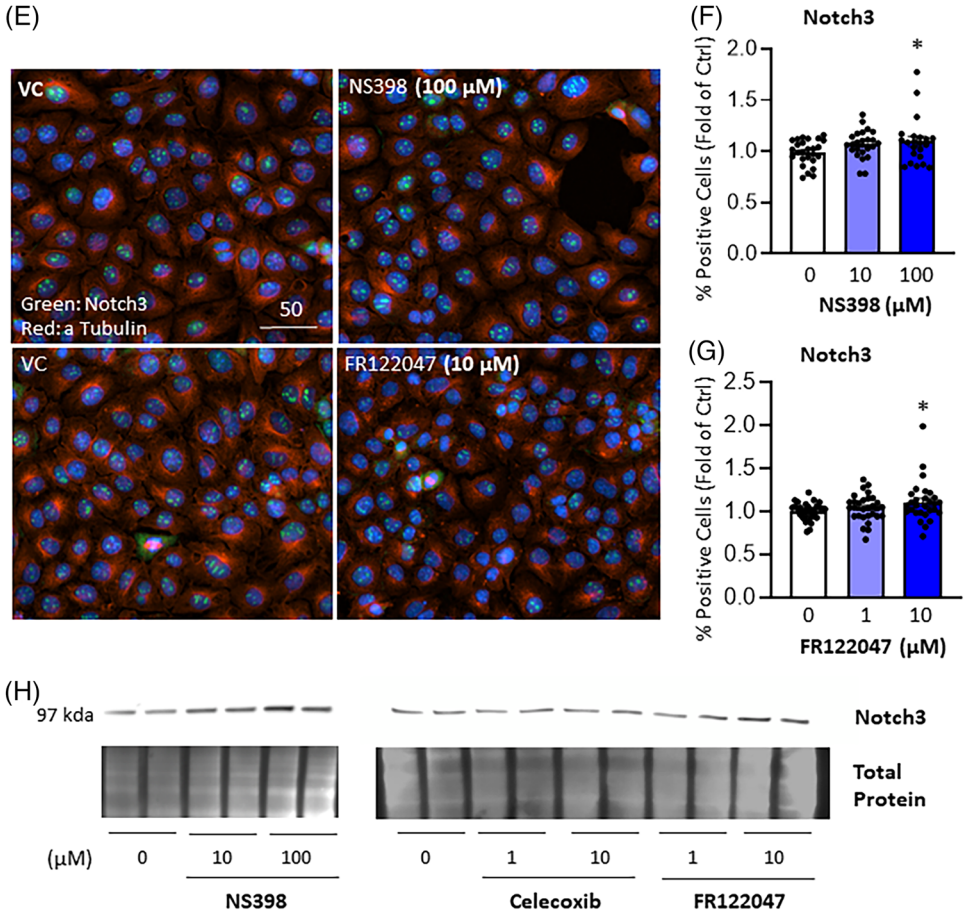


FIGURE 6 Continued

was found to be positively associated with differentiation, while Cox1 was negatively associated with differentiation.

Previous studies conducted by our lab found that ACE and IBU also provoked functional changes in neonatal gonocytes, and we reported that treatments of these drugs were able to induce gonocyte proliferation and partially inhibit differentiation after 24 h. In the current study, we report that reducing Cox1 levels in spg promotes differentiation, whereas in gonocytes, reducing Cox2 levels decreases differentiation. Cox2 was positively associated with differentiation during the gonocyte and spermatogonia stages of development. However, more studies will be needed in gonocytes to determine whether Cox1 has similar roles in both cell types. It is also important to note that gonocytes and SSCs represent two unique stages of development with the establishment of germ cells on the basement membrane being the major differentiator between these two cell types. These cells also exhibit unique roles, with the gonocytes' main purpose being to establish the foundational SSC pool and SSCs being to maintain the lifelong production of sperm. Their unique roles are reflected in the distinctive molecular profile of these two populations of cells and their different fate trajectories.[7,25–27] Therefore, it is possible that drugs targeting the same enzymes within the gonocyte and spermatogonial populations may have different effects on regulating differentiation. However, we cannot disregard that our current findings are based on a cell-line model, which, despite being able to exhibit differentiating properties that are characteristic of SSCs, may not be able to recapitulate all the properties of a non-immortalized mammalian germ cell population.

From the total RNAseq analysis, we identified several signaling pathways that were altered by Cox1 silencing. Of those, the Notch signaling pathway was consistently upregulated in both knockdown and drug studies, suggesting that this pathway is a major downstream target of eicosanoids. This is confirmed with the treatment of PGD2 on wild-type C18-4 cells, illustrating that PGD2 can negatively regulate the expression of Notch pathway genes. By suppressing PGD2 levels, Cox inhibitors may be releasing a brake on Notch pathway activity, which subsequently results in constitutive activation of the pathway. The Notch signaling pathway is a well-conserved pathway that has been widely characterized for its involvement in cell fate determination.[28] Upon ligand binding, the Notch receptor undergoes proteolytic cleavage leading to the cytoplasmic release of its intracellular domain (NICD) and subsequent translocation into the nucleus where it interacts with transcriptional regulators and activate target genes such as Hairy/Enhancer of Split (Hes) and Hes-related (Hey) genes.[29] Cox1 silencing led to increased expression of both Notch3 and Hes1, while pharmacological Cox inhibitors induced differential effects on Notch receptors and their downstream targets.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

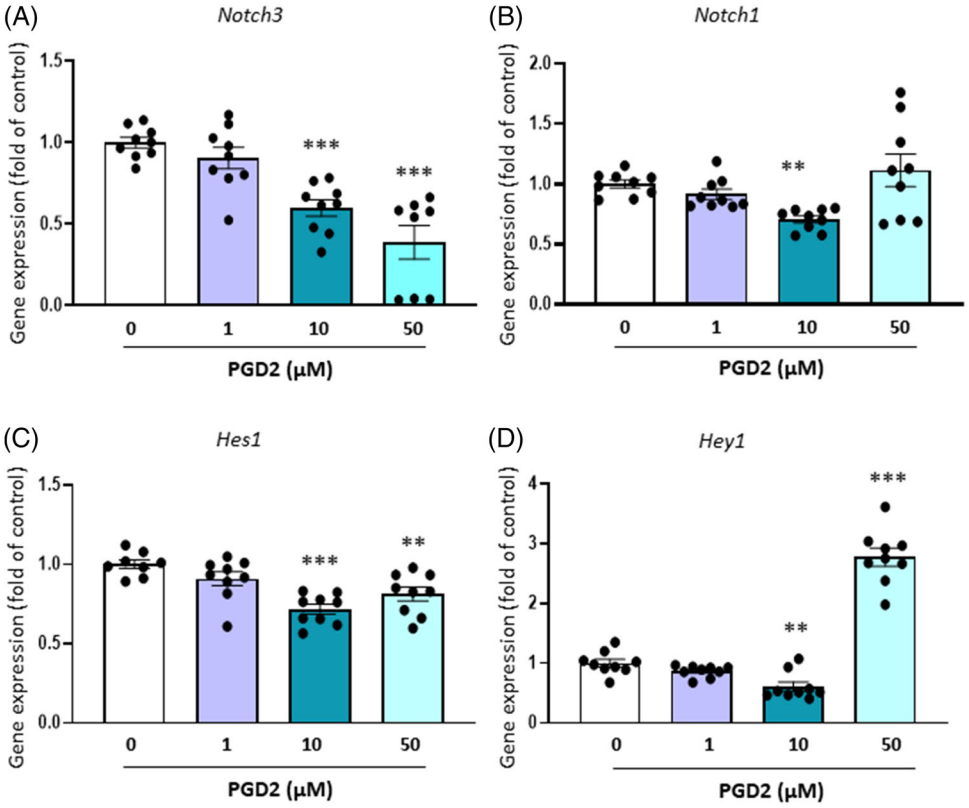


FIGURE 7 PGD2 negatively regulates Notch signaling in C18-4 spermatogonial cells. mRNA expression of (A) *Notch3*, (B) *Notch1*, (C) *Hes1*, and (D) *Hey1* after 24 h treatment of 1, 10, and 50 μM PDG2. Results are presented as fold change of control (0). mRNA expression data were normalized to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Significant difference relative to control medium with one-way ANOVA test and multiple comparisons: * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Silencing of Cox1 in the C18-4 cell line prompted morphological changes that seemed to reflect an epithelial to mesenchymal transition (EMT) both in the alteration of C18-4$^{Cox1\text{-}KD1}$ cell physical appearance, decreases in the expression of cellular adhesion and matrix metalloproteinase genes, and in the molecular pathways that were predicted to be altered from IPA. EMT is characterized by a transition from an epithelial phenotype to an elongated fibroblastic or mesenchymal phenotype leading to increased motility and invasion, which is also characteristic of tumors. During embryonic development, EMT is related to cellular plasticity, and this process plays a critical role in stem cell behavior and induction of pluripotency.[30] While more studies will have to be conducted to evaluate downstream Notch signaling effects before definitively determining a role of this pathway's involvement with EMT observed in the C18-4$^{Cox1\text{-}KD1}$ cells, we can presently turn to the literature to speculate a possible relationship.

The Notch pathway has been found to be implicated in many human cancers and several Notch pathway receptors have been shown to be prognostic markers in different types of cancers such as breast, gastric, and prostate.[31,32] Whereas many studies found positive correlations between Notch signaling activation and cancer progression, some studies evaluating the involvement of Notch signaling on EMT showed that this pathway is negatively correlated with the EMT transition of breast cancer cells.[33,34] The overexpression of the Notch intracellular domain (N3ICD) decreased mesenchymal marker vimentin and increased levels of tight junctional proteins E-cadherin and E-catenin. Perhaps in spgs, this signaling pathway can also play a significant role in the EMT of the SSC and may be involved in the malignant transformation of spgs, which may aid in the pathogenesis of SS.[8]

Other studies support the involvement of the Notch signaling pathway in spermatogenesis either in affecting the development of germ cells themselves or being involved in the regulation of germ cell development by supporting Sertoli cells. Notch3 and Hes1 were found to be expressed in juvenile and prepuberal spermatogonia, consistent with our findings, and only weakly in adult spermatogonia.[35] As for the role of Notch signaling in Sertoli cells, the cells responsible for supporting the proper maturation of spgs, the Notch-Hes1 signaling axis was dispensable for spermatogenesis as Notch knockout mice retained normal spermatogenesis and fertility.[36] However, studies have shown that the activation of Notch signaling in Sertoli cells is important for the regulation of SSC development.[37,38] Undifferentiated spgs can modulate the activation of the Notch1-Hey1 axis to downregulate CYP26b1 and remove its blockade on RA production to drive SSC differentiation.[39] Our study indicates that the Notch signaling pathway is important to the development of SSCs, supporting a similar role to that of Sertoli cells.

Alternatively, one cannot rule out the role of TGFb signaling in our model, as several TGFb signaling family members were upregulated in

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

the C18-4$^{Cox1\text{-}KD1}$ cells as well. TGFb signaling pathway plays a critical role in establishing the germline during mouse embryogenesis, in addition to steroidogenesis, cord formation, and gonocyte behavior[40,41] Furthermore, TGFb was able to induce Cox2 expression during EMT in breast cancer progression, and Cox2 had the ability to enhance oncogenic TGFb signaling by inhibiting Smad 2/3 activity via PGE2.[42] Both the Notch and TGFb signaling pathways are known to converge on the regulation of differentiation events during development,[43,44] and significant crosstalk exists between them in various tissues and cell types.[45–47] While we chose to focus our efforts on characterizing Notch signaling with respect to pharmacological Cox inhibitors, we note that TGFb signaling may play an important role as well, and its ability to crosstalk with Notch signaling pathways can also contribute to downstream functional effects.

In future studies, we aim to explore the mechanistic interactions between eicosanoid and Notch signaling pathways that are supported by the literature. In a gastric cancer cell line, characteristics of cancer progression that were enhanced by Notch1 pathway activation were suppressed when the cells were treated with NS398 or by Cox2 knockdown.[48] It was also found that the PGE2 receptor acts downstream of the Notch signaling pathway to inhibit differentiation of human skeletal muscle progenitors.[49] Interestingly, while these studies suggest that the eicosanoid pathway acts downstream of the Notch signaling pathway, we show that eicosanoids can also regulate the expression of Notch signaling pathway genes. Indeed, PGs may play crucial roles as biochemical mediators that regulate molecular signaling pathways that are important for cell fate determination of SSCs.

The roles of non-selective Cox inhibitors on male germ cell development are only beginning to be deciphered. While several groups have reported that Cox inhibitors can decrease the expression of fetal germ cell markers,[50–53] no reports currently exist on the effects of Cox inhibitors on SSCs, which are critical for sperm formation and fertility beyond puberty. Infancy represents a critical period for SSC formation and disrupting SSCs or their precursors may be associated with infertility and testicular cancer.[54–56] The development of germ cells is highly dynamic during this period of development, and disruptions to the balance between self-renewal, proliferation, and apoptosis have been proposed to initiate testicular germ cell tumor formation.[57] Therefore, a greater understanding of how Cox inhibitors, such as ACE and IBU, might impact the development of neonatal germ cells could provide insights into the origins of testicular cancer and male infertility that have been afflicting young men at an alarming rate.

5 | CONCLUSION

In this study, we report that Cox inhibition can alter the differentiation of juvenile undifferentiated spgs, including SSC, with Cox1 preventing differentiation and Cox2 promoting it. Cox1 silencing leads to the activation of the Notch signaling pathway primarily via the Notch3-Hes1 axis (see Graphic Abstract). We speculate that the Notch3 pathway underlies a mechanism behind the ability of pharmacological Cox inhibitors to disrupt the normal fate determination by inducing the morphological transformation of SSCs. Future studies will explore interactions between eicosanoid and Notch signaling pathways to further understand the role of Notch3 in regulating undifferentiated spgs and SSC development.

AUTHOR CONTRIBUTIONS

Conceptualization: Amy Tran-Guzman and Martine Culty; Methodology and investigation: Amy Tran-Guzman and Amina Khan; Formal analysis: Amy Tran-Guzman and Martine Culty; Resources: Martine Culty; Writing—original draft preparation: Amy Tran-Guzman; Writing—review and editing: Martine Culty; Supervision: Martine Culty; Funding acquisition: Martine Culty. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Marie-Claude Hofmann for the generous gift of the C18-4 mouse spermatogonia cell line. We would also like to thank the bioinformatics team at USC Norris Library for aiding with our total RNAseq analysis, the USC Norris Molecular Genomics Core for sequencing our samples for the Cox1 knockdown study, and Ari Gritsas and the team at the McGill University Health Centre for conducting the prostaglandin LC-MS studies. This research was supported by funds from the USC Alfred E. Mann School of Pharmacy and Pharmaceutical Sciences to Martine Culty.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings are included in the manuscript.

ORCID

Martine Culty https://orcid.org/0000-0002-8295-7716

REFERENCES

1. Kristensen DM, Mazaud-Guittot S, Gaudriault P, et al. Analgesic use — prevalence, biomonitoring and endocrine and reproductive effects. *Nat Rev Endocrinol*. 2016;12(7):381-393. doi:10.1038/nrendo.2016.55
2. Hernandez RK, Werler MM, Romitti P, Sun L, Anderka M. Nonsteroidal antiinflammatory drug use among women and the risk of birth defects. *Am J Obstetr Gynecol*. 2012;206(3):228.e1-228.e8. doi:10.1016/j.ajog.2011.11.019
3. Hurtado-Gonzalez P, Anderson RA, Macdonald J, et al. Effects of exposure to acetaminophen and ibuprofen on fetal germ cell development in both sexes in rodent and human using multiple experimental systems. *Environ Health Perspect*. 2018;126(4):047006. doi:10.1289/ehp2307
4. Kristensen DM, Lesné L, Le Fol V, et al. Paracetamol (acetaminophen), aspirin (acetylsalicylic acid) and indomethacin are anti-androgenic in the rat foetal testis. *Int J Androl*. 2012;35(3):377-384. doi:10.1111/j.1365-2605.2012.01282.x
5. Dean A, Mungall W, McKinnell C, Sharpe RM. Prostaglandins, masculinization and its disorders: effects of fetal exposure of the rat to the cyclooxygenase inhibitor- indomethacin. *PLoS ONE*. 2013;8(5):e62556. doi:10.1371/journal.pone.0062556

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

6. Skakkebaek NE, Holm M, Hoei-Hansen C, Jorgensen N, Rajpert-De Meyts E. Association between testicular dysgenesis syndrome (TDS) and testicular neoplasia: evidence from 20 adult patients with signs of maldevelopment of the testis. *APMIS*. 2003;111(1):1-9. doi:10.1034/j.1600-0463.2003.11101031.x. discussion 9–11.
7. Manku G, Culty M. Mammalian gonocyte and spermatogonia differentiation: recent advances and remaining challenges. *Reproduction*. 2015;149(3):R139-R157.
8. Waheeb R, Hofmann MC. Human spermatogonial stem cells: a possible origin for spermatocytic seminoma. *Int J Androl*. 2011;34(4pt2):e296-e305. doi:10.1111/j.1365-2605.2011.01199.x
9. Bloor M, Paech M. Nonsteroidal anti-inflammatory drugs during pregnancy and the initiation of lactation. *Anesth Analg*. 2013;116(5):1063-1075.
10. Reese J, Zhao X, Ma W-G, Brown N, Maziasz TJ, Dey SK. Comparative analysis of pharmacologic and/or genetic disruption of cyclooxygenase-1 and cyclooxygenase-2 function in female reproduction in mice*. *Endocrinology*. 2001;142(7):3198-3206. doi:10.1210/endo.142.7.8307
11. Simmons DL, Botting RM, Hla T. Cyclooxygenase Isozymes: The biology of prostaglandin synthesis and inhibition. *Pharmacol Rev*. 2004;56(3):387-437. doi:10.1124/pr.56.3.3
12. Manku G, Papadopoulos P, Boisvert A, Culty M. Cyclooxygenase 2 (COX2) expression and prostaglandin synthesis in neonatal rat testicular germ cells: effects of acetaminophen and ibuprofen. *Andrology*. 2020;8(3):691-705.
13. Tran-Guzman A, Moradian R, Cui H, Culty M. In vitro impact of genistein and mono(2-ethylhexyl) phthalate (MEHP) on the eicosanoid pathway in spermatogonial stem cells. *Reprod Toxicol*. 2022;107:150-165. doi:10.1016/j.reprotox.2021.12.007
14. Hofmann MC, Braydich-Stolle L, Dym M. Isolation of male germ-line stem cells; influence of GDNF. *Dev Biol*. 2005;279(1):114-124. doi:10.1016/j.ydbio.2004.12.006
15. Mi H, Muruganujan A, Thomas PD. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic Acids Res*. 2012;41(D1):D377-D386.
16. Thomas PD, Campbell MJ, Kejariwal A, et al. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res*. 2003;13(9):2129-2141.
17. Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, Otomo S. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. *Prostaglandins*. 1994;47(1):55-59.
18. Penning TD, Talley JJ, Bertenshaw SR, et al. Synthesis and biological evaluation of the 1, 5-diarylpyrazole class of cyclooxygenase-2 inhibitors: identification of 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1 H-pyrazol-1-yl] benzenesulfonamide (SC-58635, celecoxib). *J Med Chem*. 1997;40(9):1347-1365.
19. Ochi T, Motoyama Y, Goto T. The analgesic effect profile of FR122047, a selective cyclooxygenase-1 inhibitor, in chemical nociceptive models. *Eur J Pharmacol*. 2000;391(1-2):49-54.
20. Kato M, Nishida S, Kitasato H, Sakata N, Kawai S. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of non-steroidal anti-inflammatory drugs: investigation using human peripheral monocytes. *J Pharm Pharmacol*. 2001;53(12):1679-1685.
21. Hinz B, Cheremina O, Brune K. Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. *FASEB J*. 2008;22(2):383-390.
22. Brown RD, Wilson JT, Kearns GL, Eichler VF, Johnson VA, Bertrand KM. Single-dose pharmacokinetics of ibuprofen and acetaminophen in febrile children. *J Clin Pharmacol*. 1992;32(3):231-241.
23. Calabrese EJ. Biphasic dose responses in biology, toxicology and medicine: Accounting for their generalizability and quantitative features. *Environ Pollut*. 2013;182:452-460. doi:10.1016/j.envpol.2013.07.046
24. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Ann Rev Biochem*. 2000;69(1):145-182.
25. Culty M. Gonocytes, the forgotten cells of the germ cell lineage. *Birth Defects Res Part C: Embryo Today: Rev*. 2009;87(1):1-26.
26. Oatley JM, Brinster RL. Regulation of spermatogonial stem cell self-renewal in mammals. *Ann Rev Cell Dev Biol*. 2008;24:263-286.
27. Law NC, Oatley MJ, Oatley JM. Developmental kinetics and transcriptome dynamics of stem cell specification in the spermatogenic lineage. *Nat Commun*. 2019;10(1):1-14.
28. Harper JA, Yuan JS, Tan JB, Visan I, Guidos CJ. Notch signaling in development and disease. *Clin Genetics*. 2003;64(6):461-472.
29. Klüppel M, Wrana JL. Turning it up a Notch: cross-talk between TGFβ and Notch signaling. *Bioessays*. 2005;27(2):115-118.
30. Kim DH, Xing T, Yang Z, Dudek R, Lu Q, Chen Y-H. Epithelial mesenchymal transition in embryonic development, tissue repair and cancer: a comprehensive overview. *J Clin Med*. 2018;7(1):1.
31. Aburjania Z, Jang S, Whitt J, Jaskula-Stzul R, Chen H, Rose JB. The role of notch3 in cancer. *Oncologist*. 2018;23(8):900.
32. Giuli MV, Giuliani E, Screpanti I, Bellavia D, Checquolo S. Notch signaling activation as a hallmark for triple-negative breast cancer subtype. *J Oncol*. 2019;2019:8707053. doi:10.1155/2019/8707053
33. Tan J, Zhang X, Xiao W, et al. N3ICD with the transmembrane domain can effectively inhibit EMT by correcting the position of tight/adherens junctions. *Cell Adhesion Migration*. 2019;13(1):203-218.
34. Zhang X, Liu X, Luo J, et al. Notch3 inhibits epithelial–mesenchymal transition by activating Kibra-mediated Hippo/YAP signaling in breast cancer epithelial cells. *Oncogenesis*. 2016;5(11):e269-e269.
35. Okada R, Fujimagari M, Koya E, Hirose Y, Sato T, Nishina Y. Expression profile of NOTCH3 in mouse Spermatogonia. *Cells Tissues Organs*. 2017;204(5-6):283-292.
36. Huang Z, Rivas B, Agoulnik AI. NOTCH1 gain of function in germ cells causes failure of spermatogenesis in male mice. *PLoS One*. 2013;8(7):e71213.
37. Garcia TX, DeFalco T, Capel B, Hofmann M-C. Constitutive activation of NOTCH1 signaling in Sertoli cells causes gonocyte exit from quiescence. *Dev Biol*. 2013;377(1):188-201.
38. Garcia TX, Hofmann M-C. NOTCH signaling in Sertoli cells regulates gonocyte fate. *Cell cycle*. 2013;12(16):2538-2545.
39. Parekh PA, Garcia TX, Waheeb R, et al. Undifferentiated spermatogonia regulate Cyp26b1 expression through NOTCH signaling and drive germ cell differentiation. *FASEB J*. 2019;33(7):8423-8435.
40. Young JC, Wakitani S, Loveland KL. TGF-beta superfamily signaling in testis formation and early male germline development. *Semin Cell Dev Biol*. 2015;45:94-103. doi:10.1016/j.semcdb.2015.10.029
41. Loveland KL, Dias V, Meachem S, Rajpert-De Meyts E. The transforming growth factor-β superfamily in early spermatogenesis: potential relevance to testicular dysgenesis. *Int J Androl*. 2007;30(4):377-384.
42. Neil JR, Johnson KM, Nemenoff RA, Schiemann WP. COX-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE 2-dependent mechanisms. *Carcinogenesis*. 2008;29(11):2227-2235.
43. Kuroda K, Tani S, Tamura K, Minoguchi S, Kurooka H, Honjo T. Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J Biol Chem*. 1999;274(11):7238-7244.
44. Massagué J, Cheifetz S, Endo T, Nadal-Ginard B. Type beta transforming growth factor is an inhibitor of myogenic differentiation. *Proc Nat Acad Sci*. 1986;83(21):8206-8210.
45. Blokzijl A, Dahlqvist C, Reissmann E, et al. Cross-talk between the Notch and TGF-β signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J Cell Biol*. 2003;163(4):723-728.
46. Dahlqvist C, Blokzijl A, Chapman G, et al. Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. *Development*. 2003;130(24):6089-6099. doi:10.1242/dev.00834

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

47. Itoh F, Itoh S, Goumans MJ, et al. Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. *EMBO J*. 2004;23(3):541-551.
48. Yeh T-S, Wu C-W, Hsu K-W, et al. The activated Notch1 signal pathway is associated with gastric cancer progression through cyclooxygenase-2. *Cancer Res*. 2009;69(12):5039-5048.
49. Sakai-Takemura F, Ki Nogami, Elhussieny A, et al. Prostaglandin EP2 receptor downstream of Notch signaling inhibits differentiation of human skeletal muscle progenitors in differentiation conditions. *Commun Biol*. 2020;3(1):1-13.
50. Ben Maamar M, Lesné L, Hennig K, et al. Ibuprofen results in alterations of human fetal testis development. *Sci Rep*. 2017;7(1):44184. doi:10.1038/srep44184
51. Dean A, Van Den Driesche S, Wang Y, et al. Analgesic exposure in pregnant rats affects fetal germ cell development with inter-generational reproductive consequences. *Sci Rep*. 2016;6(1):1-12.
52. Moniot B, Ujjan S, Champagne J, et al. Prostaglandin D2 acts through the Dp2 receptor to influence male germ cell differentiation in the foetal mouse testis. *Development*. 2014;141(18):3561-3571.
53. Albert O, Desdoits-Lethimonier C, Lesné L, et al. aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. *Human Reprod*. 2013;28(7):1890-1898.
54. Singh SR, Burnicka-Turek O, Chauhan C, Hou SX. Spermatogonial stem cells, infertility and testicular cancer. *J Cell Mol Med*. 2011;15(3):468-483. doi:10.1111/j.1582-4934.2010.01242.x
55. Kristensen DM, Sonne SB, Ottesen AM, et al. Origin of pluripotent germ cell tumours: the role of microenvironment during embryonic development. *Mol Cell Endocrinol*. 2008;288(1-2):111-118. doi:10.1016/j.mce.2008.02.018
56. Almstrup K, Sonne SB, Hoei-Hansen CE, et al. From embryonic stem cells to testicular germ cell cancer—should we be concerned? *Int J Androl*. 2006;29(1):211-218. doi:10.1111/j.1365-2605.2005.00643.x
57. Hayes-Lattin B, Nichols CR. Testicular cancer: a prototypic tumor of young adults. *Semin Oncol*. 2009;36(5):432-438. doi:10.1053/j.seminoncol.2009.07.006

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Tran-Guzman A, Khan A, Culty M. Differential roles of cyclooxygenase enzymes in the regulation of murine juvenile undifferentiated spermatogonia. *Andrology*. 2024;12:899–917. https://doi.org/10.1111/andr.13537

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13537 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Check for updates

Received: 7 December 2022 | Revised: 21 July 2023 | Accepted: 16 August 2023

DOI: 10.1111/andr.13517

ANDROLOGY

WILEY

ORIGINAL ARTICLE

Cryopreservation of equine spermatozoa reduces plasma membrane integrity and phospholipase C zeta 1 content as associated with oocyte activation

Raul A. Gonzalez-Castro[1] | Luke A. Whitcomb[2] | Emma C. Pinsinski[2] | Elaine M. Carnevale[1]

[1]Equine Reproduction Laboratory, Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado, USA

[2]Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado, USA

Correspondence
Elaine M. Carnevale, Equine Reproduction Laboratory, Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado, USA.
Email: Elaine.Carnevale@colostate.edu

Abstract

Background: Phospholipase C zeta (PLCZ1) is considered the major sperm-borne oocyte activation factor. Cryopreserved stallion spermatozoa are commonly used for intracytoplasmic sperm injection (ICSI). However, plasma membrane damage and protein modifications caused by cryopreservation could impair sperm structure and function, leading to a reduction of PLCZ1 and oocyte activation after ICSI.

Objectives: We compared membrane integrity and PLCZ1 abundance in populations for fresh, frozen, and refrozen stallion spermatozoa, either thawed and refrozen at room or low temperature; and examined the effect of relative PLCZ1 content on cleavage after ICSI.

Materials and methods: Western blotting, ELISA, and immunofluorescence were conducted in stallion spermatozoa, freezing extenders, and detergent-extracted sperm fractions to detect and quantify PLCZ1. Retrospectively, PLCZ1 content and cleavage rate were analyzed. Fresh, frozen, and refrozen at room and low temperatures spermatozoa were evaluated for acrosomal and plasma membrane integrity and PLCZ1 content using flow cytometry.

Results: Western blotting, ELISA, and immunofluorescence revealed significant reduction of PLCZ1 in spermatozoa after cryopreservation and confirmed PLCZ1 detection in extenders. After detergent extraction, a PLCZ1-nonextractable fraction remained in the postacrosomal region of spermatozoa. Plasma membrane integrity was significantly reduced after freezing. Acrosomal and plasma membrane integrity were similar between frozen and refrozen samples at low temperature, but both were significantly higher than samples refrozen at room temperature. Acrosomal and plasma membrane integrity significantly correlated to PLCZ1 content. Percentages of PLCZ1-labeled spermatozoa and PLCZ1 content were reduced after freezing but not after refreezing. Relative content and localization of PLCZ1 were associated with cleavage rates after ICSI.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2023 The Authors. *Andrology* published by Wiley Periodicals LLC on behalf of American Society of *Andrology* and European Academy of *Andrology*.

Discussion and conclusion: Sperm PLCZ1 content associates with cleavage rates after ICSI. Cryopreservation is detrimental to sperm plasma membrane integrity and PLCZ1 retention. However, refreezing did not result in additional PLCZ1 loss. Refreezing stallion spermatozoa at a low temperature resulted in better survival but did not improve PLCZ1 retention.

KEYWORDS
ELISA, flow cytometry, freezing, PLCZ1, refreezing, viability

1 | INTRODUCTION

During fertilization, the spermatozoon delivers oocyte activating factors into the ooplasm, inducing oocyte activation and, consequently, cleavage, and embryo development.[1] Phospholipase C zeta (PLCZ1) is considered the main sperm-borne oocyte activation factor in several mammalian species.[2-11] In stallion spermatozoa, PLCZ1 has been identified as a 73 kDa protein, immunolocalized in the acrosomal and equatorial regions, connecting piece, and principal piece of the tail.[12] After gamete membrane fusion, PLCZ1 is released into the ooplasm, hydrolyzing organelle membrane-bound phosphatidylinositol 4,5-bisphosphate to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). In the endoplasmic reticulum, IP_3 binds to the IP_3 receptor, causing calcium oscillations and oocyte activation events. In addition, DAG activates protein kinase C.[13] In various species, a relationship between male infertility and deficiencies of PLCZ1 has been established. Reduced levels, abnormal localization, or genetic mutations of PLCZ1 are associated with intracytoplasmic sperm injection (ICSI) failure or low fertilization rates in men, mice, and cats.[4,8,14–21] High content and appropriate localization of PLCZ1 in human and equine spermatozoa are positively related to cleavage rates and ICSI outcome,[11,22] supporting the role of PLCZ1 for oocyte activation.

The initial application of ICSI was focused on producing offspring from mares with compromised fertility[23]. Currently, indications for ICSI include poor sperm quality for artificial insemination or limited availability of frozen sperm stores.[23] In clinical ICSI, frozen-thawed stallion spermatozoa are commonly used. A small quantity of spermatozoa is required for injection; thus, sperm injection could theoretically be used to produce multiple in vitro embryos from a single straw of frozen spermatozoa, maximizing its use. Advantageously, unused frozen-thawed spermatozoa can be refrozen, maintaining sufficient sperm quality and fertility potential for later microinjections,[24,25] or a single straw of frozen spermatozoa could be thawed and refrozen into straws with reduced number of spermatozoa for use with ICSI. A sperm attribute that is highly linked to embryo development and pregnancy after ICSI is plasma membrane integrity.[25] However, sperm membranes are damaged during cryopreservation when lipids undergo a phase change from a fluid to gel state during cooling and freezing and by osmotic stress and extracellular ice formation during freezing.[26] The membrane lipid phase change is implicated in cooling damage caused by irreversible conformational modifications of membrane proteins.[26] In several species, proteins involved in sperm physiology and fertilization are reduced or lost after cryopreservation.[27] Therefore, modifications in the sperm plasma membrane and PLCZ1 could result in reduction of oocyte activation and ICSI success.

We hypothesized that PLCZ1 quantity in stallion spermatozoa is reduced after cryopreservation, and this is exacerbated with thawing; however, thawing and refreezing spermatozoa at a low temperature would reduce plasma membrane damage and aid in the retention of PLCZ1. In the present study, we assessed the effect of single and double freezing cycles and sequential detergent extraction on the abundance and localization of PLCZ1 in stallion spermatozoa using immunoblotting, immunofluorescence, and quantitative sandwich enzyme-linked immunosorbent assay (QS–ELISA). Retrospectively, we analyzed the relative abundance of PLCZ1 assessed by immunofluorescence and flow cytometry, and the extent that relative PLCZ1 content was associated with cleavage rates after equine ICSI. Finally, we evaluated the loss of membrane integrity and PLCZ1 abundance in stallion sperm populations for fresh, frozen, and refrozen sperm samples, either thawed and refrozen at room or low temperature in an effort to reduce plasma membrane damage.

2 | MATERIAL AND METHODS

2.1 | Study design, antibodies, and reagents

Three experiments were conducted. In Experiment 1, the loss of PLCZ1 after single or double cryopreservation cycles or detergent exposure was determined using immunoblotting, immunofluorescence, and QS–ELISA. Experiment 2 was a retrospective study to assess the extent that immunofluorescence and flow cytometry end points for PLCZ1 are associated with the ability of spermatozoa to activate the oocyte, as determined by cleavage rates after ICSI. Finally, Experiment 3 was performed using flow cytometry of spermatozoa from a single ejaculate of 12 stallions to assess acrosomal and plasma membrane integrity and PLCZ1 content for fresh, frozen, and refrozen spermatozoa. Spermatozoa were also refrozen at room or low temperature prior to the assessment of membrane integrity and PLCZ1 content. Single ejaculates were collected from stallions (Experiment 1, $n = 6$ and Experiment 2, $n = 12$ stallions), processed and analyzed for Experiments 1 and 3 during the equine breeding seasons of 2023 and 2022, respectively. Methods for Experiments 1 and 3 are described below. For Experiment 2, materials and methods were previously

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

published.[22,28,29] Data collected for spermatozoa ($n = 12$) from a previous study were reanalyzed for correlation analysis.[22] Cleavage rates obtained subsequent to the original study and including six of the spermatozoa samples were compiled to determine the relationship between relative abundance of PLCZ1 and cleavage rates after equine ICSI.[28,29]

The primary antibody used in the experiments was a rabbit polyclonal antibody raised against amino acids 500–549 of PLCZ1 of human origin (Santa Cruz Biotechnology, Dallas, TX) and characterized in detail in a previous study.[22] Secondary antibodies for flow cytometry, immunofluorescence, and immunoblotting were goat anti-rabbit IgG-H+L-Alexa Fluor488 (Invitrogen, Eugene, OR) and goat anti-rabbit IgG H+L HRP (Abcam, Boston, MA), respectively. Unless stated otherwise, all chemicals and reagents were purchased from MilliporeSigma (Burlington, MA).

2.2 | Semen collection and cryopreservation procedures

Semen was collected using an artificial vagina (CSU Model; Animal Reproduction Systems, Chino, CA) with an in-line nylon micromesh filter to remove the gel fraction. A portion of semen from each ejaculate was used for a fresh sperm evaluation, and the remaining semen was frozen as previously described.[30] Briefly, filtered semen was extended 1:1 in prewarmed INRA96 (IMV Technologies, Maple Grove, MN) and centrifuged at 400×*g* for 15 min at room temperature. The sperm pellet was resuspended to 200×10^6 spermatozoa/mL in lactose-EDTA extender containing 20% egg yolk and 5% glycerol (LEDTA).[31] Spermatozoa were loaded into 0.5-mL polyvinylchloride straws (IMV Technologies), sealed, and cooled at 5°C for 30 min. The straws were frozen 4 cm above liquid nitrogen for 15 min and then plunged into liquid nitrogen. Frozen straws were stored for at least 60 days prior to analyses or refreezing. Frozen spermatozoa were thawed at 37°C for 60 s and processed for analysis within 10 min.

For Experiments 1 and 3, eight straws of frozen spermatozoa were thawed using one of two protocols. For the first protocol, frozen spermatozoa were thawed at 37°C water bath for 60 s. Frozen thawed spermatozoa from individual straws were pooled for each stallion at room temperature (22°C; RT; refrozen room temperature) and diluted 10-fold in E-Z Freezin CryoMax MFR5 (Animal Reproduction Systems). Spermatozoa were packaged into straws, sealed, cooled at 5°C for 30 min and refrozen above liquid nitrogen as described above. For the second protocol, all procedures (thawing, diluting, and packing) occurred in a 5°C cold room.[32] Eight straws of frozen spermatozoa from each stallion were thawed individually immersed in a water bath at 37°C for 12 s and quickly removed into a cold-water bath at 5°C (LT, refrozen low temperature). Thawed spermatozoa were pooled and diluted 10-fold in E-Z Freezin CryoMax MFR5. Spermatozoa were packaged into straws, sealed, and refrozen above liquid nitrogen. Refrozen spermatozoa were thawed at 37°C for 60 s and processed for analysis within 10 min.

2.3 | Immunoblotting

A single ejaculate from each of six stallions was split for cryopreservation and detergent extraction. Protein lysates from fresh, frozen, and refrozen spermatozoa from were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described previously with some modifications.[33] Approximately 500×10^6 frozen and refrozen spermatozoa were thawed at 37°C for 60 s and centrifugated at 500×*g* for 8 min to collect the freezing and refreezing extenders. Fresh, frozen, and room temperature refrozen spermatozoa were washed in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4; filtered 0.22 μm) at 22°C. Supernatant was removed and the pellet was resuspended in radioimmunoprecipitation assay buffer (RIPA: 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0), sonicated (medium intensity level, 3 s × 5: Bioruptor Standard, Liege, Belgium) and centrifuged at 14,000×*g* for 10 min.

Fresh stallion spermatozoa were sequentially extracted by incubation in nonionic (Nonidet-P40, USBiological, Salem, MA; NP40) and ionic detergents (sodium dodecyl sulfate; SDS) to indirectly examine the extent of PLCZ1 binding to internal sperm structures. Nonionic and ionic detergents can differentially remove surface and internal sperm components, respectively.[34] Briefly, approximately 500×10^6 fresh spermatozoa were washed in PBS twice to remove seminal plasma and incubated in 400 μL of 1% NP40 at room temperature for 2 h. After incubation, spermatozoa were centrifugated at 500×*g* for 8 min, and the supernatant (NP40 extractable fraction) was recovered for analysis. The pellet was washed twice in PBS, and spermatozoa were incubated in 400 μL of 2% SDS for 2 h. Spermatozoa were centrifuged to recover the SDS extractable fraction. The remaining spermatozoa (nonextractable fraction) were washed twice, resuspend in 400 μL of PBS and sonicated for protein extraction. Nonextractable, NP40 and SDS fractions were centrifuged at 14,000×*g* for 10 min, and supernatants were collected for immunoblotting. Spermatozoa after incubation with NP40 and SDS were washed and fixed for immunofluorescence.

A Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) was used to assess protein concentration in fresh, frozen, and refrozen spermatozoa; the corresponding freezing and refreezing extenders; and extractable (NP40 and SDS fractions) and nonextractable detergent fractions. Protein (60 μg) was suspended in 2X Laemmli buffer (Bio-Rad, Hercules, CA) and distilled water up to a final volume of 40 μL and heated at 95°C for 10 min. Samples and molecular weight markers (Precision Plus Protein Dual Color Standards, Bio-Rad) were loaded into SDS-PAGE gradient gels (BOLT™ 4%–12% Bis-Tris Plus) and separated at 150 V for 40 min at 4°C, followed by transfer to a polyvinylidene difluoride membrane (PVDF; Immun-Blot PVDF Membrane; Bio-Rad) at 40 V for 90 min at 4°C. The PVDF membrane was blocked in 5% skim milk in tris-buffered saline with Tween (TBST: 137 mM NaCl, 2.7 mM KCl, 19 mM Tris base, 0.1% Tween 20, pH 7.4) for 60 min at room temperature and then incubated overnight with anti-PLCZ1 antibody (1:40) at 4°C under continuous agitation. After incubation, the PVDF membrane was washed with TBST and incubated

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

with secondary antibody (1:3000; goat anti-rabbit IgG H+L HRP) for 60 min at room temperature. After washing, immunoreactivity was captured using enhanced chemiluminescence (Super Signal West Dura; Thermo Scientific). Amido Black Staining Solution (Millipore Sigma) was used to assess total protein for the loading control. Individual PLCZ1 densitometric measurements from each sample group were standardized to total protein as the loading control, then expressed relative to the average standardized PLCZ1 band density for the fresh sample.[35] Densitometry analysis was performed using Vision Works Software (Analytik Jena, Germany) and ImageJ (NIH, Bethesda, MD).

2.4 | Immunofluorescence

Immunofluorescence of PLCZ1 in stallion spermatozoa was conducted as previously described with some modifications.[36] Two replicates for each sperm sample from the ejaculates of six stallions for Experiment 1 were evaluated. Fresh, frozen, refrozen at room temperature, and detergent-extracted spermatozoa were washed in PBS, and pellets were fixed in 2% paraformaldehyde for 40 min at room temperature. Fixed spermatozoa were washed and permeabilized using 0.1% Triton X-100 in PBS for 40 min. After washing, spermatozoa were resuspended to 5×10^6 spermatozoa/mL in PBS. A 30-μL aliquot was pipetted onto a 0.1% poly-L-lysine-coated slide and allowed to dry for 15 min on a slide warmer at 37°C. Spermatozoa were blocked (5% normal goat serum in PBS) for 60 min in a humidified chamber. The blocking solution was discarded, and spermatozoa were incubated with anti-PLCZ1 antibody (1:100; Santa Cruz Biotechnology) overnight at 5°C in a humidified chamber. Slides were washed in PBS and incubated with Alexa Fluor488-conjugated goat anti-rabbit IgG-H+L (1:200) for 60 min. Slides were washed, and spermatozoa were counterstained with 2 μg/mL Hoechst 33342 (Invitrogen) for 10 min. Slides were washed and prepared (Fluoromount Aqueous Mounting Media) for analysis. To evaluate background and nonspecific binding, permeabilized spermatozoa were incubated overnight in blocking solution without the primary antibody and only with the secondary antibody. Slides were imaged at X1000 magnification using an epifluorescence microscope equipped with 40,60-diamidino-2- phenylindole (DAPI) and fluorescein isothiocyanate (FITC) filters (BX53; Olympus Scientific, Waltham, MA) and using cellSens Dimension 1.6 Software (Olympus Scientific). Fluorescence intensity and localization of PLCZ1 were examined in > 200 spermatozoa per sample in each replicate for quantitative and proportional distribution analyses, respectively. Sperm heads were analyzed using ImageJ (NIH) and the regions of interest tool. Integrated density was calculated for the mean level of fluorescence and the area of each sperm head. Corrected total cell fluorescence (CTCF) was obtained after subtraction of background fluorescence.[37]

2.5 | Quantitative sandwich enzyme-linked immunosorbent assay

A commercially available QS–ELISA(MBS072674, MyBioSource, San Diego, CA) was used to quantify PLCZ1 in sperm extracts from fresh, frozen, and refrozen spermatozoa and their corresponding freezing and refreezing extenders from six stallions for Experiment 1. Protein sperm extraction and procedures for QS–ELISA were performed following the manufacture's guidelines. Briefly, 4 mL of semen from each of the six ejaculates containing approximately 800×10^6 spermatozoa was washed in PBS, and the seminal plasma was removed to obtain fresh spermatozoa. Frozen and room temperature refrozen spermatozoa from the same ejaculate were cryopreserved as described previously. Approximately 800×10^6 frozen and refrozen spermatozoa were thawed at 37°C for 60 s and centrifugated at 500×*g* for 8 min to recover the freezing and refreezing extender for analysis. Pellets from fresh, frozen, and refrozen spermatozoa were washed in PBS, and the supernatants were discarded. Spermatozoa were resuspended in 400 μL of PBS and sonicated at 5°C for protein extraction. Sonicated sperm samples and extenders were centrifuged at 10,000×*g* for 10 min at 5°C, and the supernatants were collected for analysis.

In brief, 50 μL of sperm samples were placed into each well in duplicate, with the provided two-fold serial dilution of standard at concentrations of 20, 10, 5, 2.5, 1.25, and 0.625 ng/mL added in triplicate. A blank (0 ng/mL protein) control was used to assess the enzymatic reaction without sample or HRP-conjugated reagent. Samples and standards were incubated at 37°C for 1 h with 100 μL HRP-conjugated reagent (excluding blank wells). After washing four times with a supplied wash buffer, 50 μL of Chromogen Solution A and B were added. The microplate was incubated at 37°C for 15 min, and the reaction was ended by adding 50 μl of Stop Solution into each well. The microplate was evaluated within 5 min at the optical density of 450 nm using a VersaMax Microplate Reader (Molecular Devices, San Jose, CA). Triplicates of blank and standard optical density values were fitted in a linear curve ($y = 0.017 + 0.070x$, $R^2 = 0.997$) to estimate the protein concentration of the samples.

2.6 | Flow cytometric assessment

Fresh, frozen, and refrozen at room or low-temperature spermatozoa from single ejaculates from 12 stallions (Experiment 3) were washed in 10 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4; filtered 0.22 μm) and centrifuged at 500×g for 8 min. The pellet was resuspended in PBS, and an aliquot containing approximately 5×10^6 spermatozoa was incubated with 1 μL of fixable viability stain (LD; LIVE/DEAD Fixable Far-Red Kit, Molecular Probes, Eugene, OR) for 30 min in the dark, following the manufacturer's guidelines. Stained spermatozoa were washed in PBS, fixed with 2% paraformaldehyde for 40 min at room temperature in the dark, washed in PBS, and permeabilized with 0.1% Triton-X-100 for 1 h. Spermatozoa were blocked with 5% normal goat serum for 1 h, and then incubated with anti-PLCZ1 antibody (1:100). After overnight incubation at 5°C, spermatozoa were washed in PBS and incubated with goat anti-rabbit IgG-H+L-Alexa Fluor488 (1:200) for 60 min at room temperature. Then, 5.6 μg/mL of lectin from Arachis hypogaea (peanut) Alexa Fluor594 conjugate (PNA594; Invitrogen) was added, incubated for 15 min, and washed in PBS for flow cytometric analysis.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Samples were analyzed in duplicate using ZE5 Cell Analyzer (Bio-Rad) and FlowJo v10.2 Software (Ashland, OR). Fluorochromes were excited with 488, 561, and 640 nm lasers and 525/25, 615/24, and 670/30 nm band pass filters were used for green, red, and far-red fluorescence detection. Spermatozoa unstained, single-stained with viability stain (LD) or PNA594 and incubated only with secondary antibody were used to set voltages, compensations, and quadrants. Acquired events were gated into FSC-area/FCS-height plot to exclude cell aggregates. Single events were gated into an LD histogram and acquisition stopped at 20,000 positive events for LD stain. Events included in the LD gate were considered spermatozoa. Spermatozoa displaying low LD intensity were considered as viable with intact plasma membrane (MI+), and spermatozoa exhibiting high LD intensity were determined as nonviable with disrupted plasma membrane (MI−). Spermatozoa included in the LD gate (MI+ and MI−) were brought into a PNA594 histogram to identify spermatozoa with intact acrosome (intact, PNA+) or damaged acrosome (reacted, PNA−). Spermatozoa then were gated into LD/PNA594 plot to identify three main sperm subpopulations: (1) spermatozoa with intact plasma and acrosomal membranes (MI+/PNA+); (2) spermatozoa with disrupted plasma membrane and intact acrosome (MI−/PNA+); and (3) spermatozoa with damaged plasma and acrosomal membranes (MI−/PNA−). Fluorescence emitted by spermatozoa incubated only in secondary antibody was used to assess nonspecific binding, setting a marker at the end of the nonspecific binding. Spermatozoa displaying fluorescence from the primary−secondary antibody complex to the right of the marker were considered positive spermatozoa for PLCZ1 (PLCZ1+). Mean fluorescence intensity (MFI) emitted for the whole and each sperm subpopulation were recorded for analysis (Figure 1).

2.7 | Oocyte collection and intracytoplasmic sperm injection procedures

Equine oocyte collection and ICSI procedures are described in detail in previous studies.[28,29] Briefly, oocytes were collected from light-horse mares by transvaginal, ultrasound-guided follicular aspiration during the follicular phase and after follicle maturation induction of dominant follicles > 35 mm in diameter. Cumulus oocyte complexes were incubated in TCM199 with Earle's salts (GibcoTM, ThermoFisher) with 10% fetal calf serum, 25 μg/mL of gentamicin and 0.2 mM pyruvate at 38.2°C in 5% CO_2 and air for approximately 26 h, before being denuded of cumulus cells. Extrusion of the first polar body was confirmed. Prior to ICSI, approximately one-tenth of a 0.5-mL straw of frozen-thawed spermatozoa (n = 6), evaluated for PLCZ1 from a previous study,[22] was cut under liquid nitrogen and thawed by placing directly into 1 mL of MOPS-buffered medium (G-MOPS, Vitrolife, Sweden) with 0.04% BSA at 38.2°C. Selected spermatozoa were injected into oocytes (n = 227) using a micromanipulator (Narishige Group, Amityville, NY) with a piezo-driven injection system (Prime Tech, Japan). Presumptive zygotes were placed into the embryo culture medium (Global, LifeGlobal Group, Guilford, CT) and incubated at 38.2°C in 5% O_2, 7% CO_2 and 88% N_2. Cleavage (≥2 apparent cells) was assessed at approximately 24 and 48 h after ICSI.

2.8 | Statistical analysis

Statistical analyses were conducted using R Core Team Software (2020), R Foundation for Statistical Computing. In Experiment 1, densitometry, quantitative immunofluorescence analysis and protein concentration assessed by QS-ELISA were evaluated using a one-factor mixed model for repeated measures with sample type (spermatozoa state, extender, and detergent-extracted fraction) as a fixed effect and stallion as a random effect. For comparison of relative PLCZ1 abundance, Dunnett's pairwise comparison was conducted to examine differences among fresh sperm lysates, used as the control group, and the other experimental groups. For quantitative immunofluorescence and protein concentration, Tukey's pairwise comparison was performed to detect differences among experimental groups. In Experiment 2, the association of fluorescence intensity of PLCZ1 acquired by immunofluorescence and flow cytometry was estimated by a Pearson's correlation. Cleavage rates were compared by the Chi-square test. Spermatozoa were examined and categorized into four patterns of PLCZ1 localization: (1) acrosomal, (2) acrosomal and postacrosomal combined, (3) postacrosomal region, or (4) the absence of PLCZ1 immunostaining. The proportional distribution of PLCZ1 localization was analyzed by the generalized linear model and Tukey-adjusted pairwise comparison. The likelihood of cleavage by PLCZ1 content or localization pattern was estimated using odd ratio (OR) with a 95% confidence interval (CI). For Experiment 3, data obtained from flow cytometry assessments were tested for normality and homogeneity of variances using the Shapiro and Levene test. The one-factor mixed model for repeated measures with sample state (fresh, frozen, refrozen at room temperature, and refrozen at low temperature) as fixed effect and stallion as a random effect was fit for each parameter evaluated. Tukey-adjusted pairwise comparison was carried out to identify differences among sample states. Pearson's correlation was used to determine the association between flow cytometric sperm parameters. Significance was considered at $p < 0.05$.

3 | RESULTS

3.1 | Experiment 1

Equine PLCZ1 was detected from the spermatozoa of six stallions by immunoblotting in protein extracts prepared from fresh, frozen, and refrozen spermatozoa; freezing and refreezing extenders; and extractable and nonextractable sperm proteins by detergents. Samples exhibited an immunoreactive band at ~71 kDa, the expected molecular weight for equine PLCZ1 (Figure 2A). The band density of PLCZ1 of each sample group was standardized by total protein content and then expressed as relative to the average band density of the fresh sample, which was used as the control group, for Dunnett's comparisons. The relative abundance of PLCZ1 was lower in frozen (p = 0.01) and refrozen (p < 0.01) sperm isolates than fresh isolates. Successive detergent extraction using NP40 and SDS was performed to assess the

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

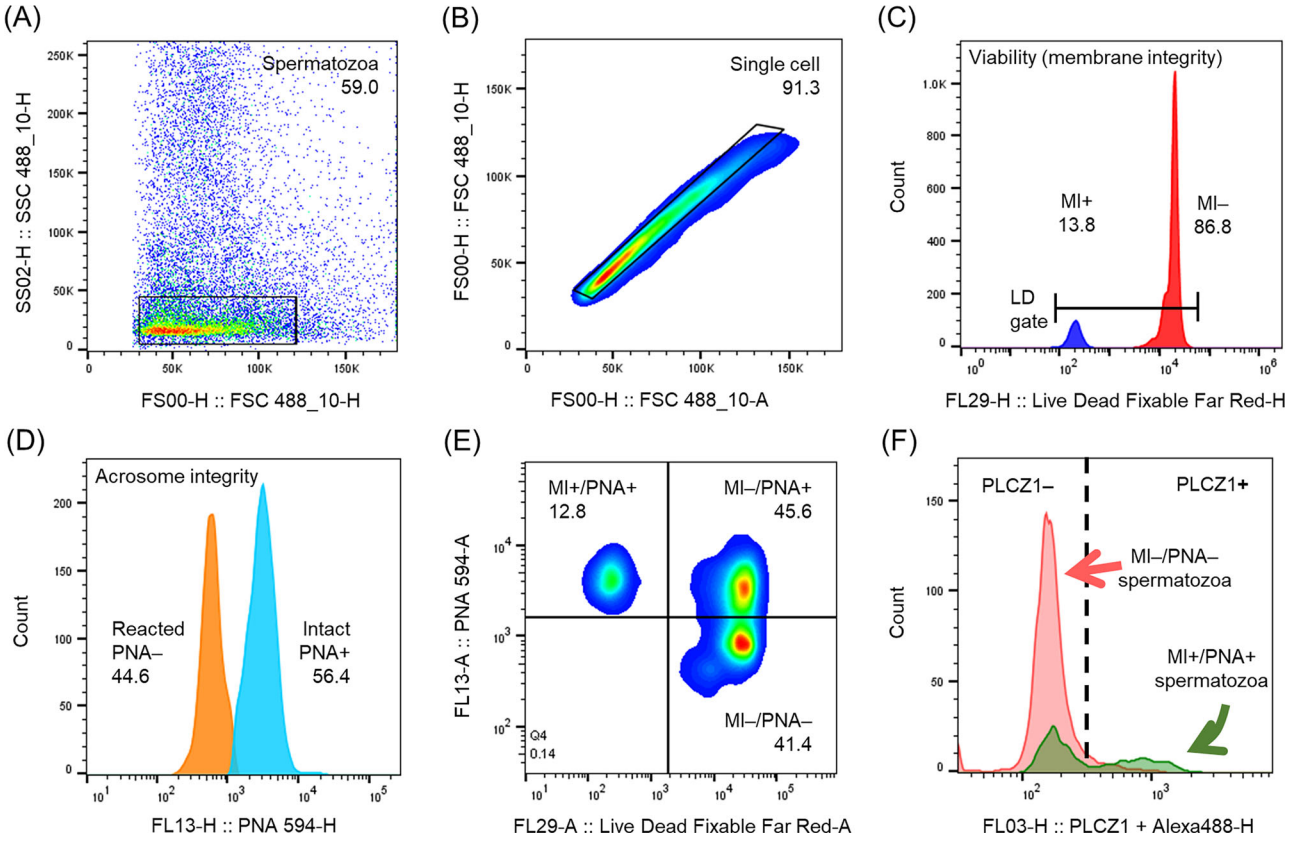


FIGURE 1 Representative cytograms and gating strategy for acrosome and plasma membrane integrity and PLCZ1 content in refrozen–thawed equine spermatozoa. Spermatozoa were stained with LIVE/DEAD Fixable Far-Red Kit, and then fixed, permeabilized, blocked, and incubated with rabbit anti-PLCZ1 antibody of human origin and goat anti-rabbit IgG-H+L-Alexa Fluor488 as primary and secondary antibody, respectively. (A) Event acquisition. (B) Single cells were selected and gated into Live/Dead Fixable stain histogram. (C) Events within LD gate were identified as spermatozoa with intact plasma membranes (viable, MI+) or disrupted plasma membranes (nonviable, MI−). (D) Acrosome integrity of spermatozoa within LD gate were detected using Lectin-PNA Alexa Fluor594 conjugate and classified with intact acrosome (intact, PNA+) or damaged acrosome (reacted, PNA−). (E) Spermatozoa were gated into a Live/Dead Fixable stain/PNA-594 plot to identify spermatozoa with intact acrosomal and plasma membrane (MI+/PNA+), or disrupted plasma membrane with intact (MI−/PNA+) or reacted acrosome (MI−/PNA−). (F) Spermatozoa displaying fluorescence from the primary–secondary antibody complex to the right of the marker were considered positive spermatozoa for PLCZ1 (PLCZ1+) and mean fluorescence intensity was recorded for each sperm subpopulation. LD, LIVE/DEAD; PLCZ1, phospholipase C zeta 1.

extent of PLCZ1 binding to surface and/or internal sperm structures, respectively.[34] No difference was observed between fresh samples and spermatozoa treated with NP40 detergent or the relative PLCZ1 abundance in freezing and refreezing extenders. Nonextractable samples and those treated with SDS contained lower ($p < 0.01$) PLCZ1 than fresh preparations (Figure 2B). The relative PLCZ1 abundance tended to be greater ($p = 0.051$) in NP40 extracts than in refreezing extenders.

Representative immunofluorescence images for PLCZ1 of fresh, cryopreserved, and detergent-extracted spermatozoa are shown in Figure 3. Quantitative immunofluorescence analysis of PLCZ1 in equine sperm heads demonstrated a reduction ($p < 0.01$) in CTCF values after the first freezing cycle but not after the second freezing cycle (Figure 3S). In addition, we observed an apparent decline in tail fluorescence after each freezing cycle and detergent extraction (Figure 3A–R). In spermatozoa subjected to detergent extraction, NP40-extracted spermatozoa displayed lower ($p < 0.01$) CTCF than fresh spermatozoa, but similar CTCF when compared to frozen and refrozen spermatozoa at room temperature. Spermatozoa extracted with SDS after NP40 exhibited lower ($p < 0.01$) values of CTCF than fresh and frozen spermatozoa but did not differ from refrozen and NP40-extracted spermatozoa (Figure 3S). After SDS extraction, remaining spermatozoa retained a weak PLCZ1 signal in the base of the postacrosomal region, midpiece, and tail (Figure 3M–O).

Using a commercial QS–ELISA, PLCZ1 concentration was estimated in sperm extracts from fresh, frozen, and refrozen spermatozoa and their corresponding freezing and refreezing extenders. Freezing and refreezing were associated with reduced ($p < 0.01$) sperm PLCZ1 protein concentration when compared to fresh preparations. No difference was observed in PLCZ1 concentration between frozen and refrozen sperm samples at room temperature. The concentration of PLCZ1 was similar in freezing and refreezing extenders when compared to fresh spermatozoa (Figure 3T).

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

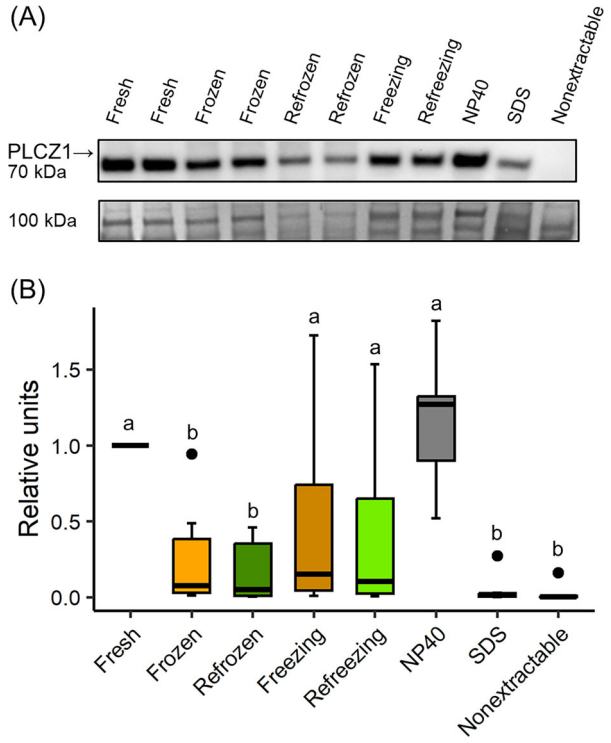


FIGURE 2 Immunoblotting of PLCZ1 and densitometry analysis in fresh and cryopreserved equine spermatozoa and detergent-extracted fractions using anti-PLCZ1 antibody of human origin. Ejaculates ($n = 6$) were split for cryopreservation and detergent extraction. The first split of fresh spermatozoa (Fresh) was frozen (Frozen), thawed, diluted, and refrozen (Refrozen). Freezing (Freezing) and refreezing (Refreezing) extenders were recovered and tested for PLCZ1. The second split of fresh spermatozoa were sequentially incubated in Nonidet-P40 (NP40) and sodium dodecyl sulfate (SDS) to obtain detergent-extractable, sperm-free fractions. Spermatozoa after detergent extraction were considered the nonextractable fraction (Nonextractable). (A) Anti-PLCZ1 antibody labeled an immunoreactive band of ~71 kDa corresponding to equine PLCZ. Amido Black Staining Solution was used to assess total protein as loading control. (B) Densitometry analysis was performed by standardization of total protein load and expressed as relative to the average band density of the fresh sample (mean ± SD, relative units) for Dunnett's comparisons. Relative to fresh samples, sperm PLCZ1 abundance was reduced after cryopreservation and the presence of PLCZ1 was confirmed in cryodiluents after each freezing cycle. Nonionic detergent extraction by NP40 largely removed PLCZ from equine spermatozoa.[a,b] Superscripts differ at $p \leq 0.01$. PLCZ1, phospholipase C zeta 1.

3.2 | Experiment 2

Retrospective analysis of sperm samples from 12 stallions for fluorescence intensity of PLCZ1 evaluated by flow cytometry and immunofluorescence was performed. MFI of PLCZ1 acquired by flow cytometry strongly correlated ($r = 0.63$, $p = 0.02$) to CTCF obtained by immunofluorescence (Figure 4M). Six stallions from the 12 stallions were grouped by high ($n = 2$) and low ($n = 4$) relative abundance of PLCZ1 before ICSI ($n = 227$). Representative differences of spermatozoa with high (Stallion Q and R) and low (Stallion D and E) PLCZ1 fluorescence intensity are presented in Figure 4A–L. Equine oocytes ($n = 192$) injected with spermatozoa from samples with high PLCZ1 fluorescence intensity had a higher ($p < 0.01$) cleavage rate (Stallion Q, 145/182, 79.7% and Stallion R, 9/10, 90.0%) than did equine oocytes ($n = 35$) injected with spermatozoa from samples with low PLCZ1 fluorescence (Stallion D, 4/12, 33.3%; Stallion E, 4/12, 33.3%; Stallion F, 3/8, 37.5%, and Stallion J, 1/3, 33.3%) (Figure 4N). Mare oocytes microinjected with spermatozoa from sperm samples displaying low PLCZ1 fluorescence were 76.7% (CI: 0.05–0.29) less likely to cleave when compared to oocytes injected with a spermatozoon from a sample with high fluorescence intensity.

Proportional distribution analysis of PLCZ1 revealed that sperm populations exhibiting high PLCZ1 had a greater ($p = 0.01$) proportion of spermatozoa with PLCZ1 in the acrosomal–postacrosomal and postacrosomal localization than in the acrosomal region or absent PLCZ1. In sperm populations with low PLCZ1, the proportion of spermatozoa exhibiting PLCZ1 in the acrosomal–postacrosomal localization was greater ($p = 0.01$) than spermatozoa lacking PLCZ1 but did not differ to spermatozoa displaying acrosomal ($p = 0.06$) or postacrosomal localization ($p = 0.5$). When compared by PLCZ1 content, samples with high content had a greater ($p = 0.01$) proportion of spermatozoa displaying PLCZ1 in the acrosomal–postacrosomal localization than samples with low content. Samples with low content tended to have higher proportion of spermatozoa without PLCZ1 ($p = 0.08$) or exhibiting PLCZ1 only in the acrosomal region ($p = 0.06$) when compared to samples showing high PLCZ1 content (Figure 4O). Cleavage rates were negatively associated with acrosomal localization ($p < 0.01$, OR: 0.91, CI: 0.86—0.95), but positively associated with acrosomal–postacrosomal ($p < 0.01$, OR: 1.13, CI: 1.07–1.20) and postacrosomal localizations ($p < 0.01$, OR: 1.08, CI: 1.03–1.14).

3.3 | Experiment 3

Flow cytometry was used to assess PLCZ1 content and integrity of the plasma membrane and acrosome. The percentage of spermatozoa with intact plasma membrane was reduced ($p < 0.01$) in frozen and refrozen spermatozoa at room temperature, but refrozen spermatozoa at low temperature did not differ with frozen spermatozoa. Acrosome integrity was reduced ($p < 0.01$) only in refrozen samples at room temperature when compared to fresh, frozen, and refrozen at low temperature samples (Table 1). When both sperm attributes were assessed simultaneously, percentages of spermatozoa with intact plasma membrane and acrosome were reduced ($p < 0.01$) after freezing and RT refreezing. However, refrozen spermatozoa at low temperature had similar plasma membrane and acrosome integrity to frozen spermatozoa. Percentages of positive-labeled spermatozoa for PLCZ1 (PLCZ1+) were reduced ($p < 0.01$) after freezing, but frozen and refrozen samples at room or low temperature did not differ (Table 1). MFI of PLCZ1 in the sperm population was reduced ($p < 0.01$) after freezing, but

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by meyam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

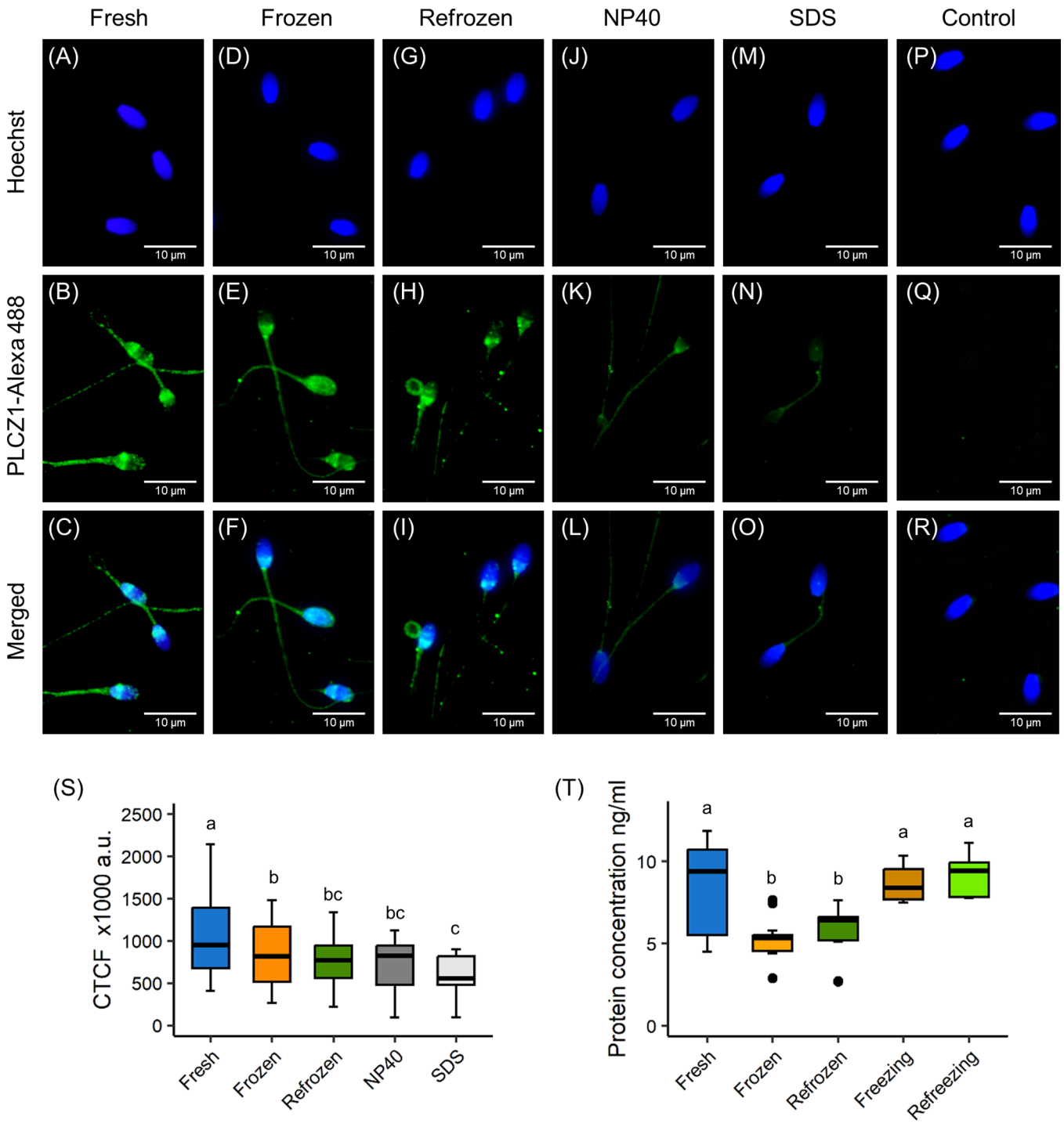


FIGURE 3 Representative immunofluorescence images (X1000) of fresh, frozen, refrozen, and detergent-extracted spermatozoa from one of six stallions for PLCZ1, quantitative immunofluorescence analysis and protein concentration of sperm PLCZ1 assessed by QS–ELISA. A single ejaculate from each of six stallions was split for cryopreservation and detergent extraction. Fresh spermatozoa (Fresh) were frozen (Frozen), thawed, diluted, and refrozen (Refrozen) at room temperature. Freezing (Freezing) and refreezing (Refreezing) extenders were collected for QS–ELISA. A split of fresh spermatozoa was sequentially incubated in Nonidet-P40 (NP40) and sodium dodecyl sulfate (SDS) to obtain detergent-extracted spermatozoa. (A–R) Images of spermatozoa incubated with rabbit anti-PLCZ1 antibody of human origin and goat anti-rabbit IgG-H+L-Alexa Fluor488 and counterstained with Hoechst 33342 (Hoechst). (P–R) Control spermatozoa incubated only with the secondary antibody. (S) Corrected total cell fluorescence [CTCF, mean ± SD of arbitrary units (a.u.)] of PLCZ1 for fresh spermatozoa was reduced after cryopreservation. Fluorescence of PLCZ1 was also reduced after detergent extraction and similar to cryopreserved samples. (T) Protein concentration (mean ± SD) of PLCZ1 in fresh sperm samples determined by QS–ELISA was also reduced after cryopreservation, with similar PLCZ1 quantity in both freezing and refreezing extenders.[a,b,c] Superscripts differ at $p < 0.01$. QS–ELISA, quantitative sandwich enzyme-linked immunosorbent assay; PLCZ1, phospholipase C zeta 1.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by meryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

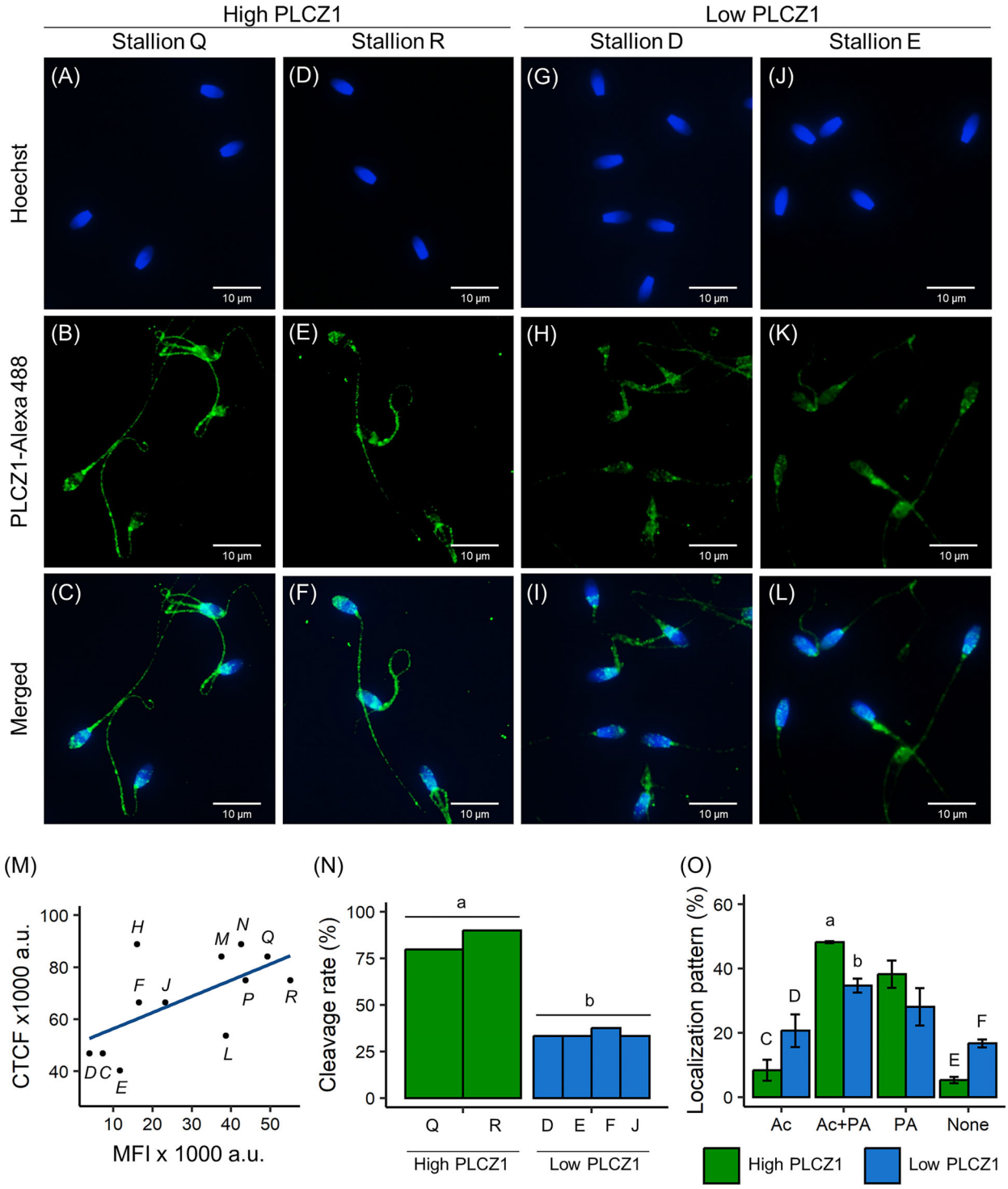


FIGURE 4 Immunodetection of sperm PLCZ1 by immunofluorescence and flow cytometry and its association with cleavage rate after ICSI. Spermatozoa from 12 stallions, identified by different capital letters, were incubated with rabbit anti-PLCZ1 antibody of human origin and goat anti-rabbit IgG-H+L-Alexa Fluor488, and counterstained with Hoechst 33342 (Hoechst). Representative immunofluorescence intensity for PLCZ1 of frozen–thawed spermatozoa from stallions with relatively high (A–F) and low PLCZ1 content (G–L). (M) Relative abundance of sperm PLCZ1 acquired by flow cytometry [mean fluorescence intensity, MFI in arbitrary units (a.u.)] and immunofluorescence [CTCF, corrected total cell fluorescence in arbitrary units (a.u.)] were significantly correlated. (N) Cleavage rates after ICSI of equine oocytes using frozen-thawed sperm samples of stallions grouped as high ($n = 2$ stallions; $n = 192$ oocytes; stallion Q, 145/182 and R, 9/10) or low PLCZ1 ($n = 4$ stallions, $n = 35$ oocytes; stallion D, 4/12; stallion E, 4/12; stallion F, 3/8 and stallion J, 1/3). (O) Percentage (mean ± SEM) of localization patterns of PLCZ1 on frozen-thawed spermatozoa from stallions with sperm population containing relatively high ($n = 2$, Q and R) or low ($n = 4$, D, E, F and J) PLCZ1 content. Ac, acrosomal region; Ac+PA, acrosomal and postacrosomal regions; PA, postacrosomal region; None, absence of PLCZ1.[a,b] Superscripts differ at $p \leq 0.01$.[C,D] Superscripts differ at $p = 0.06$.[E,F] Superscripts differ at $p = 0.08$. ICSI, intracytoplasmic sperm injection; PLCZ1, phospholipase C zeta 1.

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by meryam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Plasma membrane and acrosome integrity and positive-labeled spermatozoa for PLCZ1 (PLCZ1+) of fresh, frozen, or refrozen stallion spermatozoa at room temperature (RT) or low temperature (LT).

Sperm subpopulation	Fresh	Frozen	Refrozen RT	Refrozen LT
Membrane intact	67.9 ± 2.8^{a}	34.0 ± 2.8^{b}	17.7 ± 2.7^{c}	28.2 ± 2.9^{b}
Acrosome intact	79.5 ± 3.6^{a}	74.1 ± 3.6^{a}	58.1 ± 3.4^{b}	79.0 ± 3.7^{a}
Membrane and acrosome intact	62.8 ± 3.0^{a}	26.7 ± 3.0^{b}	15.9 ± 2.9^{c}	25.5 ± 3.1^{b}
Membrane disrupted-acrosome intact	22.2 ± 3.5^{a}	48.9 ± 3.6^{ab}	42.3 ± 3.4^{b}	55.8 ± 3.7^{c}
Membrane and acrosome disrupted	12.7 ± 2.3^{a}	23.4 ± 2.3^{b}	40.8 ± 2.2^{c}	18.0 ± 2.4^{bc}
PLCZ1+ spermatozoa (%)	84.3 ± 1.9^{a}	58.8 ± 1.9^{b}	56.9 ± 1.9^{b}	53.0 ± 1.9^{b}

Notes: Sperm samples from 12 stallions were stained with LIVE/DEAD Fixable Far-Red Kit, fixed, permeabilized, blocked, incubated with rabbit anti-PLCZ1 antibody of human origin, goat anti-rabbit IgG-H+L-Alexa Fluor™488 and lectin from Arachis hypogaea (peanut) Alexa Fluor594 conjugate, and analyzed by flow cytometry.

[a, b, c] Superscripts within a row differ at $p < 0.01$. Results shown as percentage (mean ± SEM).

TABLE 2 Mean fluorescence intensity (MFI) of PLCZ1 in sperm subpopulations for fresh, frozen, and refrozen spermatozoa at room (RT) or low temperature (LT).

Sperm subpopulation	Fresh	Frozen	Refrozen RT	Refrozen LT
Whole sperm population	329.2 ± 19.3^{a}	201.1 ± 19.3^{b}	176.7 ± 19.3^{b}	133.6 ± 19.3^{b}
Membrane intact	325.3 ± 29.6^{a}	228.5 ± 29.6^{b}	230.1 ± 28.3^{bx}	136.1 ± 12.1^{b}
Membrane disrupted	314.0 ± 19.7^{a}	200.5 ± 19.7^{b}	180.4 ± 18.8^{by}	128.5 ± 20.7^{b}
Acrosome intact	328.4 ± 23.6^{ax}	218.3 ± 23.6^{byx}	205.5 ± 22.5^{bx}	133.2 ± 24.8^{bx}
Acrosome disrupted	295.1 ± 19.4^{ay}	170.2 ± 19.4^{by}	163.3 ± 18.5^{by}	120.1 ± 20.4^{by}
Membrane and acrosome intact	322.4 ± 27.6^{ax}	224.3 ± 27.6^{bx}	223.1 ± 26.3^{bx}	133.9 ± 29.0^{bx}
Membrane disrupted-acrosome intact	337.0 ± 22.0^{ax}	214.2 ± 22.0^{bx}	200.4 ± 21.0^{bx}	133.1 ± 23.2^{bx}
Membrane and acrosome disrupted	287.4 ± 16.2^{ay}	169.2 ± 16.2^{by}	158.8 ± 15.5^{by}	116.3 ± 17.1^{by}

Notes: Sperm samples from 12 stallions were stained with LIVE/DEAD Fixable Far-Red Kit, fixed, permeabilized, incubated with rabbit anti-PLCZ1 antibody of human origin, goat anti-rabbit IgG-H+L-Alexa Fluor488 and lectin from Arachis hypogaea (peanut) Alexa Fluor594 conjugate, and evaluated by flow cytometry.

[a,b] Superscript within a row differ at $p < 0.01$.[x,y] Superscripts within a column for membrane integrity, acrosome integrity, or both attributes differ at $p < 0.01$. Results shown as arbitrary units of mean fluorescence intensity (mean ± SEM).

similar among frozen and refrozen samples at room or low temperature (Table 2). Within sperm subpopulations, MFI of PLCZ1 was reduced ($p < 0.01$) after freezing within the subpopulations of spermatozoa with intact plasma membranes or acrosomes or both attributes simultaneously. The MFI did not differ between sperm populations from frozen and refrozen at room or low-temperature samples (Table 2).

Mean percentages of membrane-intact spermatozoa for all samples correlated positively to the percentage of PLCZ1+ spermatozoa ($r = 0.74$, $p < 0.01$). Similarly, percentages of spermatozoa with intact acrosomal or plasma membrane correlated positively to the mean fluorescent intensity of PLCZ1 (Figure 5).

When, MFI of PLCZ1 was compared within a sperm treatment group, sperm subpopulations with intact acrosomes displayed higher ($p < 0.01$) MFI than sperm subpopulations with damaged acrosomes in all sample types (Table 2). Sperm subpopulations exhibiting intact acrosomes with intact or disrupted plasma membrane showed similar MFI, but both were higher ($p < 0.01$) than the MFI observed in sperm subpopulation with disrupted plasma membrane and damaged acrosomes (Table 2).

4 | DISCUSSION

Frozen and refrozen spermatozoa are routinely used for equine ICSI. Frozen-thawed spermatozoa undergo membrane damage and protein conformational changes,[26] that can lead to a loss or modification of sperm-borne PLCZ1 and a reduction in oocyte activating potential. Therefore, the development of techniques to maintain and identify spermatozoa with high plasma membrane integrity after cryopreservation are important for ICSI success.

In the present study, we wanted to determine to what extent PLCZ1 is lost during cryopreservation or sperm manipulations. Using immunoblotting, we detected equine PLCZ1 as a ~71 kDa protein in fresh, frozen, and refrozen spermatozoa as previously described.[2,12,22] Our results demonstrate that cryopreservation leads to significantly lower abundance of PLCZ1 in stallion spermatozoa. To further confirm the extent that equine PLCZ1 is reduced after cryopreservation, we quantified PLCZ1 using a commercial QS–ELISA kit. ELISA is comparatively a more sensitive assay than immunoblotting. We found congruent and quantitative lower PLCZ1 in cryopreserved sperm samples

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

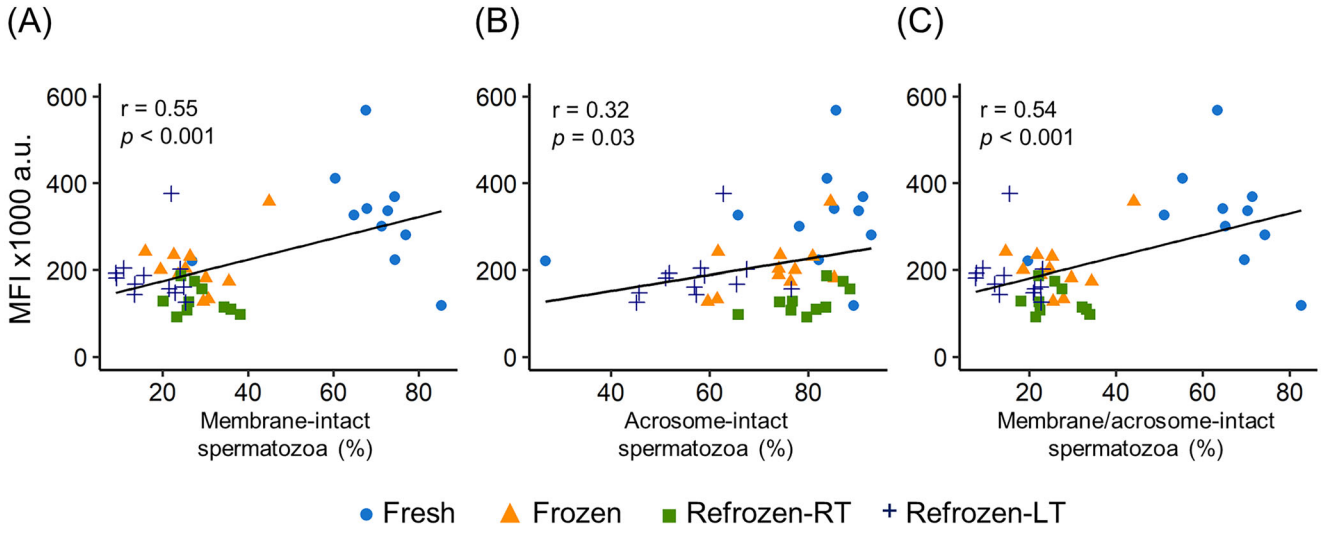


FIGURE 5 Correlation of mean florescence intensity [MFI, arbitrary units (a.u.)] of positive-labeled spermatozoa for PLCZ1 (PLCZ1+) with percentage of membrane intact, acrosome intact, and membrane and acrosome intact spermatozoa. Different dot shapes indicate fresh, frozen, and refrozen spermatozoa at room temperature (RT) and low temperature (LT). Sperm samples from 12 stallions were stained with LIVE/DEAD Fixable Far-Red Kit, fixed, permeabilized, blocked, incubated with rabbit anti-PLCZ1 antibody of human origin, goat anti-rabbit IgG-H+L-Alexa Fluor488 and lectin from Arachis hypogaea (peanut) Alexa Fluor594 conjugate, and assessed by flow cytometry. PLCZ1, phospholipase C zeta 1.

when compared to fresh samples. The presence of PLCZ1 in cryodiluents confirmed that PLCZ1 was removed from stallion spermatozoa after cryopreservation.

Sequential protein extraction using nonionic and ionic detergents did not totally extract PLCZ1 from stallion spermatozoa. A large proportion of PLCZ1 was removed by a nonionic detergent (NP40), suggesting that PLCZ1 interacts with sperm surface structures and to a lesser extent with internal structures. However, a nonextractable fraction of PLCZ1 was retained in the postacrosomal region. In mouse and hamster spermatozoa treated with a nonionic detergent, Triton X-100, the immunofluorescent signal of PLCZ1 was retained similar to untreated spermatozoa in the postacrosomal region in the full-length form of ~74 kDa. However, the acrosomal localization of PLCZ1 was eliminated, and the Triton-soluble fraction showed a smaller prominent band of‘ ~55–60 kDa corresponding to acrosomal PLCZ1.[38] Congruently, we observed reduced PLCZ1 by flow cytometry in spermatozoa with damaged acrosomes when compared to spermatozoa with intact acrosomes. Using immunogold labeling and electron microscopy, PLCZ1 localizes in the perinuclear theca (PT) and inner acrosomal membrane at the end of the base of the acrosomal vesicle in human spermatozoa.[16] This localization is consistent with the immunofluorescence images obtained in this study from cryopreserved and detergent-extracted stallion spermatozoa. The PT is a condensed cytosolic protein layer that forms the major cytoskeletal component in the sperm head. It is a nonionic detergent-resistant structure, divided structurally and compositionally in the subacrosomal layer and postacrosomal sheath (PAS), which are involved in the assembly of the acrosome and sperm–oocyte interactions during fertilization, respectively.[39] It is anticipated that the sperm oocyte activation factor locates in the PAS-PT which is the first component of the sperm cytosol to enter the oocyte cytoplasm and its disassembly triggers oocyte activation.[39,40] Interestingly, mouse spermatozoa with chemically misshapen acrosomes injected into homologous oocytes kept oocyte activating ability.[41] Local solubilization of the PAS is sufficient to elicit oocyte activation during murine and bovine in vitro fertilization.[40] Proteins located in PAS–PT such as WBP2NL (PAWP) and WBP2 of mouse, bull, and human sperm have also been demonstrated to be capable of initiating oocyte activation.[34,42–44] These findings support the hypothesis that the PAS is the region of the PT where the sperm–oocyte activating factor is located.[39,40,45,46] In stallion spermatozoa, freezing and thawing procedures cause cryodamage, reducing the abundance of PLCZ1. However, a fraction of PLCZ1 is retained in the postacrosomal region, potentially strategically located to be available for sperm-oocyte interactions, as seen in human spermatozoa.[16]

From the flow cytometric evaluations, we observed that the proportion of acrosomal- and plasma membrane-intact spermatozoa within the total sperm population was reduced with each freezing cycle when the refreezing was conducted at room temperature, confirming that cryopreservation results in plasma membrane damage. However, we also thawed and refroze spermatozoa in low temperature (5°C) conditions without letting spermatozoa warm to room temperature. Compared to the initial freeze, refreezing spermatozoa at a low temperature did not result in more disruption of the acrosomal and plasma membranes or reduction of spermatozoa expressing PLCZ1. During cryopreservation of mammalian spermatozoa, a membrane–phase transition occurs when transitioning from physiological temperatures to 0°C. This is the result of the lipid fractions within the cell membranes going into a gel phase and initiating lateral phase separation of membrane components.[47] Phase changes are implicated in cold shock injury caused by permanent conformational changes of membrane proteins.[26] At subzero temperatures, extracellular ice formation

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

occurs with a fluid–to–gel membrane phase transition, exposing spermatozoa to strong osmotic gradients that cause cell dehydration.[48] In the low-temperature protocol for refreezing, thawing to only 5°C prevents the sperm plasma membranes from undergoing an additional cycle of phase changes, resulting in higher percentages of spermatozoa with intact plasma membranes after the second freeze–thaw cycle; but content of PLCZ1 in spermatozoa refrozen at a low temperature did not differ to frozen or refrozen spermatozoa at room temperature.

Possibly the time elapsed between the moment of sperm freezing occurred and the thawing to prepare spermatozoa for ICSI may have a negative impact on the relative abundance of PLCZ1. In human spermatozoa, the variations in protein abundance after cryopreservation are most likely caused by protein degradation, post-translational processing, changes in secondary or tertiary structure, and/or translocation to other cellular compartments or outside the spermatozoon, but also by membrane damage.[27,49] However, stallion spermatozoa do not form intracellular ice at subzero temperatures using current freezing protocols.[50] Therefore, the main source of sperm cryodamage results from osmo-tic stress, especially during the thawing.[51]

In our study, the PLCZ1 content measured by MFI and the proportion of spermatozoa positively labeled for PLCZ1 were reduced after freezing but refreezing at room or low temperature did not result in additional loss. Among sperm subpopulations within frozen or refrozen samples, spermatozoa with intact acrosomes had higher PLCZ1 content than spermatozoa with damaged acrosomes. PLCZ1 is thought to be a soluble protein, as soluble sperm extracts trigger calcium oscillations in oocytes consistent with the PLC activity.[52] Because PLCZ1 is a soluble protein, modifications after the freeze–thaw are not unexpected. In ruminants and human, sperm proteins involved in transport, membrane stabilization, and capacitation are reduced in abundance, lost, or translocated after cryopreservation with a consequent loss of function.[27,49] In the horse, protein denaturation in spermatozoa is evident in the hydrated, frozen, or dried state.[53,54] Congruently, cryopreservation of human and stallion spermatozoa has been associated with reduced PLCZ1 content using immunofluorescence and flow cytometric assessment, respectively.[8,55–57] In the retrospective analysis, relative content of PLCZ1 was positively correlated with cleavage rates after equine ICSI. Although numbers were limited, sperm samples with a higher proportion of spermatozoa exhibiting PLCZ1 at the acrosomal–postacrosomal or postacrosomal regions versus the acrosomal region were positively associated with cleavage rates after ICSI. Similarly, PLCZ1 fluorescence intensity and localization patterns have been associated with cleavage rates after sperm injections in human and equine oocytes.[20,22,58–60] In retrospective data analysis of three consecutive breeding seasons (2006–2008) of clinical ICSI, we observed a higher (p = 0.04) cleavage rate in equine oocytes injected with spermatozoa from cooled samples (71.1%, n = 114) when compared to oocytes injected with frozen–thawed spermatozoa (60.9%, n = 463) (unpublished data). Consequently, oocytes injected with spermatozoa from frozen–thawed samples were 36.6% (95% CI: 0.4—0.9) less likely to cleave when compared to oocytes injected with spermatozoa from cooled samples. Our results indicate that PLCZ1 content is associated with the likelihood of cleavage. Cryopreservation induces changes in the sperm plasma membrane integrity, facilitating the loss of PLCZ1 and, consequently, reducing the likelihood of cleavage. However, a sperm subpopulation within the cryopreserved sperm sample retains enough PLCZ1 to potentially maintain oocyte activating capability. Further investigation is needed to characterize variation in spermatozoa populations between and within stallion ejaculates in larger numbers for PLCZ1 abundance and localization and their effects on ICSI success.

In clinical practice, individual sperm selection for ICSI in horses is performed under X200 or X400 magnification and based on gross morphology and motility of individual spermatozoon.[61] In a previous study, motility was less affected when frozen spermatozoa were thawed at 5°C and then refrozen in diluents containing glycerol and methylformamide.[32] In this study, refrozen spermatozoa had better survival after thawing at low temperature in the first cycle. Subsequently, refrozen sperm samples that exhibit higher proportions of motile spermatozoa can be further sorted by swim-up, density gradient centrifugation, or microfluidics,[62] facilitating the selection of individual spermatozoon for ICSI, which potentially has higher PLCZ1 content.[57.] Further research needs to be completed to elucidate the impact of spermatozoa refrozen at low temperature on cleavage and embryo development after ICSI.

In conclusion, acrosomal and plasma membrane damage caused by cryopreservation procedures result in a reduction of PLCZ1 in stallion spermatozoa. Cryopreservation is deleterious to sperm plasma membrane integrity and PLCZ1 retention. Refreezing stallion spermatozoa for ICSI at a low temperature result in better sperm survival rates during the second freeze cycle, although retention of PLCZ1 is not improved.

AUTHOR CONTRIBUTIONS

RGC designed the study, conducted the sperm processing, cryopreservation, immunofluorescence, and flow cytometry, performed the formal data analysis, and drafted the manuscript. LAW performed the BCA and QS-ELISA assays and assisted with manuscript editing. ECP performed immunoblotting. EMC obtained funding, designed, administered the study, and critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We acknowledge the Cecil and Irene Hylton Foundation for funding this project, and Dr. J. Rodriguez, H. Grim, and Vista Equine Colorado LLC for sample donations.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Raul A. Gonzalez-Castro https://orcid.org/0000-0002-6494-7557

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

REFERENCES

1. Dozortsev D, Qian C, Ermilov A, Rybouchkin A, De Sutter P, Dhont M. Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 minutes after injection as a result of the sperm–oocyte interaction. *Human Reprod*. 1997;12(12):2792-2796. doi:10.1093/humrep/12.12.2792
2. Sato K, Wakai T, Seita Y, et al. Molecular characteristics of horse phospholipase C zeta (PLCζ). *Animal Sci J*. 2013;84(4):359-368. doi:10.1111/asj.12044
3. Amdani SN, Yeste M, Jones C, Coward K. Phospholipase C zeta (PLCζ) and male infertility: clinical update and topical developments. *Adv Biol Regul*. 2016;61:58-67. doi:10.1016/j.jbior.2015.11.009
4. Villaverde AISB, Fioratti EG, Fissore RA, et al. Identification of phospholipase C zeta in normospermic and teratospermic domestic cat sperm. *Theriogenology*. 2013;80(7):722-729. doi:10.1016/j.theriogenology.2013.06.005
5. Nozawa K, Satouh Y, Fujimoto T, Oji A, Ikawa M. Sperm-borne phospholipase C zeta-1 ensures monospermic fertilization in mice. *Sci Rep*. 2018;8(1):1315. doi:10.1038/s41598-018-19497-6
6. Yu Y, Saunders CM, a LaiF, Swann K. Preimplantation development of mouse oocytes activated by different levels of human phospholipase C zeta. *Hum Reprod*. 2008;23(2):365-373. doi:10.1093/humrep/dem350
7. Gonzalez-Castro R, Graham J, Carnevale EM. Flow cytometric expression levels of phospholipase C zeta in capacitated and acrosome reacted stallion sperm. *Anim Reprod Sci*. 2018;194:e2. doi:10.1016/j.anireprosci.2018.04.009
8. Heytens E, Parrington J, Coward K, et al. Reduced amounts and abnormal forms of phospholipase C zeta (PLCζ) in spermatozoa from infertile men. *Human Reprod*. 2009;24(10):2417-2428. doi:10.1093/humrep/dep207
9. Schrimpf R, Dierks C, Martinsson G, Sieme H, Distl O. Genome-wide association study identifies phospholipase C zeta 1 (PLCz1) as a stallion fertility locus in Hanoverian Warmblood horses. *PLoS One*. 2014;9(10):e109675. doi:10.1371/journal.pone.0109675
10. Nakai M, Suzuki SI, Ito J, et al. Efficient pig ICSI using Percoll-selected spermatozoa; evidence for the essential role of phospholipase C-ζ in ICSI success. *J Reprod Dev*. 2016;62(6):639-643. doi:10.1262/jrd.2016-103
11. Yelumalai S, Yeste M, Jones C, et al. Total levels, localization patterns, and proportions of sperm exhibiting phospholipase C zeta are significantly correlated with fertilization rates after intracytoplasmic sperm injection. *Fertil Steril*. 2005;104(3):561-568. doi:10.1016/j.fertnstert.2015.05.018 e4
12. Bedford-Guaus SJ, McPartlin LA, Xie J, Westmiller SL, Buffone MG, Roberson MS. Molecular cloning and characterization of phospholipase C zeta in equine sperm and testis reveals species-specific differences in expression of catalytically active protein1. *Biol Reprod*. 2011;85(1):78-88. doi:10.1095/biolreprod.110.089466
13. Nomikos M, Kashir J, Swann K, Lai FA. Sperm PLCζ: from structure to Ca^{2+} oscillations, egg activation and therapeutic potential. *FEBS Lett*. 2013;587(22):3609-3616. doi:10.1016/j.febslet.2013.10.008
14. Chithiwala ZH, Lee HC, Hill DL, et al. Phospholipase C-zeta deficiency as a cause for repetitive oocyte fertilization failure during ovarian stimulation for in vitro fertilization with ICSI: a case report. *J Assist Reprod Genet*. 2015;32(9):1415-1419. doi:10.1007/s10815-015-0531-1
15. Durban M, Barragán M, Colodron M, et al. PLCζ disruption with complete fertilization failure in normozoospermia. *J Assist Reprod Genet*. 2015;32(6):879-886. doi:10.1007/s10815-015-0496-0
16. Escoffier J, Yassine S, Lee HC, et al. Subcellular localization of phospholipase Cz in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. *Mol Hum Reprod*. 2014;21(2):157-168. doi:10.1093/molehr/gau098
17. Kashir J, Konstantinidis M, Jones C, et al. Characterization of two heterozygous mutations of the oocyte activation factor phospholipase C zeta (PLCζ) from an infertile man by use of minisequencing of individual sperm and expression in somatic cells. *Fertil Steril*. 2012;98(2):423-431. doi:10.1016/j.fertnstert.2012.05.002
18. Lee HC, Arny M, Grow D, Dumesic D, Fissore RA, Jellerette-Nolan T. Protein phospholipase C Zeta1 expression in patients with failed ICSI but with normal sperm parameters. *J Assist Reprod Genet*. 2014;31(6):749-756. doi:10.1007/s10815-014-0229-9
19. Tavalaee M, Nasr-Esfahani MH. Expression profile of PLCζ, PAWP, and TR-KIT in association with fertilization potential, embryo development, and pregnancy outcomes in globozoospermic candidates for intra-cytoplasmic sperm injection and artificial oocyte activation. *Andrology*. 2016;4(5):850-856. doi:10.1111/andr.12179
20. Meng X, Melo P, Jones C, et al. Use of phospholipase C zeta analysis to identify candidates for artificial oocyte activation: a case series of clinical pregnancies and a proposed algorithm for patient management. *Fertil Steril*. 2020;114(1):163-174. doi:10.1016/j.fertnstert.2020.02.113
21. Cardona Barberán A, Boel A, vanden Meerschaut F, Stoop D, Heindryckx B. Sperm factors and egg activation: fertilization failure after human ICSI and the clinical potential of PLCZ1. *Reproduction*. 2022;164(1):F39-F51. doi:10.1530/REP-21-0387
22. Gonzalez-Castro RA, Amoroso-Sanches F, Stokes JE, Graham JK, Carnevale EM. Localisation of phospholipase Cζ1 (PLCZ1) and postacrosomal WW-binding protein (WBP2 N-terminal like) on equine spermatozoa and flow cytometry quantification of PLCZ1 and association with cleavage *in vitro*. *Reprod Fertil Dev*. 2019;31(12):1778-1792. doi:10.1071/RD19217
23. Carnevale EM. Clinical considerations regarding assisted reproductive procedures in horses. *J Equine Vet Sci*. 2008;28(11):686-690. doi:10.1016/j.jevs.2008.10.005
24. Choi YH, Love CC, Varner DD, Hinrichs K. Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology*. 2006;65(4):808-819. doi:10.1016/j.theriogenology.2005.04.035
25. Gonzalez-Castro R, Carnevale E. Association between equine sperm parameters and ICSI outcome. *J Equine Vet Sci*. 2016;41(2016):62. doi:10.1016/j.jevs.2016.04.041
26. Sieme H, Oldenhof H, Wolkers WF. Sperm membrane behaviour during cooling and cryopreservation. *Reprod Domest Anim*. 2015;50:20-26. doi:10.1111/rda.12594
27. Peris-Frau P, Soler AJ, Iniesta-Cuerda M, et al. Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. *Int J Mol Sci*. 2020;21(8). doi:10.3390/ijms21082781
28. Catandi GD, LiPuma L, Obeidat YM, et al. Oocyte metabolic function, lipid composition, and developmental potential are altered by diet in older mares. *Reproduction*. 2022;163(4):183-198. doi:10.1530/REP-21-0351
29. Catandi GD, Obeidat YM, Broeckling CD, Chen TW, Chicco AJ, Carnevale EM. Equine maternal aging affects oocyte lipid content, metabolic function and developmental potential. *Reproduction*. 2021;161:399-409. doi:10.1530/REP
30. Kirk ES, Squires EL, Graham JK. Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. *Theriogenology*. 2005;64(6):1422-1439. doi:10.1016/j.theriogenology.2005.03.006
31. Cochran D, Amann RP, Froman DP, et al. Effects of centrifugation, glycerol level, cooling to 5°C, freezing rate and thawing rate on the post-thaw motility of equine sperm. *Theriogenology*. 1984;22(1):25-38. doi:10.1016/0093-691X(84)90470-9
32. Gonzalez-Castro RA, Trentin JM, Carnevale EM, Graham JK. Effects of extender, cryoprotectants and thawing protocol on motility of frozen-thawed stallion sperm that were refrozen for intracytoplasmic sperm injection doses. *Theriogenology*. 2019;136:36-42. doi:10.1016/j.theriogenology.2019.06.030

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

33. McPartlin LA, Littell J, Mark E, Nelson JL, Travis AJ, Bedford-Guaus SJ. A defined medium supports changes consistent with capacitation in stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and high rates of acrosomal exocytosis. *Theriogenology*. 2008;69(5):639-650. doi:10.1016/j.theriogenology.2007.11.016
34. Hamilton LE, Suzuki J, Acteau G, et al. WBP2 shares a common location in mouse spermatozoa with WBP2NL/PAWP and like its descendent is a candidate mouse oocyte-activating factor. *Biol Reprod*. 2018;99(6):1171-1183. doi:10.1093/biolre/ioy156
35. Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ, The use of total protein stains as loading controls: an alternative to high-abundance single protein controls in semi-quantitative immunoblotting. *J Neurosci Methods*. 2008;172(2):250-254. doi:10.1016/j.jneumeth.2008.05.00
36. Grasa P, Coward K, Young C, Parrington J. The pattern of localization of the putative oocyte activation factor, phospholipase Cζ, in uncapacitated, capacitated, and ionophore-treated human spermatozoa. *Human Reprod*. 2008;23(11):2513-2522. doi:10.1093/humrep/den280
37. Liarski VM, Kaverina N, Chang A, et al. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med*. 2014;6(230):230ra46. doi:10.1126/scitranslmed.3008146
38. Young C, Grasa P, Coward K, Davis LC, Parrington J. Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. *Fertil Steril*. 2009;91(5):2230-2242. doi:10.1016/j.fertnstert.2008.05.021
39. Oko R, Sutovsky P. Biogenesis of sperm perinuclear theca and its role in sperm functional competence and fertilization. *J Reprod Immunol*. 2009;83(1-2):2-7. doi:10.1016/j.jri.2009.05.008
40. Sutovsky P, Manandhar G, Wu A, Oko R. Interactions of sperm perinuclear theca with the oocyte: implications for oocyte activation, anti-polyspermy defense, and assisted reproduction. *Microsc Res Tech*. 2003;61(4):362-378. doi:10.1002/jemt.10350
41. Suganuma R, Walden CM, Butters TD, et al. Alkylated imino sugars, reversible male infertility-inducing agents, do not affect the genetic integrity of male mouse germ cells during short-term treatment despite induction of sperm deformities. *Biol Reprod*. 2005;72(4):805-813. doi:10.1095/biolreprod.104.036053
42. Wu A, Sutovsky P, Manandhar G, et al. PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem*. 2007;282(16):12164-12175. doi:10.1074/jbc.M609132200
43. Aarabi M, Balakier H, Bashar S, et al. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *FASEB J*. 2014;28(10):4434-4440. doi:10.1096/fj.14-256495
44. Aarabi M, Balakier H, Bashar S, et al. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. *Fertil Steril*. 2014;102(2):440-447. doi:10.1016/j.fertnstert.2014.05.003
45. Sutovsky P, Oko R, Hewitson L, Schatten G. The removal of the sperm perinuclear theca and its association with the bovine oocyte surface during fertilization. *Dev Biol*. 1997;188(1):75-84. doi:10.1006/dbio.1997.8618
46. Sutovsky P, Navara CS, Schatten G. Fate of the sperm mitochondria, and the incorporation, conversion, and disassembly of the sperm tail structures during bovine fertilization. *Biol Reprod*. 1996;55:1195-1205. doi:10.1095/biolreprod55.6.1195
47. Gousset K, Wolkers WF, Tsvetkova NM, et al. Evidence for a physiological role for membrane rafts in human platelets. *J Cell Physiol*. 2002;190(1):117-128. doi:10.1002/jcp.10039
48. Wolkers WF, Balasubramanian SK, Ongstad EL, Zec HC, Bischof JC. Effects of freezing on membranes and proteins in LNCaP prostate tumor cells. *Biochim Biophys Acta*. 2007;1768(3):728-736. doi:10.1016/j.bbamem.2006.12.007
49. Bogle OA, Kumar K, Attardo-Parrinello C, et al. Identification of protein changes in human spermatozoa throughout the cryopreservation process. *Andrology*. 2017;5(1):10-22. doi:10.1111/andr.12279
50. Oldenhof H, Gojowsky M, Wang S, et al. Osmotic stress and membrane phase changes during freezing of stallion sperm: mode of action of cryoprotective agents. *Biol Reprod*. 2013;88(3):1-11. doi:10.1095/biolreprod.112.104661
51. Peña FJ, Macías García B, Samper JC, Aparicio IM, Tapia JA. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? *Theriogenology*. 2011;76(7):1177-1186. doi:10.1016/j.theriogenology.2011.06.023
52. Nomikos M, Blayney LM, Larman MG, et al. Role of phospholipase C-ζ domains in Ca^{2+}-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca^{2+} oscillations. *J Biol Chem*. 2005;280(35):31011-31018. doi:10.1074/jbc.M500629200
53. Wolkers WF, Oldenhof H. In situ FTIR studies on mammalian cells. *Spectroscopy*. 2010;24(5):525-534. doi:10.3233/SPE-2010-0474
54. Wolkers WF, Oldenhof H. Use of in situ Fourier transform infrared spectroscopy to study freezing and drying of cells. *Methods Mol Biol*. 2015;1257:147-161. doi:10.1007/978-1-4939-2193-5_5
55. Moreau J, Fargeon S, Gatimel N, Parinaud J, Léandri RD. Expression of phospholipase PLC Zeta in human spermatozoa: impact of cryopreservation. *Andrology*. 2019;7(3):315-318. doi:10.1111/andr.12593
56. Gonzalez-Castro RA, Carnevale EM. Phospholipase C zeta quantification by flow cytometry in fresh, frozen and refrozen stallion sperm. *J Equine Vet Sci*. 2020;89:103033. doi:10.1016/j.jevs.2020.103033
57. Kashir J, Heynen A, Jones C, et al. Effects of cryopreservation and density-gradient washing on phospholipase C zeta concentrations in human spermatozoa. *Reprod Biomed Online*. 2011;23(2):263-267. doi:10.1016/j.rbmo.2011.04.006
58. Cheung S, Xie P, Parrella A, Keating D, Rosenwaks Z, Palermo GD. Identification and treatment of men with phospholipase Cζ-defective spermatozoa. *Fertil Steril*. 2020;114(3):535-544. doi:10.1016/j.fertnstert.2020.04.044
59. Kashir J, Mistry B, BuSaleh L, et al. Phospholipase C zeta profiles are indicative of optimal sperm parameters and fertilisation success in patients undergoing fertility treatment. *Andrology*. 2020;8(5):1143-1159. doi:10.1111/andr.12796
60. Kashir J, Jones C, Mounce G, et al. Variance in total levels of phospholipase C zeta (PLC-ζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. *Fertil Steril*. 2013;99(1):107-117. doi:10.1016/j.fertnstert.2012.09.001 e3
61. Sessions-Bresnahan DR, Graham JK, Carnevale EM. Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using invitro fertilization, perivitelline, and intracytoplasmic sperm injections. *Theriogenology*. 2014;82(2):274-282. doi:10.1016/j.theriogenology.2014.04.002
62. Gonzalez-Castro RA, Carnevale EM. Use of micro fluidics to sort stallion sperm for intracytoplasmic sperm injection. *Anim Reprod Sci*. 2019;202(2018):1-9. doi:10.1016/j.anireprosci.2018.12.012

How to cite this article: Gonzalez-Castro RA, Whitcomb LA, Pinsinski EC, Carnevale EM. Cryopreservation of equine spermatozoa reduces plasma membrane integrity and phospholipase C zeta 1 content as associated with oocyte activation. *Andrology*. 2024;12:918–931. https://doi.org/10.1111/andr.13517

20472927, 2024, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/andr.13517 by myam mohammadi - Ondokuz Mayis Universitesi Kutuphane Ve Dokumantasyon , Wiley Online Library on [28/07/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License