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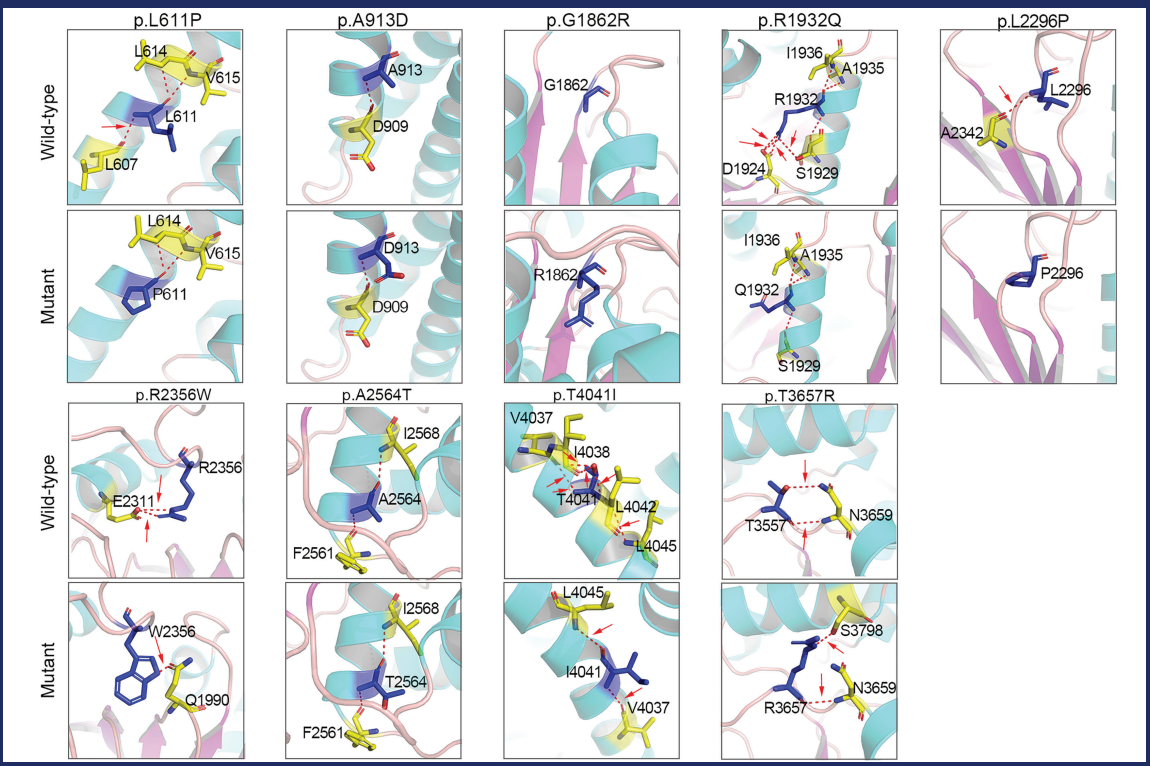

Cover Illustration: Bioinformatic protein conformation predictions of dynein axonemal heavy chain 1 (DNAH1) variants in spermatozoa. The dashed lines represent hydrogen bonds, and the arrows indicate hydrogen bond changes. From Long *et al: Novel biallelic variants in DNAH1 causemultiplemorphological abnormalities of sperm flagella with favorable outcomes of fertility after ICSI in Han Chinese male*. pp 349-364 in this issue.

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From Waterloo to the Great Wall: A retrospective, multicenter study on the clinical practice and cultural attitudes in the management of premature ejaculation, in China

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Premature ejaculation (PE), despite its wide prevalence, is largely underdiagnosed and undertreated. Being a multifactorial dysfunction with strong cultural characteristics, PE requires skillful attitudes in the psychosexological support, necessary to manage the patient's and the couple's expectations, as well as in the medical treatment. Dapoxetine is a short-acting selective serotonin reuptake inhibitor approved for use in lifelong and acquired PE in a number of countries. Opinions, not always generated by the evidence-based medicine, impacted the attitudes of Western andrologists, as a nocebo

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effect which produced a drug's Waterloo, characterized by low prescription rates much more built on the patients' and doctors' expectations than on costs, side effects, and efficacy.

In the present study, we retrospectively reviewed real-life data from eight Andrology and Sexual Medicine Public Centers in China to assess the prevalence of PE among attending patients, its association with erectile dysfunction, its subtype, and the proposed treatments. In 2019, among 156,486 patients coming to the centers, 32,667 visits having PE as the chief complaint were performed (20.9%). Almost all patients received treatment prescriptions (32,641 patients, 99.92%); 23,273 patients came back for a follow-up visit in the subsequent 12 months (71.2% of those who initially received treatment). Dapoxetine, either alone or in combination with another therapy, was the most prevalent treatment, prescribed to 22,767 patients (69.7% of treated patients), followed by traditional Chinese medicine (TCM) (39.4%). At follow-up, 8174 patients were unsatisfied with treatment, and a new treatment was proposed (35.12%). Dapoxetine was the best treatment, with an overall 27.1% switching rate when used either alone or in combination: Although the switching rate for Dapoxetine alone was 44.2%, the association of the same drug with psychotherapy resulted in much lower rates (19.5%) and reached a minimum of 12% when also combined with TCM demonstrating how cultural aspects and medical attitudes may dramatically impact on the therapy of a multifaceted, complex, and culture-grounded sexual symptom such as PE.

In conclusion, taking switching rates as surrogate markers of treatment failure, this real-life study—the largest in the field—shows that in a more patient-oriented (as in Chinese medical culture), and less symptom-oriented (as in Western medical attitudes), Dapoxetine is a successful treatment for PE patients, with higher reliability when used alone or as part of combined and integrated therapies.

KEYWORDS
dapoxetine, premature ejaculation, SSRI, traditional Chinese medicine, treatment

1 | INTRODUCTION

Although the field of erectile dysfunction (ED) and related guidelines is pigeonholed in a substantial uniformity and homogeneity of the evidence and positions[1–3] (with relatively little debate on the use of old, such as surgery, and new, such as shockwaves therapies[4,5]), that one of premature ejaculation (PE) results characterized by the admission that the main pathophysiological mechanisms, the diagnostic workups, and managements are not universally accepted.[6–8] This is also clearly demonstrated by the mono-dimensional definition of the former[9] (inability to reach/maintain erection), facing a tridimensional definition of the latter[7] (loss of control on the ejaculatory mechanism, stress-induced/inducing, and short time from penetration to ejaculation).[10] For example, the neurobiological hypothesis on a genetic involvement of the serotoninergic pathway on lifelong forms of PE (LPE), although popular in the last 20 years, has been found confirmed in rodents (animals with a peculiar copulatory behavior and penile anatomy),[11] but never, with robust findings, in humans, whereas in the patient's clinical history, both sexual symptoms are bona fide anticipated by subclinical forms[12,13] (surprisingly not too frequently explored[14]), PE appears as more multifaced than ED.[6,7]

For a long time, PE has been considered merely a psychorelational sexual dysfunction to be exclusively treated by psychotherapies,[15] until the revolutionary proposal of the Dutch psychiatrist Marcel Waldinger to therapeutically exploit the common anti-ejaculatory side effects of the serotoninergic antidepressants in the management of PE.[16] Although this genial intuition opened a huge highway of investigations,[17] improving the sexual health of a large number of patients, it produced the erroneous scientific perception that the patients with PE must have a serotoninergic central derangement, leading to ineffectual research. In these regards, the idea that PE could be generated by an impaired, absolute or relative, serotoninergic activity, possibly genetic in nature,[18] has been used to justify the use of serotoninergic drugs in the PE treatment. However, exactly as in ED

patients, the symptom can be resolved by inhibiting PDE5, in subjects with PE the symptom can be overcome by increasing the presence of the serotonin in the synaptic cleft. But this simple evidence seems not as easy to be accepted by a number of researchers still looking for a neurobiological/morphological alteration in PE.[19]

In fact, the chapter of PE therapy is still under debate. Again, although PDE5 inhibitors are universally accepted for ED therapy,[20] there definitely is no "one-size-fits-all" strategy for the management of PE. Some forms of PE, most frequently included among acquired forms of PE (APE), can successfully be treated by administering specific treatments, such as thyrostatic drugs for patients diagnosed with hyperthyroidism[3,6,21–23] or anti-inflammatory/antibiotic therapies for patients diagnosed with prostatitis.[23–26] However, a definite, treatable organic factor for PE is rarely identified, leading to the widespread, abovementioned idea that PE is mostly "psychogenic" in nature. Although it is in fact undeniable that PE is often associated with performance anxiety,[3,27,28] in the broader context of the "loss of control of erection and ejaculation" (LCEE),[13] as well as in patients with poor psychological health,[29,30] it should be considered that the biological mechanisms regulating ejaculation are obviously organic in nature: Therefore, the multifactorial origin of PE should always be considered during the diagnostic and therapeutic process.[15,31] Therefore, current guidelines suggest providing both psychosexological support and medical treatment for PE.[3,6,8] Unsurprisingly, some patients report beneficial outcomes in terms of ejaculatory latency when using PDE5 inhibitors: Indeed, such drugs can potentially improve the sense of control over performance anxiety in some patients. The concept of LCEE provides a clear explanation of the efficacy of these treatments: Each sexual dysfunction can potentially contribute to the onset or worsening of the other, and therefore, acting on either one can also have beneficial effects on the other.[13,32]

A fundamental milestone in the history of PE was the development and marketing in several countries of a short-acting selective serotonin reuptake inhibitor (SSRI) derived from fluoxetine,[33] named Dapoxetine.[34] The drug was approved by several medical agencies not as an antidepressant, being exempt from mood-regulating effects owing to its very short half-life which prevents accumulation,[35] but as the first (and so far unique) oral treatment for PE. Dapoxetine is now recognized as the gold standard of the medical treatments for both LPE and APE,[36] whereas local anesthetics have been more recently approved for use only in the case of LPE.[37,38] Apparently, all PE treatments, however, are burdened with varying degrees of efficacy and high discontinuation rates, also owing to their costs and posology, and patient's expectations.[39,40] In fact, for a number of reasons, interesting to explore and to study in deep, despite excellent clinical outcomes in randomized controlled trials,[41,42] in Western Countries, the prescriptions of the new short-acting SSRI have been so low that the scenario has been depicted as the "Waterloo" of the Dapoxetine.[43] However, the perception of a low efficacy of the drug, relatively diffused in the Western urological milieu on the basis of opinions and not on published and controlled evidence,[44,45] was not universal. In oriental countries, such as China or Vietnam, the use of Dapoxetine has been much more successful, and it is currently much largely diffused than in Europe or in other Western countries.[46–48] The dose-dependent efficacy in the three dimensions of PE (increasing the feeling of control on ejaculatory mechanisms, reducing the distress produced by PE, as measured by well validated psychometric tools exploring patient reported outcomes, and the increase in the intravaginal ejaculatory latency time [IELT], as measured by the stopwatch)[49] does not justify the low prescription rate in Western doctors. Similarly, the Waterloo scenario appears not justified by the Dapoxetine safety profile, which is much more tolerable than all other SSRIs, frequently prescribed off-label for PE.[50] In the Waterloo effect, cultural factors and doctors' opinions may have played and may still play a major role. For example, it has been claimed that the majority of a small number of Dutch patients with LPE prefer daily treatment with high-risk SSRIs with respect to on-demand treatment with a better risk/benefit ratio.[51] This is surprising also considering the large number of SSRI-induced side effects but also considering the possibility of a severe post-SSRI sexual disease described after chronic use of this drug class, but never in Dapoxetine users.[52–54]

On the basis of this complex background, in the present study, we retrospectively reviewed data from several Chinese centers active in PE management in order to highlight culturally driven clinical behaviors and to measure, by means of follow-up and treatment switch rates, to what extent different treatments were considered reliable by doctors and patients, and by extension, the prevalence of PE, its duration (LPE vs. APE) and its association with ED, as in the LCEE.

2 | MATERIALS AND METHODS

2.1 | Survey design

A letter and blank survey were sent to 10 major centers in China describing the project and requesting their participation. Centers have been selected as active in basic and clinical research on PE and participating to international meetings of sexual medicine and andrology. They do not represent the whole Chinese scientific community in the field, but a large part of it, and are all located East of the "Heihe–Tengchong Line," that is, in the area where 94% of China's population live. Among participating centers, only two are mainly traditional Chinese medicine (TCM)-oriented, namely, the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine and the Beijing Xiyuan Hospital of the China Academy of Chinese Medical Sciences.

The main objective was to carry out a real-life retrospective, multicenter survey on the prevalence of PE and on the use of different therapies for its treatment in China.

Participants were asked to refer to the International Society of Sexual Medicine (ISSM) definitions of LPE and APE[8] and to the National Institutes of Health definition of ED.[9] Severity of ED was graded as mild, moderate, and severe, using the simplified International Index of Erectile Function (IIEF-5) scores.[55] In terms of statements, "continuous/repetitive" was to be used when the condition occurs ≥25% of times, "non-occasional" between 10% and <25% of times, and "occasional" for <10% of times. Sexological criteria were used to define

TABLE 1 Participating centers

Center name	Overall visits in 2019	PE visits in 2019
Peking University 3rd Hospital	*N* = 50,250 (32.11%)	*n* = 8000 (24.5%)
First Teaching Hospital of Tianjin University of Traditional Chinese Medicine	*N* = 8200 (5.24%)	*n* = 2380 (7.3%)
Xiyuan Hospital of China Academy of Chinese Medical Sciences	*N* = 47,996 (30.67%)	*n* = 10,559 (32.3%)
Shenzhen Key Laboratory of Fertility Regulation	*N* = 1500 (0.96%)	*n* = 360 (1.1%)
First Affiliated Hospital of Anhui Medical University, Hefei	*N* = 37,000 (23.64%)	*n* = 9120 (27.9%)
Xijing Hospital, Fourth Military Medical University, Xi'an	*N* = 2200 (1.41%)	*n* = 736 (2.3%)
3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou	*N* = 7490 (4.79%)	*n* = 1000 (3.1%)
Xi'an Daxing Hospital	*N* = 1850 (1.18%)	*n* = 512 (1.6%)

Abbreviation: PE, premature ejaculation.

subclinical ED (SED), defined as a continuous or repetitive inability to achieve or maintain an erection sufficient for satisfying sexual activity.[12] SED is diagnosed when at least one major and two minor criteria are met (Table S1).

To be included in the study, it was requested that patients have an age of at least 18 years and a diagnosis of PE.

Being the clinical definition of subclinical PE (SPE)[13] not available at the time of the present survey, it was not considered here. However, for the sake of clarity, criteria for diagnosis of SPE have been included as part of the supplementary material (Table S2).

Data from 01.01.2015 to 31.12.2019 were collected, divided by year; where unavailable, centers collected data for a shorter period, always indicating year by year.

2.2 | Statistical analysis

Descriptive statistics were used to summarize pertinent study information. Associations between categorical variables were analyzed according to the Pearson chi-square test. The statistical software R (version 4.1.1, R core team, Vienna, Austria) was used for all analyses. Figure 2 is drawn using SankeyMATIC (www.sankeymatic.com).

3 | RESULTS

Among the 10 invited centers, 9 agreed to participate in the survey, and 8 returned completed questionnaires (Table 1). In all, from the 5-year period from 2015 to 2019, a total of 654,590 patients were seen in the centers actively participating to the survey.

Being the time of clinical activity different from Center to Center, for the present analysis, the data refer to 2019 (January 1–December 31), unless otherwise specified.

In all, the 8 centers reported a total of 156,486 patients observed during the pre-pandemic year 2019 (Figure 1). Of these, 32,667 (20.9%) referred to PE (Figure 2). APE accounted for almost 46.8% of cases and LPE in nearly 36.1%. The remaining cases (17.1%) were undefined or were found ex-post with other complaints related to the lack of control of ejaculation.

TABLE 2 Prevalence of premature ejaculation (PE) in combination with different forms of erectile dysfunction (ED)

Type	%
No ED	34.6
Comorbid ED + PE	65.4
PE + subclinical ED[a]	24.3
PE + mild ED	35.7
PE + moderate ED	23.6
PE + severe ED	16.3

[a]Data on subclinical ED[12] available from six centers only.

The association of ED in combination with PE, later defined as LCEE, was also addressed (Table 2). Overall, among all patients included in analysis, almost two out of three subjects (65.3%) had comorbid ED of varying severity, and only 34.6% of subjects did not complain of ED. Among subjects having LCEE, most had mild ED (35.7%), and progressively lower rates were found for moderate and severe ED (23.6% and 16.3%, respectively). The remaining 24.3% patients were classified as SED.

In 2019, as stated, 32,667 visits with PE as the chief complaint were performed in the 8 participating centers. A total of 32,641 patients received treatment (99.92%), and 23,273 came back for a follow-up visit in the subsequent 12 months (71.2% of those who initially received treatment). According to the study protocol, patients were treated according to the clinicians' experience, their practice, and their clinical judgment on the patient's needs and requests.

Dapoxetine, either alone or in combination with another therapy, was the treatment of choice in 22,767 patients (69.7% of treated patients). TCM was the second most used treatment, accounting for about 39.4% of all administered treatments (4693 patients treated with TCM alone, and 8176 in combination with Dapoxetine), whereas 15.9% were treated with other treatments, either alone or in combination (Table 3). As shown in Table 4, clinicians in different centers have different treatment approaches to their patients. As an example, Dapoxetine was used in close to 100% of patients in some Western medicine-oriented centers, whereas its prevalence of use was around 20% in other more TCM-oriented centers. Among the 12,869

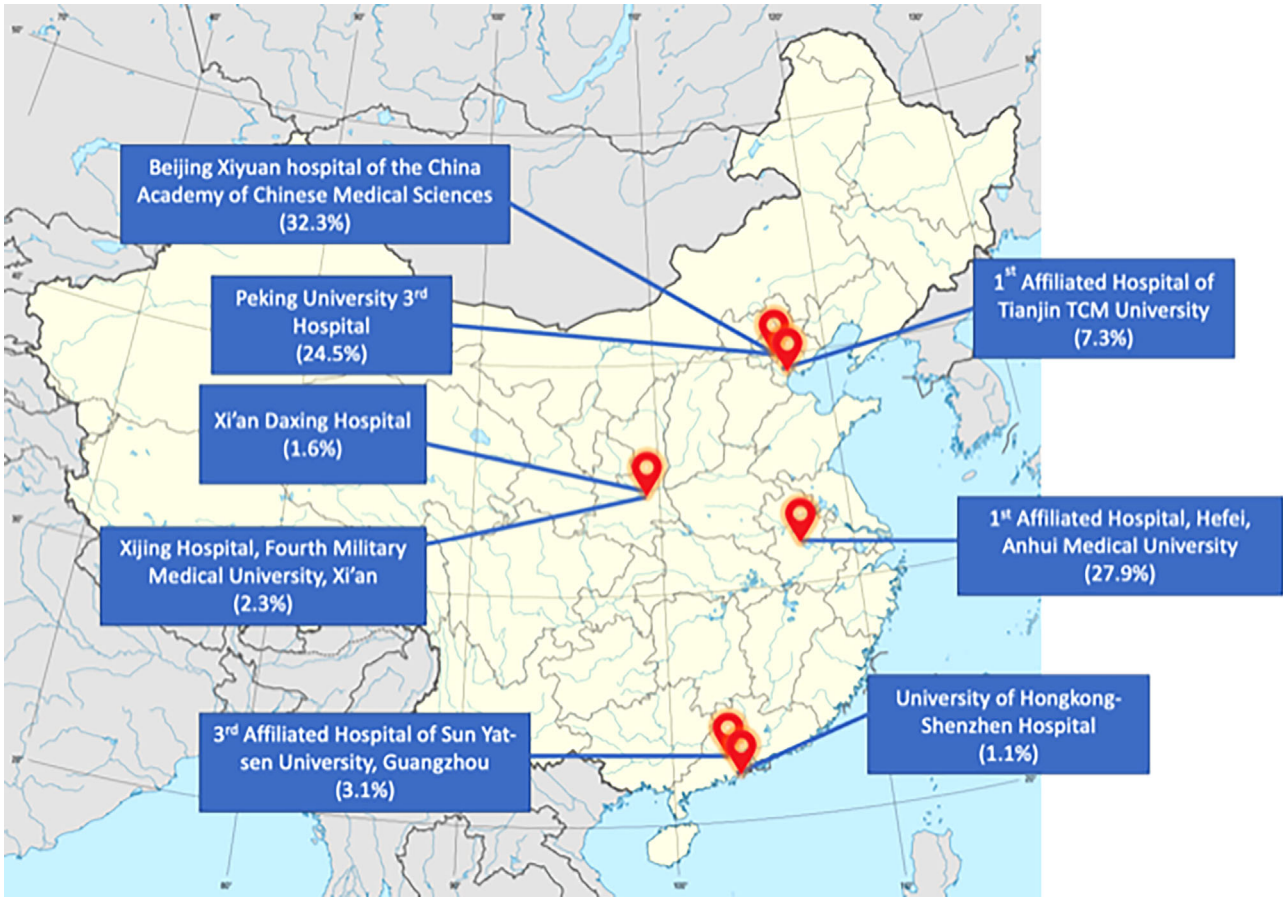

FIGURE 1 Map of China showing the approximate location of all participating centers.

TABLE 3 Treatments administered for premature ejaculation (PE), including follow-up and treatment switch rates

Treatment	Treated ($n = 32,667$)	Follow-up ($n = 23,273$)	Switch ($n = 8174$)
Dapoxetine (all)	22,767 (69.69%)	15,887 (69.78%)	4299 (27.06%)
D. alone	3190 (9.77%)	2162 (67.8%)	955 (44.2%)
D. + psychotherapy	1812 (5.55%)	822 (45.4%)	160 (19.5%)
D. + psychotherapy + local anesthetics	6630 (20.3%)	4607 (69.5%)	1860 (40.4%)
D. + psychotherapy + PDE5i	2959 (9.06%)	1410 (47.7%)	498 (35.3%)
D. + psychotherapy + TCM	8176 (25.03%)	6886 (84.2%)	826 (12%)
Local anesthetics alone	1185 (3.63%)	882 (74.4%)	487 (55.2%)
No treatment	26 (0.08%)	6 (23.1%)	4 (66.7%)
Other combinations without Dapoxetine	1679 (5.14%)	1075 (64%)	554 (51.5%)
Other treatments	55 (0.17%)	55 (100%)	39 (70.9%)
PDE5i alone	755 (2.31%)	330 (43.7%)	223 (67.6%)
Psychotherapy alone	786 (2.41%)	548 (69.7%)	350 (63.9%)
SSRIs or other antidepressants alone	622 (1.9%)	436 (70.1%)	257 (58.9%)
Surgery	99 (0.3%)	73 (73.7%)	57 (78%)
TCM alone	4693 (14.37%)	3981 (84.8%)	1904 (47.8%)

Abbreviations: SSRIs, selective serotonin reuptake inhibitors; TCM, traditional Chinese medicine.

TABLE 4 Use of different treatments according to Center

Treatment	Beijing III	Beijing Xiyuan	Guangzhou	Hefei	Shenzhen	Tianjin	Xian Daxing	Xian Xijing
Dapoxetine (all)	6160 (77%)	6524 (61.8%)	1000 (100%)	7200 (78.9%)	300 (83.3%)	1310 (55%)	113 (22.1%)	160 (21.7%)
D. alone	160 (2%)	225 (2.1%)	0 (0%)	1920 (21.1%)	300 (83.3%)	360 (15.1%)	82 (16%)	143 (19.4%)
D. + psychotherapy	800 (10%)	0 (0%)	810 (81%)	0 (0%)	0 (0%)	200 (8.4%)	1 (0.2%)	1 (0.1%)
D. + psychotherapy + local anesthetics	1200 (15%)	0 (0%)	0 (0%)	5280 (57.9%)	0 (0%)	150 (6.3%)	0 (0%)	0 (0%)
D. + psychotherapy + PDE5i	1600 (20%)	869 (8.2%)	190 (19%)	0 (0%)	0 (0%)	300 (12.6%)	0 (0%)	0 (0%)
D. + psychotherapy + TCM	2400 (30%)	5430 (51.4%)	0 (0%)	0 (0%)	0 (0%)	300 (12.6%)	30 (5.9%)	16 (2.2%)
Local anesthetics alone	80 (1%)	55 (0.5%)	0 (0%)	960 (10.5%)	20 (5.6%)	70 (2.9%)	0 (0%)	0 (0%)
No treatment	0 (0%)	26 (0.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Other combinations without Dapoxetine	800 (10%)	310 (2.9%)	0 (0%)	0 (0%)	0 (0%)	230 (9.7%)	145 (28.3%)	194 (26.4%)
Other treatments	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	30 (5.9%)	25 (3.4%)
PDE5i alone	400 (5%)	210 (2%)	0 (0%)	0 (0%)	20 (5.6%)	120 (5%)	4 (0.8%)	1 (0.1%)
Psychotherapy alone	160 (2%)	46 (0.4%)	0 (0%)	480 (5.3%)	0 (0%)	100 (4.2%)	0 (0%)	0 (0%)
SSRIs or other antidepressants alone	80 (1%)	38 (0.4%)	0 (0%)	384 (4.2%)	20 (5.6%)	100 (4.2%)	0 (0%)	0 (0%)
Surgery	0 (0%)	0 (0%)	0 (0%)	96 (1.1%)	0 (0%)	0 (0%)	0 (0%)	3 (0.4%)
TCM alone	320 (4%)	3350 (31.7%)	0 (0%)	0 (0%)	0 (0%)	450 (18.9%)	220 (43%)	353 (48%)

Abbreviation: SSRIs, selective serotonin reuptake inhibitors; TCM, traditional Chinese medicine.

FIGURE 2 Prevalence of different subtypes of premature ejaculation (PE) in the study population.

patients being treated with TCM, either alone or in combination, most were followed in the Beijing Xiyuan Hospital of the China Academy of Chinese Medical Sciences (8780 patients, 68.2%) and the Beijing University Third Hospital (2720 patients, 21.1%). The two mainly TCM-oriented centers had significantly lower dropout (1902/12,939 vs. 7508/19,728, $p < 0.001$) and switch rates (3395/11,037 vs. 4795/12,220, $p < 0.001$) rates.

Table 3 and Figure 3 also show the follow-up rates and the switching from one therapy to another, for the specified therapies. Overall, the follow-up rate for all patients coming to the different centers involved was 71.2% (23,273 patients), suggesting that almost 3 out of 10 PE patients are lost to follow-up. Follow-up rates were significantly different among treatments ($p < 0.0001$); most notably, follow-up rates for TCM were remarkably high (84.8%), and likewise the inclusion of TCM in combination therapies was associated with similarly high follow-up rates (84.2%). A treatment switch was prescribed in 8174 patients among those coming at follow-up visits, meaning that slightly more than one out of three patients coming to follow-up was not fully satisfied by the first treatment proposed (35.12%). As for follow-up, the rate of treatment switch was significantly different among centers ($p < 0.0001$). Surgery was the treatment with the highest rates of switching (78%), followed by PDE5i used alone (67.6%) and psychotherapy alone (63.9%). Among patients exclusively treated with TCM, almost half requested a treatment switch (47.8%). The lowest rates of treatment switch were instead found for Dapoxetine: Overall, when considering Dapoxetine alone and in combination, switching rate was about 27.1%. Combination use was more successful than the

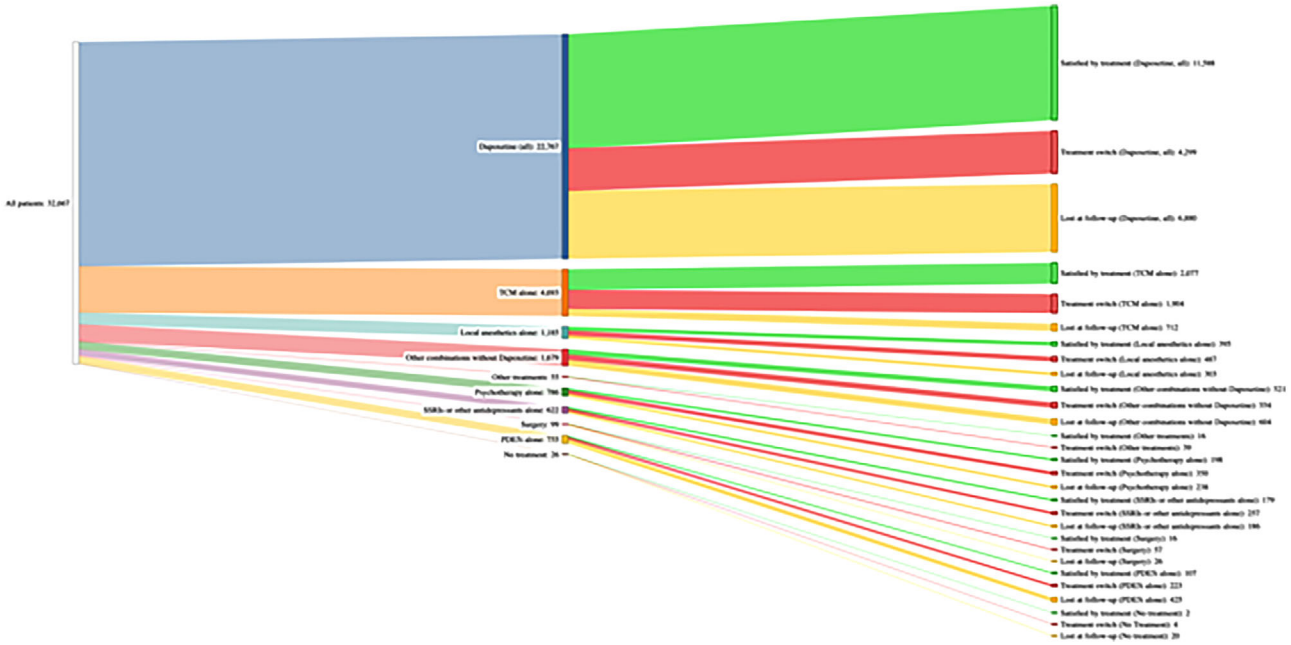

FIGURE 3 Sankey diagram describing treatment flow for the study population. Colors in the rightmost part of the diagram provide a visual representation of the different outcomes of treatment (*green: satisfied with treatment; red: treatment switch necessary; yellow: patient lost at follow-up*).

use of Dapoxetine alone: Indeed, although switching rate for Dapoxetine alone was 44.2%, association of the same drug with psychotherapy resulted in much lower requests for switching (19.5%) and reached a minimum of 12% when also combined with TCM.

4 | DISCUSSION

Although limited by the retrospective design and by the obvious heterogeneity of the clinical behavior of the clinical centers, this is one of the largest real-life studies in the field of PE management.

We found that objective PE (APE + LPE) is one of the more frequent sexual complaints, being present in more than 1 out of 5 patients attending 8 of major Chinese outpatient clinics practicing either Western or TCM. Interestingly, this percentage mirrors other previous epidemiological studies in different countries.[28,56–64] A similar prevalence (28%) has recently been found in a single-center Italian report.[14] Subclinical forms of PE[13] or subjective PE[65] have not been considered in this retrospective study, which is one of the largest strictly based on the ISSM criteria and not on other definitions of PE and PE-like, or subjective, conditions.

Our study focused on follow-up and treatment switch rates in order to show a picture of the clinical behaviors and attitudes in China in the PE management and to have surrogate indications on the efficacy of different available therapies examining the rate of refilling or changes of drugs/treatment in the follow-up. However, to fully evaluate the study outcomes, it is mandatory to consider the "true" meaning of follow-up rates and requests for treatment switch. Patients might be lost at follow-up for a plethora of reasons, which include, but are not limited to, treatment inefficacy (e.g., they moved to another city, they were ill on that day, they were no longer interested in addressing this issue, they forgot, etc.). On the other hand, it could also be quite the opposite: The patient might disregard the follow-up visit because the proposed treatment was perfectly effective, and therefore, subsequent evaluation might not be deemed as necessary as before. Therefore, judging the efficacy of a treatment by the frequency of follow-up visits does not seem, apparently, a viable strategy. On the other hand, it can be assumed that patients who show up at such follow-up visits are the most reliable population upon which to assess treatment efficacy. Those who did not ask for a treatment switch were, most likely, completely satisfied with their prescription; on the other hand, those who found their initial treatment to be inadequate came back asking for a new therapy, for example, as shown by the high rates of switching after initial surgery treatment. Therefore, treatment switch can be considered a surrogate marker for assessing the efficacy of different therapies, even more in the context of real-life studies for conditions such as PE, which are largely underdiagnosed and undertreated.[13,66] PE is a sexual symptom, that is, by definition, largely dependent on sociocultural and relational contexts, and as such, it is largely expected that the efficacy and compliance to treatment are similarly dependent on the same factors. Our study results show that Dapoxetine, when administered either alone or in combination with other treatments, was the most successful treatment for PE in China, owing to the lowest rates of treatment switch requested by patients.

Real-life studies on a large population, such as the present one, prove that the same treatments can have different rates of success in different centers. On a broader scale, our results prove that some treatments, at least in the Chinese population, are unequivocally associated with better follow-up and fewer requests for treatment change. Combination therapy is generally more likely to succeed in

treating PE, acting synergistically on the different factors involved in the pathogenesis of this sexual dysfunction and/or on the patients' expectations. Such factors can possibly explain why Dapoxetine, while potentially being the most "targeted" treatment for management of PE, has had varying rates of success in clinical trials. The clearest example of this comes from an Italian study from 2013, performed on just 120 patients complaining of PE, which showed poor efficacy of Dapoxetine and poor compliance to treatment.[43] Several factors were considered likely culprits of this "Waterloo," including drug costs, side effects, and perceived lack of efficacy. However, as IELT remarkably and significantly increased in subjects deciding to continue treatment, the perceived poor efficacy can also be attributed to doctors' prejudices, inadequate management of expectations, and lack of psychosexological consultations. Studies without any psychosexological assessment, in fact, show remarkably higher treatment discontinuation rates and lower satisfaction.[43,67] This is unsurprising, as perception of any improvement for sexual health could be differently judged according to patient's expectations.[6] A common clinical scenario depicts a patient complaining of LPE, having an IELT of about 1 min since his first sexual experiences, who is able to delay ejaculation for up to 3.5 min while undergoing an effective treatment. The same patient could be satisfied by his more than threefold increase in IELT, but at the same time, his time to ejaculation might be too short to provide enjoyable sexual intercourse for both partners. Undoubtedly, this clinical situation represents a possible target for careful counseling, if not for a tailored sexological treatment, possibly involving the couple rather than the man alone in order to adequately manage the couple's distress and expectations.[6,31] Failing to address this issue can potentially decrease the compliance to treatment, as the therapy itself would be deemed ineffective by the couple,[13] therefore leading to either loss at follow-up or subsequent visits for new treatments. To an extent, likelihood of treatment success can possibly be predicted by the use of nomograms[68]: These nomograms frequently rely upon Clinical Global Impression of Change, rather than on changes to IELT or patient-reported outcomes, in order to provide reliable assessment on treatment efficacy.[69]

Establishing a good doctor–patient relationship is another item to be considered in the management of many conditions, and particularly in the case of PE. Treatment compliance can also be affected by the stigma associated with SSRIs[66,67,70]: In fact, the idea that an antidepressant drug is necessary to treat PE might be misinterpreted by patients, who might be worried about the effects on mental health and behavior of these substances, or who might also be skeptical of their doctor's suggestions. Most SSRIs can impair sexual health,[6,52] and such effects are commonly (and correctly) reported on the drug leaflets: Patients finding out that the same drug they have been prescribed for treatment of PE can impair erection might be doubtful of the prescription's adequacy and might therefore refrain from correct use. It is therefore unsurprising that many patients, when being proposed treatment with off-label SSRIs, are more reluctant to start and more likely to discontinue treatment.[43,67] These side effects are very much less frequent for Dapoxetine than for other SSRIs, but stigmatization of the whole drug class might mislead patients, who may receive wrong explicit or non-explicit wrong messages from the doctor, regarding efficacy and/or possible side effects, resulting in unwillingness to pursue treatment. This possible nocebo effect,[71] better defined as "drucebo" effect[72] could be produced by the prejudices of the doctors based not on evidence, but on opinions, and/or on the ignorance that the safety profile of antidepressant SSRIs is largely because of their pharmacokinetics and to their ability to cross the blood–brain barrier. In these regards, cultural differences might become once again one of the main reasons for different rates of treatment compliance. It is likely that a greater tendency toward self-care might be an additional reason for poor compliance in some countries: A good doctor–patient relationship can improve adherence to treatment,[73] whereas relying too much on information provided by Internet can potentially become harmful.[74] In the field of andrology and sexual medicine, it is quite common for doctors to visit patients who have "self-prescribed" pro-erectile drugs (i.e., PDE5 inhibitors, such as sildenafil, tadalafil, vardenafil, and avanafil), either buying these treatments online or getting them second-hand from friends or colleagues. This behavior carries several risks, from the possible contamination with other pharmacologically active compounds to the delayed diagnosis of any underlying condition affecting sexual function, for example, male hypogonadism or cardiovascular diseases.[75–77] As such, establishing a reliable doctor–patient relationship is necessary to increase compliance to treatment and prevent the progression of subclinical to overt forms of sexual dysfunctions.[12,13] Cultural differences can exist in these regards, and although they have not been adequately investigated so far, it is fairly likely that Eastern and Western cultures might have different approaches to sexual health, and to PE in particular. Additionally, it should be considered that sexual health can be considered a reliable clinical biomarker, or rather a surrogate marker, of systemic health.[78] Sexual medicine is, in fact, a fundamental part of systems medicine,[78] that is, the interdisciplinary field of research that considers the interaction between the human body and genomic, behavioral and environmental factors, and sexual dysfunctions, sharing the same risk factors as most non-communicable diseases, can be considered the proverbial "canary in the gold mine" for subsequent clinical evaluation.[79] To an extent, it can be hypothesized that sexual medicine might play a role in more complex theories focusing on overall health, such as the developmental origin of health and diseases[80–82] or the "one health" approach.[83] China is among the most active countries in PE research[17]: It can be supposed that such dynamic interest in research is an echo chamber for the concerns of the general population, and that therefore request for medical support for management of PE is higher than elsewhere despite similar prevalence.[84] This can possibly be because of the high importance given to female sexuality in East Asia,[7,13,85] or to the perception of PE as a more thorough health concern.[86] In fact, a well-consolidated tradition of positive attitudes toward sexuality is present in the Chinese culture grounded in Taoism, which is "one of the few [indigenous religions in the world] that has stressed the importance of using sexual techniques for individual benefits".[87] For example, the notorious Chinese intellectual Zhang Jingsheng (1888–1970) theorized the "Third Kind of Water" (*disanzhong shui*) theorizing the pursuit of sexual pleasure.[88] Moreover, it is a characteristic of the TCM and of the Chinese doctors in general

to discuss problems with the patients with a closer attention to their needs and expectations than in a typical hasty urological visit in Western countries, usually more concentrated on the symptom (in this case the PE) than on the patient (in this case the couple). Unlike Western medicine, TCM requires a dialectical treatment based on the overall condition of the patient's body. Depending on each person's condition, there may even be cases where the principles of medication are completely opposite. This depends to a large extent on the doctor's judgment of the symptoms, which so far cannot be fully standardized and objectified, and is one of the major reasons why it is difficult to conduct clinical research in TCM. Some symptoms are more frequently associated with particular "phenotypes" (e.g., Yin deficiency, kidney Qi deficiency, damp-heat syndrome) and can be treated by administering different herbal preparations (e.g., Cinnamomi Cortex, Rehmanniae Radix, Common Macrocarpium Fruit, etc.) following careful evaluation by a TCM expert clinician. Formulas are often prepared with different ingredients and can be taken in different shapes, such as teas, pills, and tinctures. Additionally, other non-herbal treatments are available, such as acupuncture and moxibustion, which overall complement the wide spectrum of disease that can be treated by TCM. Finally, another reason for the success of the Chinese approach to PE with respect to that common in the Western countries could be found in the particular eclectic attitude to associate different treatments, even from different cultures.[89] This therapeutical strategy could be particular successful in a multifaceted and complex symptom, such as PE.[90] Clearly, such claims deserve further confirmation in future studies addressing the preferences for PE patients toward different treatments.

This study is, to our best knowledge, one of the largest ones to investigate PE in a real-life sample of patients attending different Centers with a solid knowledge of PE research. However, it also has several limitations. The study was carried out in a single Country (People's Republic of China), and as such whether these results can be generalized to a broader audience is an open question. Clinical data of patients attending these centers were only partially available, and as such addressing the presence of underlying comorbidities was not feasible. The dosage of Dapoxetine (30 or 60 mg) was not thoroughly collected, therefore adding a possible source of bias.

5 | CONCLUSIONS

PE is a complex sexual dysfunction with a multifactorial pathogenesis, which is largely underreported, under-investigated, and undertreated. Several therapies are available, including drug treatments whose efficacy has been disputed in previous research. In this real-life study on a large population of patients attending different Centers in China, 20.9% came for a primary complaint of PE. Patients were treated according to clinicians' practice and judgment: Overall, most patients received treatment with Dapoxetine, either alone or in combination, and this treatment was likewise the most reliable one, according to the fewer requests for treatment switch. Although results cannot be generalized, this study highlights how both efficacy and reliability of Dapoxetine, a Waterloo in the Western, European, and Flemish opinions and a success under the shadow of the Great Wall, is much higher than perceived by Western doctors who may find in the real-life data presented here some reasons to modify their attitudes.

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CONFLICTS OF INTEREST STATEMENT

EAJ is or has been a speaker and/or paid consultant for Bayer, Ibsa, Lundbeck, Menarini, Merk-Serono, Otsuka, Pfizer, Shionogi, and Viatris. AS has been a paid consultant for Menarini. All others declare not to have any conflict of interest for the present manuscript.

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SUPPORTING INFORMATION

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

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REVIEW ARTICLE

Effect of oestrogen modulation on semen parameters in men with secondary hypogonadism: Systematic review and meta-analysis

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Abstract

Background: Selective oestrogen receptor modulators and aromatase inhibitors stimulate endogenous gonadotrophins and testosterone in men with hypogonadism. There are no systematic reviews/meta-analyses assessing the effects of selective oestrogen receptor modulators/aromatase inhibitors on semen parameters in men with secondary hypogonadism.

Objectives: To assess the effect of monotherapy or a combination of selective oestrogen receptor modulators/aromatase inhibitors on sperm parameters and/or fertility in men with secondary hypogonadism.

Materials and methods: A systematic search was conducted in PubMed, MEDLINE, Cochrane Library and ClinicalTrials.gov. Study selection and data extraction were performed by two reviewers independently. Randomised controlled trials and non-randomised studies of interventions reporting effects of selective oestrogen receptor modulators and/or aromatase inhibitors on semen parameters or fertility in men with low testosterone with low/normal gonadotrophins were selected. The risk of bias was assessed using ROB-2 and ROBINS-I tools. The results of randomised controlled trials were summarised using vote counting while summarising effect estimates where available. Non-randomised studies of intervention meta-analysis were conducted using the random-effect model. The certainty of evidence was assessed using GRADE.

Results: Five non-randomised studies of interventions ($n = 105$) of selective oestrogen receptor modulators showed an increase in sperm concentration (pooled mean difference 6.64 million/mL; 95% confidence interval 1.54, 11.74, $I^2 = 0\%$) and three non-randomised studies of interventions ($n = 83$) of selective oestrogen receptor modulators showed an increase in total motile sperm count (pooled mean difference 10.52; 95% confidence interval 1.46–19.59, $I^2 = 0\%$), with very low certainty of evidence. The mean body mass index of participants was >30 kg/m^2. Four randomised controlled

trials ($n = 591$) comparing selective oestrogen receptor modulators to placebo showed a heterogeneous effect on sperm concentration. Three included men with overweight or obesity. The results were of very low certainty of evidence. Limited pregnancy or live birth data were available. No studies comparing aromatase inhibitors with placebo or testosterone were found.

Discussion and conclusion: Current studies are of limited size and quality but suggest that selective oestrogen receptor modulators may improve semen parameters in those patients, particularly when associated with obesity.

KEYWORDS
aromatase inhibitors, fertility, hypogonadotropic hypogonadism, secondary hypogonadism, selective oestrogen receptor modulators, spermatogenesis

1 | INTRODUCTION

Reduced semen quality (male infertility) is one of the most common reasons a couple cannot conceive within 12 months of regular unprotected intercourse. Male infertility may result from secondary hypogonadism, in which gonadotropin levels are low or inappropriately normal. Secondary hypogonadism may result from organic (irreversible) or functional (potentially reversible) hypothalamic–pituitary factors.[1–4] Accordingly, unlike primary hypogonadism, gonadotrophin therapy (GnT) has been proven to restore fertility as well as normal testosterone levels in men with secondary hypogonadism.[5] Unfortunately, GnT is quite expensive and therefore unaffordable in most healthcare systems worldwide.

Selective oestrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) can increase endogenous gonadotrophin-releasing hormone (GnRH), gonadotrophin and testosterone secretion in men by reducing oestrogenic negative feedback on the hypothalamic–pituitary axis even regardless of a man's age.[6–10] Due to their putative mechanism of action, SERMs/AIs require the presence of an intact hypothalamic–pituitary–testis axis and they should be considered unsuitable in patients with irreversible damage at the central level or in those with primary hypogonadism.[1–4] These agents can potentially improve semen quality as well as the likelihood of paternity in men with idiopathic infertility.[11–13] These agents have also been shown to be beneficial in improving testosterone deficiency in men with secondary hypogonadism, mostly data limited to men with dysmetabolic conditions with functional hypogonadism such as obesity, metabolic syndrome and functional hypogonadism.[8,14,15] Nevertheless, it remains unresolved whether SERMs/AIs might have utility in men with infertility associated with milder cases of secondary hypogonadism, or cases of functional, secondary hypogonadism unresponsive to lifestyle intervention. In addition, these drugs are not approved for the treatment of male hypogonadism and their use must be considered an 'off-label' approach. Finally, SERM agonistic effect on venous vessels could facilitate the development of venous thromboembolic diseases in predisposed men.[16]

If SERMs/AIs could be used to improve semen quality in a subset of men with secondary hypogonadism-related infertility, this would enable many affected couples to access fertility treatment, otherwise unaffordable because of the high costs of GnT. There currently exists no consensus on the effectiveness of SERMs/AIs to treat men with secondary hypogonadism-related infertility. We conducted a systematic review and meta-analysis investigating the effect of monotherapy or a combination of SERMs/AIs on sperm parameters and/or fertility in men with secondary hypogonadism, and to identify predictors of successful treatment if observed.

2 | MATERIALS AND METHODS

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines in conducting this systematic review. The study was registered in PROSPERO under the registration number CRD42022306535.

2.1 | Search strategy

We performed an electronic search in the Cochrane Library, MEDLINE and PubMed in January 2022. We searched using the search strategy ('hypogonadotropic hypogonadism' OR 'secondary hypogonadism') AND ('selective estrogen receptor modulator' OR SERM OR clomiphene OR tamoxifen OR enclomiphene OR aromatase inhibitor OR anastrozole OR letrozole) in PubMed and MEDLINE from inception to 11 January 2022 without additional filters. All search results were exported into EndNote, and duplicates were removed before screening. The search was updated on 11 November 2022.

To identify additional papers, we performed citation searching by manually screening references of selected articles. We performed an electronic search in ClinicalTrials.gov for registered studies of potential relevance, but without publications. We contacted the principal investigator for data; studies were excluded if the results were not available or there was no response from the investigators. The detailed search strategy is available in Supporting Information S1.

2.2 | Study selection

Titles and abstracts of all records were screened independently by two reviewers (N. L. de S. and H. D.) to identify publications eligible for full-text review. The same reviewers screened selected full-text articles independently to identify eligible studies to include in the review. When there were discrepancies in selected full-text articles, two reviewers discussed and reached a consensus. Conflicts were resolved by a third reviewer (C. J.).

All randomised-controlled trials (RCTs) and non-randomised studies of intervention (NRSIs; prospective or retrospective) reporting the effect of SERMs/AI as monotherapy or in combination on semen parameters in men with secondary hypogonadism of any aetiology (defined as hypogonadism with low or inappropriately normal gonadotrophins)[1] irrespective of fertility history were included in the analysis. We included any RCT reporting semen parameters (sperm concentration/motility/total motile sperm count/morphology/volume) compared to placebo or other treatment modalities for secondary hypogonadism. We also included NRSIs reporting semen parameters before and after the intervention with SERMs and/or AIs. Additionally, our inclusion criteria included RCTs and NRSIs reporting conception or live birth. We searched for full-text original articles published in English. We excluded case reports.

When available data in the full-text article were inadequate to define eligibility, corresponding authors were contacted for clarification. If there was no response, the studies were excluded.

2.3 | Data extraction

Two reviewers (N. L. de S. and H. D.) independently extracted data from all selected full-text articles on study details (year of publication, country), design, participant characteristics (age at commencement, cause of secondary hypogonadism, body mass index [BMI], co-morbidities, hormone profile, baseline semen parameters), intervention (agent, dose, duration) and control, outcomes, data analysis and any additional concerns. Funding details and conflicts of interest were assessed to determine if there was a notable concern.

Primary outcomes were the effects of SERMs/AIs on semen parameters either in RCTs or NRSIs. Secondary outcomes included clinical pregnancy and live birth rates. We assessed serious adverse effects as safety outcomes. The data extraction form is available in Supporting Information S2. We have not analysed outcomes related to reproductive hormone levels because they have been addressed in previous systematic reviews[8,14] and because it is beyond the review's primary focus.

2.4 | Risk of bias analysis and assessment of the quality of evidence

The risk of bias (ROB) in RCTs was assessed using a revised version of the Cochrane risk of bias (ROB2) tool.[17] When assessing 'the bias because of deviations from intended interventions', we were

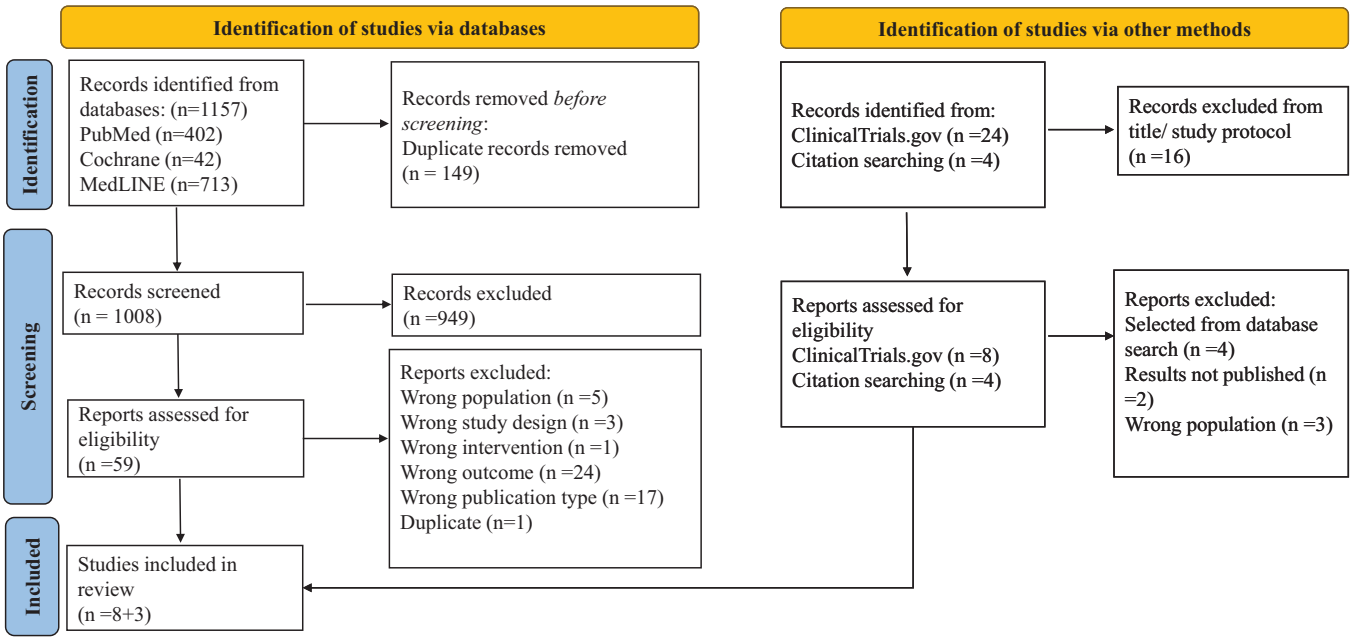

FIGURE 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram for the study selection. *Source*: Page MJ, McKenzie JE, Bossuyt PM, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ*. 2021;372:71. https://doi.org/10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/. Database search yielded 1157 studies, which was reduced to 1008 after removing duplicates. Fifty-nine studies were selected to review full-text articles. Eight studies from the database search were included in the review. Three additional studies were included through citation searching and ClinicalTrials.gov.

interested in the effect of the assignment to the intervention at baseline (intention-to-treat effect) rather than the effect of adhering to the intervention (per-protocol effect). We reviewed the published articles and records from Clinicaltrials.gov to obtain information on reporting bias.

The ROBINS-I tool was used to assess the ROB in NRSIs.[18] Potential confounders considered were as follows: severity and cause of hypogonadism; baseline sperm concentration; previous testosterone replacement therapy (TRT) stopped at the time of intervention; other interventions that could have improved the underlying secondary hypogonadism.

The quality of evidence was assessed according to the 'GRADE' approach using GRADEproGDT software. The certainty of evidence was graded based on five main considerations: ROB, consistency of effect, imprecision, indirectness and publication bias.[19]

2.5 | Data analysis

The planned meta-analysis of effect estimates from the available studies could not be performed for RCTs because outcome measures differed across studies and there was incomplete reporting of effect estimates. Therefore, we adapted the vote counting method to determine the direction of effect.[20] Vote counting was performed without considering the statistical significance as per the standard method in data synthesis. The benefit or harm of the intervention was defined based on the direction of the effect.

Available NRSIs were pre- and post-intervention studies with critical ROB. The statistical heterogeneity was calculated using I^2 statistics. Even when low heterogeneity was detected, a random-effect model was applied because the validity of tests of heterogeneity can be limited with a small number of component studies.

While acknowledging the limitation in synthesising data from NRSIs with a critical ROB, we decided to conduct a meta-analysis because available NRSIs and their data were directly relevant in answering the research question of the review. Meta-analysis was performed in Comprehensive Meta-Analysis V4, with single-group pre- and post-intervention data calculation and random effect model.

3 | RESULTS

Database searches yielded 1157 records. After removing duplicates, 1008 records remained for screening. After excluding 949 abstracts, 59 full-text articles were screened for eligibility. After excluding 51 studies, eight studies were selected for the systematic review. The updated search did not yield any additional eligible studies. One additional study was identified through citation searching. Two studies identified through ClinicalTrials.gov were included using the results published in the database. Attempts to obtain published full-text articles of these studies by contacting the investigators were unsuccessful. Other studies were excluded because they were either already included through database search (n = 4) or had no published results and could not be accessed even after contacting the principal investigators (n = 2). Details of studies excluded after full-text review are summarised in Supporting Information S3. PRISMA flow diagram summarising the search strategy is given in Figure 1.

3.1 | Randomised controlled studies

Six RCTs including 747 participants were eligible for inclusion: among included studies, two compared the effects of SERMs versus placebo versus TRT (n = 377),[21,22] or just with placebo (n = 332).[23,24] In addition, one study investigated the role of SERMs compared to TRT (n = 12),[25] or AIs (n = 26).[26] The characteristics of the included studies are summarised in Table 1. All studies recruited participants with mean serum testosterone <300 ng/dL and mean serum luteinising hormone (LH) within the reference range; however, the minimum levels of serum LH were reported only in one study.[26] Three of the six studies were restricted to men with overweight/obesity[22–24]; the aetiology of secondary hypogonadism was not specified in the remaining studies.[21,25,26] However, the mean BMI of participants in two other studies was >30 kg/m^2,[21,26] and the mean weight was 105 kg in the other study.[25] Normospermia was an inclusion criterion for two studies,[23,24] and another study excluded men with a sperm concentration <1 million/mL.[26] All participants had previously received TRT in one study,[25] whereas in another study, 23% of the participants had received TRT.[22] TRT within the last 3[26] or 6 months[23,24] was an exclusion criterion in three studies. Data on TRT were not available in one.[21] Semen analyses were performed 3–6 months after starting treatment in all included studies. All included studies, except one, had a high ROB, as summarised in Figure 2.

The main RCT results related to semen outcome are summarised in Table 2. Outcomes and reporting differed across studies, so it was not possible to compute summary statistics. Therefore, we adapted the vote counting method to adjudicate the overall direction of effect. Table 5 summarises findings, including the assessment of the certainty of evidence using the GRADE tool. Four RCTs with 591 participants compared SERMs against placebo. Two published studies showed evidence towards the benefit; however, two unpublished studies suggested that SERMs were inferior to placebo (possible overlap of patients not excluded). Overall, the certainty of evidence was very low because of the high ROB and imprecision because of the small total sample size.

Three RCTs comparing SERMs against testosterone gel (n = 275) showed higher sperm concentration with SERMs compared to testosterone gel in men with secondary hypogonadism. Further statistical analyses were not carried out because exogenous testosterone is already known to suppress spermatogenesis.

The study comparing anastrozole to clomiphene suggested better sperm concentration outcomes with clomiphene (mean difference −15.00 [−25.40, −4.60]).[26]

Only the study by Helo et al. mentioned fertility outcomes reporting the conception of one and two partners of men on anastrozole and

TABLE 1 Characteristics of selected randomised controlled studies.

			Participant baseline characteristics													
Study (country)	Inclusion criteria	Exclusion criteria	Group	Number (number with semen parameters)	Aetiology	Mean age (SD) [years]	Mean BMI (SD) (kg/m2)	Mean testosterone (SD) [ng/dl]	Mean FSH (SD) [IU/L]	Mean LH (SD) [IU/L]	Mean sperm-concentration (SD) [million/mL]	Intervention	Control	Duration	Outcome	Funding, conflict of interest	Notes
Selective oestrogen receptor modulators versus placebo versus testosterone gel																	
Wiehle, 2014 (USA)	Diagnosis of secondary hypogonadism, morning testosterone <250 ng/dL	NR	Enclomiphene 12.5 mg	27 (16)	NR	49.7 (11.6)	32.6 (5.17)	217.2 (58.8)	6.4 (4.2)	4 (1.8)	3/16[a]	Enclomiphene 12.5, 25 mg	1% testosterone gel, placebo	6 months (semen analysis at 3 months)	Sperm concentration < 15 million/mL (*p* values for total sperm count, volume, motility)	Repros therapeutics, notable concerns	No details on previous testosterone use
			Enclomiphene 25 mg	33 (19)		49.2 (10.9)	31.7 (4.9)	209.8 (55.4)	9.4 (10.9)	5.3 (4)	1/19[a]						
			Testosterone gel	33 (19)		52 (10.6)	33.1 (5.87)	210 (54)	6 (2.9)	3.9 (1.8)	3/19[a]						
			Placebo	28 (13)		51.6 (11.7)	30.9 (4.17)	213.7 (74.9)	6.1 (4.8)	3.9 (2.6)	1/13[a]						
Kim, 2016 (USA)	Overweight men, aged 18–60 years, with secondary hypogonadism (morning serum TT of <300 ng/dL and LH <9.4 IU/L)	NR	Enclomiphene	41 + 44	Possible overweight/obesity	49.1 (7.4), 47.3 (8.8)	33.1 (4.4), 33.8 (4.6)	203.3 (52.4), 212.9 (48)	4.9–6.1	3.3–3.8	98.3 (87.2), 79 (55.2)	Enclomiphene 12.5 mg/day increased to 25 mg/day if TT did not increase >450 ng/dL after 4 weeks	1.62% testosterone gel adjusted according to the manufacturer, placebo	16 weeks	Sperm concentration percentage change from the baseline, adverse effects	Repros therapeutics, notable concerns	Combination of two studies, previous testosterone use 21–23%. Study complete by 217.
			Testosterone gel	43 + 42		47.4 (7.2), 45 (8.2)	34 (4.4), 33.1 (4.6)	208.6 (54), 229.8 (44)	4.9–6.1	3.3–3.8	78.9 (68.5), 75.1 (45.8)						
			Placebo	45 + 41		47.2 (9), 47.5 (8.9)	32.6 (4.3), 33.5 (4.4)	200 (43.1), 206 (48.2)	4.9–6.1	3.3–3.8	95 (90.8), 80.5 (62.3)						

(Continues)

TABLE 1 (Continued)

			Participant baseline characteristics													
Study (country)	Inclusion criteria	Exclusion criteria	Group	Number (number with semen parameters)	Aetiology	Mean age (SD) [years]	Mean BMI (SD) (kg/m2)	Mean testosterone (SD) [ng/dl]	Mean FSH (SD) [IU/L]	Mean LH (SD) [IU/L]	Mean sperm-concentration (SD) [million/mL]	Intervention	Control	Duration	Outcome	Funding, conflict of interest	Notes
Selective oestrogen receptor modulators versus testosterone gel																	
Kaminetsky, 2013 (USA)	Secondary hypogonadism, TT <300 mg/dl	Kallman syndrome, primary hypogonadism, testicular failure, significant medical illness. Unwilling to stop testosterone or other hormones for ?30 days (not clear)	Enclomiphene Testosterone gel	7 (6) 5	NR	46 years (range 41–59 years)	Mean weight 105 kg	165 (66)	NR	NR	NR	Enclomiphene 25 mg daily	Testosterone gel (dose NR)	6 months	Post-intervention sperm concentration difference between groups	Repros therapeutics, notable concerns	All used topical testosterone for at least 6 months, stopped for 7—14 days before baseline assessment

(Continues)

TABLE 1 (Continued)

Study (country)	Inclusion criteria	Exclusion criteria	Participant baseline characteristics: Group	Number (number with semen parameters)	Aetiology	Mean age (SD) [years]	Mean BMI (SD) (kg/m2)	Mean testosterone (SD) [ng/dl]	Mean FSH (SD) [IU/L]	Mean LH (SD) [IU/L]	Mean sperm-concentration (SD) [million/mL]	Intervention	Control	Duration	Outcome	Funding, conflict of interest	Notes
Selective oestrogen receptor modulators versus placebo																	
NCT01739595 (USA)	Overweight/obese men with secondary hypogonadism (TT <300 ng/dL, LH <9.4 mIU/L) and sperm concentration >15 million/mL	Prior use of testosterone treatments within the last 6 months, irreversibly infertile or compromised fertility (cryptorchism, Kallman syndrome, primary hypogonadism, vasectomy, or tumours of the pituitary)	Enclomiphene 12.5 mg	112 (99)	Possible overweight/obesity	44.6 (9.6)	NR	NR	NR	NR	NR	Enclomiphene 12.5 mg daily, dose increase to 25 if TT not increased to 300 in 6 weeks	Placebo	12 weeks, extended to 18 weeks if dose increased	Proportion of subjects with a 50% or greater decrease in sperm concentration from baseline after 12 weeks of treatment, adverse events	Repros therapeutics, notable concerns	Published article not available
			Enclomiphene 25 mg	22 (21)		45.8 (8.6)											
			Placebo	47 (45)		43.6 (10.5)											
NCT01532414 (USA)	Same as above	Same as above	Enclomiphene 12.5 mg	92 (85)	Possible overweight/obesity	47.2 (9.6)	NR	NR	NR	NR	NR	Same as above	Same as above	Same as above	Same as above	Repros therapeutics, notable concerns	Protocol similar to the study described above. Published article not available. Possible overlapping population.
			Enclomiphene 25 mg	21 (19)		43.6 (10.1)											
			Placebo	38 (35)		47.8 (9.5)											

(Continues)

TABLE 1 (Continued)

Study (country)	Inclusion criteria	Exclusion criteria	Participant baseline characteristics: Group	Number (number with semen parameters)	Aetiology	Mean age (SD) [years]	Mean BMI (SD) (kg/m2)	Mean testosterone (SD) [ng/dl]	Mean FSH (SD) [IU/L]	Mean LH (SD) [IU/L]	Mean sperm-concentration (SD) [million/mL]	Intervention	Control	Duration	Outcome	Funding, conflict of interest	Notes
Selective oestrogen receptor modulator versus aromatase inhibitors																	
Helo, 2015 (USA)	Men 18–50 years, infertility, testosterone 150–350 ng/dL (average of two consecutive samples), LH 1.2–8.6 mIU/L	Sperm concentration 1 million/mL, BMI >40, haematocrit <36 or >52, previous oral or inhaled steroid use, opioid use. Known pituitary or testicular disease	Anastrozole	13 (12)	NR	35 (6.5)	33 (9.8)	248 (18)	9.9 (1.9)	4.8 (0.48)	32.7 (12)	Anastrozole 1 mg daily	Clomiphene 25 mg daily	12 weeks	Semen volume, concentration and motility before and after intervention, fertility, adverse events	Not reported, no notable concerns	Six participants with secondary subfertility
			Clomiphene	13 (12)		33 (3.9)	32 (7.5)	253 (17)	4.2 (1.7)	3.9 (0.45)	32 (12)						

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinising hormone; NR, not reported; TT, total testosterone.
[a]Number of men with sperm concentration <15 million/mL out of the participants with semen parameters.

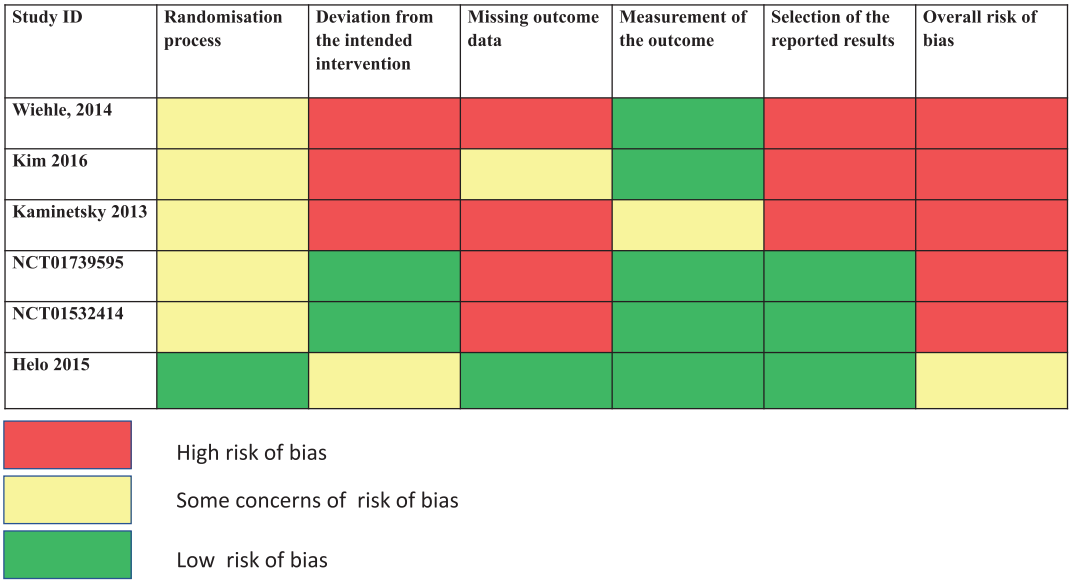

FIGURE 2 Summary of risk of bias assessment of randomised controlled studies. Based on the overall assessment, the first five studies a have high risk of bias and the other study has some concerns on the risk of bias.

clomiphene, respectively, conceiving during the study period. However, its intervention period was only 12 weeks.

Two studies did not report safety data,[21,25] and one study reported that there were no serious adverse effects in participants.[23] One study reported one fatal stroke in a participant on enclomiphene, although causality was not established.[22] One serious adverse event with enclomiphene was reported in another study; however, the adverse effect was not specified.[24] In the remaining study, anastrozole treatment was associated with skin rash in one patient, and deep vein thrombosis in another patient (who had a previous episode of deep vein thrombosis).[26]

3.2 | Non-randomised studies

We identified five NRSIs including 105 participants reporting semen parameters with SERMs.[27–31] Four studies were uncontrolled retrospective observational, while the study by Lima et al. was uncontrolled prospective. Although details of patients treated with human chorionic gonadotropin (hCG) are also reported in two studies[29,31] the authors did not consider this a comparator group. There were no eligible studies reporting data on AIs. Characteristics of included studies are summarised in Table 3.

All studies included men with infertility. Whitten et al. included men with idiopathic secondary hypogonadism ($n = 3$) or panhypopituitarism ($n = 1$).[29] The aetiology of secondary hypogonadism was not reported in the other four studies. The mean/median BMI of participants in all four studies reporting BMI was >28 kg/m^2.[27,28,30,31] In two studies, previous TRT was an exclusion criterion,[27,28] whereas in one study, previous TRT was not reported,[30] and in the other, one patient had been on TRT.[29] In one study, patients who had been on TRT were given a washout period of 8 weeks.[31] Except in the study by Patel et al.,[28] mean/median sperm concentration at baseline was low or close to the lower limit of the normal. The approximate follow-up duration was 3 months for all the studies.

All five studies had a critical ROB because of the risk of confounding because all of them had a single pre-intervention outcome measurement and a single post-intervention outcome measurement. Other domains of bias were variable between the studies (Figure 3). The publication bias could not be assessed because less than 10 studies were included in the quantitative analysis. The main results of the studies in relation to semen parameters and the overall ROB judgment are summarised in Table 4.

All five studies ($n = 105$) reported changes in sperm concentration. Mean differences were suggestive of benefit (6.64 [95% confidence interval, CI 1.54, 11.74], $I^2 = 0\%$) (Table 5). Semen volume and total motile sperm count were reported in three studies with a total population of 83. The overall mean differences were −0.03 (95% CI −0.44, 0.38), $I^2 = 0\%$ and 10.52 (95% CI 1.46–19.59), $I^2 = 0\%$, respectively. The certainty of evidence was very low because of the critical ROB in these studies.

Surbone et al. reported fertility data, with three female partners becoming pregnant during the study period out of 57. In the study by Whitten et al., female partners of two study participants (out of four) conceived. The other three studies did not report fertility data.

Safety data were not reported in three studies.[27,29,31] Surbone et al. reported that there were no significant adverse effects.[30] In the other study, three patients had a paradoxical drop in total testosterone and switched to hCG.[28] One patient had fatigue and mood swings.

Overall, little AI data were found; specifically, neither RCTs comparing AI with placebo nor NRSIs of AI were identified. There were inadequate data on fertility because only three studies reported fertility outcomes, and all were of short follow-up. It was not possible to analyse determinants of response to SERM or AI because of the limited

TABLE 2 Summary of results from randomised controlled trials.

Study	Outcome	Overall ROB judgment	Available data	Summary statistics	Vote counting (without considering statistical significance)
Selective oestrogen receptor modulators versus placebo					
Wiehle, 2014	Sperm concentration <15 million/mL	High risk	Intervention 2/35 Control 2/13	OR 0.33 (95% CI 0.04–2.66)	Benefit
	Sperm concentration (million/mL)		EC 12.5 mg: $p = 0.95$ EC 25 mg: $p = 0.78$		NR
	Sperm motility		NS		NR
	Total sperm count		EC 12.5 mg: $p = 0.45$ EC 25 mg: $p = 0.77$		NR
Kim, 2016	Percentage change of the sperm concentration from the baseline	High risk	Intervention 11.7 (80.3), 15.2 (55.8) Control 4.1 (57.2), 7.6 (89.6)	MD 7.60 (−22.12, 37.32), MD 7.60 (−24.40, 39.60)	Benefit
NCT01739595	Proportion of subjects with a 50% or greater decrease in sperm concentration	High risk	Intervention 19/134 Control 2/47	OR 3.72 (0.83, 16.61)	Harm
NCT01532414 (? Overlapping population)	Proportion of subjects with a 50% or greater decrease in sperm concentration	High risk	Intervention 16/113 Control 1/38	OR 6.10 (0.78, 47.67)	Harm
Selective oestrogen receptor modulator versus aromatase inhibitors					
Helo, 2015	Sperm concentration (million/mL)	Some concerns	AZ 26 (13) CC 41 (13)	MD −15.00 (−25.40, −4.60)	CC benefit
	Semen volume (mL)		AZ 3.15 (0.51) CC 2.2 (0.54)	MD 0.95 (0.53, 1.37)	AZ benefit
	Sperm motility (%)		AZ 35 (5.4) CC 41 (5.4)	MD −6.00 (−10.32, −1.68)	CC benefit
	Fertility		AZ 1/12 CC 2/12	Inadequate data	

Abbreviations: AZ, anastrozole; CC, clomiphene citrate; CI, confidence interval; EC, enclomiphene citrate; MD, mean difference; NR, not reported; NS, not significant; OR, odds ratio; ROB, risk of bias.

number of studies, low total numbers of participants, and unavailability of disaggregated data from subgroups of interest. Safety data were not reported in many studies. Few major adverse events were observed, but causality could not be attributed to SERM/AI treatment.

4 | DISCUSSION

Our systematic review was conducted to appraise for the first time whether SERMs/AIs might improve semen quality in men with secondary hypogonadism. Non-comparative cohort studies suggest that SERM treatment is associated with improved sperm concentration and total motile sperm count in men with low testosterone with low/normal gonadotrophins and infertility; however, RCTs show heterogeneous effects of SERMs compared with placebo. Although studies comparing SERMs to testosterone gel were also summarised in our review, we have not pursued to draw any conclusions using their data because of known suppressive effects of exogenous testosterone on spermatogenesis.

Although we aimed to review studies including men with secondary hypogonadism of diverse aetiology, there was an overrepresentation of men with low testosterone with low/normal gonadotrophins in the background of obesity. From a pathophysiological point of view, a greater response is expected with SERMs in men with obesity-related low testosterone because oestradiol produced through increased aromatase activity from adipose tissue is thought to mediate suppression of the hypothalamus and pituitary.[32] There remains controversy on whether this can be strictly defined as a type of hypogonadism because their hypothalamic–pituitary–gonadal axis has no pathology and this is considered an adaptive response to another illness.[33–35] In contrast, if there are symptoms of hypogonadism and low testosterone with low/normal gonadotrophins, making a diagnosis of secondary

TABLE 3 Characteristics of selected non-randomised studies of intervention with selective oestrogen receptor modulators.

		Participants			Participant characteristics									
Study (country)	Design	Inclusion	Exclusion	Number of participants	Aetiology	Mean age (SD) (years)	Mean BMI (SD) (kg/m^2)	Mean TT (SD) (ng/dL)	Mean FSH (SD) (IU/L)	Mean LH (SD) (IU/L)	Mean sperm concentration (SD) (million/mL)	Intervention, duration	Outcome of interest	Funding, conflicts of interest	Notes
Lima, 2021 (USA)	Prospective	Men with primary infertility, morning TT <300 ng/dL on two occasions, abnormal semen parameters in two occasions (TMSC <9)	IHH being on testosterone/hCG/CC at the time of baseline assessment (stopped for 8 weeks)	16	NR	40.9 (4.3)	34.7 (7.9)	190.7 (63.7)	6.1 (4.6)	4.5 (2.6)	3.1 (3.8)	CC 25 mg every other day, 3 months	Sperm concentration, TMSC, semen volume and sperm motility before and after intervention	NR, no notable concerns	Additional group received CC + hCG Primary objective was to assess 17-OHP as a predictor of success
Sharma, 2019 (USA)	Retrospective	Hypogona (TT <300 ng/d and/or infertility	Previous treatment with testosterone Opted varicocele repair	57	NR	Median[a] 35, IQR 31–40	Median[a] 28, IQR 26–32	Median[a] 242, IQR 191–317	Median[a] 3, IQR 2–6	Median[a] 4, IQR 2–6	15 (27)	CC 25 mg/day titrated to 50 mg/day (if TT <300 mg/dL after 4 weeks), median 2.8 months (IQR 1.8–4.4)	Semen volume, sperm concentration, motile percentage, TMC before and after intervention	NR, notable concerns	Hypogonadotropic hypogonadism not mentioned as an inclusion criteria, but participant characteristics suggestive

(Continues)

TABLE 3 (Continued)

		Participants			Participant characteristics									
Study (country)	Design	Inclusion	Exclusion	Number of participants	Aetiology	Mean age (SD) (years)	Mean BMI (SD) (kg/m^2)	Mean TT (SD) (ng/dL)	Mean FSH (SD) (IU/L)	Mean LH (SD) (IU/L)	Mean sperm concentration (SD) (million/mL)	Intervention, duration	Outcome of interest	Funding, conflicts of interest	Notes
Surbone, 2019 (Switzerland)	Retrospective	Men with infertility, low plasma TT, low or normal FSH and LH, sperm concentration >0.1 million/mL	Secondary causes of hypogonadism like Kallman, cranial surgery, tumour, radiotherapy	18	NR	36 (6.8)	28.2 (3.9)	8.5 (2.3)	4.2 (2.3)	4.8 (3.3)	14.7 (18.2)	CC 50 mg every 48 h, at least 3 months	Sperm concentration, progressive motility before and after, spontaneous pregnancy, adverse effects	NR, no notable concerns	
Patel, 2015 (USA)	Retrospective	Men aged 18–55 years with hypogonadism and/or subfertility TT <300 ng/d and/or bioavailable testosterone <155 ng/d	Previous testosterone/tamoxife hCG/anastrozole use	47 (baseline sperm parameters only in 27, 3 month data only in 10)	NR	34.5 (NR)	30.2 (NR)	246.8 (97.6)	5.8 (4.8)	9.6 (10.7)	50.7 (71.6)	CC 50 mg every other day, increased to daily if testosterone rise <50 ng/dL in 2 weeks, median follow-up 3 months (IQR 2.3–3.7)	Semen volume, sperm concentration, progressive motile percentage, total motile count, percentage normal morphology before and after, adverse effects	American medical systems, no notable concerns	

(Continues)

TABLE 3 (Continued)

Study (country)	Design	Participants		Number of participants	Participant characteristics						Intervention, duration	Outcome of interest	Funding, conflicts of interest	Notes
		Inclusion	Exclusion		Aetiology	Mean age (SD) (years)	Mean BMI (SD) (kg/m^2)	Mean TT (SD) (ng/dL)	Mean FSH (SD) (IU/L)	Mean LH (SD) (IU/L)	Mean sperm concentration (SD) (million/mL)				
Whitten, 2006 (England)	Retrospective	Men with hypogonadotropic hypogonadism	NR	4 (six other patients treated with hCG)	IHH-3, panhypopituitarism-1	31, 30, 28, 33	NR	194, 117, 41, 1477	1, 0.8, 0.9, 0.6	NR	0.6, 0, 0, 0	CC 50 mg 3 times a week for 3 months	Sperm concentration before and after, fertility	NR, no notable concern	Patient with panhypopituitarism was on testosterone replacement. One additional patient (IHH) had taken clomiphene and remained azoospermic before the study

Abbreviations: 17–OHP, 17–hydroxyprogesterone; BMI, body mass index; CC, clomiphene citrate; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; IHH, idiopathic hypogonadotropic hypogonadism; IQR, interquartile range; LH, luteinising hormone; NR, not reported; TMC, total motile count; TMSC, total motile sperm count; TT, total testosterone.

[a] Mixed with 20 eugonadal men.

FIGURE 3 Summary of risk of bias assessment of non-randomised studies of intervention on selective oestrogen receptor modulators. All included studies have a critical risk of bias in the overall assessment with bias of confounding been critical in all the studies.

TABLE 4 Summary of results from non-randomised studies of intervention on selective oestrogen receptor modulators.

Study	Outcome	Overall risk of bias judgement	Pre-intervention	Post-intervention	Mean difference (95% confidence interval)	Significance
Lima, 2021	Sperm concentration (million/mL)	Critical	3.1 (3.8)	10.14 (14.27)	7.04 (−0.20, 14.28)	0.006
	Semen volume (mL)		2.7 (1.6)	2.61 (1.31)	−0.09 (−1.1, 0.2)	0.43
	Total motile sperm count (million)		3.75 (6.6)	13.33 (18.45)	9.58 (−0.02, 19.18)	0.03
Sharma, 2019	Sperm concentration (million/mL)	Critical	15 (27)	21 (25)	6 (−3.55, 15.55)	0.22
	Semen volume (mL)		2.4 (1.5)	2.4 (1.2)	0.00 (−0.50, 0.50)	1
	Total motility (%)		31 (21)	35 (26)	−4.00 (−12.68, 4.68)	0.37
	Total motile sperm count (million)		14 (26)	30 (112)	16.00 (−13.85, 45.85)	0.29
Surbone, 2019	Sperm concentration (million/mL)	Critical	14.7 (18.2)	19.3 (16.6)	4.60 (−6.78, 15.98)	0.024
	Progressive motility (%)		20.5 (17.5)	22.2 (18.5)	1.70 (−10.06, 13.46)	0.395

(Continues)

TABLE 4 (Continued)

Study	Outcome	Overall risk of bias judgement	Pre-intervention	Post-intervention	Mean difference (95% confidence interval)	Significance
Patel, 2015	Sperm concentration (million/mL)	Critical	50.7 (71.6)	72.4 (60.1)	21.70 (−24.31, 67.71)	0.009
	Semen volume (mL)		3.3 (1.9)	3.2 (1.3)	−0.10 (−1.18, 0.98)	0.28
	Sperm motility (%)		33.1 (20.5)	38.5 (24.3)	5.40 (−11.53, 22.33)	0.53
	Total motile sperm count (million)		59.7 (110.8)	90.99 (92.9)	31.29 (−39.86, 102.44)	0.09
	Sperm morphology (normal %)		8.6 (17.2)	8.8 (16.8)	0.20 (−12.07, 12.47)	0.45
Whitten, 2006	Sperm concentration (million/mL)	Critical	0.6, 0, 0, 0	10, 33, 163, 0	51.35 (−22.74, 125.44)	0.17

TABLE 5 Summary of findings for effects of selective oestrogen receptor modulators/aromatase inhibitors in men with hypogonadotropic hypogonadism.

Outcome	Number of studies	Number of participants	Summary of results[a]	Certainty of evidence (GRADE)
Selective oestrogen receptor modulators versus placebo				
Sperm concentration	4	591	Benefit: 2 studies Harm: 2 studies (? overlapping population)	Very low[b]
Other sperm parameters	1	88	No benefit	Very low[b]
Selective oestrogen receptor modulators versus aromatase inhibitors				
Sperm concentration	1	26	Clomiphene benefit	Very low[c]
Non-randomised studies of selective oestrogen receptor modulators reporting before and after intervention data				
Sperm concentration	5	105	6.64 (1.54, 11.74), $p = 0.01$	Very low[d]
Semen volume	3	83	−0.03 (−0.44, 0.38), $p = 0.89$	Very low[d]
Total motile sperm count	3	83	10.52 (1.46, 19.59), $p = 0.02$	Very low[d]
All studies				
Fertility	3	Inadequate data		
Serious adverse events	6		Only few events	Very low

[a]For randomised-controlled trials (RCTs)—summary according to vote counting; for non-randomised studies of intervention (NRSIs)—summary is pooled mean difference.
[b]Studies with high risk of bias, imprecision because of small sample size.
[c]Some concern in risk of bias, small sample size.
[d]Very serious risk of bias because of critical risk of bias in all non-randomised studies of intervention.

hypogonadism in these men may be recommended.[36] Additionally, hypogonadism associated with obesity or type 2 diabetes is considered an increasingly prevalent cause of male infertility.[37,38] Considering these factors, we included these studies in our review acknowledging the limitation in generalising these data to men with organic secondary hypogonadism and we have identified the broad patient population in our review as having low testosterone with low/normal gonadotrophins.

There is a gap in the evidence between the androgenic and spermatogenic effects of oestrogen modulators among men considered in our review because the evidence on the improvement of testosterone seems to be more robust.[8,14] It is not clear whether this is because of the true inefficacy of these agents to promote spermatogenesis or the lack of high-quality studies. This ambiguity is further intensified by some data that suggest that SERMs enhance Leydig cell function but not Sertoli cell action.[39] Another important factor is the possible

short duration of intervention and follow-up in the studies. The total duration for spermatogenesis and delivery of spermatozoa to ejaculatory ducts is approximately 120 days.[40] Therefore, follow-up of about 3 months is likely to miss the slow response of spermatogenesis even if the Sertoli cells are responding. On the other hand, spermatogenesis is an intricate process dependent on many factors other than hormone regulation.[41] This could limit the spermatogenic response despite the rise of intra-testicular testosterone synthesis. Oestrogen signalling is implicated in the regulation of spermatogenesis,[6] so its blockade might negatively impact semen quality. In keeping with this, paradoxical declines of spermatogenesis have been reported in a minority of men treated with SERMs.[42] However, some studies have suggested that SERMs/AIs may improve semen parameters in men with idiopathic infertility.[9,11,13]

It is important to consider the limitations of the current analysis and its constituent studies. Most studies, particularly RCTs, did not consider fertility history in the inclusion criteria. Additionally, several studies had enrolled normospermic men with normal fertility. Measures to optimise results of semen analysis and assessment of patient adherence to drug treatment during the follow-up were underreported in the available studies. Safety data and pregnancy rates were not reported adequately. The lack of disaggregated data and small numbers limited any subgroup analysis. Due to the heterogeneity in the methods used in reporting outcomes within the included studies, we did not conduct a formal meta-analysis to synthesise available RCT data. Of the available options, we used vote counting because that was the only method that could be used across studies. However, we have summarised effect estimates wherever possible. Vote counting has a few inherent limitations, such as disregarding the magnitude of the effect and differences in the relative sizes of the studies.[20] Ideally, vote counting would be better suited to a larger number of studies than identified by our review. Multiple studies have reported the effects of AIs on semen parameters in men with low testosterone:oestradiol ratio. However, gonadotrophin levels in these studies were not clearly consistent with secondary hypogonadism,[43–45] so these studies did not meet the inclusion criteria of our review.

SERMs have been used off-label in clinical practice for men with secondary hypogonadism, especially when fertility is a concern. According to the findings of our review, it seems that this practice is not supported by high-quality evidence. Therefore, we believe that high-quality studies on men with secondary hypogonadism and abnormal semen parameters should be performed with an adequate follow-up duration to clarify this dilemma. It is also important to define standardised objective outcome measures to determine improvement in semen parameters and assess clinically relevant outcomes such as pregnancy rate in future studies evaluating the role of SERMs and/or AIs in the treatment of male subfertility because of secondary hypogonadism.

5 | CONCLUSIONS

Although low-quality evidence suggests a possible improvement of semen parameters with selective oestrogen receptor modulators in men with low testosterone associated with low/normal gonadotrophins, there is inadequate evidence on its efficacy to improve semen parameters compared to placebo or other fertility options. The available evidence is mostly limited to men with overweight/obesity. There is no evidence on the role of aromatase inhibitors. Data on the effects of selective oestrogen receptor modulators/aromatase inhibitors on fertility in the population of interest are limited. Larger randomised-controlled trials with well-defined inclusion criteria on the aetiology of secondary hypogonadism, cut-off values for testosterone, gonadotrophin and semen parameters and fertility status are required to ascertain the effects of selective oestrogen receptor modulators/aromatase inhibitors on the fertility of these men. Outcomes such as semen parameters and fertility should be assessed with a longer follow-up considering the time taken to normalise spermatogenesis before these agents can be recommended for clinical practice, even for off-label use.

AUTHOR CONTRIBUTIONS

Channa N. Jayasena conceived and Nipun Lakshitha de Silva and Ranga Eshaka Wickramarachchi planned the review. Giovanni Corona and Suks Minhas supervised the methodological aspects of the review. Nipun Lakshitha de Silva and Harsha Dissanayake performed the search, screening and study selection. Nipun Lakshitha de Silva, Harsha Dissanayake and Camila Suarez performed data extraction and analysis. Channa N. Jayasena, Waljit S. Dhillo and Ranjith Ramasamy guided and supervised data extraction and analysis. Nipun Lakshitha de Silva drafted the initial manuscript. All authors revised and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information.

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SUPPORTING INFORMATION

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

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REVIEW ARTICLE

Is telomere length a biomarker of sperm quality? A systematic review and meta-analysis of observational studies

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Abstract

Background: Telomeres are essential for the integrity of chromosome ends during cell division and their involvement in different processes linked to aging has been established. These chromosome components are involved in spermatogenesis and seem to play an important role in fertilization and embryo development. Telomere length is shortened with each cell division. Recently, short sperm telomere length has been proposed as a potential biomarker of male infertility.

Objectives: To conduct a systematic review and meta-analysis of studies exploring the association between spermatozoa and/or leukocyte telomere length with sperm quality parameters and different infertility conditions.

Material and methods: A systematic review and meta-analysis was conducted with studies from Medline-PUBMED and Cochrane Library databases until May 2022. Eligible studies included cohort, cross-sectional and case–control studies, and telomere length in spermatozoa and/or leukocytes cells was defined as the exposure. Semen quality parameters or infertility conditions (e.g., oligozoospermia, asthenozoospermia, teratozoospermia, or other spermatogenic impairment combinations) were defined as the outcomes.

Results: Twenty-three observational studies were included. In the qualitative analysis, high heterogeneity was observed between studies regarding the associations between telomere length and semen parameters in different normozoospermic/fertile and oligozoospermic/infertile populations. In the meta-analysis, spermatozoa and leukocyte telomere length were shorter in infertile individuals than in fertile individuals (mean difference [95% confidence interval]: −1.43 [−1.66 to −1.21], p-value <0.001 and −1.67 [−2.02 to −1.31], p-value <0.001, respectively). Moreover, in terms of sperm telomere length, these differences were also significant between individuals with a normal seminogram and individuals with a low quantity of spermatozoa in the ejaculate (−0.97 [−1.32, −0.61], p-value <0.001).

Conclusion: The current systematic review and meta-analysis suggests the potential role of spermatozoa or leukocyte telomere length as a reliable biomarker of semen

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quality, which may help distinguish between infertility conditions beyond the routine semen analysis.

KEYWORDS
biomarker, leukocyte, spermatozoa, sperm quality, telomere length

1 | INTRODUCTION

Infertility is a disease of the reproductive system leading to the failure to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse, according to the World Health Organization (WHO).[1] Currently, infertility affects 8%–12% of the world's population and over the last years this condition has increased.[2] Globally, of couples affected by infertility, male factors are responsible for 40%–50% of cases.[3] Research on infertility is therefore important and quality of life and mental health of populations may also benefit.[4]

Oxidation and inflammation processes have a recognized influence on sperm quality parameters and consequently on male fertility because of the susceptibility of spermatozoa to oxidative stress (OS).[5] The pathological characteristics of oxidative molecules, such as reactive oxygen species (ROS), can lead to impairments in the male reproductive system resulting from an imbalance between oxidant production and antioxidant capacity. A comparative study showed that seminal ROS concentrations were higher in infertile men than in healthy sperm donors and in infertile men with all abnormal sperm parameters than in infertile men with normal sperm parameters.[6] Therefore, investigating the biological mechanisms implicated in infertility beyond conventional sperm parameter analysis and identifying reliable biomarkers of infertility is of clinical relevance.

Telomeres are repetitive DNA sequences and specialized proteins at the end of the eukaryote chromosome, whose main function is to maintain genome integrity.[7] With each cell division under normal conditions, a small fragment of telomeric DNA is lost, leading to the activation of a DNA damage response that induces replicative senescence, anticipating the onset of age-related diseases.[8] Telomeric structures are susceptible to oxidation processes because of their high guanine content, thus leading to accelerated telomere shortening.[9] Telomere length (TL) is mainly regulated by a reverse transcriptase called telomerase, which adds 5′-TTAGGG-3′ sequences in tandem[10] and whose activity decreases progressively during embryonic differentiation in somatic tissues. However, in male germ cells, this activity is maintained until spermatogenesis, resulting in longer telomeres in spermatozoa than other cell types.[10]

TL has been consolidated as a hallmark of processes linked to aging such as oxidation, inflammation, epigenetic regulation, or mitochondrial dysfunction, among others.[11] Furthermore, in recent decades, telomere function has been investigated as a potential biomarker of infertility because of its apparently important role in fertilization and embryo development.[12] A recent systematic review and meta-analysis of observational studies evaluating sperm TL as a biomarker of embryonic development revealed that higher sperm TL was associated with a higher probability of pregnancy but not with fertilization rate.[13] However, the study has design limitations in the evaluation of the associations between sperm TL and sperm quality.

Recently, observational studies measuring TL as a marker of infertility in men[14,15] or evaluating the relationship between TL and spermatozoa-related parameters[16,17] have increased. The main aim of the present study is to conduct a systematic review and meta-analysis of: (a) cross-sectional studies exploring the association between spermatozoa and/or leukocyte TL with sperm quality parameters, and (b) case–control studies comparing TL in populations with different semen abnormalities or (in)fertility. Our main hypothesis is that TL is a biomarker of semen quality and male infertility and can complement the analysis of other parameters predicting semen quality.

2 | METHODS

2.1 | Protocol and registration

The protocol of the systematic review and meta-analysis was registered in the international prospective register of systematic reviews PROSPERO (https://www.crd.york.ac.uk/prospero) with the code CRD42021227690.

2.2 | Literature search strategy

A literature search of human studies published in English was carried out in both MEDLINE-PubMed and Cochrane Library databases from the earliest available indexing year until May 2022. In order to obtain a reference list of the articles we performed a systematic search of two subsets of Medical Subject Heading terms and keywords: the first subset comprised telomere-related terms (telomere OR telomere shortening OR telomere homeostasis OR telomerase OR telomere length OR telomerase activity OR telomere maintenance) and the second subset comprised keywords related to seminogram alterations or infertility (spermatozoa OR spermatogenesis OR sperm motility OR sperm count OR sperm maturation OR sperm capacitation OR semen OR semen analysis OR infertility, male OR oligospermia OR aspermia OR asthenozoospermia OR azoospermia OR teratozoospermia OR sperm OR semen quality OR oligozoospermia OR oligoasthenozoospermia OR oligoasthenoteratozoospermia OR male fertility OR sperm dysfunction OR spermatogenesis OR protamine deficiency OR

sperm parameters OR sperm DNA fragmentation OR sperm DNA damage OR varicocoele OR non-obstructive azoospermia OR erectile dysfunction OR sperm DNA extraction OR spermatozoa abnormality OR sperm chromosomal abnormalities). In Supporting Information S1 the complete search strategy is available.

2.3 | Eligibility criteria and study selection

In a preliminary screening, two independent researchers screened titles and abstracts for eligibility (M. F., C. V. H.), and the discrepancies were re-evaluated by two other authors (A. S.-H. and S. C.). Eligible studies included in the systematic review and meta-analysis were those with a cohort design and cross-sectional and case–control studies. Articles defining TL in spermatozoa and/or leukocyte cells as exposure, and semen quality parameters (volume, ejaculate pH, total sperm count or concentration, sperm vitality, sperm motility, sperm morphology) or seminogram alterations (oligospermia, aspermia, asthenozoospermia, azoospermia, teratozoospermia, oligozoospermia, oligoasthenozoospermia, oligoasthenoteratozoospermia, varicocoele, non-obstructive azoospermia) as outcomes were included in this systematic review and meta-analysis when the number of studies was three or more. Studies including data regarding sperm DNA fragmentation, sperm DNA damage or protamine deficiency were included in this systematic review with these endpoints as secondary outcomes. Study exclusion criteria were as follows: ecological, retrospective, methodological or case report studies; review or meta-analysis articles; animal or in vitro studies; studies without describing TL as exposure and without semen quality parameters or fertility outcomes as endpoints. Finally, non-original articles (letters, commentaries, viewpoints, summaries, editorials), abstracts, symposium presentations or invited lectures, guidelines or scientific statements, and special articles were also excluded.

2.4 | Data extraction

A standardized model was used to extract the information from each study: title, type of publication, first author, journal and year of publication, study design and period, sample size and participant's disease status, age, country of origin and city or place of recruitment, endpoint data, statistical analysis performed, and main conclusions.

The main exposure of this study, TL, can be reported in relative (telomere [T] to single-copy gene [S] sequence [T/S] ratio) or absolute (bp) units. TL was mainly reported in relative units, so we contacted the corresponding authors of studies reporting absolute units to transform them into relative units to standardize data.

2.5 | Study quality assessment

The National Heart, Lung, and Blood Quality Assessment Tool for observational cohort and cross-sectional and case–control studies was used to evaluate the risk of bias of the articles included in this systematic review and meta-analysis (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools; accessed on 6 March, 2023). Two independent researchers (M. F., C. V–H.) assessed the risk of bias, and the discrepancies were re-evaluated by two other authors (A. S-H. and S. C.). The studies were categorized as good, fair or poor depending on the overall quality score. Cross-sectional studies with a score between 0 and 5 points and case–control studies with a score between 0 and 4 were considered low-quality and therefore excluded.

2.6 | Statistical and sensitivity analysis

When three or more articles analyzed the same exposure and outcome, the results were meta-analyzed. Meta-analyses were conducted using the *meta* package for R 4.2.2 statistical software and Review Manager 5.4 in accordance with Cochrane guidelines.[18] The mean difference (MD) and 95% confidence interval (CI) were computed from the mean and standard deviation (SD) extracted in each study included. If other data distribution values (e.g., median, standard error of mean, or interquartile range) were presented in the original studies, they were recalculated to mean and SD. Values were obtained by two authors (M. F. and C. V. H.) and checked by another author (A. S–H.). Fixed effect models were used to obtain summary MD and 95% CI of the studies analyzed. The statistical significance level was set at $p < 0.05$ (two-tailed). Chi-square tests and the I^2 index were used to evaluate heterogeneity between studies, and in this case, the significance level was set at $p < 0.1$. I^2 values <50% were considered moderate, ≥50% to <75% were considered substantial, and ≥75% were considered of considerable heterogeneity. To evaluate the robustness of our findings, sensitivity analyses using random effect models were performed.

3 | RESULTS

3.1 | Article selection

Study selection, identification, screening, inclusion and exclusion processes are summarized in Figure 1. Following a primary search, screening of the titles and abstracts of 681 articles led to 37 studies that were eligible for inclusion. At this point, two studies not in English or unavailable for download were excluded. Following full-text screening, 12 studies were excluded for (a) only including processed sperm data (density gradient centrifugation and/or swim-up); (b) being in vitro experiments; (c) including a specific selected population highly exposed to pollutants; or (d) being reviews or hypothesis analysis articles. Finally, 23 cross-sectional or case–control studies were included for analyses.

3.2 | Study characteristics

The characteristics of the cross-sectional and case–control studies included in the analyses are presented in Tables 1 and 2, respectively. The studies were conducted mainly in China ($n = 3$), the UK ($n = 1$), India

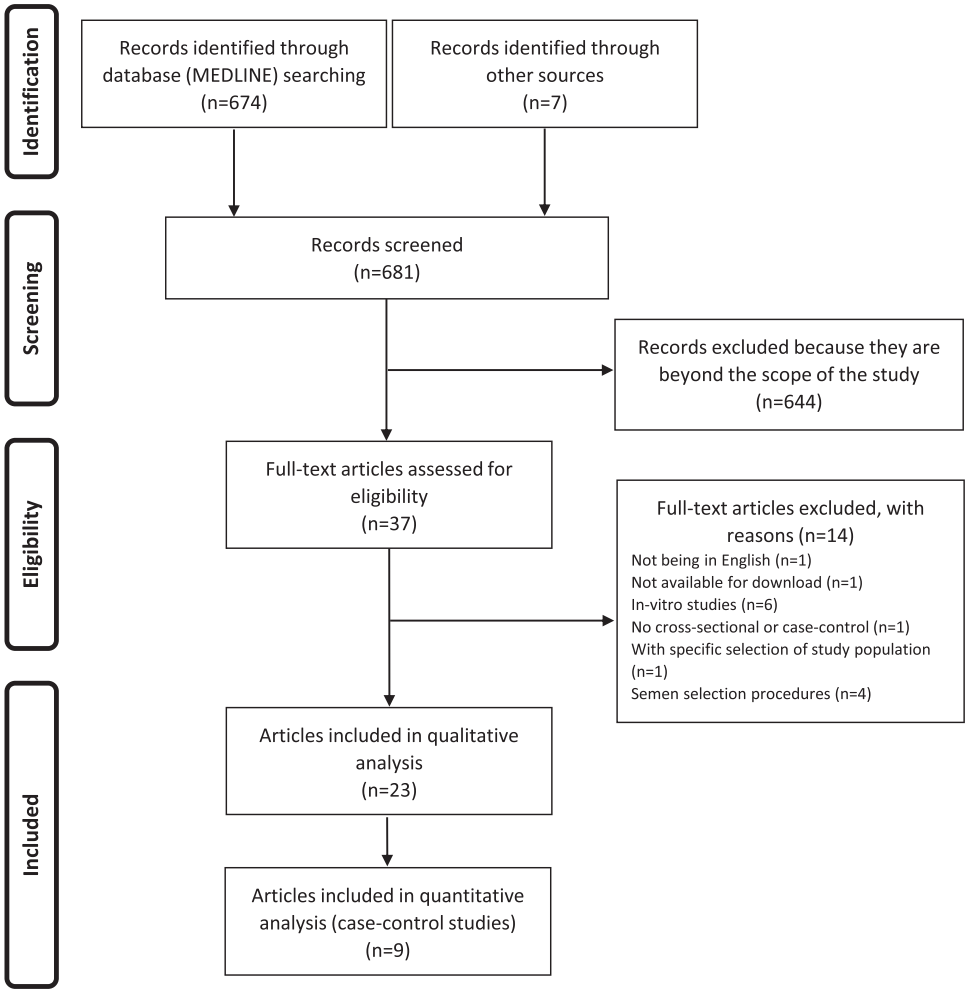

FIGURE 1 Flowchart of the literature search and selection process.

($n = 3$), Iran ($n = 5$), Israel ($n = 1$), Italy ($n = 5$), France ($n = 1$), and Spain ($n = 4$) with sample sizes ranging from 20 to 239 participants in the case of cross-sectional studies, and from 20 to 866 for case–control studies. Fertile and/or infertile populations were included with ages ranging from 18 to 52 years.

3.3 | Qualitative analysis

3.3.1 | Results of cross-sectional studies

A total of 17 cross-sectional studies were selected for the qualitative analysis. Four articles included fertile men only, four included infertile men, and 9 included both.

Fertile or normozoospermic men

The study of the relationship between sperm TL and sperm parameters in fertile or normozoospermic populations revealed contradictory results among the papers. In relation to sperm vitality and motility, Rocca et al.[19] reported positive correlations between sperm TL and vitality and progressive motility in 100 normozoospermic subjects. No associations between sperm TL and motility were reported in the other two studies including semen samples from 65 normozoospermic and from 60 donor participants.[16,20] A positive correlation between sperm TL and sperm concentration was reported by Torra-Massana et al.,[20] but this association was not reported in the other two studies.[16,19] Regarding other spermatozoa-related parameters, no associations were observed between sperm TL and volume, count or morphology in any of the three aforementioned studies. A study by Balmori et al.[21] only observed a positive correlation between sperm TL and sperm count, as well as total progressive motility, in a group of 20 normozoospermic participants under 25 years of age.

Concerning secondary outcomes, a negative correlation between sperm TL and sperm DNA fragmentation, and a positive correlation with normal sperm protamination were reported.[19]

Infertile population

In relation to semen parameters, the study performed by Lafuente et al.[17] showed a positive correlation between sperm TL measured before sperm selection and percentage of immotile spermatozoa in 42 infertile participants. The investigators also reported a negative correlation between sperm TL and progressive motility.[17] However, Zhao

TABLE 1 Summary of the cross-sectional studies investigating the association between sperm telomere length (STL) and/or leukocyte telomere length (LTL) and sperm quality parameters or fertility outcomes.

Reference	Location	Population studied	Age (years)	Cell type	Primary outcomes	Secondary outcomes
Fertile or normozoospermic populations						
Rocca et al.[19]	Italy	100 normozoospermic fertile participants	34.0 (8.6)	STL	Semen parameters (volume, total count, concentration, progressive motility, vitality, and morphology)	Sperm DNA fragmentation and normal protamination
Berneau et al.[16]	UK	66 normozoospermic participants	35.5 (4.5); [25–45]	STL	Semen parameters (volume, total count, concentration, progressive and grade A motility, and immotile spermatozoa)	No
Torra-Massana et al.[20]	Spain	60 donor participants	24.3 (5.0); [18–35]	STL	Sperm parameters (concentration and motility)	No
Balmori et al.[21]	Spain	20 normozoospermic subjects	21.2 (2.4)	STL	Semen parameters (total count and total progressive motility)	No
Infertile populations						
Lafuente et al.[17]	Spain	42 infertile patients	NR	STL	Sperm parameters (concentration, progressive motility, and immotile spermatozoa)	Sperm DNA fragmentation
Thilagavathi et al.[24]	India	25 iRPL subjects	33.2 (5.2)	LTL	Sperm parameters (pH, total count, and motility)	Sperm DNA fragmentation
Zhao et al.[22]	China	150 normozoospermic infertile subjects	31.8 (6.1)	STL	Semen parameters (volume, total count, concentration, progressive motility, and normal morphology)	Sperm DNA fragmentation
Sun et al.[23]	China	105 infertile subjects	31.2 (6.1)	STL	Semen parameters (total count)	No
Fertile and infertile or normozoospermic and oligozoospermic populations						
Ferlin et al.[14]	Italy	61 normozoospermic and 20 oligozoospermic subjects	[18–19]	STL and LTL	Semen parameters (total count)	No
Cariati et al.[15]	Italy	54 normozoospermic and 19 oligozoospermic	Normozoospermic 39.4 (5.5); oligozoospermic 39.3 (5.3); [31–52]	STL	Semen parameters (total count, motility, and normal morphology)	No
Thilagavathi et al.[26]	India	32 idiopathic infertile and 25 fertile participants	NR	STL	Semen parameters (pH, volume, total count, motility, and normal morphology)	Sperm DNA fragmentation
Amirzadegan et al.[25]	Iran	10 fertile and 10 oligozoospermic subjects	Fertile: 35.5 (5.6); oligozoospermic: 40.3 (3.8)	STL and LTL	Semen parameters (total count, concentration, motility, abnormal morphology)	Sperm DNA fragmentation, protamine deficiency

(Continues)

TABLE 1 (Continued)

Reference	Location	Population studied	Age (years)	Cell type	Primary outcomes	Secondary outcomes
Darmishonnejad et al.[27]	Iran	10 fertile and 10 infertile subjects	Fertile: 40.1 (3.1); infertile: 38.1 (4.2)	STL and LTL	Semen parameters (total count, concentration, motility, and abnormal morphology)	No
Darmishonnejad et al.[28]	Iran	19 fertile and 38 infertile subjects	Fertile: 40.5 (3.8); infertile 32.6 (6.6); [20–50]	STL	Semen parameters (concentration, motility, and abnormal morphology)	Sperm DNA fragmentation, protamine deficiency
Mishra et al.[32]	India	102 fertile participants and 112 infertile	Fertile: 32.2 (4.0); infertile:31.7 (4.4); [18–45]	STL	No	No
Tahamtan et al.[30]	Iran	20 fertile and 18 infertile varicocoele patients	Fertile: 41.4 (3.6), varicocoele: 28.5 (5.5)	STL and LTL	Semen parameters (sperm count, concentration, motility, and abnormal sperm morphology)	Sperm DNA fragmentation, protamine deficiency
Gentiluomo et al.[29]	Italy	239 participants	34.8 (7.5)	STL	Semen parameters (total count, concentration, motility, morphology)	No

Note: The studies are ordered as: fertile population, infertile population, and fertile and infertile population. Age is given as mean (SD), or [range] where such data are available.
Abbreviations: iRPL, idiopathic recurrent pregnancy loss; NR, not reported.

et al.[22] found a significant but positive correlation between sperm TL and progressive motility in unprocessed semen samples of 150 normozoospermic infertile patients. Lafuente et al.[17] reported a negative correlation between sperm TL and sperm concentration. Nevertheless, this association was not significant in the case of sperm concentration or morphology in the analysis performed by Zhao et al.[22] A positive correlation between sperm TL and sperm count was reported in two of the four studies that have evaluated this endpoint.[22,23]

On the other hand, Thilagavathi et al.[24] did not report any significant association between leukocyte TL and sperm count or motility in 25 men from couples with a history of idiopathic recurrent pregnancy loss.

Three studies investigated the associations between sperm TL and secondary outcomes in infertile populations. While Zhao et al.[22] reported a negative correlation between sperm TL and sperm DNA fragmentation index, no association between these two parameters was reported by Lafuente et al.[17] Besides, no significant correlations between leukocyte TL and sperm DNA fragmentation index were observed.[24]

Populations including a mix of fertile and infertile or normozoospermic and oligozoospermic subjects

Nine studies have analyzed the association between sperm TL and sperm quality parameters in populations including fertile and infertile or normozoospermic and oligozoospermic subjects. A positive correlation between sperm TL and sperm count was found in 81 subjects (61 normozoospermic and 20 idiopathic oligozoospermic).[14] This was also true in a similar population including 54 normozoospermic and 19 oligozoospermic adults,[15] as well as in the study performed by Amirzadegan et al.,[25] in which 10 fertile and 10 oligozoospermic men were included. On the contrary, in a group of 32 idiopathic infertile men and 25 fertile controls, sperm TL and sperm count were not correlated.[26] No associations were reported between sperm TL and motility or morphology in the aforementioned studies.[15,25,26] Two studies reported a positive correlation between sperm TL and sperm concentration,[25,27] but Darmishonnejad et al.[27] did not observe correlations between sperm TL and sperm count, motility, or abnormal morphology in 10 fertile and 10 infertile subjects. In Darmishonnejad et al.'s study,[28] no associations between relative sperm TL and different semen-related parameters (concentration, motility, and abnormal morphology) in 38 infertile and 19 fertile participants mixed together were found. Besides, Gentiluomo et al.'s study,[29] performed in 239 participants, revealed no associations between sperm TL and semen-related parameters (concentration, total number, motility, and morphology). Only Tahamtan et al.[30] reported a positive correlation between sperm TL and spermatozoa motility in 20 fertile and 18 infertile men with grade II or III varicocoele. In relation to leukocyte TL, positive correlations with concentration and sperm count were also shown in two studies.[25,30] Amirzadegan et al.[25] also reported negative associations between leukocyte TL and abnormal morphology. In contrast, leukocyte TL was not significantly related to sperm count[14] or motility.[25]

TABLE 2 Summary of case–control results investigating the differences on sperm telomere length (STL) and/or leukocyte telomere length (LTL) between normozoospermic (controls) and oligozoospermic (cases) or fertile (controls) and infertile (cases) subjects.

Reference	Location	Population studied	Age (years)	Cell type	Main analyses
Normozoospermic (controls) and oligozoospermic (cases) subjects					
Ferlin et al.[14]	Italy	61 normozoospermic and 20 oligozoospermic subjects	[18–19]	STL and LTL	Differences in STL and LTL between normozoospermic and oligozoospermic men
Cariati et al.[15]	Italy	54 normozoospermic and 19 oligozoospermic subjects	Normozoospermic 39.4 (5.5); oligozoospermic 39.3 (5.3); [31–52]	STL	Differences in STL between normozoospermic and oligozoospermic men
Amirzadegan et al.[25]	Iran	10 fertile and 10 oligozoospermic subjects	Fertile: 35.5 (5.6); oligozoospermic: 40.3 (3.8)	STL and LTL	Differences in STL and LTL between fertile and oligozoospermic men
Balmori et al.[21]	Spain	Younger group: 20 normozoospermic and 17 oligozoospermic Older group: 20 normozoospermic and 20 oligozoospermic	Younger group: 20 normozoospermic, 21.2 (2.4) and 17 oligozoospermic, 21.4 (2.3) Group ≥40 years: 20 normozoospermic, 43.3 (3.4) and 20 oligozoospermic, 43.6 (4.0)	STL	Differences in STL between normozoospermic and oligozoospermic men under 25 years and between normozoospermic and oligozoospermic men over 40 years
Fertile (controls) and infertile (cases) populations					
Mishra et al.[32]	India	102 fertile participants and 112 infertile patients	Fertile: 32.2 (4.0); infertile: 31.7 (4.4); [18–45]	STL	Differences in STL between fertile and infertile men
Darmishonnejad et al.[28]	Iran	19 fertile and 38 infertile subjects	Fertile: 40.5 (3.8); infertile 32.6 (6.6); [20–50]	STL and LTL	Differences in STL between fertile and infertile men
Berby et al.[34]	France	20 control men and 30 infertile patients	Control: 35.1 (5.7); infertile: 35.2 (8.0)	STL	Differences in STL between control and infertile men
Rocca et al.[33]	Italy	30 healthy controls and 35 men undergoing ART	Control: 36.1 (6.8); ART group: 39.6 (5.4)	STL	Differences in STL between control and ART men
Thilagavathi et al.[26]	India	32 idiopathic infertile and 25 fertile men	NR	STL	Differences in STL between fertile and idiopathic infertile men
Darmishonnejad et al.[27]	Iran	10 fertile and 10 infertile patients	Fertile: 40.1 (3.1); infertile: 38.1 (4.2)	STL and LTL	Differences in STL between fertile and infertile men
Biron-Shental et al.[31]	Israel	10 fertile and 16 sub-fertile subjects requiring ICSI	Fertile: 36.5 (7.0); sub-fertile: 37.4 (5.0)	STL	Differences in STL between fertile and sub-fertile subjects
Yang et al.[37]	China	270 normal men, 247 with obstructive azoospermia and 349 with non-obstructive azoospermia patients	Normal: 33.0 (29.0–38.0); OA: 27.0 (25.0–31.0); NOA: 27.0 (25.0–31.0)	LTL	Differences in LTL between normal, OA and NOA men
Thilagavathi et al.[24]	India	20 fertile controls and 25 iRPL patients	Controls: 31.5 (5.3); iRPL subjects: 33.2 (5.2)	LTL	Differences in STL between fertile and iRPL patients
Tahamtan et al.[30]	Iran	20 fertile controls and 18 infertile subjects with grade II or III varicocoele	Fertile: 41.4 (3.6); varicocoele: 28.5 (5.5)	STL and LTL	Differences in STL between fertile and varicocoele patients
Lara-Cerrillo et al.[35]	Spain	12 fertile donors and 20 patients with unilateral or bilateral grade II or higher varicocoele	17–44	STL	Differences in STL between fertile controls, before and after surgery in varicocoele patients
Heidary et al.[36]	Iran	30 fertile participants and 30 idiopathic non-obstructive azoospermic patients	35.4 (4.5)	LTL	Differences in LTL between fertile controls and idiopathic non-obstructive azoospermic men

Note: Age is given as mean (SD), [range], or median (25th–75th percentile) where such data are available.

Abbreviations: ART, assisted reproductive technology; ICSI, intracytoplasmic sperm injection; iRPL, idiopathic recurrent pregnancy loss; NOA, non-obstructive azoospermia; NR, not reported; OA, obstructive azoospermia.

With respect to secondary outcomes, one study did not observe a significant relationship between sperm TL and sperm DNA fragmentation.[26] However, in three other articles, negative correlations between sperm TL and DNA fragmentation[25,28,30] were observed. In addition, two studies reported negative correlations between spermatozoa or leukocyte TL and sperm protamination deficiencies.[25,30]

3.3.2 | Results of case–control studies

Sixteen studies evaluated the differences in spermatozoa and leukocyte TL between controls and cases.

Differences in sperm TL between normozoospermic (controls) and oligozoospermic (cases) subjects

Three studies clearly showed that the normozoospermic men had significantly longer telomeres than the oligozoospermic patients.[14,15,25] Similarly, Balmori et al.[21] reported significant differences (in the same direction) in sperm TL between older normozoospermic and older oligozoospermic participants, between younger normozoospermic and older normozoospermic participants and between younger oligozoospermic and older normozoospermic participants.

Differences in sperm TL between fertile (controls) and infertile (cases) subjects

Four studies reported significantly longer sperm TL in fertile men than in infertile men,[26–28,31] even after adjustment for age.[32] Besides, Rocca et al.[33] observed longer sperm TL in 30 controls than in 35 men who underwent their first assisted reproductive procedure. On the contrary, Berby et al.[34] reported no differences between control men and infertile men. While significant differences in sperm TL were observed between 18 infertile men with grade II or III varicocoele and 20 fertile men,[30] no differences were reported by Lara-Cerrillo et al.[35] between 12 fertile donors and 20 patients before microsurgical varicocelectomy (MV) and after MV.

Differences in leukocyte TL between fertile and infertile or normozoospermic and oligozoospermic subjects

Regarding TL in leukocytes, three studies reported lower leukocyte TL in infertile patients than in fertile men.[24,28,36] Similarly, longer leukocyte TL in 10 fertile men than in 10 oligozoospermic patients was reported by Amirzadegan et al.[25] On the other hand, Yang et al.[37] reported shorter leukocyte TL in 349 non-obstructive azoospermia patients than in 247 obstructive azoospermia patients and 270 normospermic men, and results from the Tahamtan et al.[30] study showed significantly lower leukocyte TL in men with infertility resulting from varicocoele than fertile controls. Only two studies found no differences in leukocyte TL between normozoospermic and oligozoospermic men[14] or between fertile and infertile subjects.[27]

3.4 | Quantitative analysis

The relatively high number of case–control studies providing sufficient data and the homogeneity between them led us to conduct three meta-analyses to test the associations between TL in male sexual cells (spermatozoa) and somatic cells (leukocytes) and different case and control populations. No quantitative analysis was carried out with the cross-sectional studies because of the heterogeneity and insufficient data from these.

3.4.1 | Infertile versus fertile populations

Sperm telomere length

Data from five studies were meta-analyzed to test the associations between sperm TL and fertility group. In summary, the infertile group of patients had significantly shorter sperm TL than the fertile group (MD [95% CI]: −1.43 [−1.66, −1.21], p-value < 0.001). However, there was evidence of considerable interstudy heterogeneity (I^2 = 94%, p-value < 0.001) (Figure 2A). The sensitivity analysis was consistent with the primary analysis (Figure S1A).

Leukocyte telomere length

Analyzing data from four different studies testing the associations between leukocyte TL and fertility group showed that the infertile group had significantly shorter leukocyte TL than the fertile group (MD [95% CI]: −1.67 [−2.02, −1.31], p-value < 0.001). This comparison displayed a considerable interstudy heterogeneity (I^2 = 86%, p-value < 0.001) (Figure 2B). The results were consistent with those of the sensitivity analysis (Figure S1A).

3.4.2 | Oligozoospermic versus normozoospermic populations

Sperm telomere length

Data from three studies were meta-analyzed to test associations between sperm TL and the WHO 2010 reference limits in sperm concentration or total sperm count; oligozoospermic versus normozoospermic patients. In summary, the oligozoospermic group of patients had shorter sperm TL than the normozoospermic group (MD [95% CI]: −0.97 [−1.32, −0.61], p-value < 0.001). Interstudy heterogeneity was non-significant in this evaluation (I^2 = 0, p-value = 0.51) (Figure 3). The sensitivity analysis result was identical to the primary analysis (Figure S2).

4 | DISCUSSION

This systematic review and meta-analysis of observational studies provides the most comprehensive and up-to-date analysis of the associations between spermatozoa and leukocyte TL and sperm quality

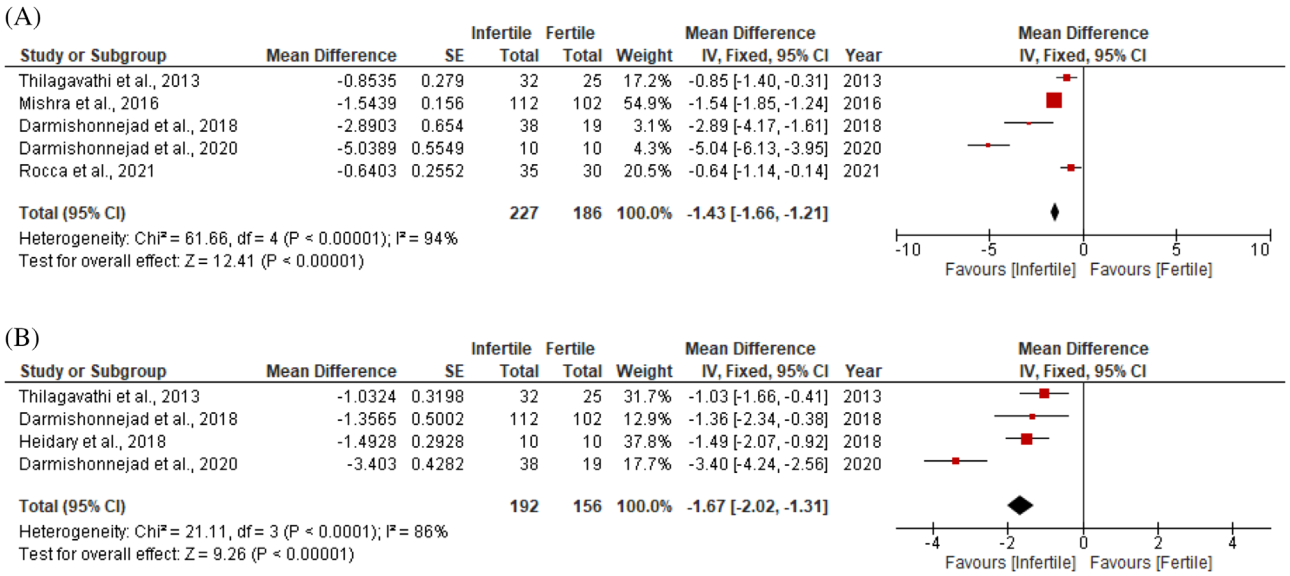

FIGURE 2 Forest plot of mean differences (MD) and 95% confidence intervals (CI) for studies evaluating the association between (A) sperm telomere length and (B) leukocyte telomere length in infertile versus fertile participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represent the pooled MD and 95% CI. Overall estimates were obtained using fixed-effect models. An MD value <0 indicates a negative association between telomere length and fertility group; infertile group had lower telomere length than fertile group.

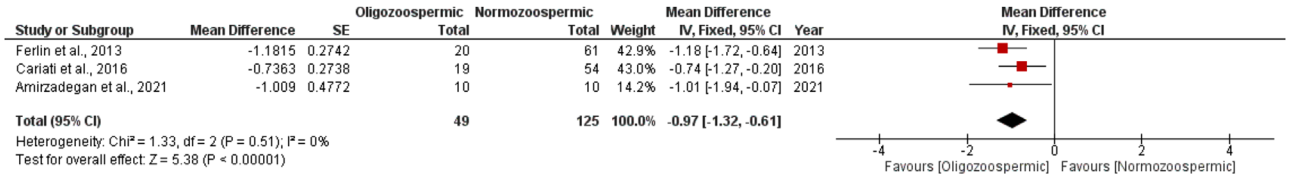

FIGURE 3 Forest plot of mean differences (MD) and 95% confidence intervals (CI) for studies evaluating the association between sperm telomere length in oligozoospermic versus normozoospermic participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represent the pooled MD and 95% CI. Overall estimates were obtained using fixed-effect models. An MD value <0 indicates a negative association between telomere length and normal seminogram result; oligozoospermic group had lower telomere length than normozoospermic group.

parameters and male fertility to date. Our qualitative results showed high heterogeneity in methodology evaluation and contradictory outcomes, making the identification of clear patterns difficult. However, when data were comparable, the quantitative analysis revealed that spermatozoa and leukocyte TL are shorter in infertile than in fertile men. Moreover, these differences, in terms of sperm TL, are also significant between men with a normal seminogram and those with a low quantity of ejaculate spermatozoa. This systematic review and meta-analysis suggests the potential role of spermatozoa and leukocyte TL as a biomarker of semen quality, which may help distinguish between different spermatogenic alterations beyond the routine semen analysis. These results suggest that spermatozoa and leukocyte TL may also be relevant biomarkers to help discriminate fertility potential in cases with sperm quality parameters that are within or close to the threshold values established by the WHO.

To the best of our knowledge, only Yuan et al.[13] performed a systematic review and meta-analysis evaluating the role of sperm TL as biomarker of male infertility and embryonic development. However, only the data from studies that they meta-analyzed were summarized, the qualitative results were omitted, and important limitations to their meta-analysis should be highlighted. For example, in the main analysis, all normozoospermic individuals were considered as fertile population and all oligozoospermic men as the infertile population without considering their fertility status. However, in a secondary analysis, they appropriately compared fertile men versus unexplained infertile men and reported similar results. Although the MD and 95% CI were completely different, they also concluded that sperm TL is shorter in infertile than in fertile men.

In our study, we also compared sperm TL between normozoospermic and oligozoospermic men, and it was shorter in the oligozoospermic population. It is important to mention that in the study performed by Yuan et al., on some occasions, untransformed data to mean and SD were incorrectly included in their meta-analysis. However, in our study we carefully estimated for each meta-analyzed study the mean and SD

values using the original data. Finally, in our study, we analyzed not only studies measuring sperm TL but also those measuring TL in leukocytes, and our results also revealed that infertile men had shorter TL in leukocytes than fertile men.

Although this was not the scope of our study, in the Yuan et al. systematic review and meta-analysis, the capacity of sperm TL to predict the success of pregnancy outcome and embryo development was also explored. A relationship between longer sperm TL and clinical pregnancy was reported, whereas higher fertilization probabilities were not observed in men with higher sperm TL. We recognize that in light of medical and social concerns, identifying biomarkers not only of sperm quality but also of infertility and reproductive outcomes is essential. This will provide valuable insights into possible clinical implications.

Two other studies not included in Yuan et al.'s meta-analysis should also be mentioned. First, in normozoospermic male partners of couples undergoing assisted reproductive technology (ART) treatments, Berneau et al.[16] reported positive significant associations between sperm TL and the percentage of fertilization rate. Second, regarding embryological parameters, sperm TL was positively associated with good embryo quality and transplantable embryo rates in the men of couples undergoing in vitro fertilization (IVF).[38] On the contrary, no associations between sperm TL and embryological parameters (e.g., good embryo cleavage, implantation rates or biochemical, clinical and ongoing pregnancy rates) were found.[16] Likewise, no associations were observed with clinical pregnancy and fertilization rates.[38] These contradictory results might be explained by differences in the study design, population studied, and/or unconsidered factors related to female partners, suggesting that evidence exploring the associations between sperm TL and clinical and embryological outcomes is still needed.

The effect of parental age at conception has been studied because offspring conceived by older parents have been found to have longer telomeres. Kimura et al.[39] confirmed that paternal age was positively associated with leukocyte TL from the offspring in four cohorts, and this may be related to telomere characteristics in paternal spermatozoa. Longer sperm TL was observed in older than younger men. Moreover, when comparing the effect of maternal and paternal age at conception, the major determinant of offspring leukocyte TL was paternal age, with longer leukocyte TL observed in the offspring of older fathers.[40] Ferlin et al.[14] also explored the relationship between maternal and paternal age and offspring sperm TL and reported longer sperm TL in the offspring of older fathers and mothers. However, because there was a high correlation between paternal and maternal ages, which contributed the most could not be determined. The fact that paternal age may act as a determining factor of human telomere dynamics because the inheritance of TL to the offspring demonstrates the important role of these structures in reproduction. Therefore, sperm selection procedures for spermatozoa with longer telomeres in ART practices may play a role in IVF success, but this must be extensively explored. Unfortunately, we could not evaluate this because the studies did not report maternal and paternal age at conception.

Several methods are currently available for TL measurement and quantification, each with its own advantages and limitations. These methods can be broadly categorized into four groups: hybridization, polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), and mixed methods (e.g., hybridization/PCR combination), each providing different information about TL. Terminal restriction fragment analysis, the "gold standard," is based on hybridization techniques, and it is considered more accurate in providing absolute TL measurements. However, it requires large amounts of genomic DNA and laborious processes, making it less practical for large-scale population studies. PCR-based methods have become the most popular method for large-scale studies because of their high-throughput capability and simplicity and because small amounts of DNA are required. These methods determine the ratio between the telomere (T) and single-copy gene (S) signals, providing a proportional measure of relative average TL. Nevertheless, these PCR-based methods often exhibit difficulties in standardization. Methods based on FISH (e.g., quantitative FISH, flow FISH) are also used to determine TL. Because of their low detection limit capacity, these techniques are particularly useful for detecting short telomeres, which are highly indicative of cellular senescence. These procedures can be suitable for large-scale studies to determine TL on fixed lymphocytes but are time consuming and limited to specialized laboratories. Additionally, other techniques, such as single telomere length analysis or telomere shortest length assay, are also used to quantify the shortest telomeres but with low throughput.[41]

This study has several strengths that should be highlighted. This is the first comprehensive systematic review and meta-analysis that has separately meta-analyzed spermatozoa and leukocyte TL differentiating between fertility conditions and seminogram alterations: fertile versus infertile and normozoospermic versus oligozoospermic individuals. Second, in the meta-analysis, we only included studies measuring TL by quantitative PCR as a mode of standardizing the results. Third, in this systematic review and meta-analysis, we did not include studies using sperm selection procedures as we understand that TL measurement would not be representative of the whole sperm population. However, our work also has some limitations that should be considered. Relatively few studies evaluating spermatozoa and leukocyte TL as a biomarker of infertility have been carried out. Besides, we could not meta-analyze cross-sectional data because of the lack of information provided despite trying to obtain this through contact with the corresponding authors. Unfortunately, because of the limited number of published studies, we cannot conduct a sensitivity analysis to consider differences between the populations of the studies included with regard to age, sample size, or other factors that could affect TL. Finally, it is worth mentioning that although positive associations were observed between TL and conventional sperm quality parameters, its translation into better reproductive outcomes (e.g., clinical pregnancy or live birth) remains unanswered and deserves further studies.

In conclusion, our study presents a global review of the evidence available regarding the associations between TL and semen quality parameters and differences in TL between different populations based on their seminogram abnormalities or fertility. Our results suggest that TL has the potential to be used as a biomarker of sperm quality and male infertility. However further and larger studies are warranted in the future to increase the certainty of evidence of a potential relationship between TL, semen quality and fertility. Finally, if TL is a proxy of

sperm quality or fertility potential (or vice versa) should be tested using molecular and in vitro experiments.

AUTHOR CONTRIBUTIONS

Conceptualization: María Fernández de la Puente, Albert Salas-Huetos, Cristina Valle-Hita, Silvia Canudas, and Jordi Salas-Salvadó. *Data curation*: María Fernández de la Puente and Cristina Valle-Hita. *Formal analysis*: María Fernández de la Puente, Albert Salas-Huetos, and Cristina Valle-Hita. *Investigation*: María Fernández de la Puente, Albert Salas-Huetos, and Cristina Valle-Hita. *Methodology*: María Fernández de la Puente, Albert Salas-Huetos, and Cristina Valle-Hita. *Visualization*: María Fernández de la Puente and Albert Salas-Huetos. *Writing original draft*: María Fernández de la Puente. *Writing review and editing*: Albert Salas-Huetos, Cristina Valle-Hita, Nancy Babio, Michelle M. Murphy, Silvia Canudas, and Jordi Salas-Salvadó. *Funding acquisition*: Jordi Salas-Salvadó. *Supervision*: Albert Salas-Huetos, Nancy Babio, Michelle M. Murphy, Silvia Canudas, and Jordi Salas-Salvadó. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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SHORT COMMUNICATION

CAG and GGN repeat polymorphisms in the androgen receptor gene of a Chilean pediatric cohort with idiopathic inguinal cryptorchidism

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Abstract

Background: Cryptorchidism is one of the most common congenital disorders in boys and it is associated with a higher risk of sub-fertility and testicular cancer. Testicular descent occurs during embryo-fetal development in two phases, transabdominal and inguino-scrotal. In the latter process, androgens play a leading role. The androgen receptor has in its N-terminal domain, two aminoacidic repeats encoded by polymorphic nucleotide repetitions: (CAG)nCAA and GGN. The number of repetitions of these trinucleotides has been associated with different transactivation capacities and sensitivities of the androgen receptor response.

Objective: To determine whether pediatric Chilean individuals with idiopathic inguinal cryptorchidism have a different number of CAG and/or GGN repeats polymorphisms compared with controls.

Materials and methods: A total of 109 cases with idiopathic inguinal cryptorchidism (26 bilateral and 83 unilateral) were studied by polymerase chain reaction amplification from DNA extracted from peripheral blood, followed by fragment size analysis by capillary electrophoresis, which were compared with 140 controls.

Results: The CAG26 repeats allele was increased in the total cases (8.3% vs. 1.4%; $p = 0.012$; odds ratio = 6.21, 95% confidence interval 1.31–29.4), and in bilateral cases compared to controls (11.5% vs. 1.4%; $p = 0.028$; odds ratio = 9 CI 95% 1.43–56.8). Similarly, CAG > 22 alleles were increased in the total cases (62.4% vs. 49.3%, $p = 0.041$), and more significantly in bilateral cases (73.1% vs. 49.3%; $p = 0.032$; odds ratio = 2.79, 95% confidence interval 1.1–7.1). In addition, CAG < 18 alleles were not observed among cases, but were present in 5.7% of controls ($p = 0.01$). Regarding the GGN repeats, no differences were observed between cases and controls either when analyzing separately unilateral and bilateral cryptorchidism. The joint analysis of the distribution of CAG and GGN alleles showed that the CAG26 allele was present with GGN23, hence the combination CAG26/GGN23 alleles was equally increased in bilateral cases compared with controls (11.5% vs. 1.4%). In contrast, CAG < 18 was

preferably observed in the combination CAG < 18/GGN≠23 and was absent in the total cases (4.3% vs. 0%; p = 0.037).

Discussion: These results suggest that greater lengths of CAG alleles may contribute to a diminished androgen receptor function. The CAG26 allele alone or in combination with GGN23 was associated with a higher risk of bilateral cryptorchidism. On the other hand, CAG < 18 and the CAG < 18/GGN≠23 allele combination may reduce the probability of cryptorchidism.

KEYWORDS
androgen receptor, cryptorchidism, polymorphisms

1 | INTRODUCTION

The incomplete descent of one or both testis through the inguinal canal into the scrotum is a developmental anomaly known as cryptorchidism (DOID: 11383), which is seen in approximately 1%–9% of male newborns.[1,2] By the end of the first year of life, due to spontaneous descent, only 1% of boys will still have cryptorchidism.[3] The persistence of cryptorchidism represents a risk factor for testicular cancer[4] and sub-fertility.[5] The influence of different factors over cryptorchidism has been extensively studied. Potential etiological factors include maternal, gestational and hormonal disturbances, and also genetic factors. Only a few of them, however, have demonstrated significant associations, such as maternal smoking, birth weight, family history of cryptorchidism, and some genetic variants.[2] Among the genes potentially implicated in cryptorchidism is the androgen receptor gene (*AR*).[6]

Testicular descent occurs in two phases; transabdominal, mainly regulated by *INSL3*, and inguinoscrotal, which is under androgen control (testosterone and *dih*ydrotestosterone—DHT). The first phase (transabdominal) occurs between the 10th and 12th weeks of gestation in humans and is characterized by *gubernaculum* thickening which pulls the testicles from the abdominal wall into the inguinal ring. The last phase (inguinoscrotal) is characterized by migration of the *gubernaculum* with elongation of the *processus vaginalis* following its path alongside the muscular and fascial layers. This allows evagination to the scrotal protuberance and the formation of the inguinal canal, allowing the descent of the testes between the 12th and 28th weeks of gestation, to reach the scrotum by the 33rd week. Testosterone and DHT acting on the genitofemoral nerve release *C*alcitonin *G*ene-*R*elated *P*eptide, a neurotransmitter that provides a chemotactic gradient to guide the migration of the *gubernaculum* towards the fund of the *scrotum*.[7]

The AR, located on the X chromosome, is a member of the nuclear steroid receptor family. It mediates testosterone and DHT action, both through DNA binding-dependent (genomic), and independent (non-genomic) mechanisms. The AR comprises three main functional domains, the N-terminal transcriptional regulation domain, the DNA binding domain, and the ligand binding domain. The former domain (transactivation) is the most variable domain, and consequently, genomic response to androgens may be affected by this variability.[8] This variability is in part due to two polymorphic trinucleotide repeats located within the first exon. The (CAG)nCAA genetic polymorphism is followed by the (GGN)n polymorphism, compound by $(GGT)_3$ $(GGG)_1$ $(GGT)_2$ $(GGC)_n$, and encode a tract of polyglutamine followed by one of the polyglycines in the N-terminal of the protein. The normal range for these polymorphisms is 10–30 for CAG, with a mean of 22 repeats, and 10–27 for GGN, with a mean of 23 repeats.[9,10] Evidence from an in vitro transactivational activity study[11] and diverse studies in male patients with conditions related to androgen action (e.g., infertility, prostate cancer, cognitive and vitality decline, pubertal gynecomastia, and depression) have shown an association with the number of CAG repeats.[12–14] Similarly, several studies suggest an association between GGN repeat lengths and other male conditions.[15,16] Nevertheless, the effect of CAG and GGN repeat lengths, within the normal range, on AR-transactivational activation is still unclear.[10,17–19]

Until now, however, studies regarding the possible effects of AR repeat polymorphisms over testicular descent are scarce and controversial. Nevertheless, a meta-analysis of eight case-control studies by Wang and coworkers showed a significant association between longer CAG and GGN repeats and cryptorchidism risk in Caucasian populations from Europe, but not from Asia. On the other hand, three studies performed in Latin American populations, which analyze CAG repeats, have shown contradictory results.[20–22]

Consequently, our aim was to determine whether Chilean children and adolescents with isolated inguinal cryptorchidism have a different proportion of CAG and GGN repeats polymorphisms, and/or their combinations, compared with normozoospermic control subjects without a history of cryptorchidism or testicular cancer.

2 | MATERIAL AND METHODS

2.1 | Study subjects

Our study population included a subset of 109 boys consecutively selected from a previous cohort of 283 boys described by Rodríguez and co-workers.[23] This subset included all patients who had undergone

orchidopexy for isolated inguinal cryptorchidism at the San Borja Arriarán Clinical Hospital in Santiago, Chile from 2012 to 2017. We excluded eight patients with abdominal cryptorchidism and eight without true cryptorchidism (i.e. retractile or evanescent testicles); 83 patients with syndromic or apparently syndromic cryptorchidism (two or more signs associated with Noonan syndrome); 21 with short stature (height = −2 SDS for age and sex); 15 with paternal history of cryptorchidism (probably not related to AR gene polymorphism); 3 with chronic use of drugs or analgesics during pregnancy, and 34 patients without a surgical report available, 1 patient with a mutation probably associated with syndromic cryptorchidism (*NRAS* c.149 C > T p.T50I)[24] and 1 patient with one no Chilean ancestor. Height, weight and body mass index Z score were calculated using the 2000 CDC charts. Birth weight Z score was calculated according to Chilean birth weight references.[25] This study was performed according to the Declaration of Helsinki, and the Informed Consent for DNA extraction was approved by the Ethics Committees of the School of Medicine, University of Chile and the Central Metropolitan Health Service in Santiago, Chile.

The control group consisted of 140 healthy normozoospermic men without infertility history, cryptorchidism, or testicular cancer (self-reported) recruited over a period of 9 years (2004–2013), mostly during 2013 and studied for CAG and GGN repeats polymorphisms at the Reproductive and Molecular Andrology Unit of the Institute of Maternal and Child Research, University of Chile at the San Borja Arriarán Clinical Hospital in Santiago. The characterization of the controls is shown in Supplementary Table 1.

Serum testosterone, LH, FSH, AMH, and inhibin B were measured as previously described.[23] The control serum hormones were measured by Radioimmunoassay for testosterone, and by Immunoradiometric assay for FSH and LH.

2.2 | Determination of CAG and GGN repeats polymorphisms

Genomic DNA, from the patients and controls, was isolated from blood lymphocytes using the Wizard Genomic DNA Purification Kit (Promega, WI, USA). DNA purity and concentration were determined by spectrophotometric measurements of absorbance at 260 and 280 nm. The number of CAG and GGN repeats were determined by polymerase chain reaction (PCR) with primers specifics for each repeat (Genebank n° AH002624.2). The forward and reverse primers for CAG repeat were 5ť TCCAGAATCTGTTCCAGAGCGTGC 3ť and 5ť GCTGTGAAGGTTGCTGTTCCTCAT 3ť respectively; the former was marked with 6-FAM. Forward and reverse primers for GGN repeat were 5ť TCCTGGCACACTCTCTTCAC 3ť and 5ť GCCAGGGTACCACACATCAGGT 3ť respectively; the former was marked with NED. The PCR conditions were 1X AmpliTaq Gold 360 10X buffer (Applied Biosystems); 2.4 mM $MgCl_2$; 200 μM dNTPs; 0.7 μM each primer; 0.75 U of DNA polymerase (AmpliTaq Gold 360; Applied Biosystems); 2 μl de 360 GC Enhancer (Applied Biosystems) and 60 ng of genomic DNA in a total volume of 10 μl. Amplification was performed on a 2720 Thermal Cycler (Applied Biosystems), with preincubation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 60 seconds; followed by a final extension at 72°C for 10 min. Amplification products were resolved by automated capillary electrophoresis in a SeqStudio Genetic Analyzer (Applied Biosystems), and the results were analyzed with the Genemarker Analysis Software (version 2.4.0).

2.3 | Statistical analysis

Statistical analyses were performed using the SPSS v.29 software (SPSS Inc.). The Mann-Whitney or Kruskal Wallis tests were applied to analyze possible differences between two or more independent groups. To compare the proportions of the different numbers or combinations of CAG and/or GGN alleles between the control and patient groups, we performed Pearson's after the Bonferroni test and Fisher's exact test. The odds ratio (OR) was used to estimate relative risk between groups. In order to avoid type I (familywise) errors in comparisons of multiple proportions (crosstab greater than 2 × 2), the p-values obtained from the adjusted standardized residuals (z-values) of each independent cell-wise were used to obtain adjusted *p*-values multiplying the p-values obtained by the number of independent adjusted standardized residuals that were consulted in the crosstabs table (Bonferroni correction).[26]

3 | RESULTS

3.1 | Cohort characteristics

A total of 109 boys with idiopathic inguinal cryptorchidism, with a mean age at orchidopexy of 6 years (range = 1.2 - 17.1), were studied. According to the surgical report, 83 (76.2%) orchidopexies were unilateral, and 26 (23.8%) were bilateral. The anthropometric and hormonal characteristics of cases are shown in Tables 1 and in Tables S2 and S3, respectively. Regarding the anthropometric characteristics, no differences were observed between patients with unilateral and bilateral cryptorchidism. The analysis of the hormonal profile at the time of orquidopexy showed that patients in Tanner 1 with bilateral cryptorchidism had AMH and inhibin B levels significantly lower than those with unilateral cryptorchidism (Table S3).

3.2 | CAG allele distribution

The total distribution of CAG alleles in patients and controls was similar (p = 0.37, χ2 test) (Figure 1A). In order to improve the comparison of the total distribution of CAG, we grouped these alleles by 4 variables (< 18, 18–25, 26 and > 26), and statistical differences were observed in the total distribution (p = 0.003 by χ2 test), given by the allele CAG26 and by CAG < 18 (adjusted p = 0.037 and p = 0.047

TABLE 1 Anthropometric and hormonal characteristics of cases.

	Total cases	Unilateral cases	Bilateral cases	
n	109	83	26	*p*-Value[a]
Age (years)	6.0 (3.5–8.9)	6.0 (3.5–8.9)	6.3 (3.1–9.8)	0.639
Birth weight (kg.)	3.34 (3.1–3.7)	3.34 (3.1–3.7)	3.6 (3.2–3.8)	0.410
Birth height (cm.)	50 (49–51)	50 (49–51)	50 (48–52)	0.849
Weight (kg.)[b]	23.5 (18–38)	23.5 (18–38)	24.6 (16–43)	0.986
Weight (SDS) [b]	0.95 ± 1.23	0.95 ± 1.23	0.81 ± 1.07	0.593
Height (m.) [b]	1.17 (1.0–1.4)	1.16 (1.0–1.3)	1.24 (1.0–1.4)	0.837
Height (SDS) [b]	0.20 (−0.6–0.1)	0.20 (−0.7–1.1)	0.25 (−0.5–0.8)	0.706
BMI (kg./m2) [b]	18.0 (16.8–22.2)	18.1 (16.7–22.5)	17.4 (16.7–21.9)	0.670
BMI (SDS) [b]	1.3 (0.5–1.9)	1.3 (0.6–2.0)	1.0 (0.4–1.9)	0.318

Values are represented by mean ± standard deviation or median (interquartile range).
[a]Statistical differences between unilateral cases and bilateral cases were analyzed by *t*-test for parametric data or Mann-Whitney test for non-parametric data.
[b]Data obtained at orchidopexy.

based in corrected residuals) (Figure 1B). When we compared separately the CAG26 repeats, we observed statistical differences between total cases (8.3% vs. 1.4%, p = 0.012; odds ratio [OR] = 6.21, 95% confidence interval [CI] 1.31–29.4) and bilateral cases (11.5% vs. 1.4%, p = 0.028; OR = 9, CI 95% 1.43–56.8) compared to controls (Fisher's exact test). In addition, an increase of CAG > 22 repeats was observed in the total cases (62.4% vs. 49.3%, p = 0.041), and more significantly in bilateral cases (73.1% vs. 49.3%; p = 0.032; OR = 2.79, 95% CI 1.1–7.1). In contrast, CAG < 18 alleles were not observed among cases but were present in 5.7% of the controls, showing a statistical difference when compared with the total (p = 0.01) and unilateral cases (p = 0.02).

3.3 | GGN allele distribution

The total distribution of GGN alleles ranged from 10 to 25 repeats and was similar between patients and controls (p > 0.05, Fisher's exact test). As expected, the most common alleles with significantly higher prevalence in cases and controls were those with 23 and 24 GGN repeats, which also showed to have a similar prevalence between cases and controls. Similarly, there were no differences between cases and controls when we compared the GGN repeats other than 23 (GGN≠23), or equal to 24 (Table S4).

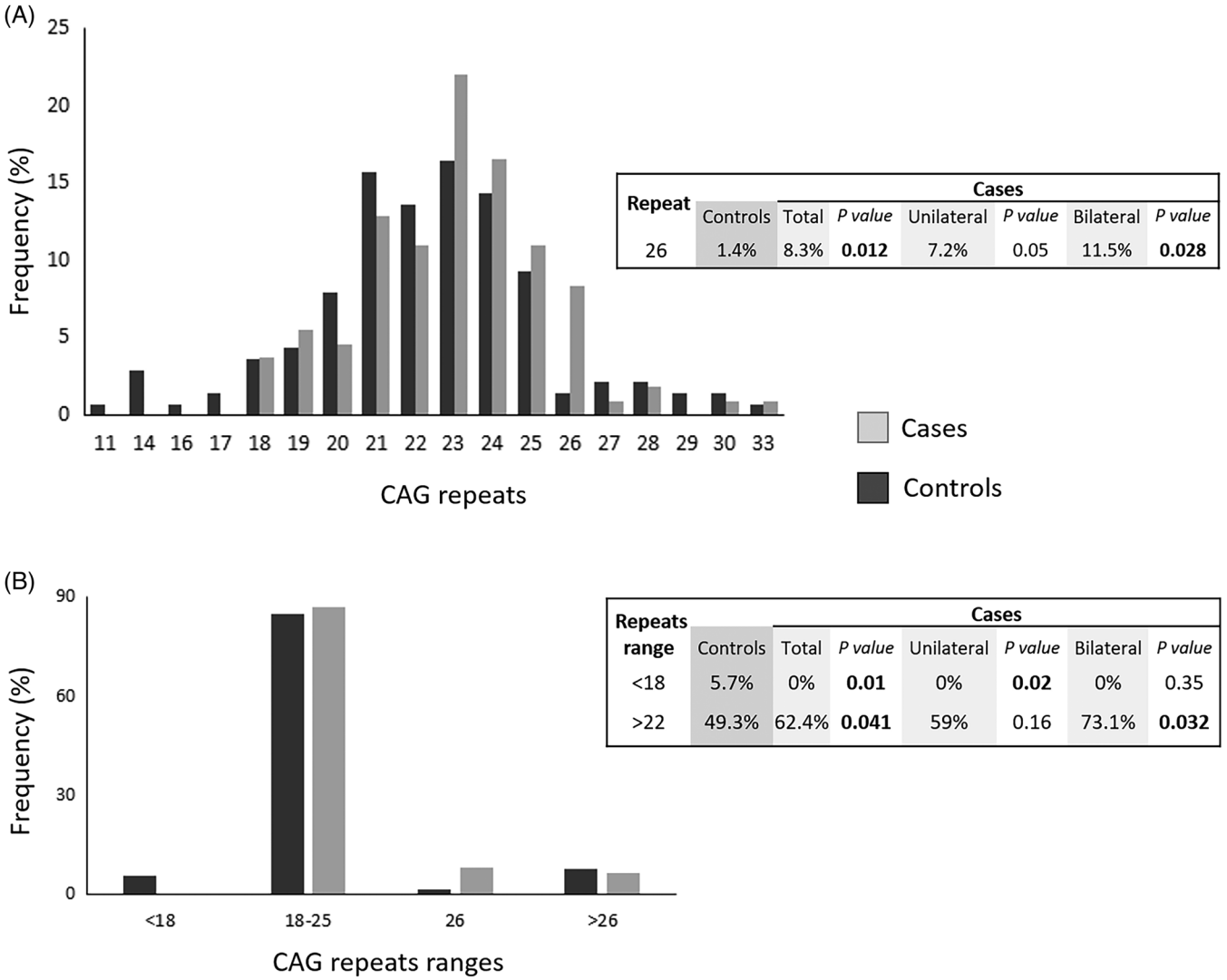
3.4 | CAG/GGN combination

When assessing the joint effect of both repeat polymorphisms, we observed that CAG26 was associated with GGN23 repeats in cases with bilateral cryptorchidism, and therefore we observed the same significant difference as for CAG26 (11.5% vs. 1.4%; p = 0.028). On the other hand, the CAG < 18 was associated preferentially with GGN≠23, and also showed a significant difference compared to controls (4.3% vs. 0%; p = 0.037). In addition, although we did not observe significant differences when evaluating the combinations of the CAG > 22 repeats with GGN23, we observed a trend for greater association with GGN23 in bilateral cryptorchidism compared with controls (42.3% vs. 25.7%, p = 0.09).

4 | DISCUSSION

The presence of undescended testicles at birth is a frequent finding and its incidence seems to be increasing according to some recent studies[1,27–31]; probably related to increased exposure to endocrine disruptors,[32] although more epidemiological studies are needed to confirm this hypothesis.[33] There are epidemiological studies in full-term newborns from Europe, the USA, and Africa, as well as in South America, showing an incidence of 1.2% to 9%[1,30,31,34–42].; which tends to decrease by one year of life,[30–32] or more precisely between 3 to 6 months due to the transient activation of the gonadal axis, in the so-called mini-puberty.[43]

The etiology of cryptorchidism is multifactorial and therefore, the effect of the CAG and GGN polymorphisms presented in this study may be considered as one of the possible causes. The information collected in different studies and a meta-analysis show a reasonable degree of agreement regarding the association of long repeats of the CAG / GGN polymorphisms with a greater risk of cryptorchidism.[20,22,44] In accordance, in vitro studies suggest a deleterious effect of long CAG and GGN repeats over androgen action.[45,46] Regarding the CAG polymorphism, prostatic and non-prostatic cell lines showed an inverse relationship between the transactivation activity of the androgen receptor and the number of CAG repeats within the normal range.[45] Although the length of GGN polymorphism shows no effect on the transactivation activity of the AR, it does affect inversely the level of AR protein expression, suggesting a role for RNA length on the translational process.[46]



Repeat	Controls	Cases					
		Total	*P value*	Unilateral	*P value*	Bilateral	*P value*
26	1.4%	8.3%	**0.012**	7.2%	0.05	11.5%	**0.028**

Repeats range	Controls	Cases					
		Total	*P value*	Unilateral	*P value*	Bilateral	*P value*
<18	5.7%	0%	**0.01**	0%	**0.02**	0%	0.35
>22	49.3%	62.4%	**0.041**	59%	0.16	73.1%	**0.032**

FIGURE 1 *CAG allele distribution*. (A) Bar chart displays the distributions of the CAG repeat lengths (11 to 33 repeats) in Controls (black bars) and Cases (grey bars). Statistical analysis by the Pearson chi-square test shows similar distribution ($p = 0.37$) between controls and total cases. Inserted table shows distribution of CAG26 allele between controls and cases (statistic by Fisher's exact test). (B) Bar chart displays the distribution of four CAG repeat ranges (< 18; 18−25; 26; > 26) in Controls (black bars) and Cases (grey bars). Statistical analysis by Pearson $\chi 2$ test shown significant different distribution ($p = 0.003$) between controls and cases. Inserted table shows the distribution of < 18CAG and > 22CAG alleles between controls and patients (statistic by Fisher's exact test).

The present study was performed in a typical Chilean mixed (European-Amerindian) population and shows a higher frequency of long CAG repeats (CAG > 22, CAG26) in the total cases, and more significantly in patients with bilateral inguinal cryptorchidism. Regarding the GGN repeats, however, we did not observe significant differences between patients and controls. When considering both polymorphisms together, we found that the combination CAG26/GGN23 seems to be a risk factor for bilateral cryptorchidism, whereas the combination CAG < 18/GGN≠23 may reduce the risk of cryptorchidism. In a similar Mexican and Brazilian population, previous studies showed that longer CAG repeats are more frequent in cases of inguinal cryptorchidism[22] most notably in bilateral cases,[20] which agree with our findings. In contrast, in a Hispanic cohort residing in California, the risk of cryptorchidism was associated with shorter CAG repeats.[21] This contradictory result, in an ethnically related population, may be explained by differences in the control subjects. The present study and those of Silva-Ramos (Brazil) and Landero-Huerta (Mexico) considered unrelated controls subject, whereas Davis-Dao and co-workers recruited cryptorchid patients and their mothers assigning the untransmitted allele of the mother as the control allele. Another difference, that might explain the discrepancy, is the inclusion of cryptorchid cases with comorbidities (15%) such as micropenis and syndromic cryptorchidism in Davis-Dao's cohort.

On the other hand, the studies by Radpour et al. in an Iranian cohort[47] and by Aschim et al.[6] in Swedish individuals showed that long GGN repeats are more frequent in patients with cryptorchidism than in controls, but this finding was not observed for CAG repeats. Furthermore, Ferlin et al., in an Italian cohort,[48] demonstrated that there

was no difference in the frequency of CAG or GGN repeats between patients with a history of cryptorchidism and controls; however, considering the combination of both repeats, they observed that in cases with bilateral cryptorchidism the combinations CAG21/ GGC18 (equivalent to GGN24) and CAG≥21/GGC≥18 were significantly more frequent compared with controls. Finally, a study in a Japanese cohort conducted by Sasagawa et al.[49] did not show any difference between patients and controls when considering CAG repeats. This variability may be related to ethnic reasons, as demonstrated by Ackerman et al. who compared CAG repeats distribution of four large cohorts from different ethnic origins: Afro-Caribbean; Caucasian (European ancestry); Hispanic (Mexican ancestry) and Thai; and observed significant differences in the distribution of this polymorphic repeat.[50] In addition, we cannot exclude that these differences between South-American and European or Asian population may be due to differences in the exposure to endocrine disrupting chemicals which might interact with predisposition genetics factors, such as AR CAG/GGN repeats, and possibly affect testicular descent. Finally, an interesting discrepancy arise from the analysis of European and Asian studies, but none of them differentiated inguinal from abdominal cryptorchidism cases.

An interesting result that merits some discussion is the observed higher frequency of the 26 CAG repeats in patients compared with controls [8.3% vs. 1.4%; p = 0.012]. It is noteworthy that there is no difference between patients and controls when similar repeats where analyzed, such as 25 or 27 CAG repeats. A meticulous study by Nenonen et al.,[17] using a AR reporter gene assay, demonstrated higher in vitro AR transactivational activity of the 22 CAG repeat variant compared with shorter (16) and longer (26) repeat variants. This observation challenges the known monotonic association between CAG repeat length with AR transactivational activity. In addition, this group showed lower amount of AR mRNA and protein from the 22 CAG variant, compared with shorter (16) and longer (26) repeats, suggesting a compensatory mechanism between gene expression and transactivational activity. Finally, we cannot rule out a dichotonic effect of CAG repeats variants over non-genomic AR actions.[51] Consequently, further studies will be necessary to clarify this possible dichotomic effect of CAG repeat length over AR transactivational activity or non-genomic AR actions.

The strengths of this study lie in the complete information available on the patients at the time of their surgical correction of cryptorchidism, including the surgical protocols, as well as the clinical and hormonal features of the subjects. This information allowed us to exclude cases of not true cryptorchidism (retractile or evanescent testicles), and possible causes not related to the androgen receptor gene polymorphisms, as well as cryptorchidism associated with a positive paternal history and syndromic cases. Thus, the detailed surgical information available allowed us to focus our study on true inguinal cryptorchidism, since androgenic action is more relevant in the second phase of testicular descent, that is, the transit of the testicles from the inguinal ring to the scrotum. In addition, for the South American / Hispanic American context, we evaluated a significant number of patients and control subjects. Finally, it should be noted that the hormonal description of the patients was in agreement with the surgical and clinical records, noting lower concentrations of AMH in bilateral compared with unilateral cases, as previously described.[52] Considering the consensus of the mutual modulatory link of genetic and environmental components in the development of testicular non-descent, it would have been desirable to have groups of cases and controls recruited at comparable ages. However, the fact that the control men were healthy and normozoospermic represents a plus.

In summary, this study, conducted in a carefully selected cohort of Chilean patients with isolated inguinal cryptorchidism, shows that long CAG repeats (CAG > 22) are associated with an increased risk of cryptorchidism, most notably for bilateral cases. In addition, we detected a higher prevalence of the allele of 26 CAG repeats. However, even CAG alleles longer than 26 might have a similar role, suggested by our finding in CAG > 22, but this cannot be determined with certainty, given the low prevalence in our population and the number of subjects studied. In addition, the combination of CAG26/GGN23 repeats may also represent a risk factor, and both the CAG < 18 and the CAG < 18/GGN≠23 allele combination may reduce the risk of cryptorchidism. The differential association of GGN23 and other than 23 with CAG alleles in this study deserves further investigation.

AUTHOR CONTRIBUTIONS

Conceptualization, funding acquisition, project administration, preparation and editing of the manuscript, and investigation: Fernando Rodríguez. Investigation: María José Godoy. Investigation: Eliana Ortiz. Data curation and editing of the manuscript: Andrés Benítez-Filselcker. Patient recruitment and characterization: María Teresa López. Editing of the manuscript: Fernando Cassorla. Conceptualization, funding acquisition, project administration, resources supervision, preparation, and editing of the manuscript: Andrea Castro.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Repositorio AcadÃmico de la Universidad de Chile at https://uchile.cl/u92726.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Meta-analysis and construction of simple-to-use nomograms for approximating testosterone levels gained from weight loss in obese men

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Abstract

Background: Obesity-induced hypogonadism, which manifests as erectile dysfunction and a lack of libido, is a less visible and under-recognized obesity-related disorder in men.

Objective: We examined the impact of weight loss on total (TT) and free testosterone (FT) levels, and constructed nomograms to provide an easy-to-use visual aid for clinicians.

Materials and methods: Meta-analysis was conducted using RevMan (v5.3) and expressed in standardized mean differences (SMD) for testosterone. Parallel-scale nomograms were constructed from baseline and target body mass index values to estimate the gain in testosterone.

Results: In total, 44 studies were included, comprising 1,774 participants and 2,159 datasets, as some studies included several datasets at different time points. Weight loss was controlled by low calorie diet (LCD) in 19 studies (735 participants, 988 datasets), by bariatric surgery (BS) in 26 studies (1,039 participants, 1,171 datasets), and by both in one study. The median follow-up was 26 weeks (interquartile range = 12–52). The range of baseline mean age was 21–68 yr, BMI: 26.2–71.2 kg/m^2, TT: 7–20.2 nmol/L and FT: 140–583 pmol/L. TT levels increased after weight loss by LCD: SMD (95%CI) = 2.5 nmol/L (1.9–3.1) and by BS: SMD = 7.2 nmol/L (6.0–8.4); the combined TT gain was 4.8 nmol/L (3.9–5.6). FT levels increased after weight reduction by LCD: SMD = 19.9 pmol/L (7.3–32.5) and by BS: SMD = 58.0 pmol/L (44.3–71.7); the combined gain was 42.2 pmol/L (31.4–52.9). Greater amounts of total and free testosterone could be gained by weight loss in men with higher baseline BMI, or lower levels of SHBG, TT and FT, while gain in TT was relatively greater in older and FT in younger age. Age-stratified nomograms revealed that compared to older men (> 40 yr), younger men (≤ 40 yr) gained less TT but more FT for a given weight loss.

Discussion and conclusion: Both TT and FT levels increased after weight loss, relatively greater with higher baseline BMI, or lower levels of SHBG, TT and FT. Nomograms

constructed from a large number of participants with a wide range of BMI and testosterone values provide an evidence-based and simple-to-use tool in clinical practice.

KEYWORDS
bariatric surgery, erectile dysfunction, Hypogonadotrophic hypogonadism, libido, low calorie diet, sexual function

1 | INTRODUCTION

Obesity, defined as a body mass index (BMI) ≥30 kg/m^2,[1] has become a global health condition over several decades. In 2016, the prevalences of obesity in American men and women were 37.5% and 39.5%, and UK men and women were 29.3% and 31.3%. By 2031, these figures are projected to reach 43.6% and 44.4% of US men and women, and 36.9% of both sexes in the UK. Corresponding figures from 17 other European countries were not far behind.[2] Obesity causes multiple health complications including type-2 diabetes, cardiovascular disease, hypertension, dyslipidaemia, osteoarthritis[3,4] and obstructive sleep apnea (OSA).[5] By contrast, obesity-induced hypogonadism, which manifests as erectile dysfunction and a lack of libido, is a less visible and under-recognized obesity-related disorder in men.

As the global prevalence of obesity continues to increase, the number of men with obesity-induced hypogonadism has also increased,[6] with progressively more men being referred to endocrine clinics.[7] Although the standard approach for hypogonadism is testosterone replacement therapy (TRT), it is essential to offer help with weight loss as the initial treatment for obese men to disrupt the obesity–hypogonadism cycle,[8] and at the same time improve or prevent other obesity-related health complications.[9] Weight loss, by lifestyle management, or more effectively by low calorie diet (LCD) or bariatric surgery (BS) in obese men, has been shown to improve androgen levels. However, most studies of weight loss, including meta-analyses,[10] have focused primarily on total testosterone (TT), while information on bioactive (unbound) free testosterone (FT) has been sparsely documented. In recent years, BS for obese men has proliferated worldwide. As a result, more studies, including randomized control trials (RCT) of large numbers of participants, have been published, providing the opportunity to examine more robustly the relationship between weight loss and changes to free testosterone, and determine factors influencing changes in testosterone induced by weight loss. In this study, we conducted a meta-analysis of the updated literature to assess the impact of weight loss on TT and in particular FT levels in obese men. We also characterized the influences of age, BMI, sex-hormone binding globulin (SHBG), luteinizing hormone (LH), and testosterone levels at baseline on changes to testosterone levels induced by weight loss. It was also possible to construct a set of nomograms to estimate the amount of testosterone gained from the extent of weight loss. This simple visual aid will be particularly useful in a clinical setting where consultation on the management of obesity-induced hypogonadism takes place, to help explain to the patient the potential improvement in gonadal function by weight loss.

2 | METHODS

After the proposal of the concept of the study (TSH), two investigators (GK and TSH) together discussed and devised the strategy for conducting a literature search and data extraction, following guidelines from the Cochrane and PRISMA recommendations for conducting meta-analyses.[11,12]

2.1 | Search strategy and data extraction

Two investigators (GK and TSH) independently performed a literature search and extracted data from papers using MEDLINE, Google Scholar, and the Cochrane Library Central Register of Controlled Trials databases up to June 2023. Their results were then compared before creating a final database for analysis key terms used were: obesity, body mass index, BMI, testosterone, sex steroids, gonadotropins, gonadotrophins, luteinizing hormone, LH, fertility, male, men, weight loss, lifestyle modification, LCD, and BS. No filters for language or data were applied and the Boolean operators "AND" and "OR" were used to combine search terms. Relevant studies were manually searched from references within the identified papers.

2.2 | Selection criteria

Studies examining the effect of weight loss on TT or FT in men were included irrespective of age, race, comorbidities, duration, or type of intervention. Those that fit the inclusion criteria were cohort studies or RCTs. Studies were excluded if they did not present numerical data for BMI or testosterone at baseline and end-point.

2.3 | Risk of bias assessment

The quality of the reports was evaluated independently by two investigators (GK and TSH) using the risk of bias in non-randomized studies of interventions (ROBINS-I) tool for cohort studies[13] and risk of bias assessed using Cochrane Collaboration's tool for RCTs.[14]

TABLE 1 Baseline characteristics in men undergoing weight management.

LCD studies (references)	Study information					Mean ± SD					
	Study year	Country (IBAN)[a]	Study design	Duration (weeks)	Datasets	Age (years)	BMI (kg/m^2)	SHBG (nmol/L)	TT (nmol/L)	FT (pmol/L)	LH (U/L)
Hoffer et al.[18]	1986	USA	CS	4	6	34 ± 7	33.1 ± 3.0	–	13.0 ± 1.0	277 ± 28	–
Pasquali et al.[19]	1988	Italy	CS	8	9	34 ± 11	43.4 ± 6.3	–	11.9 ± 4.2	–	8.4
Leenen et al.[20]	1994	NLD	CS	13	37	40 ± 6	30.7 ± 2.2	17.0 ± 6.0	12.7 ± 3.2	410 ± 80	–
Pritchard et al.[21]	1999	CAN	CS	13	14	21 ± 1	26.2 ± 5.5	–	12.3 ± 4.1	–	–
Kraemer et al.[22]	1999	USA	RCT	6	8	40 ± 6	33.1 ± 4.3	–	15.9 ± 7.7	–	–
Kraemer et al.[22]	1999	USA	RCT	12	8	40 ± 6	33.1 ± 4.3	–	15.9 ± 7.7	–	–
Kraemer et al.[22]	1999	USA	RCT	6	11	38 ± 9	31.3 ± 3.1	–	17.6 ± 4.3	–	–
Kraemer et al.[22]	1999	USA	RCT	12	11	38 ± 9	31.3 ± 3.1	–	17.6 ± 4.3	–	–
Kraemer et al.[22]	1999	USA	RCT	6	10	40 ± 6	29.2 ± 2.9	–	18.8 ± 5.9	–	–
Kraemer et al.[22]	1999	USA	RCT	12	10	40 ± 6	29.2 ± 2.9	–	18.8 ± 5.9	–	–
Kaukua et al.[23]	2003	FIN	RCT	11	19	46 ± 9	39.3 ± 3.3	29.0 ± 14.0	11.1 ± 3.4	201 ± 59	3.6 ± 2.2
Kaukua et al.[23]	2003	FIN	RCT	17	19	46 ± 9	39.3 ± 3.3	29.0 ± 14.0	11.1 ± 3.4	201 ± 59	–
Kaukua et al.[23]	2003	FIN	CS	32	19	46 ± 9	39.3 ± 3.3	29.0 ± 14.0	11.1 ± 3.4	201 ± 59	–
Niskanen et al.[24]	2004	FIN	CS	9	58	46 ± 8	36.1 ± 3.8	27.6 ± 11.9	11.2 ± 3.9	187 ± 63	–
Niskanen et al.[24]	2004	FIN	CS	52	58	46 ± 8	36.1 ± 3.8	27.6 ± 11.9	11.2 ± 3.9	187 ± 63	–
Khoo et al.[25]	2010	AUS	RCT	8	44	50 ± 11	34.4 ± 4.0	22.2 ± 8.7	20.2 ± 6.9	583 ± 222	–
Khoo et al.[26]	2011	AUS	RCT	8	19	58 ± 11	35.1 ± 4.3	22.5 ± 9.3	11.7 ± 3.6	285 ± 87	–
Khoo et al.[26]	2011	AUS	RCT	52	9	58 ± 11	35.1 ± 4.3	22.5 ± 9.3	11.7 ± 3.6	285 ± 87	–
Khoo et al.[26]	2011	AUS	RCT	8	12	62 ± 12	35.6 ± 4.8	30.4 ± 13.9	13.9 ± 3.3	296 ± 47	–
Khoo et al.[26]	2011	AUS	RCT	52	7	62 ± 12	35.6 ± 4.8	30.4 ± 13.9	13.9 ± 3.3	296 ± 47	–
Håkonsen et al.[27]	2011	DNK	CS	14	14	32 ± 10	37.5 ± 2.1	18.0 ± 7.6	9.2 ± 2.7	–	3.6 ± 1.3
Håkonsen et al.[27]	2011	DNK	CS	14	14	32 ± 10	43.9 ± 1.1	17.4 ± 7.6	8.0 ± 3.4	–	4.9 ± 2.3
Håkonsen et al.[27]	2011	DNK	CS	14	15	32 ± 10	53.5 ± 3.7	22.8 ± 9.1	7.0 ± 1.5	–	3.9 ± 1.8
Jaffar et al.[28]	2018	India	CS	52	105	33 ± 8	33.2 ± 5.1	19.2 ± 10.4	9.4 ± 7.2	–	–

(Continues)

TABLE 1 (Continued)

LCD studies (references)	Study information					Mean ± SD					
	Study year	Country (IBAN)[a]	Study design	Duration (weeks)	Datasets	Age (years)	BMI (kg/m^2)	SHBG (nmol/L)	TT (nmol/L)	FT (pmol/L)	LH (U/L)
Armamento-Villareal et al.[29]	2016	USA	RCT	26	9	68 ± 4	39.1 ± 4.5	38.8 ± 14.4	7.3 ± 4.9	–	–
Armamento-Villareal et al.[29]	2016	USA	RCT	52	9	68 ± 4	39.1 ± 4.5	38.8 ± 14.4	7.3 ± 4.9	–	–
Armamento-Villareal et al.[29]	2016	USA	RCT	26	10	72 ± 5	35.9 ± 2.8	43.9 ± 16.4	9.5 ± 5.0	–	–
Armamento-Villareal et al.[29]	2016	USA	RCT	52	10	72 ± 5	35.9 ± 2.8	43.9 ± 16.4	9.5 ± 5.0	–	–
Armamento-Villareal et al.[29]	2016	USA	RCT	26	12	69 ± 5	35.1 ± 5.2	46.6 ± 24.9	9.3 ± 4.3	–	–
Armamento-Villareal et al.[29]	2016	USA	RCT	52	12	69 ± 5	35.1 ± 5.2	46.6 ± 24.9	9.3 ± 4.3	–	–
Moran et al.[30]	2016	AUS	RCT	12	118	50 ± 10	33.3 ± 4.1	23.5 ± 11.0	13.8 ± 4.0	229 ± 74	–
Moran et al.[30]	2016	AUS	RCT	52	118	50 ± 10	33.3 ± 4.1	23.5 ± 11.0	13.8 ± 4.0	229 ± 74	–
De Lorenzo et al.[31]	2018	ITA	CS	13	14	47 ± 14	36.2 ± 7.6	–	10.4 ± 2.8	–	–
Samavat et al.[32]	2018	ITA	RCT	26	8	41 ± 7	47.2 ± 4.2	31.1 ± 11.1	8.9 ± 3.6	179 ± 68	–
Jensterle et al.[33]	2019		RCT	16	15	49 ± 10	39.0 ± 9.0	20.2 ± 5.4	7.2 ± 3.2	170 ± 70	–
Jensterle et al.[33]	2019	SVN	RCT	16	15	44 ± 12	43.2 ± 7.5	26.3 ± 13.6	7.6 ± 1.5	170 ± 40	–
Cignarelli et al.[34]	2023	ITA	CS	1	22	39 ± 12	36.4 ± 5.0	21.9 ± 10.8	8.7 ± 3.9	205 ± 55	–
Cignarelli et al.[34]	2023	ITA	CS	4	22	39 ± 12	36.4 ± 5.0	21.9 ± 10.8	8.7 ± 3.9	205 ± 55	–
Mongioi et al.[35]	2020	ITA	CS	8	34	46 ± 15	38.6 ± 6.4	–	13.3 ± 4.3	–	3.7 ± 1.7
Mongioì et al.[35]	2020	ITA	CS	8	4	46 ± 15	28.4 ± 0.8	–	12.4 ± 2.0	–	3.6 ± 0.4
La Vignera et al.[36]	2023	ITA	CS	12	20	49 ± 5	32.0 ± 3.1	–	6.2 ± 1.3	–	2.6 ± 0.6
BS studies (references)								–	–	–	–
Bastounis et al.[37]	1998	GRC	CS	52	19	35 ± 8	57.1 ± 7.4	28.3 ± 16.2	12.2 ± 6.2	416 ± 207	2.4 ± 1.6
Alagna et al.[38]	2006	ITA	CS	52	20	42 ± 11	47.3 ± 13.1	–	9.7 ± 3.7	–	2.4 ± 1.6
Hammoud et al.[39]	2009	USA	RCT	104	64	49 ± 10	46.2 ± 7.2	–	11.8 ± 5.9	224 ± 79	–
Omana et al.[40]	2009	USA	CS	52	10	48 ± –	48 ± –	–	10.6 ± 5.1	199 ± –	–
Pellitero et al.[41]	2012	ESP	CS	52	33	41 ± 10	50.3 ± 6.1	18.3 ± 11.8	8.6 ± 3.2	230 ± 83	–
Woodard et al.[42]	2012	USA	CS	13	64	48 ± 10	48.2 ± 12.0	–	9.0 ± 4.2	–	–
Woodard et al.[42]	2012	USA	CS	26	64	48 ± 10	48.2 ± 12.0	–	9.0 ± 4.2	–	–
Woodard et al.[42]	2012	USA	CS	52	64	48 ± 10	48.2 ± 12.0	–	9.0 ± 4.2	–	–
Reis et al.[43]	2012	BRA	RCT	104	10	37 ± 12	55.7 ± 7.8	–	11.8 ± 4.5	347 ± 156	4.7 ± 1.5

(Continues)

TABLE 1 (Continued)

LCD studies (references)	Study information					Mean ± SD					
	Study year	Country (IBAN)[a]	Study design	Duration (weeks)	Datasets	Age (years)	BMI (kg/m^2)	SHBG (nmol/L)	TT (nmol/L)	FT (pmol/L)	LH (U/L)
Reis et al.[43]	2012	BRA	RCT	17	10	37 ± 12	55.7 ± 7.8	–	11.8 ± 4.5	347 ± 156	4.7 ± 1.5
Zhu et al.[43]	2019	CHN	RCT	52	20	35 ± 11	37.2 ± 5.6	12.3 ± 7.4	10.4 ± 4.5	155 ± 43	5.9 ± 3.6
Zhu et al.[43]	2019	CHN	RCT	52	45	30 ± 9	43.6 ± 6.1	11.1 ± 4.5	6.9 ± 4.0	163 ± 71	4.7 ± 2.1
Mora et al.[44]	2013	ESP	CS	52	39	44 ± 10	46.9 ± 7.8	–	8.9 ± 4.2	199 ± 92	3.7 ± 1.9
Botella et al.[45]	2013	ESP	CS	24	20	40 ± 10	47.1 ± 6.0	21 ± 9.5	10.1 ± 3.5	258 ± 100	3.2 ± 2.2
Facchiano et al.[46]	2013	ITA	CS	24	20	41 ± 14	43.6 ± 5.8	19 ± 6.4	8.1 ± 2.0	204 ± 70	2.8 ± 1.9
Luconi et al.[47]	2013	ITA	CS	26	24	43 ± 11	43.9 ± 9.6	19.2 ± 9.4	8.8 ± 2.7	–	2.3 ± 1.6
Luconi et al.[47]	2013	ITA	CS	52	24	43 ± 11	43.9 ± 9.6	19.2 ± 9.4	8.8 ± 2.7	–	2.3 ± 1.6
Mihalca et al.[48]	2014	ROU	CS	52	28	43 ± 10	50.1 ± 11.2	23.4 ± 17.5	8.3 ± 3.2	–	3.8 ± 1.8
Globerman et al.[49]	2015	ISR	CS	46	17	38 ± 10	44.3 ± 6.8	–	13.4 ± 7.2	–	4.9 ± 3.2
Sarwer et al.[50]	2015	USA	CS	52	32	48 ± 10	45.1 ± 7.6	21.6 ± 8.1	11.0 ± 3.9	267 ± 87	3.8 ± 3.2
Sarwer et al.[50]	2015	USA	CS	104	32	48 ± 10	45.1 ± 7.6	21.6 ± 8.1	11.0 ± 3.9	267 ± 87	3.8 ± 3.2
Sarwer et al.[50]	2015	USA	CS	156	32	48 ± 10	45.1 ± 7.6	21.6 ± 8.1	11.0 ± 3.9	267 ± 87	3.8 ± 3.2
Sarwer et al.[50]	2015	USA	CS	209	32	48 ± 10	45.1 ± 7.6	21.6 ± 8.1	11.0 ± 3.9	267 ± 87	3.8 ± 3.2
El Bardisi et al.[51]	2016	QAT	CS	52	46	37 ± 18	71.4 ± 13.3	–	16.4 ± 6.3	–	2.0 ± 1.3
Boonchaya-anant et al.[52]	2016	THA	CS	4	29	31 ± 8	56.9 ± 11.7	21.6 ± 10.7	8.4 ± 4.7	214 ± 113	–
Boonchaya-anant et al.[52]	2016	THA	CS	26	29	31 ± 8	56.9 ± 11.7	21.6 ± 10.7	8.4 ± 4.7	214 ± 113	–
Öncel et al.[53]	2021	TUR	CS	26	40	36 ± 4	47.2 ± 6.6	–	13.9 ± 4.1	–	–
Samavat et al.[32]	2018	ITA	RCT	26	23	38 ± 9	45.8 ± 7.4	20 ± 8.8	9.0 ± 4.0	228 ± 94	2.5 ± 1.7
Calderón et al.[54]	2019	ESP	CS	104	20	40 ± 8	50.0 ± 10.0	23.6 ± 8.7	12.6 ± 5.7	277 ± 139	3.5 ± 1.0
Arolfo et al.[55]	2020	ITA	CS	52	44	43 ± 4	44.3 ± 5.8	16.5 ± 8.8	8.0 ± 4.1	267 ± 77	4.1 ± 2.2
Di Vincenzo et al.[56]	2020	ITA	RCT	4	29	41 ± 10	43.4 ± 8.5	–	10.8 ± 3.5	–	3.6 ± 1.3
Machado et al.[57]	2021	BRA	CS	26	33	36 ± 8	43.8 ± 7.8	19.8 ± 13.7	7.0 ± 3.9	201 ± 97	3.4 ± 1.8
Van de Velde et al.[58]	2021	BEL	CS	3	14	52 ± 11	42.6 ± 2.2	34.5 ± 11.0	9.3 ± 2.7	140 ± 44	4.2 ± 1.5
Van de Velde et al.[58]	2021	BEL	CS	6	14	52 ± 11	42.6 ± 2.2	34.5 ± 11.0	9.3 ± 2.7	140 ± 44	4.2 ± 1.5
Van de Velde et al.[58]	2021	BEL	CS	26	14	52 ± 11	42.6 ± 2.2	34.5 ± 11.0	9.3 ± 2.7	140 ± 44	4.2 ± 1.5
Van de Velde et al[58]	2021	BEL	CS	52	14	52 ± 11	42.6 ± 2.2	34.5 ± 11.0	9.3 ± 2.7	140 ± 44	4.2 ± 1.5
Cobeta et al.[59]	2022	ESP	RCT	26	20	46 ± 9	45.0 ± 6.9	–	12.0 ± 4.0	255 ± 60	–
Cobeta et al.[59]	2022	ESP	RCT	26	20	51 ± 9	43.7 ± 7.2	–	12.1 ± 3.0	251 ± 47	–
Chen et al.[60]	2023	CHN	CS	52	59	32 ± 7	41.3 ± 7.6	–	10.0 ± 3.5	–	–

[a]Country codes according to IBAN, International Bank Account Number (https://www.iban.com/country-codes); SD, standard deviation; BMI, body mass index; TT, total testosterone; FT, free testosterone; LH, luteinizing hormone; LCD, low calorie diet; BS, bariatric surgery.

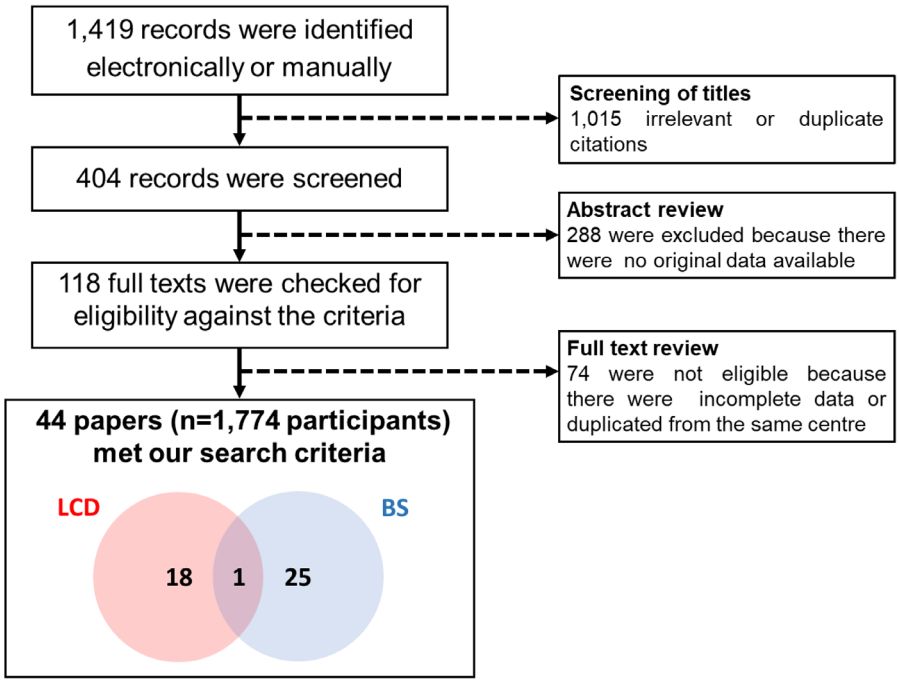


FIGURE 1 Quality of reporting of meta-analyses (QUOROM) flow chart of literature search.

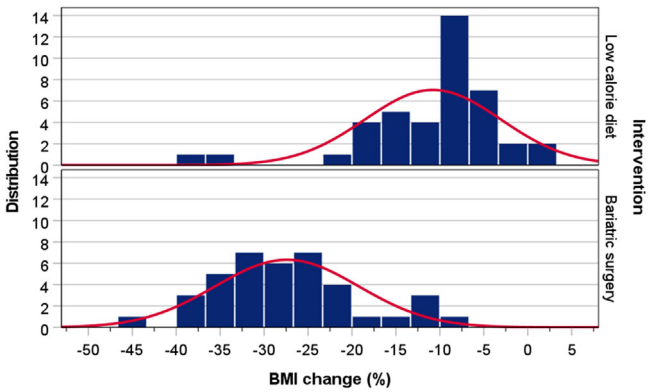


FIGURE 2 Distribution of percentage change in body mass index after intervention by low calorie diet (upper panel) and by bariatric surgery (lower panel).

2.4 | Data preparation

For consistency, all data were converted to système international d'unités (SI units). Reported standard errors (SE) were converted to standard deviations (SD) using the formula $SD = SE \times \sqrt{n}$, where n is the sample size.

After the literature search, data extraction and risk of bias assessment conducted independently by the two investigators (GK and TSH), their findings were compared and resolved where there was a disagreement. A final database was completed for analyses. Subsequently, the results generated were discussed in weekly "Lab meetings" between the pair of investigators at every stage up to the point of completion of the paper.

2.5 | Statistical analysis

Meta-analyses were conducted using Review Manager (RevMan, Version 5.3. Copenhagen: The Nordic Cochrane Centre, the Cochrane Collaboration, 2014). The standardized mean difference (SMD, also known as Cohen's *d*) was used to determine the effect size on testosterone and SHBG to accommodate for a variety of ways by which they were measured. The SMD expresses the size of the intervention effect in each study relative to the variability observed in that particular study.[15] Positive values indicated a gain of the variable with the intervention. The mean difference (MD) was used on the original scale of measurement to determine the effect size on TT and FT levels, as well as SHBG and LH. Pooled estimates of outcomes were obtained via the DerSimonian and Laird method using a random effects model.[16] Statistical significance threshold was accepted as $P < 0.05$, and heterogeneity of study results was assessed by the I^2 statistic.[17]

The influences of baseline characteristics of individuals on changes in testosterone levels induced by weight loss was assessed by multivariate regression, analyzing all predictive variables simultaneously to adjust for each other, using IBM SPSS Statistics, v28.0 (IBM Corp., Armonk, NY).

2.6 | Nomogram construction

Data were used to develop regression equations based on the values of baseline BMI and target BMI (after weight loss from an intervention) to estimate the percentage and amounts of TT (nmol/L) and FT (pmol/L)

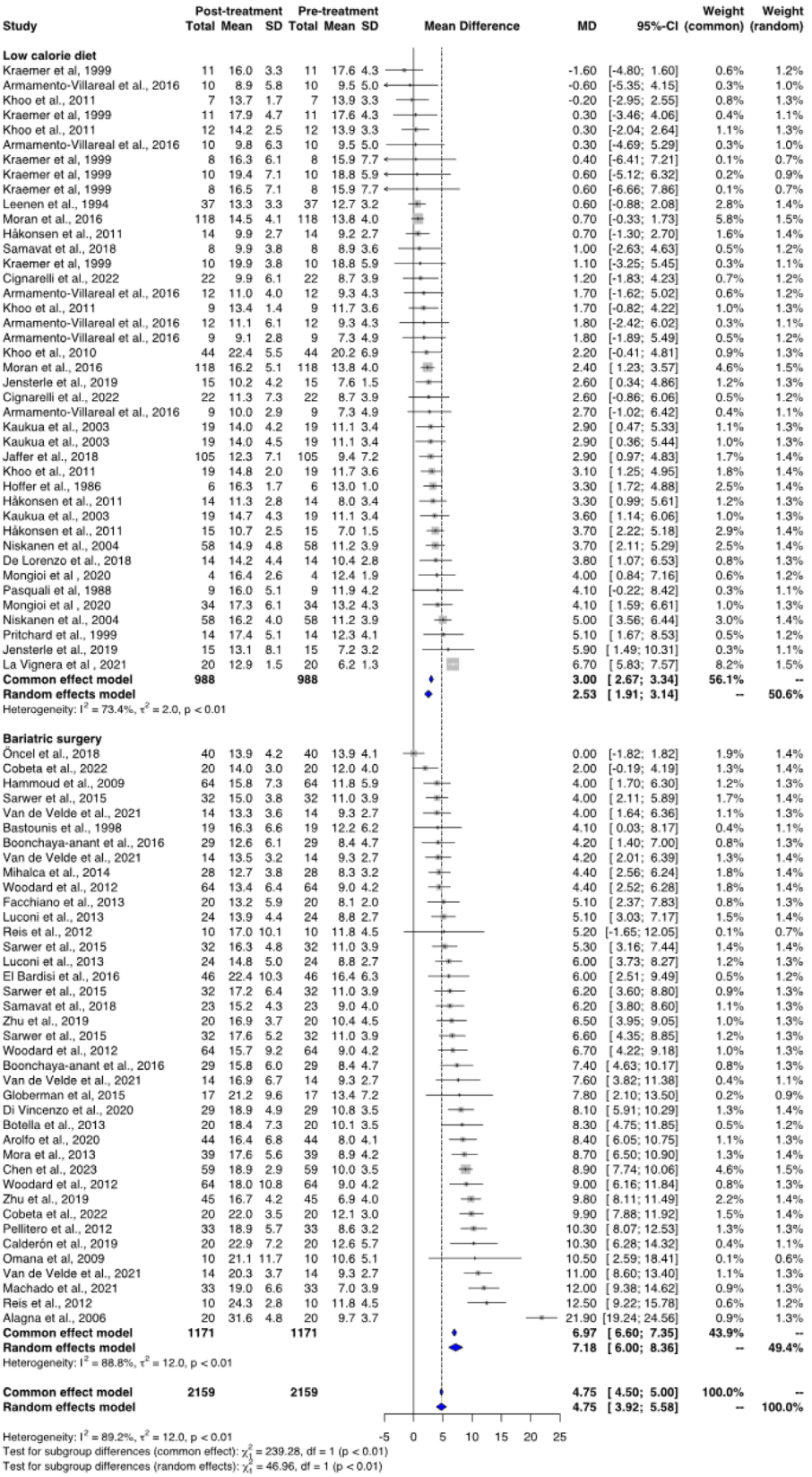


FIGURE 3 Changes in total testosterone (A), SHBG (B), free testosterone (C), and LH (D) in relation to weight loss in obese men in cohort studies and in RCTs. LH, luteinizing hormone; RCT, randomized control trials; SHBG, sex-hormone binding globulin.

gained from weight loss. The regression equations were then translated into parallel-scale nomograms using R software (https://www.r-project.org/), presented for all ages and for two separate age groups using a cut-off of 40 years.

3 | RESULTS

3.1 | Baseline characteristics

From a TT of 1,419 articles initially identified on MEDLINE, Google Scholar, and the Cochrane Library Central Register of Controlled Trials databases, 1,015 titles were found to be not relevant or duplicate. The remaining 404 articles were screened and 288 were excluded because no original data were available. Further review of the remaining 118 full texts to check for eligibility against criteria showed that 74 articles were not eligible because there were incomplete data or they were duplicated from the same center. The remaining 44 studies comprising 1,774 participants met the search criteria (Figure 1).[18–60] In total, there were 2,159 datasets as some studies included several datasets from the same group of participants, but at different time points. The ranges (lowest to highest) of baseline mean age, 21–68 years; BMI. 26.2–71.2 kg/m^2; TT, 7.0–20.2 nmol/L; and FT, 140–583 pmol/L, were recorded. Among all 44 papers, one used both LCD and BS treatments. Weight loss was controlled by LCD ($n = 735$ participants, 988 datasets) in 19 studies, and by BS in 26 studies ($n = 1{,}039$ participants, 1,171 datasets). Among LCD studies, 11 were cohort studies and eight RCT, and among BS papers, 20 were cohort studies and six RCT. Among all 44 papers, one used both LCD and BS treatments. There were 28 studies (11 controlled by LCD and 17 by BS) comprising 1,021 participants (1,317 datasets) available for analysis of free testosterone. The median and interquartile range (IQR) follow-up duration was 26 weeks (12–52) (Table 1). The most common groups studied were between 30 and 50 years old (Figure S1). The median weight reduction by LCD was 8.6% (6.2–14.0) and by BS was 28.7% (22.6–33.3) (Figure 2),

Multivariate regression analysis of baseline factors including BMI, TT and FT and SHBG levels were performed simultaneously, that is, adjusted for each other. Analysis showed that greater amounts of TT and FT could be gained by weight loss in men with higher baseline BMI (positive regression coefficients), or with lower baseline levels of SHBG, TT and FT (negative regression coefficients). A greater gain of TT was observed in older age, while a greater gain in FT was seen in younger age. SHBG increased after weight loss among men with higher baseline BMI and TT, lower baseline SHBG and FT, and increasingly older age (Table 2).

TABLE 2 Multivariate regression analysis to assess individual factors influencing a gain in total and free testosterone after weight loss in obese men.

Influencing factors of gain in total testosterone	Coefficient (β)	R^2 (%)	P
Baseline body mass index (kg/m^2)	0.266	43.4	<0.001
Baseline total testosterone (nmol/L)	−0.008		
Baseline free testosterone (pmol/L)	−0.219		
Baseline sex hormone binding protein (nmol/L)	−0.012		
Age (years)	0.056		
Intercept	−0.711		
Influencing factors of gain in SHBG			
Baseline body mass index (kg/m^2)	1.504	34.7	<0.001
Baseline total testosterone (nmol/L)	0.425		
Baseline free testosterone (pmol/L)	−0.558		
Baseline sex hormone binding protein (nmol/L)	−0.010		
Age (years)	0.687		
Intercept	−66.496		
Influencing factors of gain in free testosterone			
Baseline body mass index (kg/m^2)	1.965	47.7	<0.001
Baseline total testosterone (nmol/L)	−2.691		
Baseline free testosterone (pmol/L)	−1.794		
Baseline sex hormone binding protein (nmol/L)	−0.052		
Age (years)	−0.818		
Intercept	83.008		

Abbreviation: SHBG, sex hormone binding protein.

(B: SHBG)

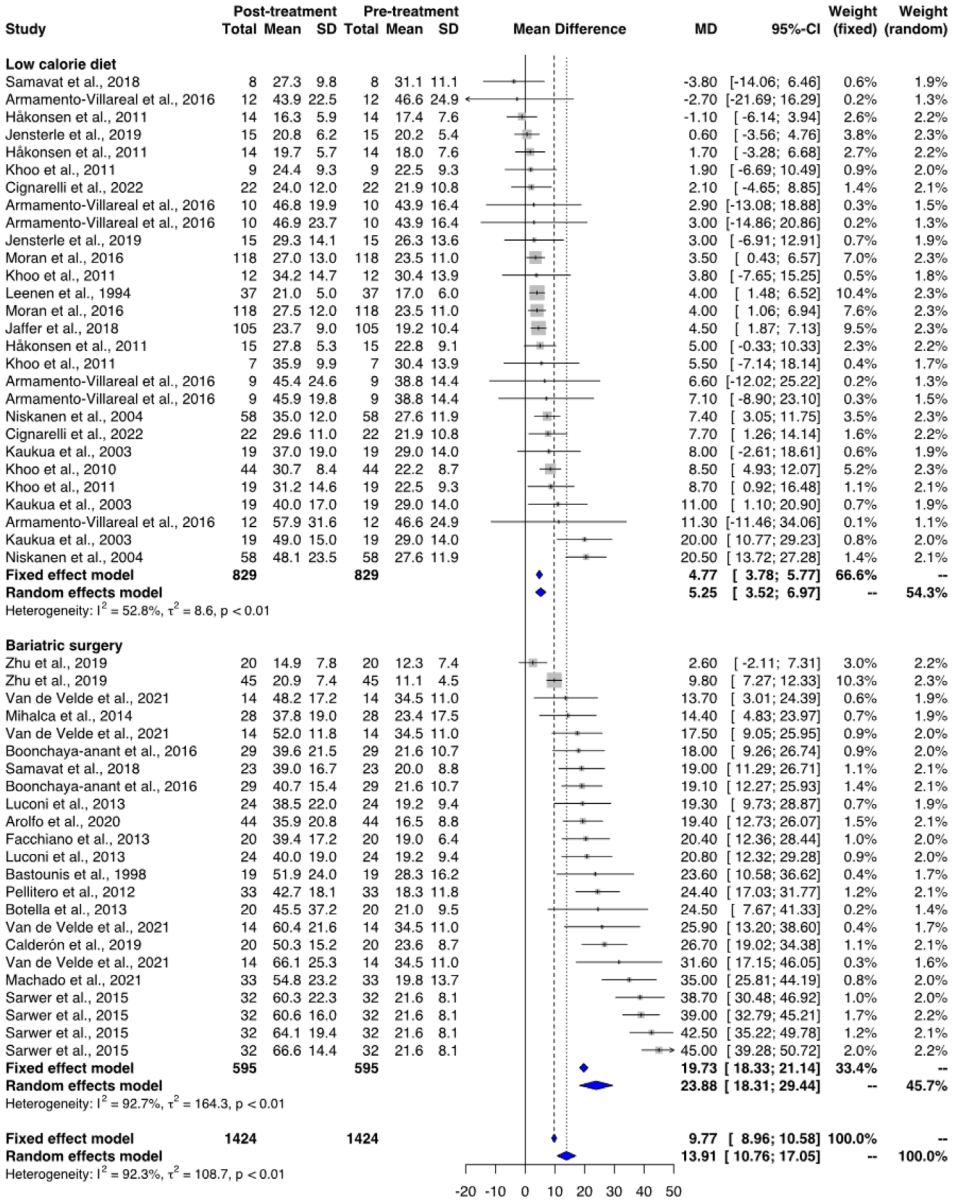


FIGURE 3 Continued

3.2 | Meta-analysis

Overall, among patients undergoing weight loss in cohort studies and RCTs, the levels of TT increased after weight loss induced by LCD: SMD = 2.5 nmol/L (95% CI = 1.9–3.1) and by BS: SMD = 7.2 nmol/L (95% CI = 6.0–8.4), giving an overall gain in TT of 4.8 nmol/L (95% CI = 3.9–5.6) (Figure 3A). This was accompanied by increases in SHBG levels through weight loss by LCD: SMD = 5.3 nmol/L (95% CI = 3.5–7.0) and by BS: SMD = 23.9 nmol/L (95% CI = 18.3–29.4), giving the overall gain in SHBG of 13.9 nmol/L (95% CI = 10.8–17.1) (Figure 3B). Similarly, the levels of free testosterone also increased after weight reduction by LCD: SMD = 19.9 pmol/L (95% CI = 7.3–32.5) and by BS: SMD = 58.0 pmol/L (95% CI = 44.3–71.7), giving an overall gain in TT of 42.2 pmol/L (95% CI = 31.4–52.9) (Figure 3C). The levels of LH did not increase with weight loss by LCD, but significantly increased after weight reduction by BS: SMD = 0.83 U/L (95% CI = 0.62–1.04), giving an overall gain in LH of 0.68 U/L (95% CI = 0.44–0.92) (Figure 3D).

With respect to analysis of RCTs only, there was a gain in TT through weight loss by LCD: SMD = 1.7 nmol/L (95% CI = 1.1–2.2) and by BS: SMD = 7.2 nmol/L (95% CI = 5.0–9.4), giving an overall gain in TT of 3.2 nmol/L (95% CI = 2.0–4.4) (Figure 4A); and a gain in free testosterone through LCD: SMD = 22.0 pmol/L (95% CI = 5.8–38.1) and by BS: SMD = 60.6 pmol/L (95% CI = 43.8–77.4), giving an overall gain in TT of 39.0 pmol/L (95% CI = 23.9–54.2) (Figure 4B).

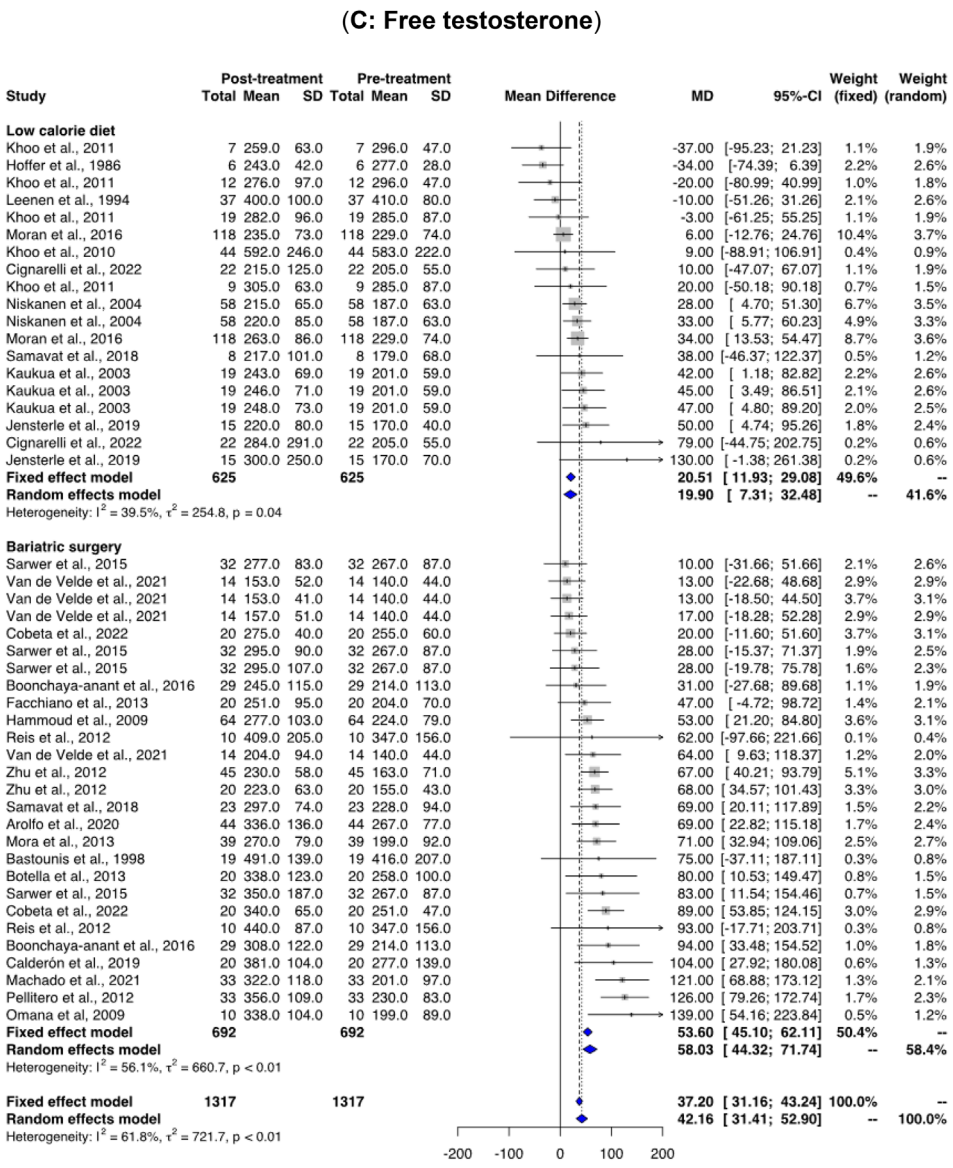


FIGURE 3 Continued

There was little evidence of bias due to confounding factors or in the selection of participants. Four studies were considered to suffer bias due to deviations from intended interventions as weight-loss treatment was relatively short (≤ 4 weeks),[18,34,52,56] and one LCD study with the addition of the glucagon-like peptide-1 agonist Exenatide),[33] and two LCD studies used very low-calorie ketogenic diet (35,36).[35,36] Data for free testosterone were not reported in 12 studies.[22,28,35,36,38,42,47–49,51,53,60] None of the studies had a bias in the measurement of outcomes, while there was insufficient information from any of the studies for assessing bias in the selection of the reported result (Figure 5).

The results of leave-one-out sensitivity analyses (Tables S1 and S2) showed that changes in TT induced by weight loss in obese men both in cohort studies and in RCTs were not substantially driven by any individual study. This indicates that the results from this meta-analysis were statistically robust.

3.3 | Nomogram construction

Regression analysis was conducted to estimate the amounts of TT and FT gained from weight loss, and used to construct a set of parallel-scale nomograms for men of all ages (Figure 6A) or for two different age groups, stratified at 40 years (Figure 6B,C). An estimated amount of TT or FT to be gained from weight loss could easily be inferred from nomograms by drawing a line (an isopleth) connecting an individual's current BMI to the desired target BMI. A similar set of nomograms was constructed for gain in free testosterone in relation to weight loss for all ages (Figure 6D) and for the two age groups (Figure 6E,F). Compared

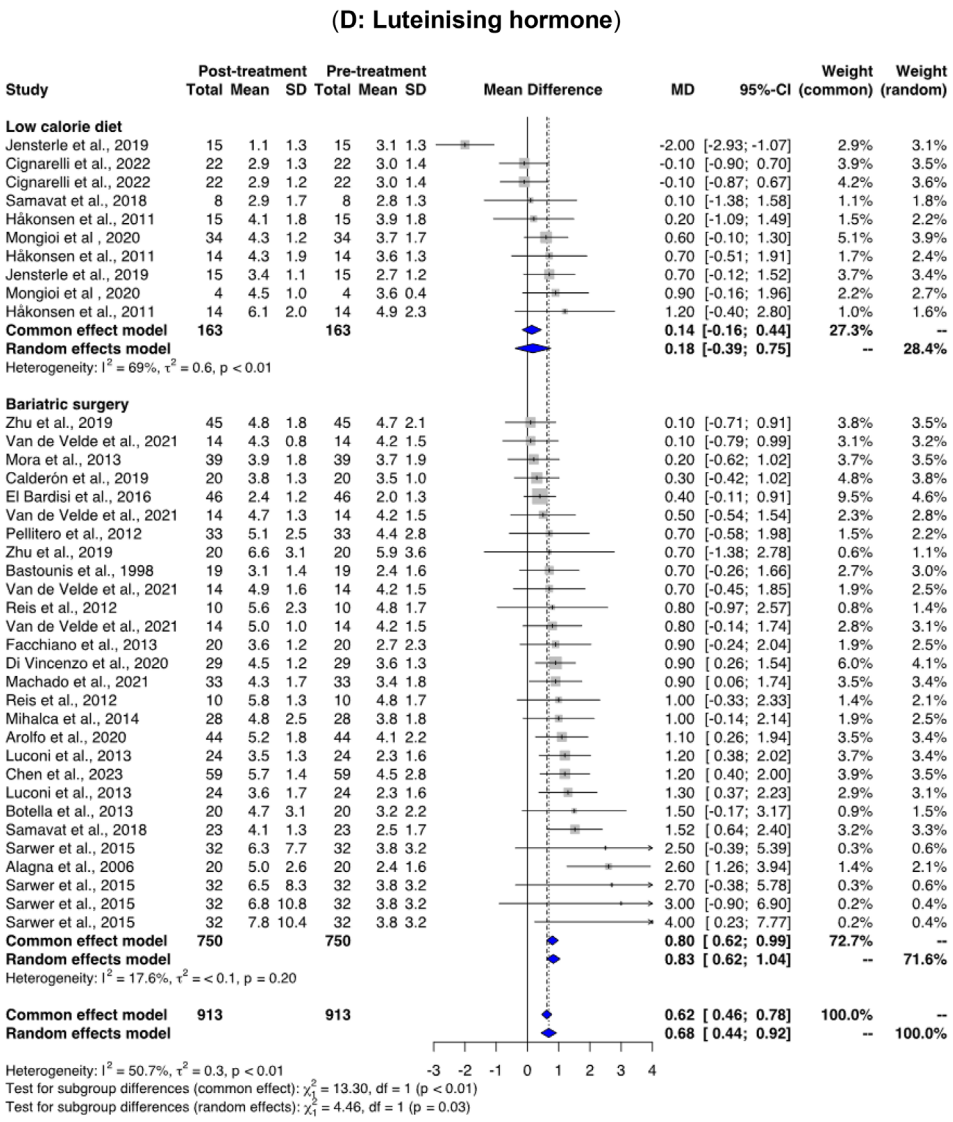


FIGURE 3 Continued

to older men (> 40 yr), younger men (≤ 40 yr) gained less TT but more FT for a given amount of weight loss.

4 | DISCUSSION

In this meta-analysis of 2,159 datasets from 1,774 participants, including 1,317 datasets from 1,021 participants with measurement of free testosterone, we observed that weight loss by LCD, and more so by BS, significantly increased the levels of TT as well as FT. Multivariate regression analysis showed that greater amounts of TT and FT could be gained by weight loss in men relatively greater with higher baseline BMI, or lower levels of SHBG, TT and FT. On the other hand, gain in TT was relatively greater in older men and FT in younger men. The set of nomograms constructed from these data provide clinicians with an evidence-based and easy-to-use visual tool in a clinical setting, for approximating the amount of testosterone a patient could gain from a certain amount of weight loss. As far as we are aware, there are no such studies available in the existing literature.

Findings from this study and previous studies[10,61] support the benefit of weight loss leading to a gain in TT and FT levels, as well as improvement in many obesity-related conditions including type-2 diabetes, metabolic syndrome,[9] OSA[62] and osteoarthritis.[63] Thus, changes in testosterone could serve as a barometer for an overall health status of a male individual. A meta-analysis conducted by Cignarelli et al. had shown that continuous positive airway pressure treatment for OSA in obese men did not alter their testosterone levels.[64] This evidence indicates that testosterone and OSA, like other metabolic disorders, are among complications of obesity rather than causally related, and all of which could be improved by weight reduction. Our findings of an overall gain in TT and FT from weight loss are broadly in agreement with older and smaller meta-analyses of 24 papers comprising 479 participants (but only seven papers available at the time for analysis of free testosterone).[10] These authors derived

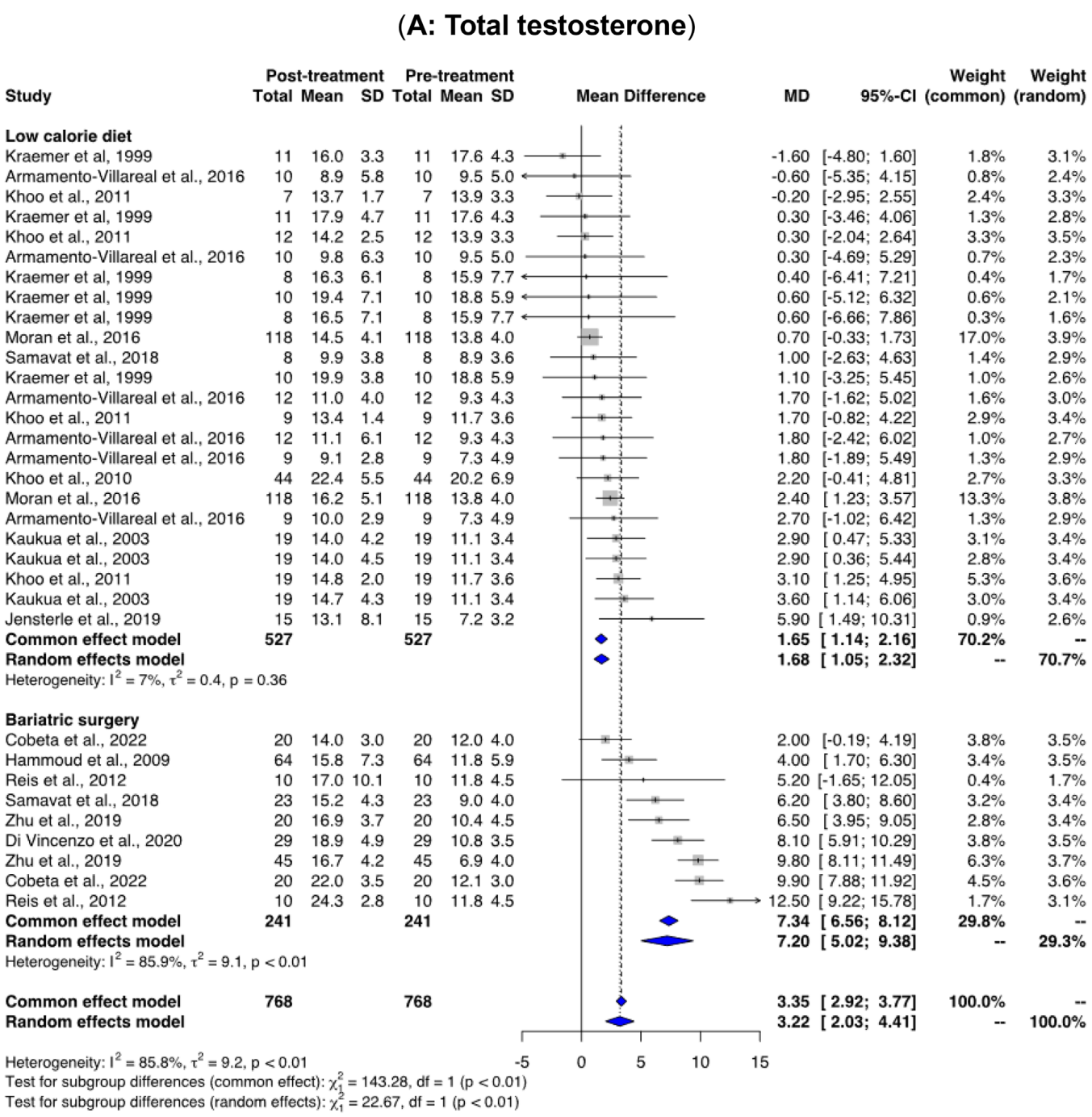


FIGURE 4 Changes in total testosterone (A) and free testosterone (B) in relation to weight loss in obese men in RCTs. RCT, randomized control trials.

several univariate regression equations and found higher amount of TT testosterone gained in younger age. Our meta-analysis comprised more up-to-date publications, with a wider age-range, and substantially more papers (n = 42) as well as participants (n = 1,736). This has enabled us to examine the effects of weight reduction on TT as well as FT in RCTs, and further extend our work to the construction of evidence-based nomograms. In a busy clinic, nomograms are convenient both for the clinician and patient in the discussion of target BMI and the expected gain in testosterone from weight loss in a realistic manner. Of importance, our study also consisted of one of the largest collections of papers (n = 28) and participants (n = 1,021) on changes to FT levels induced by weight loss. FT is the unbound and the bioactive form of sex steroid hormone which has a pleiotropic action on body tissues, thus is clinically more relevant than TT.[65,66] This is because about 70% of TT is bound with high affinity to SHBG, and about 20%–30% is bound weakly to albumin. SHBG levels progressively increase with age but decline with increasing degree of adiposity,[67] while paradoxically, weight loss induces an increase in SHBG levels as observed in our

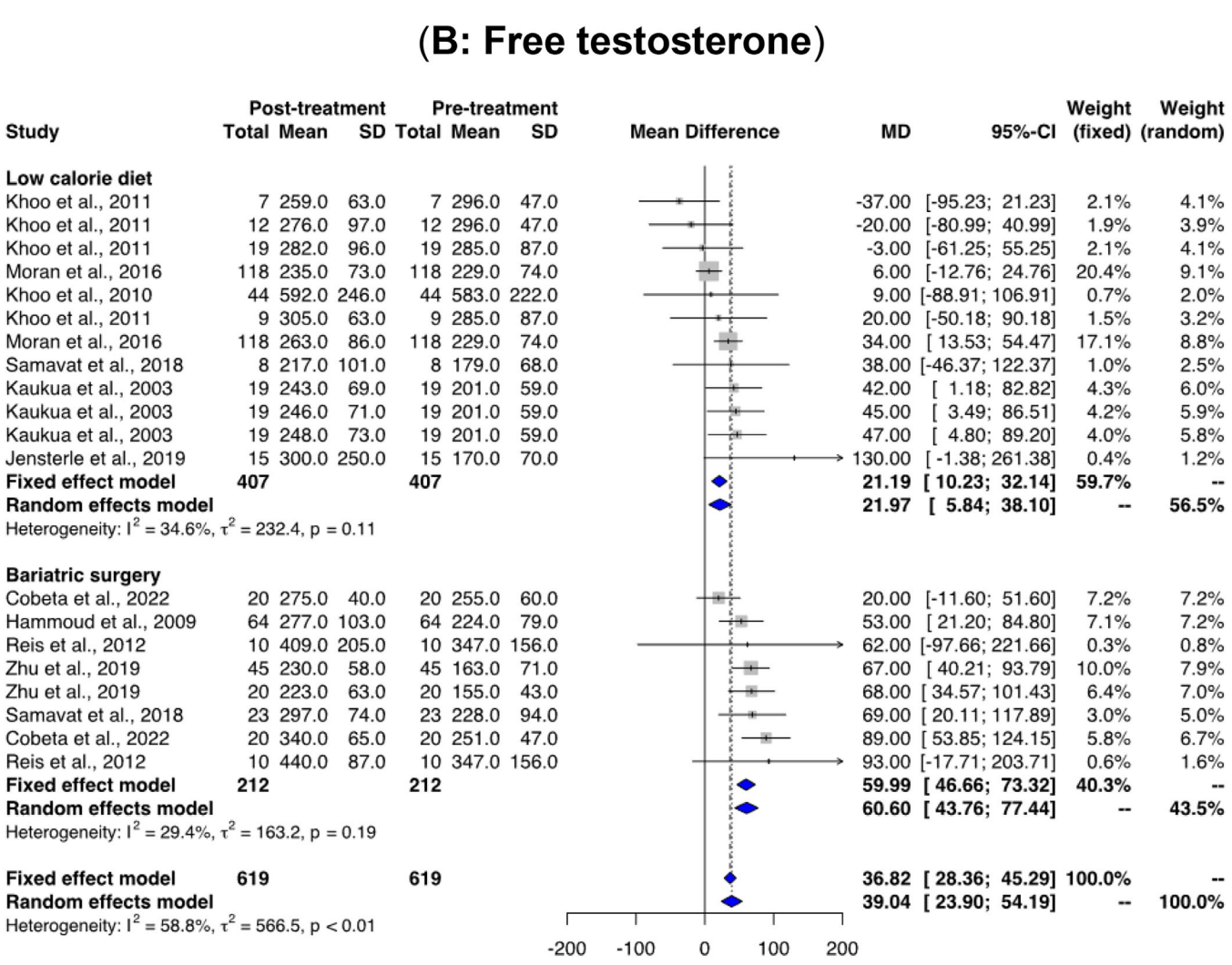
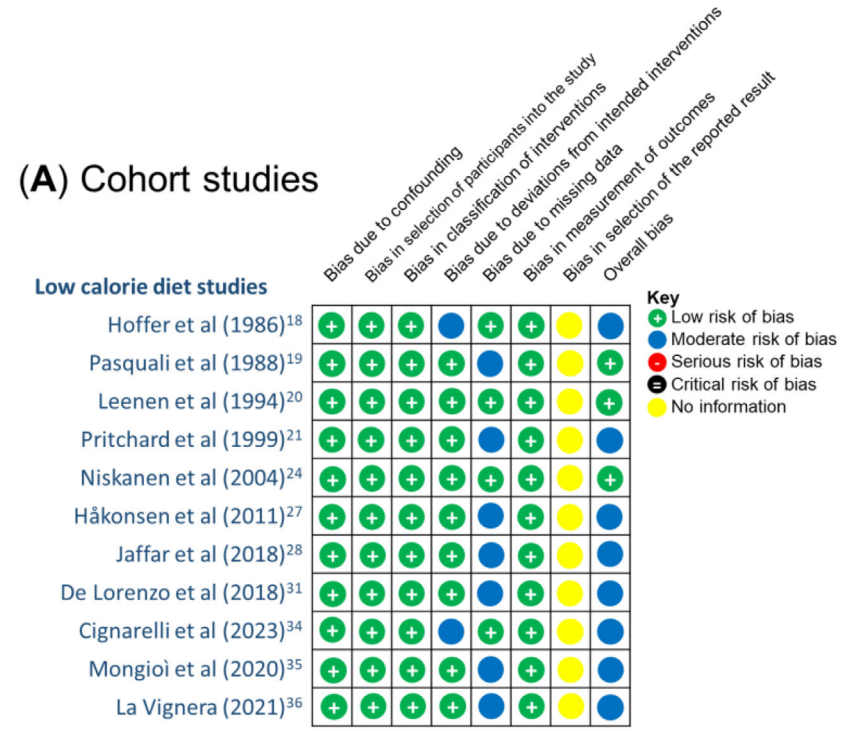


FIGURE 4 Continued

and previous studies.[68] Changes in SHBG therefore play a key role in the availability of circulating FT after weight loss. Examination of the age stratified nomograms from our study revealed that compared to men > 40 years old, men aged ≤ 40 years gained lower amount of TT (Figure 6B,C), but greater amount of free testosterone (Figure 6E,F) for any given amount of weight reduction. This observation has not been well-documented in the current literature and may be explained by the influence of age on differential increases in SHBG induced by weight loss. This notion is supported by studies showing the increase in SHBG after weight loss was not as much for younger men as for older men.[69] This is confirmed by our observation of multivariate regression analysis of age on increases in SHBG after weight loss (adjusted for baseline BMI, TT and FT), showing a positive regression coefficient of 0.687 (i.e., relatively greater increases in SHBG induced by weight loss with increasing age). Therefore, despite a smaller gain in TT, younger men gain greater amount of unbound free testosterone after weight reduction. These observations emphasize the importance of assessing FT changes after weight loss, which is more clinically relevant than TT.

Although weight loss has been shown to improve sexual function,[70] it remains unclear whether the concomitant gain in testosterone has a direct effect.[71] This relationship is complicated by conditions such as diabetes, hypertension, and dyslipidaemia that frequently coexist with obesity. Such conditions are risk factors for microvascular disease and may also contribute to sexual and erectile dysfunction. Therefore, weight loss may not reverse erectile and sexual dysfunction in all individuals, even if testosterone levels are sufficiently regained, or when exogenous testosterone is given.

The relationships between obesity and its many secondary complications, including hypogonadism, are complex and may be bidirectional. Previous studies have shown that obesity-induced hypogonadism appears to be worse in the presence of diabetes and metabolic syndrome, while hypogonadism itself is a risk for these conditions.[72,73] Hypogonadotrophic hypogonadism in obese men is thought to be due to enhanced aromatase activity from excess adipose tissue, leading to greater conversion of androgens to estrogens, which in turn inhibit LH pulse amplitude through negative feedback to the anterior pituitary. Consequently, the lack of LH signal to the testes leads to a reduction in testosterone production.[74] Increased levels of circulating estrogens also promote gynaecomastia, a condition that occurs more frequently with higher BMI.[75] The finding in this study of increased circulating LH



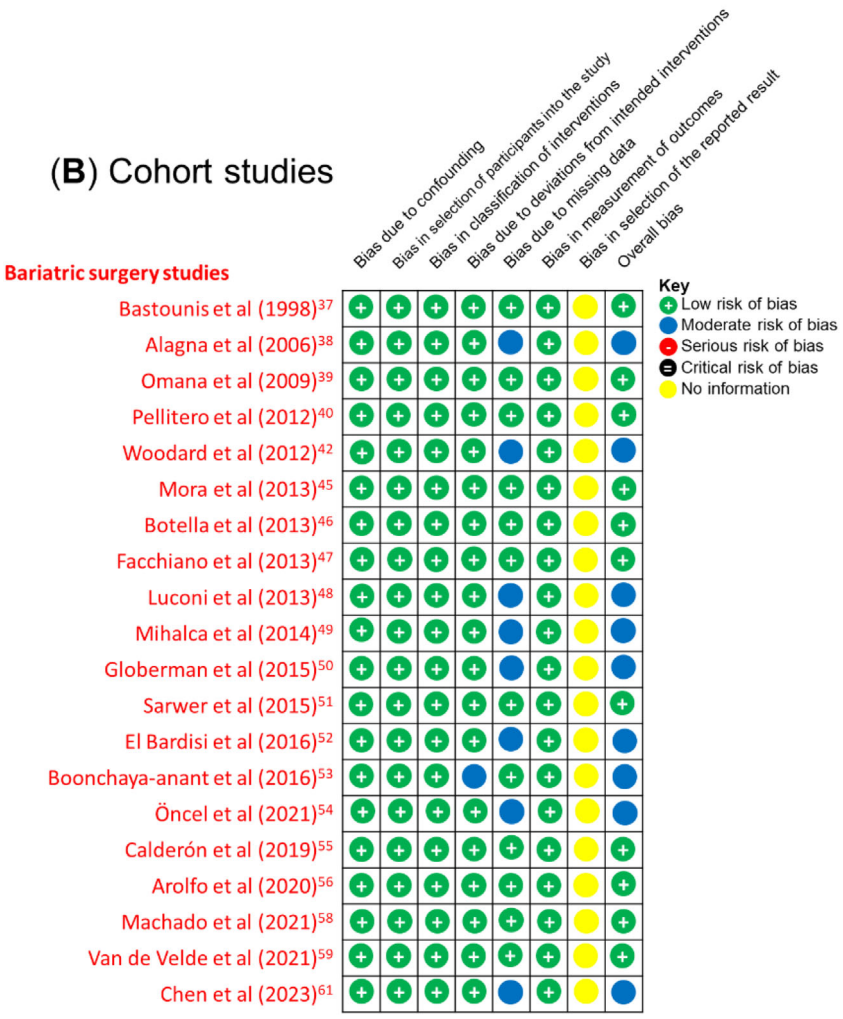


FIGURE 5 Risk of bias of cohort studies evaluated by ROBINS-I tool for low calorie diet (A) and bariatric studies (B), and RCTs evaluated by Cochrane Collaboration's tool (C). RCT, randomized control trials.

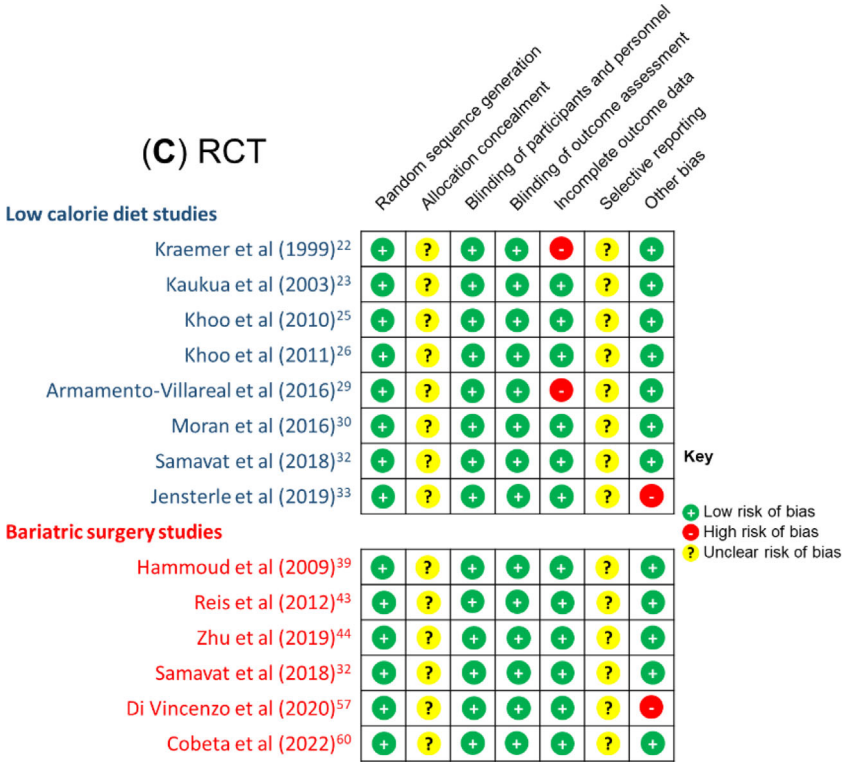


FIGURE 5 Continued

levels after weight loss is consistent with that from previous studies.[10] These observations indicate that gonadal function recovery in obese men is possible by relieving central inhibition induced by factors associated with excess adiposity, such as excess estrogens from increased aromatase activity.

We do recognize that weight loss may not always increase the levels of TT or FT to reference ranges. Thus, men who have not achieved sufficient testosterone through weight loss may benefit from TRT.[61] However, caution should be taken due to adverse effects associated with exogenous testosterone including prostate hypertrophy, which may increase the risk of prostate cancer, gynecomastia, or mood swings. Amongst obese men, OSA is common and many are undiagnosed.[76] TRT in the presence of OSA increases the risk of polycythemia,[77] which predisposes such individuals to thrombotic events.[78] Weight loss, as the first line treatment, therefore serves an additional purpose in obese hypogonadal men who consider TRT in a safer manner by reducing aromatase activity and the risk of gynecomastia, as well as OSA and related polycythemia.

As expected for a meta-analysis, certain limitations were encountered in this study, and include differences in the duration and methods of intervention, namely LDC and BS. However, the two methods provide a wide range of weight losses, making it more suitable for regression analysis and development of nomograms. Details on compliance to LCD were not reported by most studies. The impact of weight loss on testosterone has been debatable because of conflicting findings between studies. A number of factors could contribute to these discrepancies, primarily the wide variation in study designs and small-sample sizes. For example, the duration of weight management varied from a few weeks to several months. The minimum time for full recovery of pituitary–gonadal function from weight loss is not known but results from studies of a few weeks would be unlikely to have a complete effect on the pituitary–gonadal axis. Studies have indicated that TT levels increased soon after weight loss and continued to increase up the end of the study period of 52 weeks, while free testosterone started to rise from 12 weeks of weight loss.[30] The strengths of this study lie in its large number of studies and participants, comprising a wide range of age, BMI and TT and FT, as well as a wide range of weight loss. This has allowed the construction of age-stratified nomograms.

5 | CONCLUSIONS

This meta-analysis of data, accumulated from a large number of participants and datasets, showed androgens, particularly free testosterone levels increased after weight loss among men. Those with higher baseline BMI, or lower levels of SHBG, TT and FT benefit the greatest testosterone gain, while younger men gained relatively greater amounts of free testosterone induced by weight loss. Nomograms constructed from large number of participants of a wide range BMI provide an evidence-based and simple-to-use tool for clinician in a clinical setting.

AUTHORS CONTRIBUTION

Thang Sieu Han created the study concept and design. Thang Sieu Han and Gie Ken-Dror reviewed the literature and performed data collection. Gie Ken-Dror performed data analysis under the guidance of

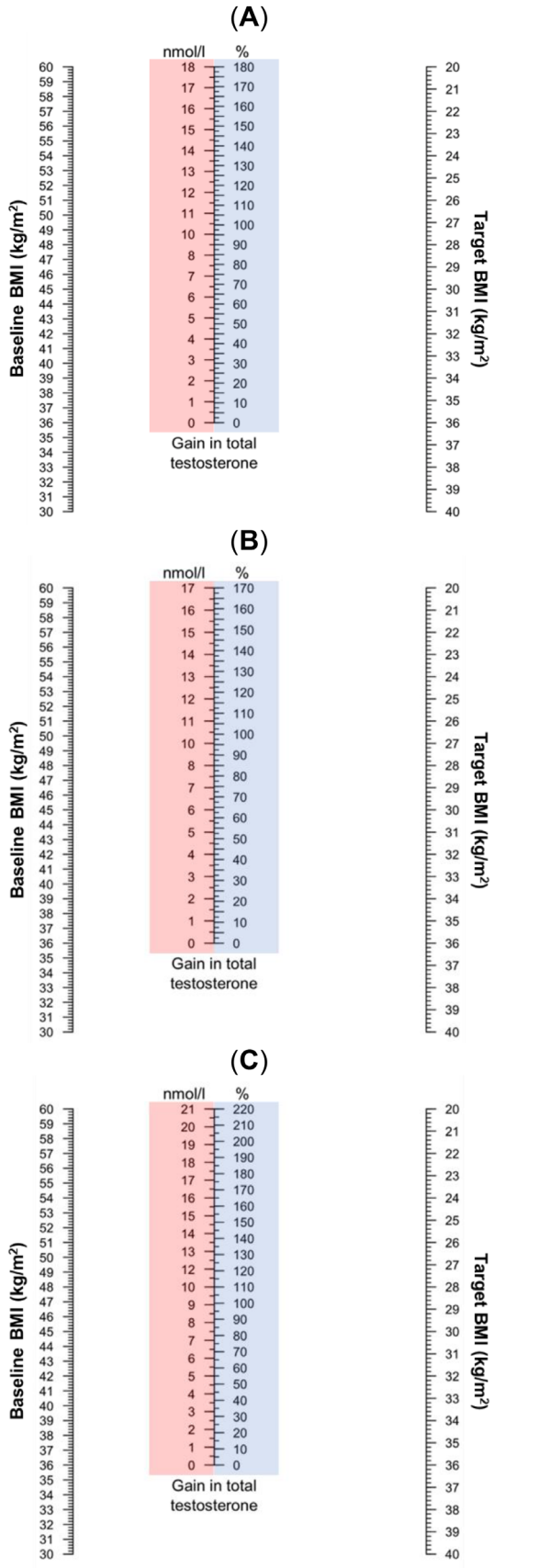


FIGURE 6 Nomograms for approximating a gain in total testosterone in obese men of all ages (A), ≤ 40 years (B) and > 40 years (C), and gain in free testosterone in obese men of all ages (D), ≤ 40 years (E) and > 40 years (F) after weight loss.

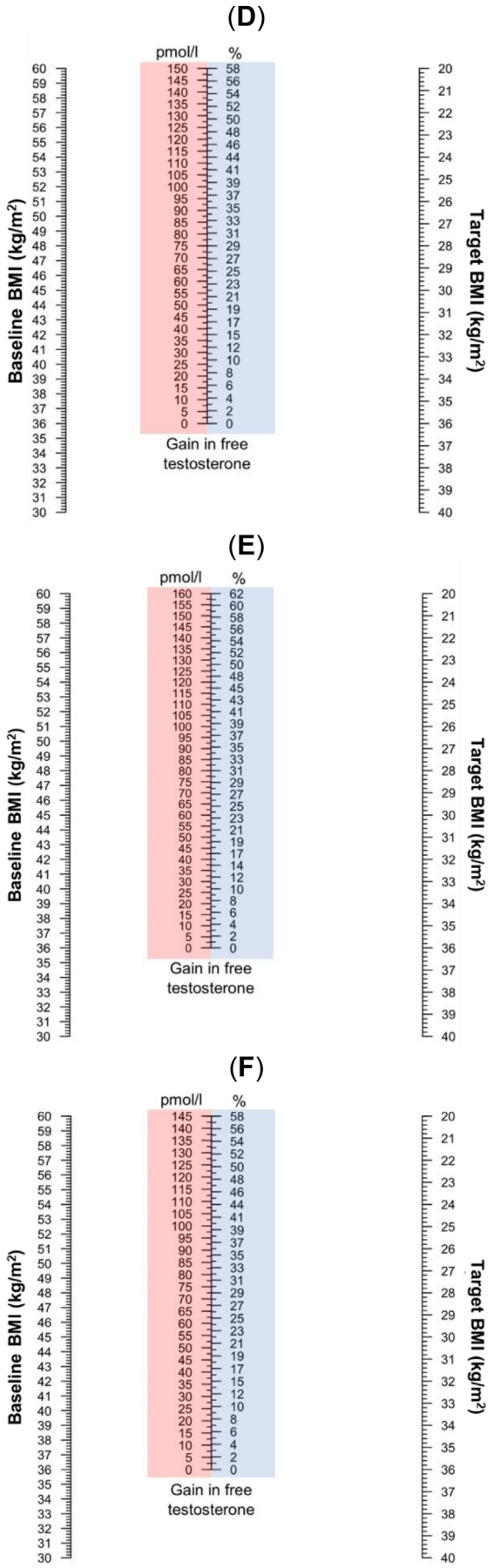


FIGURE 6 Continued

Thang Sieu Han. Thang Sieu Han wrote the first draft of the manuscript. Christopher Henry Fry, David Fluck, and Thang Sieu Han edited subsequent versions. All authors checked, interpreted the results, and approved the final manuscript.

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.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Association between central obesity and semen quality: A cross-sectional study in 4513 Chinese sperm donation volunteers

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Abstract

Background: General obesity classified by body mass index has been linked to a reduction in semen quality; however, evidence on the adverse effect of central obesity on semen quality remains limited.

Objectives: To investigate the association between central obesity and semen quality.

Materials and methods: We conducted a cross-sectional study of 4513 sperm donation volunteers in Guangdong Provincial Human Sperm Bank during 2018–2021. Three central obesity indicators, including waist circumference, waist-to-hip ratio, and waist-to-height ratio, were measured using a multi-frequency bioelectrical impedance analysis for each subject. Semen analysis was conducted according to the World Health Organization laboratory manual for the examination and processing of human semen 5th edition. Linear regression models and unconditional logistic regression models were used to quantify the association between central obesity and semen parameters.

Results: With adjustment for age, race, education level, marital status, fertility status, occupation, year of semen collection, abstinence period, ambient temperature, and relative humidity, central obesity defined as waist circumference ≥90 cm, waist-to-hip ratio ≥0.9, or waist-to-height ratio ≥0.5 was significantly associated with a 0.27 (95% confidence interval: 0.15, 0.38) mL, 14.47 (3.60, 25.34) × 10^6, 7.06 (0.46, 13.76) × 10^6, and 6.80 (0.42, 13.18) × 10^6 reduction in semen volume, total sperm number, total motile sperm number, and total progressive motile sperm number, respectively,

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and a 53% (10%, 112%) increase in odds of below the World Health Organization 2010 reference value for semen volume. These associations did not significantly vary across age. Similar results were observed for central obesity defined using each of the three indicators, except that subjects with a waist circumference ≥90 cm had a slightly higher total motility (estimated change: 1.30%; 95% confidence interval: 0.27%, 2.34%) and progressive motility (estimated change: 1.27%; 95% confidence interval: 0.23%, 2.31%).

Discussion and conclusion: We found that central obesity was significantly associated with a reduction in semen volume, total sperm number, total motile sperm number, and total progressive motile sperm number. Future studies are warranted to confirm our results in other regions and populations.

KEYWORDS

central obesity, semen quality, sperm donation volunteers, waist circumference, waist-to-height ratio, waist-to-hip ratio

1 | INTRODUCTION

Accumulating evidence has demonstrated a global declining trend of human semen quality, which has raised increasing concern on male infertility.[1,2] It was estimated that sperm concentration and total sperm number declined by 51.6% and 62.3%, respectively, in South/Central America, Asia, and Africa during 1973–2018[1]; however, the underlying reasons remain less clear.[3] Previous studies suggest that overall obesity defined by body mass index (BMI) is attributable to a higher risk of abnormal semen quality parameters, including sperm count, motility, and morphology.[4–11] However, because obesity is defined by the presence of excess fat,[12] simply using height and weight (e.g., BMI) to define obesity may lead to misclassification of obese population and fail to properly describe the body-fat distribution, especially the abdominal fat. For example, individuals with a normal BMI can be centrally obese.[13]

Compared with BMI describing overall obesity, central obesity measured by waist circumference (WC), waist-to-hip ratio (WHR), and waist-to-height ratio (WHtR) can provide a better assessment of body-fat distribution.[14] Although computed tomography or dual-energy X-ray absorptiometry (DEXA) scans can be used to directly measure body fat and aid in determining central obesity, the measurements of WC, WHR, and WHtR are simpler, more cost-effective, and safer and have been widely used in clinical and epidemiological studies to explore the detrimental metabolic effects of obesity on human health.[7,15–18] Several previous studies have investigated the association of central obesity with semen quality; however, the results remain limited and inconsistent. Some studies found that central obesity was associated with a reduction in semen quality, including semen volume, sperm concentration, total sperm number, and/or normal forms,[19–23] while other studies did not observe any associations.[24,25] The inconsistency of previous studies may result from the use of different anthropometric measures of central obesity. The use of a single indicator, including WC[20,25,26] and WHR,[27] may lead to misclassification of subjects with central obesity, which can be alleviated by combining multiple indicators (e.g., WC, WHR, and WHtR) to define central obesity. Other reasons for the inconsistent findings include different sources of study populations, various sample sizes and errors in manual measurement during data collection.[19,21,28]

Here, we conducted a cross-sectional study among 4513 sperm donation volunteers in Guangdong Provincial Human Sperm Bank in Guangzhou, China to quantitatively investigate the association between central obesity and semen quality. Central obesity was assessed using WC, WHR, and WHtR measured by a multi-frequency bioelectrical impedance analysis (BIA) with the InBody770 (InBody Co., Ltd., South Korea), which has been validated to provide body-fat measurements comparable in accuracy to those obtained via DEXA scanning.[29] We hypothesized that central obesity defined by WC, WHR, and WHtR was associated with a reduction in semen quality.

2 | METHODS

2.1 | Study population

We enrolled 4624 sperm donation volunteers who intended to donate sperm at the Guangdong Provincial Human Sperm Bank in Guangzhou, China between June 22, 2018 and December 31, 2021. According to the basic requirements for sperm donation, volunteers needed to meet the following conditions: Chinese citizens between the ages of 20 and 45 years; physically healthy with no infectious or hereditary diseases, and no tattoos; height of 168 cm or above; not color blind or color weak, and myopia not exceeding 600°. By excluding 9 (0.2%) volunteers without detailed residential addresses and 102 (2.2%) volunteers without information on WC and hip circumference, we included 4513 volunteers as the study subjects in the final analysis. This study was

approved by the Ethics Committee of School of Public Health, Sun Yat-sen University.

2.2 | Body measurement

Physical measurement for each subject was conducted from 8:00 AM to 1:00 PM. Height (m) was measured by trained technicians using an ultrasonic measuring device for medical examinations with shoes taken off. WC, WHR, and WHtR were determined using a multi-frequency BIA (InBody770). During the determination, the subjects stepped barefoot on a pedal containing individual foot electrodes and held the right and left electrodes with both hands, and all measurements were conducted according to the manufacturer's instructions.

2.3 | Covariates

We used the electronic medical records in the sperm bank to obtain the subjects' information on age, race, occupation, education level, marital status, fertility status, and abstinence period. In addition, we considered ambient temperature (°C) and relative humidity (%) exposures as covariates because previous studies have demonstrated that these meteorological conditions are associated with semen quality.[30,31] As proposed in our previous study, the daily mean grid data of temperature and relative humidity with a spatial resolution of 0.0625° × 0.0625° were obtained from the China Meteorological Administration Land Data Assimilation System (CLDAS version 2.0), which was generated by the China National Meteorological Information Center.[30] As human spermatozoa takes about 90 days to develop,[32] we used the bilinear interpolation method to extract daily temperatures at each subject's geocoded residential address during 0−90 days before semen collection and assessed exposure to temperature and relative humidity by averaging these daily values during the exposure window.

2.4 | Semen analysis

A standardized semen analysis was performed for each subject according to the fifth edition of the World Health Organization (WHO) laboratory manual for the examination and processing of human semen.[2] The subject was asked to provide a semen sample by masturbating into a sterile plastic specimen container in a private semen collection room. The semen sample was then analyzed by qualified technicians after being liquefied in a 37°C water bath for 30 min. Each semen sample was weighed to determine the semen volume (mL), and a well-mixed 10 μL sample was added to a Makler chamber to assess sperm concentration ($\times 10^6$/mL), progressive motility (%), and non-progressive motility (%) by phase-contrast optics at 200× or 400× magnification. Total sperm number ($\times 10^6$) was calculated as semen volume multiplied by sperm concentration. Total motility (%) was calculated as the sum of the progressive and non-progressive motility. Using the Papanicolaou staining method, sperm morphology of the stained slides was observed by an optical microscope to assess the normal form rate (%) of the semen sample. Total motile sperm number ($\times 10^6$), total progressive sperm number ($\times 10^6$), and total normal form sperm number ($\times 10^6$) were calculated as total sperm number multiplied by total motility (%), progressive motility (%), and normal form rate (%), respectively. To ensure the accuracy and comparability of the results, trained technicians conducted routine internal and external quality control of the semen analysis based on the WHO laboratory manual.[2,33]

2.5 | Statistical analysis

According to the WHO Asia Pacific guidelines, male central obesity was defined as WC ≥90 cm,[34] WHR ≥0.90,[35] or WHtR ≥0.50.[36–38] Chi-square tests were used to test the difference in baseline characteristics between central obese and non-central obese subjects. Student's *t*-tests were used to examine the differences in the semen parameter distributions. Linear regression models were employed to quantitatively assess the association of central obesity with semen quality, with adjustment for age (<25, ≥25 years), race (Han, other), education level (high school and lower, junior college, undergraduate or higher), marital status (unmarried, married, divorced), fertility status (ever fathered a child or not), occupation (student, non-student), year of semen collection (2018, 2019, 2020, 2021), abstinence period (≤3, 4−5, >5 days), lag 0−90 day temperature (a natural cubic spline with 3 degrees of freedom [d.f.]), and lag 0−90 day relative humidity (a natural cubic spline with 3 d.f.). We calculated the estimated changes and corresponding 95% confidence intervals (CIs) of semen parameters associated with central obesity. In addition, unconditional logistic regression models were implemented to assess the association of central obesity with the decline in semen parameters, which was defined as below the WHO 2010 reference values (semen volume, 1.5 mL; sperm concentration, 15×10^6/mL; total sperm number, 39×10^6; total motility, 40%; progressive motility, 32%; and normal forms, 4%). Similar analyses were conducted for central obesity defined with each of the three indictors (WC, WHR, and WHtR). We further included WC, WHR, or WHtR as continuous variables in the model to estimate the change in semen parameters associated with each standard deviation (SD) increase in each indicator.

We conducted stratified analysis to investigate the association between central obesity and semen quality by age. Based on the median age of the study population in our study (24.8 years), we selected 25 years as the cut-off value. Potential effect modification was tested using a likelihood ratio test by comparing nested models with and without an interaction term of age stratification by central obesity. R software (version 4.1.2) was used for all data analyses.[39] Two-sided *p*-values <0.05 were considered statistically significant. Power analysis was conducted using R package "pwr" and the estimated power was 0.99, which indicated that the sample size was sufficient.

3 | RESULTS

Our analysis included 4513 subjects in Guangdong Province, China. Characteristics of the study subjects are presented in Table 1. The

TABLE 1 Characteristics of the study subjects by central obesity in Guangdong Province, China during 2018–2021.

Characteristic	Overall	Central obesity[a]		*p*-Value
		No	Yes	
No. of subjects	4513	3371 (74.7)	742 (25.3)	
Age (years)				<0.001
<25	2184 (48.4)	1461 (43.3)	723 (63.3)	
≥25	2329 (51.6)	1910 (56.7)	419 (36.7)	
Mean (SD)	26.2 (5.3)	25.51 (4.96)	28.13 (5.84)	<0.001
Race				0.019
Han	4369 (96.8)	3276 (97.2)	1093 (95.7)	
Other	144 (3.2)	95 (2.8)	49 (4.3)	
Occupation				<0.001
Student	1410 (31.2)	1186 (35.2)	224 (19.6)	
Non-student	3103 (68.8)	2185 (64.8)	918 (80.4)	
Education level				<0.001
High school and lower	531 (11.8)	384 (11.4)	147 (12.9)	
Junior college	1664 (36.9)	1198 (35.5)	466 (40.8)	
Undergraduate or higher	2318 (51.4)	1789 (53.1)	529 (46.3)	
Marital status				<0.001
Unmarried	3732 (82.7)	2928 (86.9)	804 (70.4)	
Married	715 (15.8)	408 (12.1)	307 (26.9)	
Divorced	66 (1.5)	35 (1.0)	31 (2.7)	
Fertility status				<0.001
Never fathered a child	3918 (86.8)	3037 (90.1)	881 (77.1)	
Ever fathered a child	595 (13.2)	334 (9.9)	261 (22.9)	
Year of semen collection				<0.001
2018	727 (16.1)	496 (14.7)	231 (20.2)	
2019	1599 (35.4)	1229 (36.5)	370 (32.4)	
2020	1212 (26.9)	906 (26.9)	306 (26.8)	
2021	975 (21.6)	740 (22.0)	235 (20.6)	
Abstinence period (days)				0.657
≤3	953 (21.1)	711 (21.1)	242 (21.2)	
4–5	2070 (45.9)	1535 (45.5)	535 (46.8)	
>5	1490 (33.0)	1125 (33.4)	365 (32.0)	
BMI[b]				<0.001
Underweight	462 (10.2)	462 (12.3)	0 (0.0)	
Normal weight	3164 (70.1)	2820 (83.7)	344 (30.1)	
Overweight	788 (17.5)	89 (2.6)	699 (61.2)	
Obese	99 (2.2)	0 (0.0)	99 (8.7)	
Temperature[c] (°C)	25.1 (4.1)	25.1 (4.1)	24.9 (4.2)	0.203
Relative humidity[c] (%)	77.2 (6.8)	77.2 (6.8)	77.0 (6.8)	0.280

Note: Data are given as mean ± SD or *N* (%).

Abbreviations: BMI, body mass index; SD, standard deviation; WC, waist circumstance; WHO, World Health Organization; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio.

[a]Central obesity was defined as WC ≥ 90 cm, WHR ≥ 0.90, or WHtR ≥ 0.50.

[b]BMI was categorized using the WHO criteria: underweight (<18.5 kg/m^2), normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), and obesity (≥30.0 kg/m^2).

[c]Temperature and relative humidity refer to average temperature and relative humidity values during 0–90 days before the date of semen collection, respectively.

TABLE 2 Distribution of semen quality parameters among the study subjects in Guangdong Province, China during 2018–2021.

Semen parameters	N	Mean	SD	Percentile					IQR
				P_5	P_{25}	Median	P_{75}	P_{95}	
Semen volume (mL)	4513	4.0	1.7	1.6	2.80	3.8	5.0	7.1	2.2
Sperm concentration (10^6/mL)	4513	64.8	35.4	13.0	39.0	59.0	87.0	129.0	48.0
Total sperm number (10^6)	4513	250.8	163.2	37.9	135.0	224.0	336.7	554.0	201.7
Total motility (%)	4513	55.6	13.1	34.0	48.0	57.0	64.0	75.0	16.0
Progressive motility (%)	4513	53.2	13.1	31.0	46.0	54.0	62.0	72.0	16.0
Normal forms (%)	4299	12.7	6.7	4.3	7.7	11.7	16.6	24.9	8.9
Total motile sperm number (10^6)	4513	141.6	97.7	17.0	70.0	123.8	193.2	327.5	123.3
Total progressive motile sperm number (10^6)	4513	135.6	94.3	16.0	66.9	118.3	185.1	314.4	118.2
Total normal form sperm number (10^6)	4299	34.0	30.8	3.3	12.6	25.5	46.1	93.7	33.5

Abbreviations: IQR, interquartile range; SD, standard deviation.

mean WC, WHR, and WHtR were 80.8 (SD: 9.6) cm, 0.85 (SD: 0.06), and 0.47 (SD: 0.05), respectively. Based on three body indicators, 25.3% ($N = 742$) of the subjects were classified as having central obesity. The mean semen volume, sperm concentration, total sperm number, total motility, progressive motility, normal forms, total motile sperm number, total progressive motile sperm number, and total normal form sperm number were 4.0 mL, 64.8×10^6/mL, 250.8×10^6, 55.6%, 53.2%, 12.7%, 141.6×10^6, 135.6×10^6, and 34.0×10^6, respectively (Table 2). Significantly lower semen volume, total sperm number, total motile sperm number, and total progressive motile sperm number were observed among central obese subjects, while significantly higher total motility and progressive motility were observed among subjects with WC ≥90 cm only (Figure 1). We did not observe any significant difference in sperm concentration, normal forms, or total normal form sperm number (all $p \geq 0.05$).

In categorical analysis (Table 3), central obesity was significantly associated with a 0.27 (95% CI: 0.15, 0.38) mL, 14.47 (95% CI: 3.60, 25.34) $\times 10^6$, 7.06 (95% CI: 0.46, 13.76) $\times 10^6$, and 6.80 (95% CI: 0.42, 13.18) $\times 10^6$ reduction in semen volume, total sperm number, total motile sperm number, and total progressive motile sperm number, respectively. No significant association of central obesity with sperm concentration, total motility, progressive motility, normal forms, or total normal form sperm number was observed. Similar results were also observed for central obesity defined using each of three indicators (i.e., WC ≥90 cm, WHR ≥0.9, or WHtR ≥0.5), except that WC ≥90 cm was significantly associated with a 1.30% (95% CI: 0.27%, 2.34%) and 1.27% (95% CI: 0.23%, 2.31%) increase in total motility and progressive motility, respectively. As shown in Table 4, central obesity, WC, WHR, and WHtR were significantly associated with increased odds of semen volume decline, with odds ratios of 1.53 (95% CI: 1.10, 2.12), 1.48 (95% CI: 1.03, 2.13), 1.62 (95% CI: 1.13, 2.32), and 1.51 (95% CI: 1.08, 2.10), respectively. In continuous models (Table S1), each 1 SD increase in WC (SD: 9.62 cm) was associated with a 0.08 (95% CI: 0.03, 0.13) mL and 5.77 (95% CI: 0.98, 10.55) $\times 10^6$ reduction in semen volume and total sperm number, respectively, and a 0.58% (95% CI: 0.19%, 0.97%) and 0.56% (95% CI: 0.17%, 0.95%) increase in total mobility and progressive motility, respectively.

In the stratified analyses by age, the association of central obesity and semen volume was significant in both age groups and the association was not significantly modified by age. The associations of central obesity with total sperm number, total motility, and progressive motility were observed only among subjects <25 years. Similar results were observed for central obesity defined using each of the three indicators (i.e., WC ≥ 90 cm, WHR ≥ 0.9, and WHtR ≥ 0.5), except that WC was not associated with semen volume reduction among subjects <25 years, and the association between WHR and sperm motility was stronger among subjects <25 years (Table 5).

4 | DISCUSSION

In this cross-sectional study, we investigated the association of central obesity with semen quality among 4513 sperm donation volunteers from Guangdong Province, China. Overall, we found that central obesity and three central obesity indicators were significantly associated with a reduction in semen volume and total sperm number, and an increased odds of semen volume decline. These associations did not vary across different age groups. In contrast, we observed that central obesity was significantly associated with a slightly higher total motility and progressive motility, especially in subjects younger than 25 years.

Our results were consistent with those in most previous studies that central obesity was associated with a reduction in semen volume[19,26] and total sperm number.[19–22,26,27,40] A Hungarian study[19] estimated that central obesity was negatively correlated with semen volume (estimated change: −0.25 mL), which was close to our estimates (estimated change: −0.27 mL). However, the LIFE study conducted in the USA did not find significant association of central obesity with the odds of semen volume decline (odds ratio: 4.40; 95% CI: 0.81, 23.73).[26] The reason may be that a large proportion of the LIFE study population was overweight and obese (82%), which differs significantly from our study (19.7% for overweight and obese). In a Netherlandish study of 413 infertile men, each 1 cm increase in WC was associated with a 0.023×10^6 reduction in total sperm number, which was much lower than our results (0.600×10^6 for each 1 cm increase in WC).[20] In

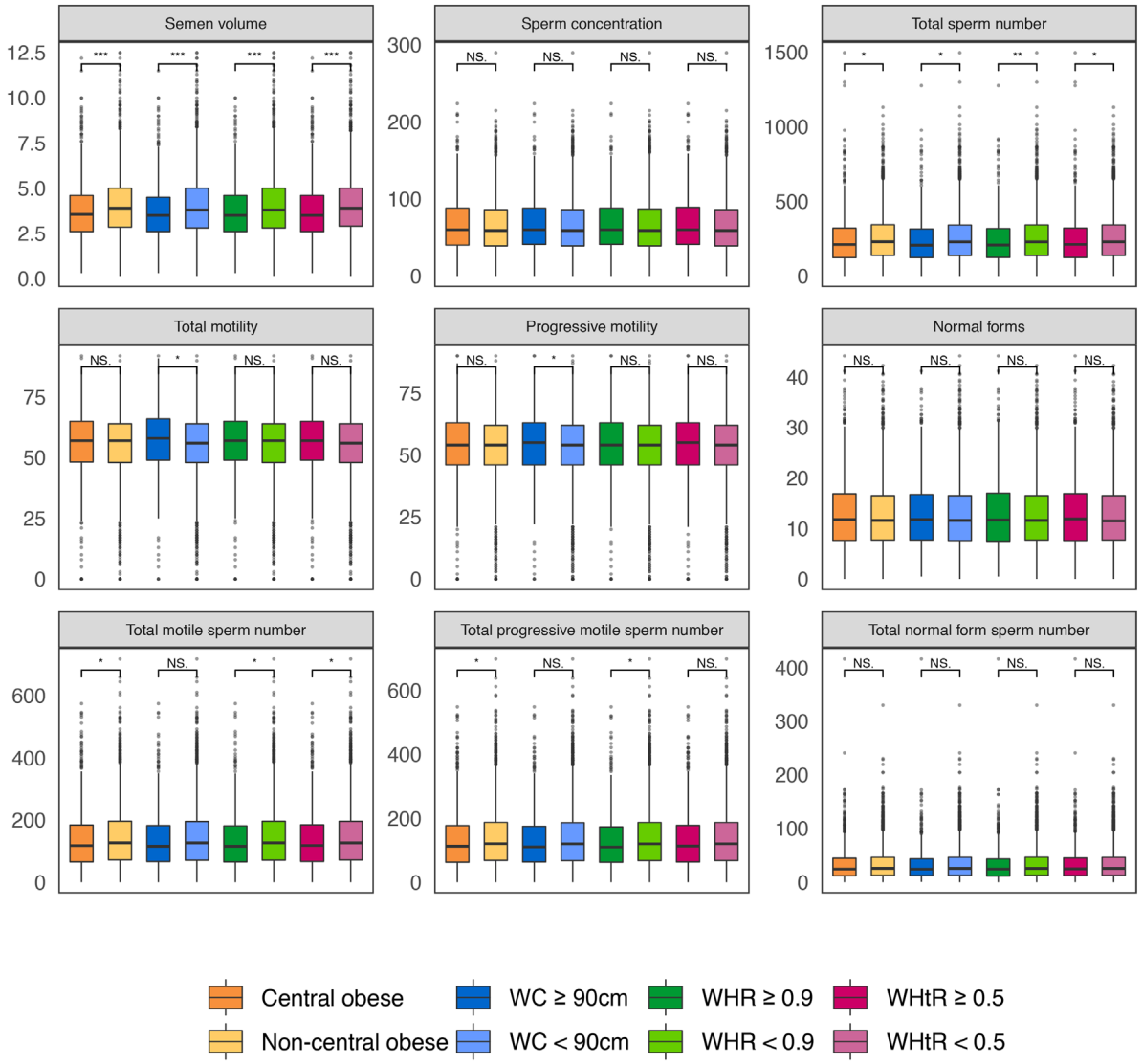

FIGURE 1 Box-plot of semen parameters by central obesity. A *t*-test was used to examine the difference of each semen parameter by central obesity, waist circumference (WC), waist-to-hip ratio (WHR), and waist-to-height ratio (WHtR). WC was categorized using the WHO Asia Pacific guidelines: WC ≥90 cm is defined as central obesity. WHR was categorized using the IDF Consensus of MetS (2006): WHR ≥0.9 is defined as central obesity. WHtR was categorized using the widely used criteria: WHtR ≥0.5 is defined as central obesity. Central obesity was defined as WC ≥90 cm, WHR ≥0.90, or WHtR ≥0.50. The six panels show the minimum, lower quartile, median, upper quartile, maximum, and outliers of the nine semen parameters in each group categorized by central obesity, WC, WHR, and WHtR criteria, respectively. NS, $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

contrast, a Brazilian study did not observe any significant associations of WC with all semen parameters. It should be noted that this study had a small sample size ($N = 153$) and did not consider possible covariates.[25] We did not find any associations of central obesity with sperm concentration or normal forms, which was consistent with results from several previous studies.[22,27]

Inconsistent with most previous studies, we found that central obesity was associated with a slight increase of total motility and progressive motility, especially among subjects <25 years. This finding is not unprecedented. A Chinese study with 990 fertile men and an US study with 483 men from infertile couples found that overweighted men had higher sperm concentration, total sperm number, or total progressive motile sperm number.[41,42] Moreover, in 2015, a Chinese study with 1231 infertile men observed that compared with normal WC (with cut-off as <90 cm) men, obese men had a higher sperm motility (estimated change: 0.06%).[24] A potential explanation is that moderate body-fat accumulation may be beneficial for improving semen quality. It was known that as an important component of spermatozoa, mitochondria are the center of energy utilization and optimal mitochondrial activity is positively associated with sperm motility.[43] Young men with high metabolism and great need for energy, including fat, may have enhanced mitochondrial activity, which can contribute to the increase in sperm motility. Nonetheless, further studies are warranted to explore the underlying biological mechanisms.

TABLE 3 Estimated change (95% confidence interval [CI]) of semen parameters associated with central obesity defined by waist circumference (WC), waist-to-hip ratio (WHR), and waist-to-height ratio (WHtR).

	Central obesity[a]		WC[b]		WHR[c]		WHtR[d]	
Semen parameter	No	Yes	<90 cm	≥90 cm	<0.9	≥0.9	<0.5	≥0.5
Semen volume (mL)	0 (ref)	−0.27 (−0.38, −0.15)	0 (ref)	−0.27 (−0.40, −0.13)	0 (ref)	−0.23 (−0.36, −0.10)	0 (ref)	−0.27 (−0.39, −0.16)
Sperm concentration (10^6/mL)	0 (ref)	0.49 (−1.92, 2.90)	0 (ref)	1.21 (−1.58, 4.01)	0 (ref)	0.03 (−2.76, 2.81)	0 (ref)	1.26 (−1.20, 3.72)
Total sperm number (10^6)	0 (ref)	−14.47 (−25.34, −3.60)	0 (ref)	−13.21 (−25.83, −0.59)	0 (ref)	−14.64 (−27.22, −2.06)	0 (ref)	−13.19 (−24.31, −2.07)
Total motility (%)	0 (ref)	0.61 (−0.29, 1.50)	0 (ref)	1.30 (0.27, 2.34)	0 (ref)	0.60 (−0.44, 1.63)	0 (ref)	0.76 (−0.15, 1.67)
Progressive motility (%)	0 (ref)	0.60 (−0.30, 1.50)	0 (ref)	1.27 (0.23, 2.31)	0 (ref)	0.61 (−0.42, 1.65)	0 (ref)	0.76 (−0.15, 1.68)
Normal forms (%)	0 (ref)	0.18 (−0.29, 0.65)	0 (ref)	0.11 (−0.43, 0.66)	0 (ref)	0.02 (−0.53, 0.56)	0 (ref)	0.26 (−0.22, 0.74)
Total motile sperm number (10^6)	0 (ref)	−7.06 (−13.67, −0.46)	0 (ref)	−4.94 (−12.61, 2.72)	0 (ref)	−7.13 (−14.77, 0.51)	0 (ref)	−6.19 (−12.95, 0.56)
Total progressive motile sperm number (10^6)	0 (ref)	−6.80 (−13.18, −0.42)	0 (ref)	−4.79 (−12.19, 2.61)	0 (ref)	−6.83 (−14.21, 0.55)	0 (ref)	−5.96 (−12.48, 0.56)
Total normal form sperm number (10^6)	0 (ref)	−0.80 (−2.95, 1.36)	0 (ref)	−1.21 (−3.71, 1.29)	0 (ref)	−1.76 (−4.25, 0.74)	0 (ref)	−0.51 (−2.71, 1.70)

Note: The estimated change and 95% CI were calculated using linear regression models, adjusting for age, race, education level, marital status, fertility status, occupation, year of semen collection, abstinence period, lag 0−90 day temperature, and lag 0−90 day relative humidity.

[a]Central obesity was defined as WC ≥ 90 cm, WHR ≥ 0.90, or WHtR ≥ 0.50.

[b]WC was categorized using the World Health Organization (WHO) Asia Pacific guidelines: WC ≥ 90 cm is defined as central obesity.

[c]WHR was categorized using the IDF Consensus of MetS (2006): WHR ≥ 0.9 is defined as central obesity.

[d]WHtR was categorized using the acknowledged criteria: WHtR ≥ 0.5 is defined as central obesity.

TABLE 4 Odds ratio (95% confidence interval [CI]) of semen quality decline defined as below World Health Organization (WHO) 2010 reference values associated with central obesity.

	Central obesity[a]		WC[b]		WHR[c]		WHtR[d]	
Semen parameter	No	Yes	<90 cm	≥90 cm	<0.9	≥0.9	<0.5	≥0.5
Semen volume (mL)	1 (ref)	1.53 (1.10, 2.12)	1 (ref)	1.48 (1.03, 2.13)	1 (ref)	1.62 (1.13, 2.32)	1 (ref)	1.51 (1.08, 2.10)
Sperm concentration (10^6/mL)	1 (ref)	1.04 (0.77, 1.40)	1 (ref)	1.02 (0.72, 1.44)	1 (ref)	1.03 (0.73, 1.46)	1 (ref)	1.04 (0.77, 1.41)
Total sperm number (10^6)	1 (ref)	1.15 (0.85, 1.57)	1 (ref)	1.11 (0.78, 1.58)	1 (ref)	1.12 (0.79, 1.60)	1 (ref)	1.12 (0.82, 1.54)
Total motility (%)	1 (ref)	0.83 (0.65, 1.05)	1 (ref)	0.86 (0.65, 1.14)	1 (ref)	0.93 (0.71, 1.22)	1 (ref)	0.88 (0.69, 1.12)
Progressive motility (%)	1 (ref)	0.77 (0.55, 1.07)	1 (ref)	0.78 (0.53, 1.15)	1 (ref)	0.79 (0.54, 1.15)	1 (ref)	0.86 (0.62, 1.20)
Normal forms (%)	1 (ref)	1.001 (0.70, 1.44)	1 (ref)	0.92 (0.60, 1.41)	1 (ref)	1.18 (0.79, 1.76)	1 (ref)	1.07 (0.75, 1.54)

Note: The odds ratio and 95% CI were calculated using logistic regression models, adjusting for age, race, education level, marital status, fertility status, occupation, year of semen collection, abstinence period, lag 0–90 day temperature, and lag 0–90 day relative humidity. The WHO 2010 reference value for semen volume, sperm concentration, total sperm number, total motility, progressive motility, and normal forms was 1.5 mL, 15×10^6/mL, 39×10^6, 40%, 32%, and 4%, respectively.

Abbreviations: WC, waist circumstance; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio.

[a]Central obesity was defined as WC ≥ 90 cm, WHR ≥ 0.90, or WHtR ≥ 0.50.

[b]WC was categorized using the WHO Asia Pacific guidelines: WC ≥ 90 cm is defined as central obesity.

[c]WHR was categorized using the IDF Consensus of MetS (2006): WHR ≥ 0.9 is defined as central obesity.

[d]WHtR was categorized using the acknowledged criteria: WHtR ≥ 0.5 is defined as central obesity.

There are several possible potential biological mechanisms linking central obesity to reduced semen quality. Hyperinsulinemia, which may accompany central obesity, can decrease the concentrations of plasma sex hormone-binding globulin, leading to a reduction in testosterone and an increase in estrogen.[44,45] In addition, excessive accumulation of white adipose tissue (WAT) and excessive activation of the aromatase cytochrome P450 promote the biotransformation of androgens to estrogens in the organism.[7,46] Meanwhile, WAT can also secrete leptin, maintain a high estrogenic state, or directly affect sperm quality through endocrine mechanisms.[47,48] These are all related with the dysfunction of the hypothalamic–pituitary–gonadal axis (HPG). HPG has a negative feedback mechanism, and men with central obesity may have higher concentrations of estrogen, which may consequently lead to lower concentrations of testosterone and further impair spermatogenesis and semen quality.[7,46] There are several other explanations for the association between central obesity and decreased semen quality, including the effect of adipose tissue on the accumulation of environmental toxicants and high scrotal temperature.[7]

Our study has some strengths. First, the sample size of our study was larger than most of the previous studies, which provided us with sufficient statistical power. Second, previous studies concerning the associations of obesity with semen quality were mainly conducted among infertile men, which were relatively incomparable to the general male population. Our study subjects were selected from sperm donation volunteers, including both qualified sperm donors and men who failed to pass the screening of semen quality, which makes our study population closer to the general male population. Third, we performed an accurate exposure assessment using the precise body-fat monitor. Most previous studies usually used manual tape measurement for waist and hip circumference and scale measurement for weight. The InBody770 device used in our study apply BIA to measure fat mass, which is more accurate and efficient and has become an important tool in daily clinical practice.

There are also several limitations. First, although our study subjects were closer to the general male population, they were likely to be healthier. The results need to be generalized with caution. Second, we were unable to consider the variables on subjects' smoking and alcohol consumption. However, in our study, sperm donation volunteers with bad habits, including alcoholism and tobacco addiction, were excluded during recruiting, and only a small proportion of subjects smoked or drank. A review investigating the association between lifestyle and infertility among infertile men noted that there was little evidence supporting that smoking and alcohol consumption were detrimental to semen quality, but whether this finding also applies to the general population remains to be studied.[49]

In conclusion, our findings indicated that central obesity was associated with a reduction in semen volume, total sperm number, total motile sperm number, and total progressive motile sperm number, and an increase in odds of semen volume decline. For subjects <25 years, central obesity was positively associated with an increase in total motility and progressive motility. Since semen quality decline is acknowledged to be associated with male infertility, our study suggests that men with central obesity are in need to examine semen quality and take actions to lose fatty mass, especially those who want to conceive. Further studies are warranted to confirm the association between central obesity and semen quality and gain insights into its underlying mechanisms.

AUTHOR CONTRIBUTIONS

Yuewei Liu and Xinzong Zhang designed this study. Xinzong Zhang, Yuewei Liu, and Qiling Wang collected the data. Tingting Wang and Qiling Wang performed the statistical analysis and drafted the manuscript. Ruijun Xu, Xinyi Deng, Yingxin Li, Sihan Liang, and Yong-Gang Duan reviewed the manuscript and commented on it. Yuewei Liu and Xinzong Zhang mainly revised the manuscript and confirmed the

TABLE 5 Estimated change (95% confidence interval [CI]) of semen quality parameters associated with central obesity defined by waist circumference (WC), waist-to-hip ratio (WHR), and waist-to-height ratio (WHtR) stratified by age.

		Semen volume (mL)	Sperm concentration (10^6/mL)	Total sperm number (10^6)	Total motility (%)	Progressive motility (%)	Normal forms (%)	Total motile sperm number (10^6)	Total progressive motile sperm number (10^6)	Total normal form sperm number (10^6)
Central obesity[a]	<25 years	−0.20 (−0.38, −0.03)	−1.10 (−4.66, 2.46)	−17.65 (−34.15, −1.14)	1.42 (0.06, 2.78)	1.43 (0.06, 2.79)	0.38 (−0.34, 1.09)	−5.33 (−15.53, 4.88)	−5.07 (−14.91, 4.78)	−0.54 (−3.72, 2.64)
	≥25 years	−0.30 (−0.45, −0.16)	1.68 (−1.63, 4.99)	−11.44 (−26.00, 3.12)	0.05 (−1.14, 1.25)	0.03 (−1.17, 1.23)	0.07 (−0.55, 0.70)	−7.81 (−16.50, 0.88)	−7.58 (−15.97, 0.81)	−0.86 (−3.83, 2.10)
	p-Value for effect modification	0.33	0.24	0.58	0.14	0.13	0.66	0.72	0.71	0.97
WC[b]	<25 years	−0.16 (−0.37, 0.05)	0.41 (−3.79, 4.61)	−7.82 (−27.32, 11.68)	2.22 (0.62, 3.83)	2.16 (0.55, 3.77)	−0.14 (−0.98, 0.71)	1.22 (−10.83, 13.27)	1.07 (−10.56, 12.70)	−1.76 (−5.50, 1.98)
	≥25 years	−0.34 (−0.51, −0.17)	1.91 (−1.88, 5.71)	−16.23 (−32.93, 0.48)	0.75 (−0.62, 2.12)	0.72 (−0.65, 2.10)	0.32 (−0.40, 1.04)	−8.60 (−18.57, 1.38)	−8.27 (−17.90, 1.36)	−0.63 (−4.03, 2.77)
	p-Value for effect modification	0.17	0.50	0.57	0.17	0.18	0.29	0.25	0.26	0.54
WHR[c]	<25 years	−0.31 (−0.52, −0.09)	−0.01 (−4.33, 4.32)	−21.78 (−41.81, −1.75)	1.97 (0.32, 3.62)	2.02 (0.37, 3.68)	−0.29 (−1.16, 0.58)	−8.26 (−20.65, 4.12)	−7.80 (−19.75, 4.15)	−4.10 (−7.95, −0.25)
	≥25 years	−0.18 (−0.34, −0.01)	0.12 (−3.59, 3.82)	−9.80 (−26.11, 6.51)	−0.18 (−1.52, 1.16)	−0.19 (−1.54, 1.15)	0.23 (−0.47, 0.93)	−6.05 (−15.78, 3.69)	−5.87 (−15.27, 3.53)	−0.20 (−3.52, 3.12)
	p-Value for effect modification	0.39	0.94	0.37	0.04	0.04	0.26	0.79	0.81	0.12
WHtR[d]	<25 years	−0.19 (−0.37, −0.01)	−0.43 (−4.10, 3.23)	−14.19 (−31.18, 2.80)	1.43 (0.03, 2.84)	1.46 (0.06, 2.87)	0.53 (−0.20, 1.27)	−3.07 (−13.57, 7.43)	−2.88 (−13.01, 7.26)	0.36 (−2.91, 3.63)
	≥25 years	−0.33 (−0.48, −0.18)	2.49 (−0.87, 5.86)	−12.03 (−26.84, 2.77)	0.31 (−0.91, 1.52)	0.29 (−0.93, 1.50)	0.10 (−0.54, 0.74)	−8.04 (−16.87, 0.80)	−7.80 (−16.33, 0.74)	−1.02 (−4.04, 2.00)
	p-Value for effect modification	0.21	0.21	0.86	0.23	0.21	0.52	0.48	0.47	0.64

Note: The estimated change and 95% CI were calculated using linear regression models, adjusting for race, education level, marital status, fertility status, occupation, year of semen collection abstinence period, lag 0–90 day temperature, and lag 0–90 day relative humidity.

[a]Central obesity was defined as WC ≥ 90 cm, WHR ≥ 0.90, or WHtR ≥ 0.50.

[b]WC was categorized using the World Health Organization (WHO) Asia Pacific guidelines: WC ≥ 90 cm is defined as central obesity.

[c]WHR was categorized using the IDF Consensus of MetS (2006): WHR ≥ 0.9 is defined as central obesity.

[d]WHtR was categorized using the acknowledged criteria: WHtR ≥ 0.5 is defined as central obesity.

final version. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Low testosterone at age 31 associates with maternal obesity and higher body mass index from childhood until age 46: A birth cohort study

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Abstract

Background: Low testosterone (T) levels in men associate with increased risks of obesity, type 2 diabetes, metabolic syndrome, and cardiovascular diseases. However, most studies are cross-sectional with follow-up-time < 10 years, and data on early growth are limited.

Objective: To compare prenatal factors and body mass index (BMI) development from birth to age 46 in relation to low T at age 31.

Materials and methods: Men with low T (T < 12.1 nmol/L, $n = 132$) and men with normal T at age 31 ($n = 2561$) were derived from the Northern Finland Birth Cohort 1966. Prenatal factors, longitudinal weight and height data from birth to age 14, and cross-sectional weight and height data at ages 31 and 46, and waist-hip-ratio (WHR) and T levels at age 31 were analyzed. Longitudinal modeling and timing of adiposity rebound (AR, second BMI rise at age 5–7 years) were calculated from fitted BMI curves. Results were adjusted for mother's pre-pregnancy BMI and smoking status, birth weight for gestational age, alcohol consumption, education level, smoking status, and WHR at age 31.

Results: Neither gestational age nor birth weight was associated with low T at age 31; however, maternal obesity during gestation was more prevalent among men with low T (9.8% vs. 3.5%, adjusted aOR: 2.43 [1.19–4.98]). Men with low T had earlier AR (5.28 vs. 5.82, aOR: 0.73 [0.56–0.94]) and higher BMI ($p < 0.001$) from AR onward until age 46. Men with both early AR and low T had the highest BMI from AR onward.

Conclusions: In men, maternal obesity and early weight gain associate with lower T levels at age 31, independently of adulthood abdominal obesity. Given the well-known health risks related to obesity, and the rising prevalence of maternal obesity, the results of the present study emphasize the importance of preventing obesity that may also affect the later reproductive health of the offspring.

KEYWORDS
adiposity rebound, childhood growth, hypogonadism, obesity, testosterone

1 | INTRODUCTION

Several studies have shown that androgen deficiency (hypogonadism) is associated with obesity in men.[1,2] In fact, obesity is currently considered as a major cause of hypogonadism, especially secondary hypogonadism with normal luteinizing hormone (LH) levels.[2–4] The association is bidirectional: obesity affects the hypothalamic–pituitary–gonad (HPG) axis by reducing testicular testosterone (T) synthesis, but T deficiency itself, for example, after androgen deprivation therapy or surgical castration, has also been shown to predispose to increased adipogenesis and accumulation of visceral obesity.[1–4]

Obesity can suppress the HPG axis via multiple pathways.[2–4] For example, aromatase enzyme activity in the adipose tissue stimulates T conversion to estradiol (E), thus increasing the negative feedback on the hypothalamus and the pituitary.[2–4] Further, regulation of LH pulse amplitude by adipocyte-secreted hormones, such as leptin, is disrupted in obese men. In addition, a decreased level of sex hormone-binding globulin, which is linked to low T, has been associated with obesity, especially increased visceral adiposity.[3,5] Low T also associates with a higher risk of abdominal obesity,[6] metabolic syndrome,[7] type 2 diabetes,[8,9] and cardiovascular diseases.[8]

Recent studies on the association between childhood obesity and cardiometabolic diseases in adulthood have focused on childhood growth trajectory data rather than on body mass index (BMI).[10–12] In the newborn, BMI increases from birth, reaching a maximum at age 7–9 months (adiposity peak, AP), after which it decreases until age 5–7 years. The second rise in BMI, the adiposity rebound (AR), occurs after this BMI decrease and lasts until adulthood.[11,12] Early AR has been associated with obesity and worse metabolic profile in adulthood both in men and women,[11,12] as well as with a diagnosis of polycystic ovary syndrome (PCOS) in women.[13] In previous studies, maternal obesity has also been shown to increase the risk of offspring's obesity,[14–17] but limited data exist on the association between maternal obesity and reproductive function in male offspring.

Low androgen levels in men are associated with several symptoms, such as gynecomastia, decreased body hair, erectile dysfunction, reduced sexual desire, hot flushes, and depression.[1–4] Physiological, gradual T decline has been reported to occur, especially after the age of 40[18,19]; however, more recently, the onset of T decline has been recognized to happen even earlier in life.[4,20] According to different guidelines, low T has been defined as serum T levels below 8–12 nmol/L, the cutoff levels being also dependent on men's age.[21–26]

In this large population-based birth cohort study, our aim was to evaluate the association of serum T levels in males at age 31 with prenatal factors, birth weight (BW), and the development of BMI from birth until late adulthood.

2 | MATERIALS AND METHODS

2.1 | Study population

The study population was derived from a general population data set, the Northern Finland Birth Cohort 1966 (NFBC1966), recruited at gestational week 24 from the two northernmost provinces of Finland. The cohort included a total of 12,058 live births (of them, 6169 males), covering 96% of all births in this area.[27,28] The study was approved by the Ethics Committee of the Northern Ostrobothnia Hospital District. All participants provided informed consent at ages 31 and 46. The flowchart depicting the study population is presented in Figure 1.

A postal questionnaire was sent at age 14 to 6022 boys and their families (of these, 5555, i.e., 92.2% answered), at age 31 to 5714 men (of these, 4285, i.e., 75% answered), and at age 46 to 5190 men (of these, 3162, i.e., 60.9% answered). The questionnaires at ages 31 and 46 included multiple questions on lifestyle, education, family history, living environment, and health.

In addition to the questionnaires, at age 31, 2906 (50.9% of contacted participants), and at age 46, 2742 (53.1%) men living in Northern Finland or in Helsinki metropolitan area participated in a clinical examination, including anthropometric measurements and blood samples for hormonal analyses. BMI was calculated as the ratio of weight (kg) and height squared (m^2). Waist and hip circumference were measured and waist-hip-ratio (WHR) was calculated at the 31- and 46-year clinical examinations. The data on current medication were verified by asking the question during the clinical examination.

2.2 | Definition of the study populations

A serum level of T < 12.1 nmol/L at age 31 was defined as a cutoff for low T levels, according to the recommendations of the European Association of Urology, the International Society for the Study of the Aging Male, and International Society for Sexual Medicine.[21,24,26] The use of T preparations (n = 0), statin medications (n = 0), and opioids (n = 8) was evaluated from a self-reported list of medications at age 31. The results did not change after excluding opioid users (data not shown). LH (cutoff value 9.4 U/L) and T were used to classify functional hypogonadism according to the literature.[29–31] Men with primary and compensated hypogonadism were excluded from the main analyses due to their small number and different etiology. This exclusion did not change the results (data not shown). A comparison of the main outcomes between the four groups is also shown in Table S1. The final study population included 2693 men, who were divided into two groups: men with low T (n = 132, 4.9% of the whole study

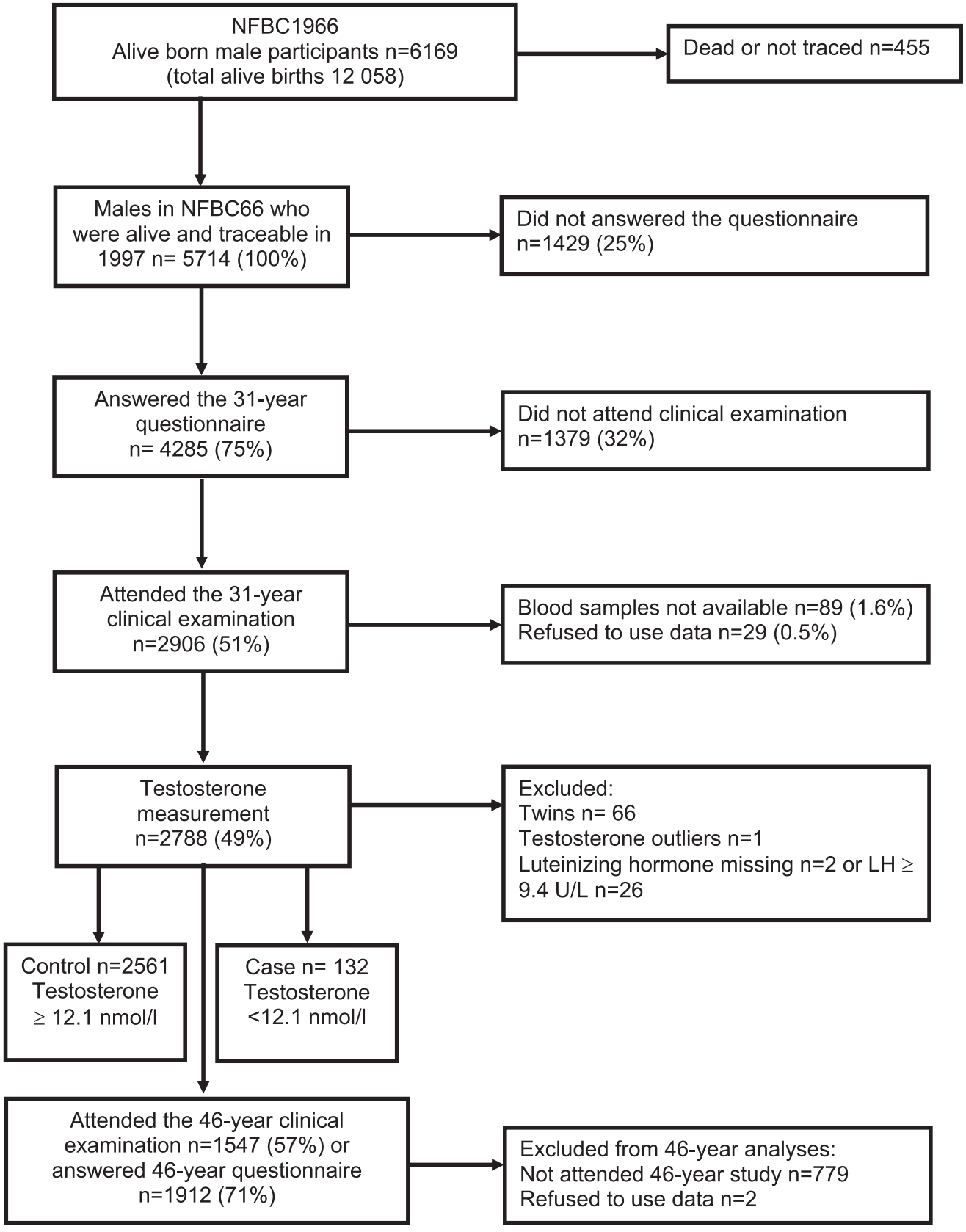


FIGURE 1 Flowchart of the study population.

population) and control men with normal T (n = 2561, 95.1% of the whole study population).

2.3 | Growth data

Weight and height from infancy to adolescence were collected from individual records reported by child health and welfare nurses, and later by school nurses, as part of the national child health program which is free for all children in Finland and covers almost 100% of the children. Growth data were available for 80% of the study population, some of the data were missing during the process of digitalization of the records from child health and welfare nurses. Twins' growth patterns differ from single-born babies, therefore, they were excluded from the analyses (n = 66).

BMI and the timing of AP and AR were derived from fitted growth curves, as described previously.[32] Previous literature has shown that BMI is an acceptable proxy for the estimation of fat mass in children.[33] Based on the natural changes observed in childhood BMI, the data were split into two age windows: infancy (2 weeks−1.5 years) and childhood (1.5−13 years). There were, on average, seven measurements during infancy and 16 during childhood for each child. Calculation of AP (1 month−1.5 years) and AR (1.5−13 years) was done only for the children having at least three measurements during childhood (AP n = 1584, AR n = 2008). We set an age cutoff value for early AR by dividing the study population into quartiles according to the age at AR. The early AR quartile limit value was ≤5.2 years in the present male population, in line with previously published data.[11,12] As late timing of AR has not been associated with adverse metabolic outcomes, the three upper quartiles (children with AR > 5.2 years) were pooled together to represent men with normal/late AR.

Height and weight at age 14 were measured and reported by the parents and BMI was calculated. According to the criteria of the World Health Organization (WHO) and Center for Disease Control and Prevention, BMI groups at the age of AR and 14 were stratified into underweight (< 5th percentile [pc]), normal weight (5th−85th pc), pre-obese, that is, overweight (85th−95th pc), and obese (> 95th pc).[34] BMI groups at ages 31 and 46 and maternal BMI were stratified according to the criteria of WHO: underweight (< 18.5 kg/m^2), normal weight (18.5−24.9 kg/m^2), pre-obese, that is, overweight (25.0−29.9 kg/m^2), and obese (> 30.0 kg/m^2).[35] If measurements were not available (46-year data), self-reported data were used. Self-reported and clinically measured BMI did not differ at age 14 (measured 19.35 vs. self-reported 19.32, p = 0.764) or at age 46 (measured 27.19 vs. self-reported 27.20 p = 0.987).

2.4 | Laboratory methods

At age 31, the serum levels of total T were measured using Agilent triple quadrupole 6410 LC/MS equipment with an electrospray ionization source, operating in positive-ion mode (Agilent Technologies). Multiple reaction monitoring was used to quantify testosterone by using tri-deuterated testosterone (d3-testosterone), with the following transitions: m/z 289.2−97 and 289.2−109 for testosterone and 292.2−97 and 292.2−109 for d3-testosterone. The intra-assay coefficient of variations (CVs) of the method were 5.3%, 1.6%, and 1.2% for testosterone at 0.6, 6.6, and 27.7 nmol/L, respectively. The inter-assay CVs were 5.3%, 4.2%, and 1.0% for the respective concentrations. LH at age 31 was determined using an immunochemiluminometric method (Advia Centaur; Siemens Healthcare Diagnostics).

2.5 | Covariates

Maternal factors (pre-pregnancy BMI, weight gain during pregnancy, maternal age, smoking at the end of pregnancy) and BW as well as gestational age were included in the confounding factors as they all associate with childhood growth.[36] Full-term, moderately preterm, and very preterm birth, as well as small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA) were defined according to the literature.[37,38] Information on the mothers was collected by the local midwifes in the antenatal clinics using a questionnaire. Smoking, educational level, and alcohol consumption at age 31 were classified based on the questionnaire. According to the literature and the study population's characteristics, maternal parameters (mothers' pre-pregnancy BMI and smoking at the end of pregnancy) as well as BW and gestational age, BMI at AR, and parameters at age 31, that is, alcohol consumption, education, smoking, and WHR (which depicts abdominal obesity and body fat distribution), were selected for the adjustment models when appropriate.

2.6 | Statistical methods

Differences in the continuous anthropometric parameters were analyzed via an independent t-test, a Mann–Whitney U-test, a one-way analysis of variance, or the Kruskal–Wallis test, as appropriate. To assess differences in the categorical parameters, a chi-square test was used. For these tests, results were reported as means or medians, standard deviations (SD) or prevalence (%), and odds ratios (OR) with a 95% confidence interval (95% CI), respectively. A p-value < 0.05 was considered statistically significant. Correlations were assessed by Pearson's correlation test. Linear association between continuous T levels, and maternal pre-pregnancy BMI, the age of AR, and BMI at AR were assessed with a regression model. Multivariable analyses were conducted using binary logistic modeling and linear regression models. IBM SPSS Statistics version 25 for Windows (SPSS, Inc., 1989, 2013, IBM Corp) was used to assess differences between the study groups and to perform regression analyses. Longitudinal BMI modeling was created with R-studio version 4.0.3. Modeling was carried out separately in infancy and adulthood, as described previously.[22] The outlier values of T were excluded using the first quartile cutoff, −1.5 × IQR (interquartile range), for the lower limit and the third quartile cutoff, +1.5 × IQR, for the upper limit.[39] Using these

TABLE 1 Baseline characteristics of the study population.

	Cases	Controls	*p*-Value
Maternal parameters			
Mother's age (y) mean ± SD (*n*)	28.83 ± 7.14 (132)	28.22 ± 6.63 (2548)	0.302
Mother's pre-pregnancy BMI (kg/m^2) mean ± SD (*n*)	**24.04 ± 4.00 (123)**	**23.19 ± 3.12 (2319)**	**0.022**
Underweight *n* (%)	3 (2.4)	60 (2.6)	0.532
Normal weight *n* (%)	**83 (67.5)**	**1747 (75.3)**	**<0.001**
Overweight *n* (%)	25 (20.3)	431 (18.6)	0.326
Obesity *n* (%)	**12 (9.8)**	**81 (3.5)**	**<0.001**
Mother's weight gain during pregnancy (kg) mean ± SD (*n*)	14.94 ± 16.68 (58)	13.95 ± 12.93 (1059)	0.577
Mothers smoking end of pregnancy *n* (%)	10 (7.9)	273 (11.1)	0.164
Index person's parameters			
Birth weight (g) mean ± SD (*n*)	3590 ± 534 (132)	3581 ± 522 (2561)	0.829
Low birth weight < 2500 g *n* (%)	3 (2.3)	62 (2.4)	0.914
Gestational age (week) mean ± SD (*n*)	39.17 ± 6.24 (132)	38.80 ± 7.12 (2561)	0.559
Prematurity (born before 37th gw) *n* (%)	7 (5.3)	179 (6.9)	0.746
Size for gestational age *n* (%)			0.137
SGA	10 (7.9)	165 (6.7)	
AGA	97 (76.4)	2033 (82.8)	
LGA	20 (15.7)	257 (10.5)	
Alcohol consumption 31 y (g/day) mean ± SD (*n*)	**10.34 ± 14.00 (125)**	**14.05 ± 20.14 (2480)**	**0.043**
Smoking 31 y *n* (%)			0.488
Non-smoker	56 (43.1)	951 (37.8)	
Former/occasional smoker	32 (24.6)	677 (26.9)	
Active smoker	42 (32.3)	885 (35.3)	
Education 31 y *n* (%)			0.673
Basic	17 (13.1)	289 (11.4)	
Secondary	91 (70.0)	1861 (73.5)	
Tertiary	22 (16.9)	381 (15.1)	

Note: The continuous data are reported as mean ± standard deviation (SD) and the comparison of differences between groups were performed using Mann–Whitney U test or Kruskal–Wallis test. Categorical data were analyzed by chi-square test.
p-values <0.05 are bolded.
Abbreviations: AGA, appropriate for gestational age; BMI, body mass index; gw, gestational week; LGA, large for gestational age; *n*, number; SGA, small for gestational age; y, year.

criteria, only one measurement (T > 50.5 nmol/L) was excluded as an outlier.

3 | RESULTS

3.1 | Maternal factors and infancy

Maternal age, gestational smoking status at the end of pregnancy, participants' own BW, or gestational age did not associate with low T at age 31 (Table 1). Maternal pre-pregnancy BMI (adjusted OR [aOR, 95% CI]: 1.06 [1.01–1.11], $p = 0.028$) and obesity (aOR: 2.43 [1.19–4.98], $p < 0.001$) associated independently with low T but weight gain during pregnancy did not (adjusted for mother's smoking at the end of pregnancy, BW and gestational age, and parameters at age 31, i.e., alcohol consumption, education, smoking, and WHR). After further adjustment for BMI at the age of AR, the associations between maternal BMI or maternal obesity with low T at age 31 lost their significance (aOR: 2.27 [0.97–5.33], $p = 0.057$ and aOR: 2.12 [0.99–4.58], $p = 0.055$, respectively). When using T level and BMI as continuous variables, maternal pre-pregnancy BMI correlated significantly with serum T levels ($r = 0.053$, $p = 0.033$) and the association between maternal pre-pregnancy BMI and serum T levels was quadratic (Figure S1A) and remained significant after adjustments ($p = 0.011$).

The proportion of AGA children did not differ between the case and the control groups (76.3% vs. 82.7%, $p = 0.061$). SGA did not associate with low T (SGA vs. AGA: OR 1.36 [0.71–1.25], $p = 0.387$), but there

TABLE 2 Anthropometric parameters in cases and controls at ages of adiposity peak, adiposity rebound, and at ages 14, 31, and 46.

	Cases mean ± SD (*n*)	Controls mean ± SD (*n*)	*p*-Value
Age			
AP (months)	8.92 ± 0.78 (72)	8.92 ± 0.71 (1512)	0.920
AR (y)	**5.28 ± 0.98 (95)**	**5.82 ± 0.88 (1913)**	**<0.001**
BMI (kg/m^2)			
AP	18.34 ± 1.04 (72)	18.29 ± 1.08 (1512)	0.804
AR	**15.80 ± 1.19 (95)**	**15.43 ± 1.00 (1913)**	**<0.001**
14 y	**20.61 ± 3.04 (117)**	**19.26 ± 2.42 (2255)**	**<0.001**
31 y	**28.91 ± 5.18 (132)**	**25.06 ± 3.40 (2543)**	**<0.001**
46 y	**29.67 ± 5.77 (94)**	**27.06 ± 3.96 (1818)**	**<0.001**
BMI change (kg/m^2)			
AR−14 y	**4.73 ± 2.23 (85)**	**3.77 ± 1.93 (1694)**	**<0.001**
14−31 y	**8.30 ± 4.03 (116)**	**5.77 ± 2.92 (2241)**	**<0.001**
31−46 y	**1.07 ± 3.95 (94)**	**2.00 ± 2.39 (1792)**	**0.029**
WHR			
31 y	**0.95 ± 0.061 (131)**	**0.91 ± 0.058 (2535)**	**<0.001**
46 y	**0.99 ± 0.051 (75)**	**0.97 ± 0.062 (1458)**	**0.033**

Note: The results are reported as mean ± standard deviation (SD) and the comparison of differences between groups were performed using Mann−Whitney U test or Kruskal−Wallis test.
p-values <0.05 are bolded.
Abbreviations: AP, adiposity peak; AR, adiposity rebound; BMI, body mass index; *n*, number; WHR, waist-hip-ratio; y, year.

was a trend toward an association between LGA and low T (OR 1.63 [0.99−2.68], $p = 0.052$).

3.2 | Adiposity peak and adiposity rebound

There were no differences between the case and the control groups regarding the age of AP (Table 2). Longitudinal, childhood BMI trajectory data revealed an earlier AR timing (before age 5.2 years) (aOR: 0.73 [0.56−0.94], $p < 0.001$) and a higher BMI at AR (aOR: 1.12 [1.01−1.45], $p = 0.012$) in cases compared to controls from AR timing onward (Table 2 and Figure 2) even after adjusting for maternal factors and for WHR at age 31 (Table S2). Supporting this finding, the case group was overrepresented in the earliest AR timing quartile (case vs. control groups: 44.2% vs. 22.5%, aOR 1.54 [1.02−2.56], $p = 0.008$). Moreover, in the whole study population, the mean T levels at age 31 were lower in men with early AR compared with normal/late AR (20.43 ± 6.32 vs. 21.94 ± 6.31, $p < 0.001$) and the prevalence of low T at age 31 was higher in the early AR group (9.3% vs. 3.5%, aOR 1.54 [1.02−2.56], $p = 0.012$). BMI at AR was higher in cases with early AR compared to controls with early AR as well as cases with normal/late AR age compared to controls with normal/late AR, but not between cases and controls with early AR (Figure 3). Age at AR and BMI at AR correlated significantly with serum T levels ($r = 0.103$, $p < 0.001$, $r = 0.049$, $p = 0.026$) and the associations were linear (Figure S1B,C). After adjustments, the association between age at AR and T levels remained significant ($p < 0.001$), but the significance was lost between BMI at AR and T levels ($p = 0.211$).

3.3 | BMI and BMI change from childhood and adolescence to late adulthood

Prevalence of pre-obesity and obesity at ages 14 and 31 and of obesity at age 46 were higher in the case group (Figure S2). Men with low T and with early AR had the highest BMI at ages 14, 31, and 46 (Figure 3) and the greatest weight gain between ages 14 and 31 (Table 3). Cases with normal/late AR compared to control men with normal/late AR also had higher BMI at ages 31 and 46 but not at age 14 (Figure 3).

BMI increased more between the age of AR and age 14 and between ages 14 and 31 in the case group, but the BMI increase between ages 31 and 46 was greater in the control group (Table 2). At ages 31 and 46, WHR was greater in the case group compared to controls (Table 2). The results did not change after adjustments (Table S2).

4 | DISCUSSION

The present prospective, population-based cohort study reports for the first time the association of serum T levels in adulthood with prenatal factors, AR timing, and BMI development from birth until age 46

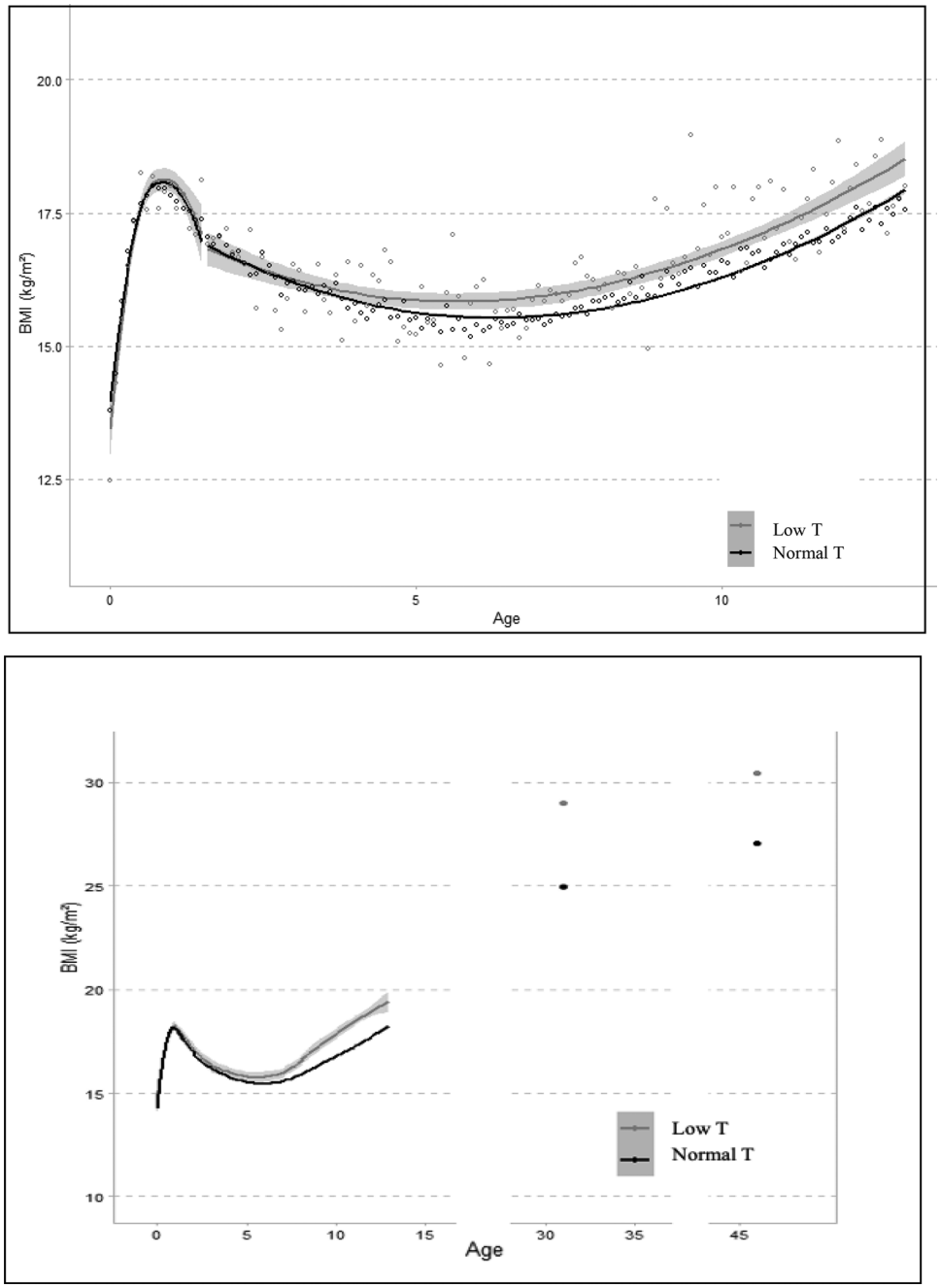
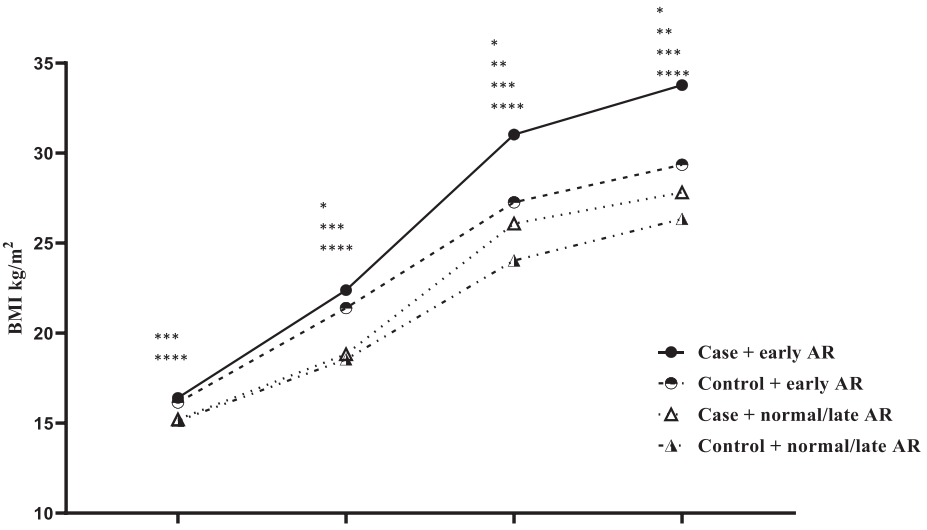


FIGURE 2 Body mass index trajectories in men with low testosterone and controls. 95% confidence intervals are shown. Men with low testosterone present early deviation in BMI trajectories from adiposity rebound onward until late adulthood.
Abbreviations: BMI, body mass index; T, testosterone.

in men. Maternal obesity was associated with an increased risk of low T levels in adulthood. Further, the BMI trajectories already started to deviate during childhood in men with low T, showing earlier AR and higher BMI from childhood until age 46.

In previous studies, low BW,[40,41] SGA,[42] and prematurity[43] have been shown to associate with decreased serum T levels in adulthood, although opposite results have also been published.[43,44] In this study, BW, gestational age, or prematurity did not associate with low T. As a novel finding, maternal obesity was associated with low T serum levels in adult men, independently of adulthood abdominal obesity, but the association vanished after adjusting for BMI in childhood. However, the inverted J-shaped association between continuous T level and maternal BMI remained significant. Developmental programming occurs in utero resulting in increased susceptibility to health problems also in adulthood.[45,46] Epigenetic modification of gene expression is a likely mechanism connecting the early development of excess adiposity with the future risk of an adverse metabolic and hormonal phenotype.[47] Indeed, these changes have long-lasting effects, not only by



	AR	14y	31y	46y
Case + early AR	16.41 ± 1.14	22.39 ± 1.11	31.03 ± 4.59	33.78 ± 6.04
Control + early AR	16.15 ± 1.11	21.39 ± 2.73	27.27 ± 3.78	29.35 ± 4.35
Case + normal/late AR	15.21 ± 0.86	18.83 ± 1.54	26.08 ± 4.56	27.82 ± 4.68
Control + normal/late AR	15.12 ± 0.86	18.54 ± 1.84	24.03 ± 2.89	26.34 ± 3.57

FIGURE 3 Body mass index at the age of adiposity rebound (AR) and ages 14, 31, and 46 in men with low testosterone at age 31 (cases) and controls. The study population was divided into four groups according to the occurrence of early or normal/late AR. Adiposity rebound was considered to be early when occurring before age 5.2 years.

TABLE 3 Body mass index changes between ages AR, 14, 31, and 46; waist-hip-ratios at ages 31 and 46 and hormonal parameters at age 31 in men with low testosterone at age 31 (cases) and controls with early versus normal/late adiposity rebound.

	Early AR ($n = 479$)			Normal/late AR ($n = 1555$)				
Mean ± SD	Cases($n = 44$)[a]	Controls ($n = 430$)[a]	p-value[b]	Cases ($n = 53$)[a]	Controls ($n = 1483$)[a]	p-value[b]	p-value[c]	p-value[d]
BMI change (kg/m^2)								
AR−14 y	5.92 ± 2.41	5.25 ± 2.41	0.083	3.63 ± 1.51	3.33 ± 1.28	0.247	**<0.001**	**<0.001**
14−31 y	8.70 ± 3.54	5.91 ± 3.47	**<0.001**	7.71 ± 3.11	5.82 ± 2.76	**<0.001**	**0.045**	0.274
31−46 y	0.52 ± 5.12	1.92 ± 2.99	0.149	1.95 ± 2.71	2.01 ± 2.18	0.882	0.256	0.582
WHR								
31 y	0.98 ± 0.045	0.93 ± 0.057	**<0.001**	0.94 ± 0.072	0.90 ± 0.054	**<0.001**	**<0.001**	**<0.001**
46 y	1.02 ± 0.057	1.00 ± 0.06	0.150	0.98 ± 0.059	0.97 ± 0.061	0.482	**<0.001**	**<0.001**

Note: The results are reported as mean ± standard deviation (SD) and the comparison of differences between groups were performed using Mann−Whitney U test or Kruskal−Wallis test. AR was considered early when occurring before age 5.2 y.
p-values <0.05 are bolded.
Abbreviations: AR, adiposity rebound; BMI, body mass index; *n*, number; WHR, waist-hip-ratio; y, year.
[a]Numbers may vary in different analysis due to some missing data.
[b]Case versus control in the early AR or normal/late AR groups.
[c]Case + early AR versus case + normal/late AR.
[d]Control + early AR versus control + normal/late AR.

increasing the risk of obesity but also the risk of metabolic and endocrine disorders.[48,49]

It has been previously reported that maternal obesity is associated with offspring's obesity later in life, and social environment has a strong impact on this association.[14–17] This association was also seen in our study population. Of note, the association between maternal pre-pregnancy BMI and low T lost its significance after adjustment with BMI in childhood, suggesting that the effect of maternal obesity might be mediated through elevated childhood BMI. In line with the present finding, extreme weight loss after bariatric surgery has been shown

to reduce the risk of offspring obesity compared to the siblings born before bariatric surgery.[50] This result might suggest that the expression of obesity in offspring can be modulated by the mother's weight loss, but the nature of the association with hormonal parameters later in life remains unclear. One putative mediator of this association is leptin, the level of which has been shown to be affected by maternal obesity and nutrition during pregnancy. Leptin concentrations are higher in the circulation of obese mothers as well as in the cord blood,[51,52] and children exposed to higher leptin levels prenatally have higher BW.[52,53] Paradoxically, higher leptin levels have been associated with slower weight gain in infancy and in early childhood,[54,55] but in later childhood and adolescence, a positive correlation between cord leptin levels and adiposity has been detected.[56] Moreover, increased leptin levels and leptin resistance are associated with obesity later in life,[3,51] and high leptin levels have been shown to act directly on the hypothalamus, pituitary, and testis by inhibiting steroidogenesis.[1–4]

Younger age and higher BMI at the time of AR onward were associated with low T at age 31. Notably, men with early AR and low T presented the highest BMI from AR onward and the greatest WHR at ages 31 and 46, suggesting a more severe phenotype for those with early AR. This finding suggests a strong positive interaction between early AR and the development of obesity with increased susceptibility to low T levels in adulthood. The association between early AR and low T remained significant after adjustment for abdominal obesity at age 31, suggesting also an independent role of AR regarding the development of hypoandrogenemia. Two previous studies in men have shown that higher BMI at prepubertal age and "rising to high" BMI growth trajectories are associated with lower T levels in adulthood.[43,44] Moreover, early AR has also been linked to adverse metabolic outcomes and obesity in men and women,[11,12,57–59] and recently with PCOS in women, independently of adulthood obesity.[13] However, studies describing the association between early AR and T levels in adult men have not previously been reported.

Interestingly, we detected lower weight gain between ages 31 and 46 among men with low T. This novel finding is perhaps surprising. One explanation could be the loss of skeletal muscle mass in late adulthood in men with hypogonadism, as shown in a previous study concerning men with functional hypogonadotropic hypogonadism.[60] More studies are needed to confirm this observation and the etiology behind it.

The strengths of our study are that it involves a large, population-based cohort and incorporates longitudinal data from birth until age 46. Our study population was also ethnically homogeneous. All measurements-except for BMI at age 14-were performed by trained professionals at every step. Additionally, we were able to evaluate the use of testosterone supplementations, statins, and opioids, albeit self-reported. We were also able to define secondary and primary hypogonadism by taking LH into account. Another novel contribution of the present work was that in addition to analyzing specific childhood and adolescent BMIs, childhood growth trajectory data were also assessed, which have been shown to be a valuable tool to evaluate the BMI and metabolic risks in adulthood.

This study had some limitations. First, only a single T sample for each subject was available for analysis.[21,26] However, to minimize potential bias, all blood tests were drawn in the morning when the physiological higher T levels occur.[61] Another limitation is that, despite high participation rates at all data collection points, full childhood growth data were not available for all participants. Further, we were not able to take paternal obesity into account. Also, the study results should also be tested in other ethnicities. Lastly, adiposity, lean body mass, T levels in childhood, or timing of pubertal onset were not available in the cohort.

5 | CONCLUSIONS

In conclusion, maternal obesity and early AR were associated with low T serum levels in men in early adulthood. The BMI growth trajectories appeared to deviate at the time of AR onward and weight gain was faster in men with low T until age 31. Men with both early AR and low T were at the highest risk of developing obesity in adulthood, suggesting a more severe phenotype in this group of men. This finding might suggest that early AR timing may be the first sign of a developmental process leading toward T deficiency in adulthood.

AUTHOR CONTRIBUTIONS

JL, PP, MN, and LM-P conceptualized the study and participated in the study design. JL and LL contributed to data acquisition and revision. JL and EK carried out the statistical analysis. JL drafted the initial manuscript. JL, PP, MO, JST, MN, and LM-P interpreted the data. All authors have reviewed several versions of the manuscript critically, participated in discussions of manuscript content, and have given final approval of the version to be published.

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CONFLICT OF INTEREST STATEMENT

The other authors have no conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

A novel SNP in *HUWE1* promoter confers increased risk of NOA by affecting the RA/RARα pathway in Chinese individuals

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Abstract

Background: The ubiquitin ligase HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 is essential for the establishment and maintenance of spermatogonia. However, the role of HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 in regulating germ cell differentiation remains unclear, and clinical evidence linking HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 to male infertility pathogenesis is lacking.

Objective: This study aims to investigate the role of *HUWE1* in germ cell differentiation and the mechanism by which a *HUWE1* single nucleotide polymorphism increases male infertility risk.

Materials and methods: We analyzed *HUWE1* single nucleotide polymorphisms in 190 non-obstructive azoospermia patients of Han Chinese descent. We evaluated HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 regulation by retinoic acid receptor alpha using chromatin immunoprecipitation assays, electrophoretic mobility shift assays, and siRNA-mediated *RARα* knockdown. Using C18-4 spermatogonial cells, we determined whether HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 participated in retinoic acid-mediated retinoic acid receptor alpha signaling. We performed luciferase assays, cell counting kit-8 assays, immunofluorescence, quantitative real-time polymerase chain reaction, and western blotting. We quantified *HUWE1* and retinoic acid receptor alpha in testicular biopsies from non-obstructive azoospermia and obstructive azoospermia patients using quantitative real-time polymerase chain reaction and immunofluorescence.

Xudong Shan, Xueguang Zhang, and Gelin Huang contributed equally to this work.

Results: Three *HUWE1* single nucleotide polymorphisms were significantly associated with spermatogenic failure in 190 non-obstructive azoospermia patients; one (rs34492591) was in the *HUWE1* promoter. Retinoic acid receptor alpha regulates *HUWE1* gene expression by binding to its promoter. HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 participates in retinoic acid/retinoic acid receptor alpha signaling pathway and regulates the expression of germ cell differentiation genes *STRA8* and *SCP3* to inhibit cell proliferation and reduce γH2AX accumulation. Notably, significantly lower levels of *HUWE1* and *RARα* were detected in testicular biopsy samples from non-obstructive azoospermia patients.

Conclusions: An *HUWE1* promoter single nucleotide polymorphism significantly downregulates its expression in non-obstructive azoospermia patients. Mechanistically, HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 regulates germ cell differentiation during meiotic prophase through its participation in retinoic acid/retinoic acid receptor alpha signaling and subsequent modulation of γH2AX. Taken together, these results strongly suggest that the genetic polymorphisms of *HUWE1* are closely related to spermatogenesis and non-obstructive azoospermia pathogenesis.

KEYWORDS

HUWE1, non-obstructive azoospermia (NOA), RA/RARα signaling pathway, single nucleotide polymorphisms (SNPs)

1 | INTRODUCTION

Infertility is a growing medical and social dilemma affecting approximately 8%–12% of couples globally,[1] with male-related factors accounting for about 30%–50% of infertile couples.[2,3] Male infertility is primarily caused by sperm abnormalities, such as asthenospermia, teratozoospermia, oligospermia, and azoospermia, with the latter being the most detrimental condition leading to male infertility.[4] Azoospermia refers to the absence of spermatozoa in ejaculation and affects 15% of infertile men.[5] It can be classified into two categories based on the pathological state: (i) obstructive azoospermia (OA) comprises 40% of azoospermia cases and is usually caused by bilateral vas deferens obstruction but with normal endocrine function and spermatogenesis[6]; (ii) non-obstructive azoospermia (NOA) affects approximately 60% of azoospermic men and is caused by congenital factor defects and other factors inducing primary or secondary testicular failure.[7,8] Unfortunately, 73% of NOA cases remain of unknown etiology because of the complexity of spermatogenesis and testicular function.[9] Genetic factors play an important role in the occurrence of NOA, and there are a few cases of NOA caused by monogenic factors. Presently, monogenic causes account for 4% of all NOA cases.[9,10] Clearly, more genetic factors could increase NOA risk by affecting normal spermatogenesis.

HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 (HUWE1) is a macromolecular protein in the HECT family and an E3 ubiquitin ligase first reported in 2005.[11] HUWE1 contains various domains, including a catalytic ubiquitin ligase domain, multiple ubiquitin-binding domains, and conserved domains of uncertain function.[12] HUWE1 regulates various physiological processes, including tumor cell growth and inhibition, apoptosis, DNA damage repair, and embryonic development.[13] Previous studies have revealed that HUWE1 is crucial in the development and function of the nervous system.[14] In addition, HUWE1 is a key factor influencing the immune system and myogenesis.[15,16] Notably, HUWE1 plays an important role in spermatogenesis. Our previous studies have shown that the expression of HUWE1 is dynamically regulated in the testis over time during spermatogenesis, consistent with its function in spermiogenesis in histone ubiquitination and degradation.[17] Deficiency in HUWE1 leads to spermatogonial degeneration, meiotic entry without synchrony, and increased H2AX and DNA damage.[18,19] Despite previous efforts, the role of HUWE1 in spermatogenesis is still not fully understood because of limited findings. Importantly, currently, there is no evidence to suggest a clinical connection between male infertility and HUWE1 polymorphisms.

Retinoic acid (RA) is a biologically active form of vitamin A that is essential in the early stages of spermatogonia differentiation and spermatocyte meiosis.[20,21] It has been proven that RA regulates crucial factors such as Retinoic Acid Activated gene 8 (STRA8), oncogene protein (KIT), Glial Cell Line-Derived Neurotraphic factor (GDNF), and Bone Morphogenetic protein 4 (BMP4) through multiple pathways, and promotes or inhibits spermatogenesis depending on context.[22] Notably, RA directly activates the *STRA8* gene during spermatogonial

differentiation, and its absence leads to the accumulation of undifferentiated spermatogonia.[23] Similarly, inactivating HUWE1 results in blocked spermatogonia differentiation in mice. However, the more nuanced role of the RA signaling pathway and HUWE1 in germ cell differentiation requires further investigation.

This study identified three single nucleotide polymorphism (SNP) loci that were significantly correlated with NOA patients and spermatogenesis disorder, one of which (located in the *HUWE1* promoter region, rs34492591) is associated with gene transcription. Functional studies indicated that retinoic acid receptor alpha (RARα) binds to the promoter region of *HUWE1* and regulates its expression. Moreover, HUWE1 deficiency leads to the accumulation of γH2AX, resulting in germ cell differentiation arrest. Furthermore, testicular biopsy samples revealed that HUWE1 deficiency was significantly associated with the pathogenesis of NOA. Collectively, our findings demonstrate that the SNP (rs34492591) on *HUWE1* impairs spermatogenesis, providing new insights into the role of HUWE1 and RA/RARα signaling in spermatogenesis.

2 | MATERIALS AND METHODS

2.1 | Patients and clinical definition

All patients and controls were recruited from West China Second University Hospital, Sichuan University, and informed consent was obtained. This study was conducted according to the tenets of the Declaration of Helsinki, and ethical approval was obtained from the Ethical Review Board of West China Second University Hospital, Sichuan University. Patients diagnosed with NOA and those with OA were included if they presented with a normal karyotype, no azoospermia factor (AZF) or chromosome Yq microdeletions, no history of testicular disorders (e.g., orchitis, dysplasia, cryptorchidism, varicocele), and normal serum hormone levels (follicle-stimulating hormone, luteinizing hormone, testosterone). Testicular biopsies obtained for sperm extraction and assisted reproduction were also used for histological analysis to confirm diagnoses and subclassify NOA patients. Patients exhibiting hypospermatogenesis (HS) showed reduced germ cell numbers at all stages, resulting in low spermatid counts. Those with maturation arrest (MA) exhibited failure of germ cells to progress beyond the spermatogonial or primary spermatocyte stage in >90% of seminiferous tubules. Sertoli cell only syndrome was defined by the presence of only Sertoli cells and the absence of germ cells within seminiferous tubules. Only patients with HS or MA were included. OA patients presented with vas deferens obstruction. Medical history, physical examination, semen analysis (World Health Organization protocol), and hormone profiling were conducted.

2.2 | SNP probe choice and SNP genotyping

SNP probe selection and SNP genotyping was conducted on 190 patients and 243 controls in 26 SNP loci regions to investigate the relationship between *HUWE1* gene abnormalities and azoospermia. The SNP lists are detailed in Table S1. MassARRAY system genotype calling was performed to process the genotyping data using MassARRAY RT software. The frequency of the nucleotide variations in control and patient groups was examined using the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) platform.

2.3 | Analysis of *HUWE1* SNPs in NOA patients using MALDI-TOF MS platform

DNA samples were prepared from peripheral blood collected from 190 NOA patients. SNPs on *HUWE1* were analyzed using the MALDI-TOF MS platform. The targeted SNPs on *HUWE1* were amplified by polymerase chain reaction (PCR) with specific primers. Amplified products were treated with shrimp alkaline phosphatase and exonuclease I to dephosphorylate remaining dNTPs and unincorporated primers. The purified single-stranded PCR products were then processed for SpectroCHIP loading. SpectroCHIPs were prepared with 384 wells and each well was loaded with a specific set of extension primers designed to anneal adjacent to the SNP site. Extension primer sets and allelic discrimination were determined according to the manufacturer's recommendations. After the extension reaction, mass spectra were acquired on a MALDI-TOF MS platform (Sequenom MassARRAY System). Data were analyzed using the SpectroTYPER software for genotype calling and SNP analysis. Statistical analysis was performed using standard methods to determine the association between *HUWE1* SNP and NOA prevalence.

2.4 | Cell culture and transfections

The human teratoma cell line NCCIT was obtained from BeNa Culture Collection (Shanghai, China). The mouse spermatogonial stem cell line C18-4 was purchased from BLUEFBIO Inc. (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640) medium was used to culture NCCIT cells, while Dulbeco's modified eagle medium (DMEM) was used for C18-4 cells. Cells were supplemented with 10% (vol/vol) fetal bovine serum and 100 nM penicillin/streptomycin and incubated in a 5% CO_2 incubator at 37°C. Transfection of cells with plasmid DNA or *RARα* siRNA (RIBOBIO Inc., Guangzhou, China) was performed using the transfection reagent Lipofectamine™ 3000 (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Following transfection, tests were conducted on the cells after a 48-h culture period.

2.5 | Plasmid construction

The *HUWE1* promoter DNA fragment was designed to encompass the rs34492591 SNP locus, consisting of 200 base pairs, and underwent amplification by PCR, utilizing the genome of NCCIT cells as a template. The primers were designed to create NheI

and NcoI restriction sites at the 5′ and 3′ ends of the amplified fragments, respectively. The forward and reverse primers were 5′-CTAGCTAGC TGCTCTGGAGGGCTGGAA-3′ and 5′-CATGCCATGG GGGCTGCGGACGACATT-3′ (Sangon Inc., Shanghai, China), respectively. Subsequently, NheI and NcoI restriction endonuclease cleavage was performed on the DNA fragment, which was then inserted into the pGL3-basic plasmid, serving as the platform for construction of the wild-type (WT) *HUWE1* promoter plasmid. The mutant plasmid was produced using the fast mutagenesis system kit (TransGen Biotech Inc., Beijing, China) based on the point polymorphism of the rs34492591 SNP locus (T to C).

2.6 | Chromatin immunoprecipitation assay

NCCIT cells were transfected with pGL3-basic plasmids containing WT *HUWE1* promoter or empty vector using commercial transfection reagents (Life Technologies). After 48 h, 10 μM RA was added and cells were incubated for another 48 h before performing chromatin immunoprecipitation (ChIP) assays. A ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY, USA) was utilized according to the manufacturer's protocol. Cells at 70%–80% confluence were crosslinked with formaldehyde and immunoprecipitated using anti-RARα antibody (Abcam, Inc., USA). The de-crosslinked and purified immunoprecipitated DNA was obtained. Quantitative real-time PCR (RT-qPCR) was performed with SYBR Green qPCR Master Mix (MCE Technologies, NY, USA) and *HUWE1* promoter-specific primers. Cycle conditions were 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 60 s, and 72°C for 60 s. Melting curve analysis confirmed specific products. Ct values calculated RARα-bound *HUWE1* promoter enrichment over control using the delta delta Ct method. The primers were as follows: forward, 5′-GCTCTGGAGGGCTGGAATTA-3′ and reverse, 5′-GAGGGGCGAAGAACTTTGAC-3′ (Sangon Inc.).

2.7 | Electrophoretic mobility shift assays

The *HUWE1* promoter DNA probe spanned the rs34492591 SNP and consisted of either WT or mutant sequence at the 3′ end (Sangon Inc.). The probe sequences of WT and mutant probes were as follows—WT: Biotin-5′-AAGACAAAAGCACTATTAAAAGAACTCCTG-3′, 3′-TTCTGTTTTCGTGATAATTTTCTTGAGGAC-5′-Biotin; MUT: Biotin-5′-AAGACAAAAGCACTACTAAAAGAACTCCTG-3′, 3′-TTCTGTTTTCGTGATGATTTTCTTGAGGAC-5′-Biotin. Nuclear extracts from NCCIT cell lines were prepared using the nuclear extraction kit (Chemicon, Merck Millipore), and protein concentration was determined by Bicinchoninic Acid (BCA) assay (Thermo Inc., USA) according to manufacturer's instructions. Electrophoretic mobility shift assay (EMSA) was performed using LightShift Chemiluminescent EMSA Kit (Thermo Inc.) and followed the protocol of the kit's instructions. Binding reactions (20 μL) contained 1× binding buffer, 50 ng/μL Poly (*dI·dC*), 2 μg nuclear extract, and 1 μL anti-RARα antibody (Abcam, Inc.), and 20 fmol biotin-labeled oligo; reactions were incubated in the dark at room temperature for 40 min. Complexes were resolved by 6% native polyacrylamide gel electrophoresis in 0.5× Tris-Borate-EDTA (TBE) buffer at 100 V for 60 min at 4°C. DNA was transferred to a nylon membrane (Merck Millipore) and crosslinked at 120 mJ/cm^2 using a UV crosslinker (HL-2000 Hybrilinker, UVP, LLC, USA). Biotin-labeled DNA was detected by chemiluminescence.

2.8 | Cell counting kit-8 analysis

For each well of a 96-well plate, 1×10^4 C18-4 cells were cultured as attached monolayers overnight. The cells were then treated with either 10 μM RA reagent or equal amounts of Dimethyl Sulfoxide (DMSO) for 0, 24, 48, or 72 h. After treatment, cell viability was assessed using the cell counting kit-8 (CCK-8) (Dojindo, Shanghai, China) in accordance with the manufacturer's instructions. Briefly, 10 μL of CCK-8 solution was added to each well, the plate was incubated for 1 h, and then the absorbance was measured at 450 nm using an automatic microplate reader (Varioskan Flash, Fisher Scientific, Inc.).

2.9 | Immunofluorescence staining

The cells were fixed with 4% PFA for 15 min at room temperature. The cell membrane permeability was increased by adding 0.3% Triton X-100 in 1× Phosphate Buffered Saline (PBS) for 20 min. The cells were then incubated overnight at 4°C with the primary antibody γH2AX (1:100, Abcam, Inc.). After washing, the cells were incubated for 60 min in the dark at room temperature with secondary fluorescent antibodies (1:1000, Invitrogen, Inc.). Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime, Inc., Shanghai, China). For testis tissues, sections were deparaffinized with xylene and rehydrated through an ethanol gradient. Antigen retrieval was performed by heating sections in 10 mM citrate buffer for 20 min. Sections were washed in 1× PBS and blocked with 5% normal goat serum for 1 h at room temperature. Sections were then incubated with a primary antibody against HUWE1 (1:100; Abcam, Inc.) and RARα (1:100; Proteintech) in a humidified chamber overnight at 4°C. After washing with 1× PBS, sections were incubated with an Alexa Fluor 594-conjugated secondary antibody for 1 h at room temperature. Nuclei were counterstained with DAPI. Images were captured using a confocal laser scanning microscope (Olympus FV3000, Olympus Corporation). Specifically, we used ImageJ software to analyze the fluorescence intensity of target protein.

2.10 | Luciferase assay

C18-4 cells were transfected with pGL3-basic plasmid containing WT or mutant *HUWE1* promoter sequences using a transfection kit (Life Technologies). The promoter sequence used was 200 base pairs, ranging from −750 to −550 relative to the transcription start site of *HUWE1*. After 48 h of cell growth, cells were treated with 10 μM RA or DMSO for 48 h and then lysed in passive lysis buffer according to the kit's instructions (Pierce, Rockford, IL, USA). The cells were then

(A)

MARKER	Allele	Case	Control	Chi^2	*P* value	Odds Ratio	95% CI	Location
rs5978179	C	14(0.069)	4(0.016)					Intron between exons 5, 6
	T	190(0.931)	243(0.984)	8.0159	0.0046	4.476	1.450-13.820	
rs34492591	C	13(0.065)	4(0.016)					672 bases upstream of
	T	188(0.935)	242(0.984)	7.0877	0.0078	4.184	1.342-13.039	transcriptional start site
rs6529689	T	14(0.069)	4(0.016)					Intron between exons 16,
	G	189(0.931)	242(0.984)	8.0286	0.0046	4.481	1.451-13.837	17

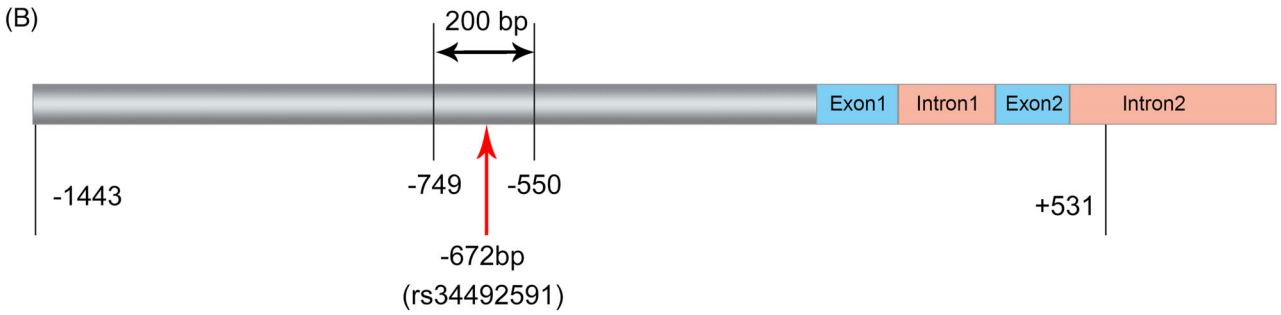

FIGURE 1 Single nucleotide polymorphism (SNP) rs34492591 in HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 (HUWE1) is associated with non-obstructive azoospermia. (A) The information of three SNP locus in *HUWE1* gene. (B) The location of rs34492591 in HUWE1 promoter region, indicated by the red arrow.

rinsed in 1× PBS and lysed in 50 L passive lysis buffer according to the kit's instructions (Pierce). Luciferase activity was measured using a microplate reader (Thermo Scientific) and expressed as relative light units.

2.11 | Western blotting

Cells were lysed on ice for 30 min in radioimmunoprecipitation assay buffer containing protease inhibitors and then centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was collected, and protein concentration was determined using a BCA assay. Proteins were separated by SDS-PAGE and transferred to Polyvinylidene Fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% bovine Serum albumin (BSA) (ThermoFisher Scientific) and incubated overnight at 4°C with primary antibodies against RARα, SCP3, γH2AX or Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Next, membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; ThermoFisher Scientific) for 60 min at room temperature. Proteins were visualized using enhanced chemiluminescence (ECL) reagent (ThermoFisher Scientific).

2.12 | Statistical analysis

The images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA), and all statistical analyses were performed using GraphPad 7.0 software (GraphPad software, Inc., La Jolla, CA, USA). Statistical differences were calculated using an unpaired two-tailed Student's *t*-test, and values of $p < 0.05$ were considered statistically significant.

3 | RESULTS

3.1 | Association of SNP rs34492591 in *HUWE1* with NOA in a Chinese cohort

To investigate associations between SNPs in the *HUWE1* gene and NOA, SNP genotyping was performed in 190 patients with NOA and 243 controls from the Yunnan cohort in China. We screened 26 SNPs in *HUWE1* exons, introns, and the promoter region. SNPs were selected from the HapMap database and genotyped using the MassARRAY system; genotype calls were made with MassARRAY RT software. The azoospermia group had substantially lower genotype and allele frequencies in rs5978179, rs34492591, and rs6529689 than the control group ($p < 0.01$).

The NOA group exhibited significantly lower genotype and allele frequencies of the C allele of rs34492591 than the control group (Figure 1A). As rs34492591 is located in the promoter region, its cytosine/thymine (C/T) variant may influence the transcriptional activity of *HUWE1* (Figure 1B). Subsequently, to validate the function of rs34492591 and its frequency in a different cohort (Sichuan Province, China), we designed a primer targeting its promoter SNP site. Denaturing high-performance liquid chromatography screening was carried

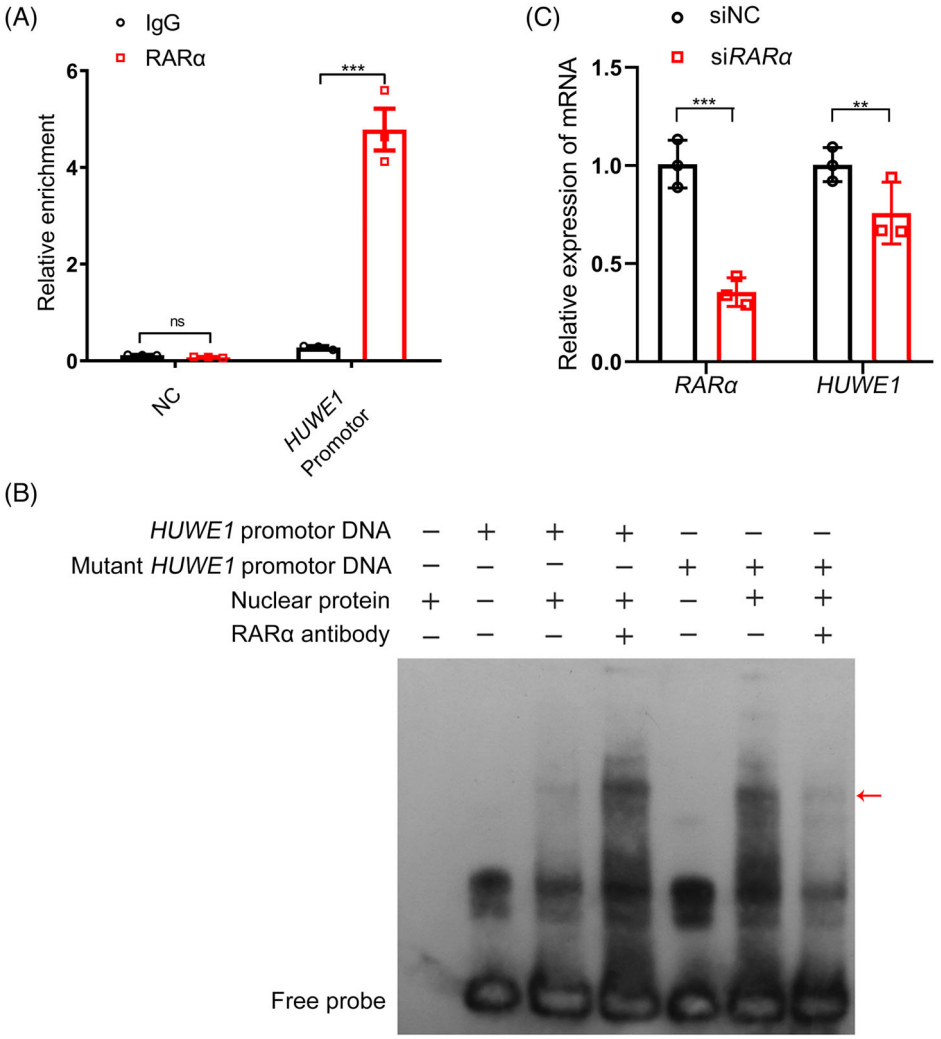

FIGURE 2 HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 (HUWE1) expression is regulated by retinoic acid receptor alpha (RARα) in spermatogenesis. (A) RARα factor binds to *HUWE1* promoter tested by chromatin immunoprecipitation (ChIP) assay in NCCIT cells. Immunoglobin G antibody was used as the negative control of RARα antibody. NCCIT cells were transfected with the plasmid containing *HUWE1* promoter gene or empty plasmid, which used as the negative control (NC). $^{***}p < 0.001$; ns: not significant; two-sided Student's *t*-test. The data are presented as the mean ± SEM. (B) RARα factor binds to *HUWE1* promoter tested by electrophoretic mobility shift assay (EMSA) in vitro. The symbol of plus and minus means adding the probe and without the probe, respectively. (C) Quantitative real-time polymerase chain reaction (RT-qPCR) test was carried out to assess the expression levels of *HUWE1* mRNA and *RARα* mRNA in C18-4 cells, which was transfected with *RARα* siRNA (si*RARα*) or negative control siRNA (siNC). $^{**}p < 0.01$, $^{***}p < 0.001$; two-sided Student's *t*-test. The data are presented as the mean ± SEM.

out to detect potential mutations in a sample size of 93 NOA patients and 144 control subjects, which were confirmed by Sanger sequencing. Our data revealed that the T to C point mutation frequency for rs34492591 in the SNP locus among patients with NOA was 0.1808/300. Collectively, these results underscore the potential significance of the rs34492591 single nucleotide polymorphism in *HUWE1* for NOA pathogenesis.

3.2 | *HUWE1* expression is regulated by RARα in germ cells

Vitamin A is instrumental to mammalian reproduction. As its metabolite, RA acts in a hormone-like manner by binding to and activating three nuclear receptor isotypes, RARα, RARβ, and RARγ.[24] In particular, RARα is paramount as an RA receptor in regulating germ cell development. First, we conducted a ChIP assay to evaluate RARα binding to the *HUWE1* promoter. RARα showed stronger binding to the *HUWE1* promoter than the immunoglobin G control (Figure 2A). To further confirm these findings, we synthesized WT and mutant variants of the *HUWE1* promoter probe containing the rs34492591 C/T polymorphism. We performed an EMSA to evaluate the binding of the RARα protein to the *HUWE1* promoter. Our results showed that the WT probe produced a noticeable binding band with nucleoprotein, which was further enhanced when treated with the RARα antibody. Additionally, we observed an upward shift in the binding band following the RARα antibody treatment. In contrast, both mutant probes showed reduced binding to the RARα antibody and

weaker band intensity, providing evidence that the rs34492591 C/T polymorphism directly regulates the binding of RARα to the *HUWE1* promoter region (Figure 2B). Subsequently, we knocked down RARα using siRNA and observed a significant reduction in *HUWE1* mRNA (Figure 2C). Together, our findings suggest that RARα plays a crucial role in regulating the expression of *HUWE1* by binding to its promoter.

3.3 | *HUWE1* participates in RA/RARα signaling pathway and promotes spermatogonia differentiation in germ cells

RA acts as a potent inducer of spermatogonia differentiation. The C18-4 cell line, a well-established spermatogonial stem cell line with the phenotype of male germ cells and undifferentiated spermatogonia, was employed to investigate the effects of RA on differentiation induction. Gene expression analysis via RT-qPCR revealed a significant increase in the expression level of the spermatogonia differentiation-related gene *STRA8*, while the expression levels of the *SCP3* gene decreased following RA induction (Figure S1A). Cell proliferation was evaluated using the CCK-8 assay, and we found a marked decrease in the proliferation capacity of cells after RA induction (Figure S1B). Finally, utilizing immunofluorescence staining, we detected a significant reduction in the phosphorylated form of H2AX (γH2AX) following RA induction (Figure S1C,D). The results suggested that RA signaling plays a critical role in spermatogenesis and supports the possible involvement of HUWE1 in this process.

Given that RARα regulates HUWE1 expression, we first assessed the effect of RA on HUWE1 expression in C18-4 cells. We transfected C18-4 cells with plasmids containing either the WT or mutant *HUWE1* promoter and performed luciferase assays. The results demonstrated that cells transfected with the WT *HUWE1* promoter had significantly increased luciferase signals following RA induction than the DMSO control group (Figure 3A), whereas those transfected with the mutant *HUWE1* promoter had significantly decreased signals than the WT *HUWE1* promoter (Figure 3A). Our findings suggest that *HUWE1* is a downstream gene of RA that is activated by RA induction. However, the mutated form of the *HUWE1* promoter was not sensitive to RA induction. To further explore the role of *HUWE1* in spermatogonia differentiation, we knocked down the *RARα* mRNA in C18-4 cells using siRNA to inhibit *HUWE1* expression (Figure 2B). RT-qPCR results showed that the differentiation of spermatogonia gene *STRA8* decreased in *RARα*-knockdown C18-4 cells, while no difference was observed in *SCP3* mRNA levels (Figure 4B). Further western blotting analysis showed that SCP3 and γH2AX protein expression levels significantly increased (Figure 3C,D). These results were consistent with those obtained from the immunofluorescence experiments of γH2AX (Figure 3E,F), suggesting that inhibiting HUWE1 expression by knocking down RARα mRNA can suppress the cell differentiation pathway. Collectively, these findings demonstrate that HUWE1 is a key downstream gene of the RA/RARα signaling pathway that upregulates the differentiating spermatogonia factor STRA8 while simultaneously downregulating SCP3 and γH2AX to promote spermatogonial differentiation.

3.4 | *HUWE1* expression decreases significantly in patients with NOA

To further evaluate the critical role of *HUWE1* in spermatogenesis and NOA etiology, we collected and analyzed testicular biopsy samples from patients with NOA and OA. RT-qPCR analyses revealed that *HUWE1* and *RARα* mRNA expression levels were significantly lower in NOA patient testes than in OA controls (*t*-test, $p < 0.01$ and <0.001, respectively) (Figure 4A,B). To further evaluate these findings, we performed immunofluorescence techniques to investigate the cellular localization and relative protein expression levels of HUWE1 and RARα in a limited number of biopsy samples. The results obtained using these methods were consistent with those of RT-qPCR, indicating that lower expression levels of both HUWE1 and RARα were observed in NOA patients than in OA controls (Figure S2A,B). These data suggest that HUWE1 plays a critical role in spermatogenesis and is a key regulatory factor in normal testicular function.

4 | DISCUSSION

HUWE1 is an important E3 ubiquitinase and multifunctional biological macromolecule that harbors multiple domains. Extant reports implicate *HUWE1* in modulating diverse physiological processes.[13,25] Moreover, *HUWE1* is crucial in spermatogonial differentiation and the meiotic prophase.[18] Here, we investigated the potential SNP polymorphisms in the regulatory sequence of *HUWE1* among patients diagnosed with NOA. Genotyping 190 Yunnan NOA patients revealed that the C allele frequency of rs34492591 was significantly lower than that in controls. This finding suggests that the SNP in the *HUWE1* gene regulatory region is partially related to NOA etiology.

RA signaling has been shown to induce germ cell differentiation and the initial stages of meiosis.[26] As a receptor of RA, *RARα* is located in the cell nucleus and cytoplasm. In this study, we focused on investigating whether RARα regulates *HUWE1* expression following RA activation and whether HUWE1 functions downstream of RA/RARα signaling. Our results demonstrate that RARα binds the *HUWE1* promoter and modulates its expression. Furthermore, the results of luciferase assay established that RA activates the expression of *HUWE1* and degrades γH2AX for normal spermatogonia differentiation (Figure 5). Bose et al. studied conditional *HUWE1* knockout mice and observed that male mice had normal fertility, and their testes and spermatozoa had no distinct defects when compared to normal male mice. The researchers inferred that *HUWE1* was not necessary for post-meiotic development during spermatogenesis,[18] which is consistent with the role of RA in inducing spermatogonia differentiation. Based on these findings, it was concluded that *HUWE1* induces germ cell differentiation and meiotic prophase through the RA/RARα signaling pathway. Alfano et al. reported increased RA signaling in Sertoli

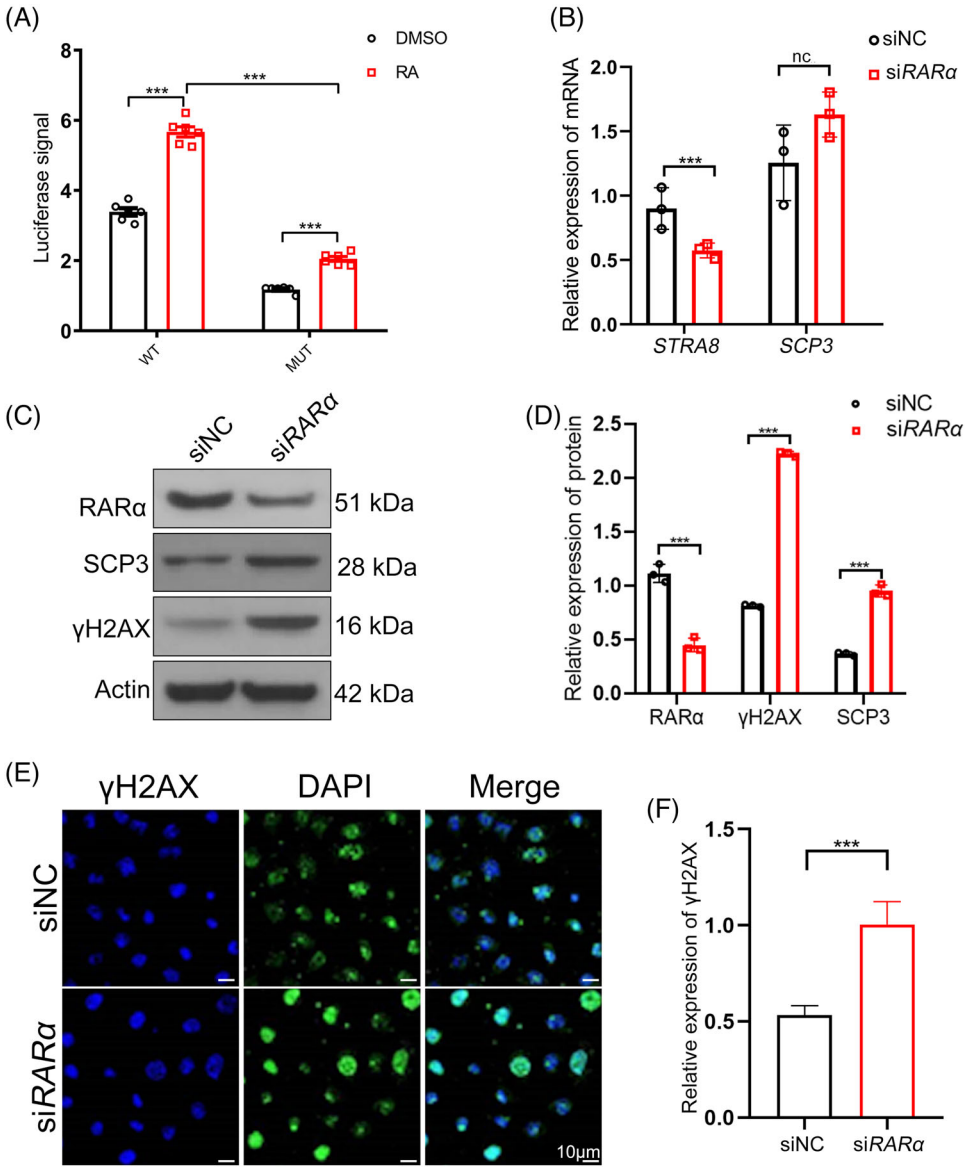

FIGURE 3 HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1 (HUWE1) promotes spermatogonia differentiation in germ cell. (A) Luciferase activity was measured to assess HUWE1 expression. C18-4 cells were transfected with *HUWE1* promoter wild-type (WT) or mutant (MUT), further induced by 10 μM retinoic acid (RA) or equal amount of DMSO for 48 h. ***$p < 0.001$; two-sided Student's *t*-test. The data are presented as the mean ± SEM. (B–F) C18-4 cells were transfected with siRARα or siNC. (B) The expression levels of *STRA8* mRNA and *SCP3*mRNA in C18-4 cells detected by quantitative real-time polymerase chain reaction (RT-qPCR) test. ***$p < 0.001$; two-sided Student's *t*-test. The data are presented as the mean ± SEM. (C and D) The protein level of γH2AX and SCP3 in C18-4 cells detected by western blotting. ***$p < 0.001$; two-sided Student's *t*-test. The data are presented as the mean ± SEM. (E and F) The immunostaining of γH2AX (green) in C18-4 cells, and nuclei were labelled with DAPI (blue). Scale bars, 10 μm. ***$p < 0.001$; two-sided Student's *t*-test. The data are presented as the mean ± SEM.

cells as well as decreased vitamin A signaling in Leydig cells of iNOA patients, leading to altered testicular extracellular matrix composition. Sertoli cell hyperresponsiveness to RA may indicate that iNOA testes resemble a prepubertal stage.[27] These results indirectly support our findings, suggesting that HUWE1 expression is closely associated with RA/RARα signaling, which promotes spermatogonial differentiation.

γH2AX, a HUWE1 substrate and DNA double-strand break (DSB) repair marker, accumulates in some physiological contexts lacking DSBs and functions as a biomarker for conditions such as cancer, age-related diseases, chronic inflammatory diseases, and ischemia–reperfusion injury.[28] However, γH2AX foci have been detected in physiological processes without DSB lesions. Fernandez-Capetillo et al. and Turner et al. established that γH2AX is necessary for chromatin remodeling and meiotic silencing in males.[29,30] In addition, γH2AX maintains the chromatin structural framework of special physiological processes such as sex chromosome inactivation in germ cells.[31] Here, we demonstrate that HUWE1 reduces γH2AX foci through RA/RARα signaling in C18-4 cells. Furthermore, siRARα-mediated loss of *HUWE1* expression led to γH2AX accumulation, consistent with findings by Fok et al. They found that in the absence of extensive DNA damage, loss

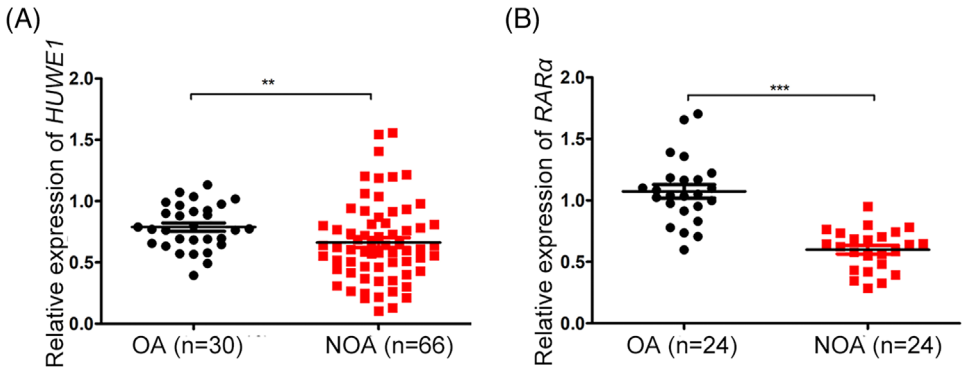

FIGURE 4 *HUWE1* expression is decreased in non-obstructive azoospermia (NOA) patients. (A) The expression of *HUWE1* mRNA in testicular tissue of NOA patients or obstructive azoospermia (OA) patients. $^{**}p < 0.01$; two-sided Student's *t*-test. The data are presented as the mean ± SEM. (B) The expression of *RARα* mRNA in testicular tissue of NOA patients or OA patients. $^{***}p < 0.001$; two-sided Student's *t*-test. The data are presented as the mean ± SEM.

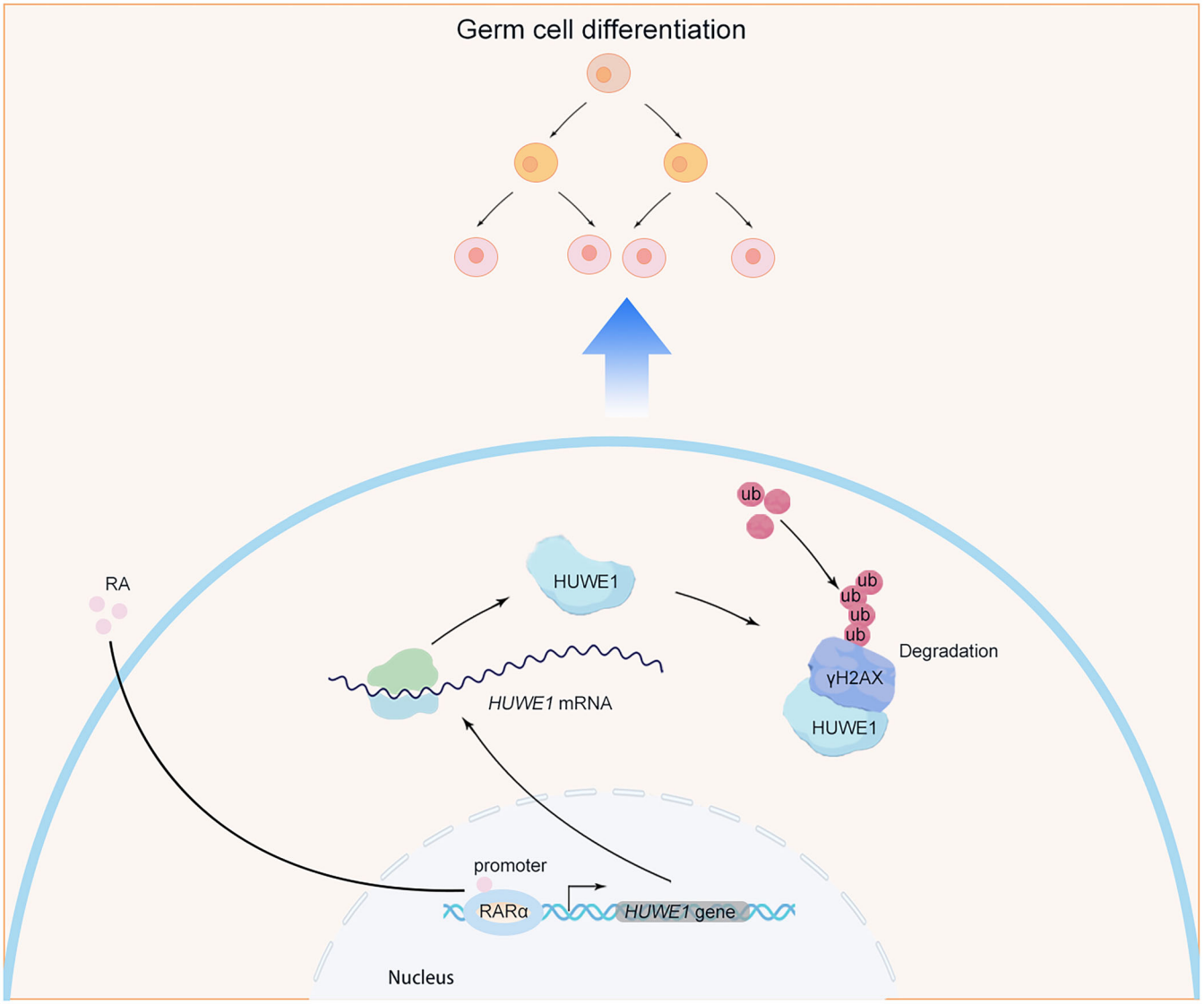

FIGURE 5 The hypothesized model to explain how the *HUWE1* variants may affect the efficiency of spermatogonia differentiation in germ cell.

of HUWE1 expression caused impaired γH2AX degradation, resulting in G2/M cell cycle arrest of spermatogonia and ultimately cell death.[19] Moreover, A previous study reported an increased amount of γH2AX in the germ cells of men with NOA. This finding is consistent with our observation that HUWE1 plays a critical role in inhibiting the accumulation of γH2AX, which suggests that reduced HUWE1 expression in men with NOA may contribute to the increased amount of γH2AX in germ cells.[32]

In this study, we investigated the molecular mechanisms that contribute to spermatogenesis disorders, with a particular focus on the role of *HUWE1* promoter polymorphisms in regulating gene expression among patients with NOA. The results of in vitro experiments showed that RARα binds to the *HUWE1* promoter region and regulates its expression. However, the regulatory effect of *RARα* weakened following introduction of the polymorphism into the *HUWE1* regulatory sequence. The expression of spermatogonial differentiation-related gene *STRA8* was impaired, and γH2AX foci accumulated following inhibition of *HUWE1* gene expression using siRARα. Overall, our results support the hypothesis that *HUWE1* expression is induced by the RA/RARα signaling pathway and inhibits γH2AX accumulation, which promotes sperm production and meiotic prophase.

AUTHOR CONTRIBUTIONS

Wenming Xu and Zhengrong Wang planned the experiments and revised the manuscript. Xudong Shan, Gelin Huang, and Xueguang Zhang performed the experiments and prepared a draft of the manuscript. Jianxing Cheng, Hui Jiang, and Haocheng Lin participated in the collection of clinical samples. Jiao Lv and Chuan Jiang performed the statistical analysis. Xiaohui Jiang and Huanxun Yue conceived the project and edited the manuscript. Zixia Ye and Wenming Xu discussed the results. All the authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

DATA AVAILABILITY STATEMENT

The dataset used and/or analyzed during the current study is available from the corresponding author upon reasonable request. Registry data are available publicly.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Novel biallelic variants in *DNAH1* cause multiple morphological abnormalities of sperm flagella with favorable outcomes of fertility after ICSI in Han Chinese males

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Abstract

Background: Multiple morphological abnormalities of sperm flagella is an idiopathic asthenoteratozoospermia characterized by absent, short, coiled, angulation, and irregular-caliber flagella. Genetic variants of *DNAH1* gene have been identified as a causative factor of multiple morphological abnormalities of sperm flagella and intracytoplasmic sperm injection is an available strategy for infertile males with dynein axonemal heavy chain 1 defects to conceive.

Objectives: To identify novel variants and candidate mutant hotspots of *DNAH1* gene related to multiple morphological abnormalities of sperm flagella and male infertility in humans.

Materials and methods: The *DNAH1* variants were identified by whole exome sequencing and confirmed with Sanger sequencing. Papanicolaou staining, scanning and transmission electron microscopy, and immunostaining were performed to investigate the morphological and ultrastructural characteristics of spermatozoa. Intracytoplasmic sperm injection was applied for the assisted reproductive therapy of males harboring biallelic *DNAH1* variants.

Results: We identified 18 different *DNAH1* variants in 11 unrelated families, including nine missense variants (p.A2564T, p.T3657R, p.G1862R, p.L2296P, p.T4041I, p.L611P, p.A913D, p.R1932Q, p.R2356W) and nine loss-of-function variants (c.2301-1G>T, p.Q1518*, p.R1702*, p.D2845Mfs*2, p.P3909Rfs*33, p.Q4040Dfs*33, p.Q4058*, p.E4060Pfs*61, p.V4071Cfs*54). A total of 66.7% (12/18) of the identified variants were novel. Morphological analysis based on Papanicolaou staining and scanning

Shunhua Long and Longlong Fu contributed equally to this work.

electron microscopy demonstrated the typical multiple morphological abnormalities of sperm flagella characteristics of dynein axonemal heavy chain 1-deficient spermatozoa. Immunostaining further revealed the absence of inner dynein arms but not outer dynein arms, which induced a general ultrastructural disorganization, such as the loss of central pair and mis-localization of the microtubule doublets and outer dense fibers. To date, seven affected couples have accepted the intracytoplasmic sperm injection treatment, and three of them have given birth to five healthy babies.

Discussion and conclusion: These findings further expand the variant spectrum of *DNAH1* gene related to multiple morphological abnormalities of sperm flagella and male infertility in humans, thus providing new information for the molecular diagnosis of asthenoteratozoospermia. The favorable fertility outcomes of intracytoplasmic sperm injection will facilitate the genetic counseling and clinical treatment of infertile males with multiple morphological abnormalities of sperm flagella in the future.

KEYWORDS
asthenoteratozoospermia, *DNAH1*, intracytoplasmic sperm injection, MMAF

1 | INTRODUCTION

Sperm flagellum is an evolutionary conserved organelle constructed around the axoneme, a typical "9 + 2" structure composed of nine peripheral microtubule doublets (MTDs) and a central microtubule pair (CP).[1] The MTDs are attached with regularly arranged inner and outer dynein arms (IDAs and ODAs) and are connected to the CP through a multi-protein T-shaped structure called radial spokes (RSs).[2] The IDAs and ODAs mainly control the amplitude, frequency, and waveform of the flagella beat, and the heterogeneous IDAs are organized into seven dynein structures (dynein a, b, c, d, e, f/I1, and g) to anchor the three subtypes of RSs (RS1, RS2, and RS3) to MTDs.[3] Defects in genes encoding the axoneme and axoneme-related components have been related to deformations of flagella ultrastructure and idiopathic male infertility in humans and mice.[4] Multiple morphological abnormalities of sperm flagella (MMAF) is a special type of asthenoteratozoospermia mainly caused by genetic factors, characterized by absent, short, coiled, angulation, and irregular-caliber flagella.[5] To date, variants of nearly 40 genes have been identified and related to approximately 60%–70% of patients with MMAF, including the genes of cilia- and flagella-associated proteins (CFAPs; e.g., *CFAP43*, *CFAP44*, *CFAP47*, *CFAP57*, *CFAP58*, *CFAP61*, *CFAP65*, *CFAP69*, *CFAP70*, *CFAP74*, *CFAP91*, and *CFAP251*), the genes of dynein axonemal heavy chain family (DNAHs; e.g., *DNAH1*, *DNAH2*, *DNAH6*, *DNAH8*, *DNAH10*, and *DNAH17*), and other genes expressed in sperm axonemal and peri-axonemal structures (*DZIP1*, *CEP78*, *CEP128*, *CEP135*, *AKAP3*, *AKAP4*, *TTC21A*, *TTC29*, *ARMC2*, *AK7*, *FSIP2*, *QRICH2*, *SPEF2*, *ODF2*).[4,6–8]

The *DNAH1* gene, which is located at chromosome 3p21.1 with a length of approximately 84.17 kb and encodes a predicted 4625-amino acid protein, had been firstly described of gene–disease relationship with the MMAF phenotype.[9] It is highly expressed in the brain, testis, and respiratory tract, with an eightfold higher level in the testis than in the trachea.[9] In spermatozoa, the DNAH1 protein is an integral part of IDA d that is responsible for anchoring RS3 to the MTDs and thereby regulates the stability of axonemal structure and the mobility of sperm flagella.[9,10] The male *Dnahc1*-knockout mice (the mouse ortholog of *DNAH1*) are sterile because of asthenozoospermia and reduced flagella beat frequency.[11,12] Biallelic variants of *DNAH1* were found to account for approximately 4.8%–57.1% of reported human MMAF cohorts.[9,13–23] Intriguingly, some variant alleles occurred more frequently in East Asian population, such as p.P3909fs*33, p.Q1518*, and p.E3284K, and higher frequencies of *DNAH1* variants were observed in Han Chinese males with MMAF.[13–16,20–22,24] Moreover, the intracytoplasmic sperm injection (ICSI) was reported as a feasible strategy for most patients with MMAF and *DNAH1* variants to obtain their genetic progenies.[25] However, more clinical cases are needed to explore the variant spectrum of *DNAH1* gene, especially its founder effect in Han Chinese males, and more investigations are needed on the phenotypes and clinical outcomes of patients affected by *DNAH1* variants.

In the current study, we recruited 45 Chinese families with males exhibiting MMAF and receiving assisted reproductive technology (ART) treatment in our clinic. Through genetic screening with whole exome sequencing (WES) and Sanger sequencing, nine missense variants and nine loss-of-function variants in *DNAH1*, including 12 novel variants and six previously reported recurrent variants, were identified in 11 unrelated families. In addition, we described the characteristics of semen parameters, changes in morphology and ultrastructure, and the clinical outcomes of ART with ICSI of the males harboring biallelic *DNAH1* variants. These findings greatly expand the variant spectrum of *DNAH1* and provide new knowledge for the genetic counseling of infertile males with MMAF in the future.

2 | MATERIALS AND METHODS

2.1 | Study participants and ethical approval

A total of 45 Han Chinese males with primary infertility and abnormal morphology of the sperm flagella were recruited at the Center for Reproductive Medicine, Women and Children's Hospital of Chongqing Medical University (Chongqing, China) from August 2019 to December 2022. All males had normal chromosomal karyotypes (46,XY), and no Y chromosome microdeletion was found. A written informed consent was signed before the collection of blood and semen samples. This study was approved by the Clinical Application and Ethics Committee of Human Assisted Reproductive Technology of Chongqing Health Center for Women and Children.

2.2 | WES, Sanger sequencing, and variant analysis

As previously reported,[26] genomic DNA was extracted from peripheral blood samples using a QIAamp DNA blood mini kit (51106, Qiagen, Hilden, Germany). Then, the gDNA was treated with an Agilent SureSelect Human All Exon V6 Kit (Agilent Technologies Inc., CA, USA) and sequenced on an Illumina NovaSeq 6000 platform (Illumina Inc., CA, USA). After quality control, the FASTQ data were aligned to the human reference genome (GRCh37/hg19) using the Burrows–Wheeler Aligner,[27] and the Genome Analysis Toolkit was applied for variant calling.[28] Subsequently, the variants were annotated with ANNOVAR.[29]

Sanger sequencing was applied for the variant verification and pedigree analysis. Briefly, the amplicons of specific primers listed in Table S1 were sequenced with ABI 3500 (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed with Chromas 2.6.5 (Technelysium Pvt. Ltd., South Brisbane, Australia). The gnomAD database (http://gnomad-sg.org/) and VarSome (https://varsome.com/) were searched for the frequencies of variants in different populations and previously reported variants. The potential pathogenicity of all identified variants was evaluated by in silico analysis with Polymorphism Phenotyping version 2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT, https://sift.bii.a-star.edu.sg/), MutationTaster (https://www.mutationtaster.org/), and Combined Annotation Dependent Depletion (CADD, https://cadd.gs.washington.edu/). A novel variant was defined as one that had not been reported in the literature.

2.3 | Amino acid conservation analysis and protein diagram

As previously reported,[30] the nomenclature of mutant DNAH1 proteins was referred to Name Checker (https://mutalyzer.nl/name-checker), and the amino acid conservation analysis of DNAH1 protein among different species was performed with Clustal Omega (http://www.clustal.org/omega/) and ESPript 3.0 (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Illustrator for Biological Sequences (http://ibs.biocuckoo.org/online.php) was used in modeling the diagrams of DNAH1 protein, and SWISS-MODEL software (https://swissmodel.expasy.org) and PyMOL (http://www.pymol.org) were used in assessing the protein structures based on Q9P2D7.

2.4 | Semen analysis

Fresh semen samples were collected from the participants after 2–7 days of sexual abstinence and tested according to the World Health Organization (WHO) guidelines.[31,32] Briefly, within 1 h of ejaculation, the liquefied samples were analyzed for semen volume, sperm concentration, round cells, normal morphology, and sperm motility via a computer-aided sperm analysis system (Jiangsu Rich Life Science Instrument Co. Ltd., Nanjing, China). Asthenozoospermia was defined by reduced motility or absent sperm motility in the fresh ejaculate (progressive motility <32%). Teratospermia referred specifically to the condition in which the frequency of spermatozoa with normal morphology was below the lower reference limit of 4%. Asthenozoospermia and teratospermia may exist at the same time, which is collectively called asthenoteratozoospermia.

2.5 | Papanicolaou staining

Modified Papanicolaou staining (Cariad Medical Technology Co. Ltd., Zhuhai, China) was used to evaluate the sperm morphology as previously reported.[30] Briefly, the spermatozoa were smeared onto slides, air-dried, and then fixed with 95% ethanol for 3 min. Then, the slides were submerged in hematoxylin for 3 min, acidic ethanol for 5 s, and eosin and bright green for 3 min. Washing was performed between each step and the prepared slides were placed onto absorbent paper for thoroughly dry. Finally, more than 200 stained spermatozoa were assessed for morphology analysis with a light microscope (TCS SP8, Leica, Wetzlar, Germany).

2.6 | Electron microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed as previously reported.[30] Briefly, the semen samples from normal individuals and males with MMAF were collected and fixed in 2.5% glutaraldehyde at 4°C overnight. For the SEM assay, the samples were washed with phosphate buffer (pH 7.4) three times and dehydrated sequentially in 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol. Subsequently, the prepared samples were dried with a CO_2 critical-point dryer (EikoHCP-2, Hitachi, Tokyo, Japan), sputter coated by an ionic sprayer meter (ACE200, Leica), and finally observed under SEM (Nova NanoSEM 450, FEI, Hillsboro, OR, USA) at an accelerating voltage of 5 kV. For the TEM assay, the samples were post-fixed in 1% buffered OsO_4, dehydrated in graded acetone solutions and embedded in Epon 812 (90529-77-4, SPI, West Chester,

PA, USA). Subsequently, ultrathin sections (70 nm) were double stained with lead citrate and uranyl acetate, and observed by TEM (TECNAI-10, Philips, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV.

2.7 | Immunofluorescence assay

The immunofluorescence assay of spermatozoa was performed as described in our previous studies.[30] Briefly, the sperm samples were smeared on glass slides, fixed with 4% paraformaldehyde for 6 min, penetrated with 0.5% Triton X-100 (Sigma, Shanghai, China) for 30 min, and pre-coated with 3% bovine serum albumin (B2064, Sigma) for 2 h at room temperature. Primary antibodies, including DNAH1 (1:100, DF13457, Affinity Biosciences, Changzhou, China), DNALI1 (1:200, HPA028305, Sigma), DNAI1 (1:100, ab171964, Abcam, Cambridge, UK), and α-TUBULIN (1:400, F2168, Sigma), were incubated at 4°C overnight. Alexa Fluor 488 (1:500, A-11001, Invitrogen, CA, USA) and Alexa Fluor 555 (1:500, A-21428, Invitrogen) were used for secondary signal amplification, and the nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (P0131, Beyotime, Shanghai, China). Finally, images were obtained through a confocal laser scanning microscope (TCS SP8, Leica).

2.8 | Ovarian stimulation and ART

Seven couples have accepted ART treatment with ICSI to conceive in our clinic. As shown in Figure S1, three different protocols of ovarian stimulation, including gonadotropin-releasing hormone (GnRH)-antagonist protocol, short-acting GnRH-agonist long protocol, and mild-stimulation protocol, were conducted based on the ovarian reserve of the female partners. As previously reported,[26] a time-lapse monitoring system (Embryoscope Plus, Vitrolife, Kungsbacka, Sweden) was applied for embryo culture to limit the effect of sub-optimal conditions. Embryos were cultured in G1 medium (509776, Vitrolife) from day 1 to day 3 and in G2 medium (509698, Vitrolife) from day 3 to day 5 or day 6 for blastocyst formation if needed. The embryo assessment was performed according to the European Society of Human Reproduction and Embryology consensus or the previously reported guidelines.[33,34] After embryo transfer, human chorionic gonadotropin (HCG) level in serum was measured at the 14th day, and a fetal heart beat observed at 7th week was classified as clinical pregnancy.

3 | RESULTS

3.1 | Biallelic variants of *DNAH1* were identified in infertile males with MMAF

We identified 18 different variants of *DNAH1* (NM_015512.5) in 11 unrelated infertile males with MMAF by WES and Sanger sequencing (Figure 1 and Table 1), including nine missense variants (c.1832T>C, p.L611P; c.2738C>A, p.A913D; c.5584G>A, p.G1862R; c.5795G>A, p.R1932Q; c.6887T>C, p.L2296P; c.7066C>T, p.R2356W; c.7690G>A, p.A2564T; c.10970C>G, p.T3657R; c.12122C>T, p.T4041I) and nine loss-of-function variants (c.2301-1G>T; c.4552C>T, p.Q1518*; c.5104C>T, p.R1702*; c.8533del, p.D2845Mfs*2; c.11726_11727delCT, p.P3909Rfs*33; c.12172C>T, p.Q4058*; c.12118_12119delCA, p.Q4040Dfs*33; c.12178_12190del, p.E4060Pfs*61; c.12210del, p.V4071Cfs*54). In silico analysis indicated that all the variants disrupted the function of DNAH1, as predicted by PolyPhen-2, SIFT, MutationTaster, and CADD, and they all had extremely rare frequencies ($<10^{-3}$) or were absent in the gnomAD database (Table 1). These variants presented as homozygous (only in the consanguineous family L010) or compound heterozygous state with an autosomal recessive inheritance pattern. Moreover, the identified variants were distributed in 14 different exons, of which the exon 76 might be a candidate mutant hotspot with five novel variants (p.Q4040Dfs*33, p.T4041I, p.Q4058*, p.E4060Pfs*61, p.V4071Cfs*54). The variant, c.11726_11727delCT (p.P3909Rfs*33), occurred in three unrelated males with MMAF, which was consistent with its allele frequency in the East Asian population as high as 0.001286 according to the gnomAD database. In addition, 66.7% (12/18) of the identified variants had not been previously reported in patients with MMAF. Thus, we speculated that these biallelic *DNAH1* variants are the primary disease-causative factors for the MMAF phenotype in these infertile males.

3.2 | Genetic analysis of *DNAH1* variants

Human *DNAH1* consists of 78 exons and the encoded DNAH1 protein (NP_056327.4) comprises an N-terminal stem that binds cargo and interacts with other dynein components, six tandemly linked AAA domains (ATPases associated with diverse cellular activities; AAA1–AAA6), and a stalk-like structure between AAA4 and AAA5.[35] The nonsense and frameshift variants identified in this study were assumed to induce premature termination codons and truncating proteins, suggesting their strong deleterious effects. The nine altered amino acids at variant positions were highly conserved among different species and mostly located at the functional domains of DNAH1 protein (Figure 2A). Assessment of the three-dimensional structure of the DNAH1 protein with PyMOL program indicated that the variants p.L611P, p.A913D, p.R1932Q, p.A2564T, and p.T4041I occurred in the α-helix region, while the variants p.G1862R, p.L2296P, p.R2356W, and p.T3657R were found in the loop region and were primarily located on the surface of DNAH1 protein (Figure 2B). The variants were all predicted to result in changes in hydrogen bonding, hydrophobic characteristics, and/or a shortened carbon chain that could affect the formation and stability of the protein structure.

Moreover, we reviewed all the *DNAH1* variants reported previously and identified in the present study (Table S2). Statistically, there have been 111 variants of *DNAH1* identified in 125 males with MMAF, including frameshift ($n = 19$), splice-site loss ($n = 19$), nonsense ($n = 18$), in-frame deletion ($n = 2$), missense ($n = 52$), and synonymous ($n = 1$). The variant, c.11726_11727delCT (p.P3909Rfs*33), occurred with

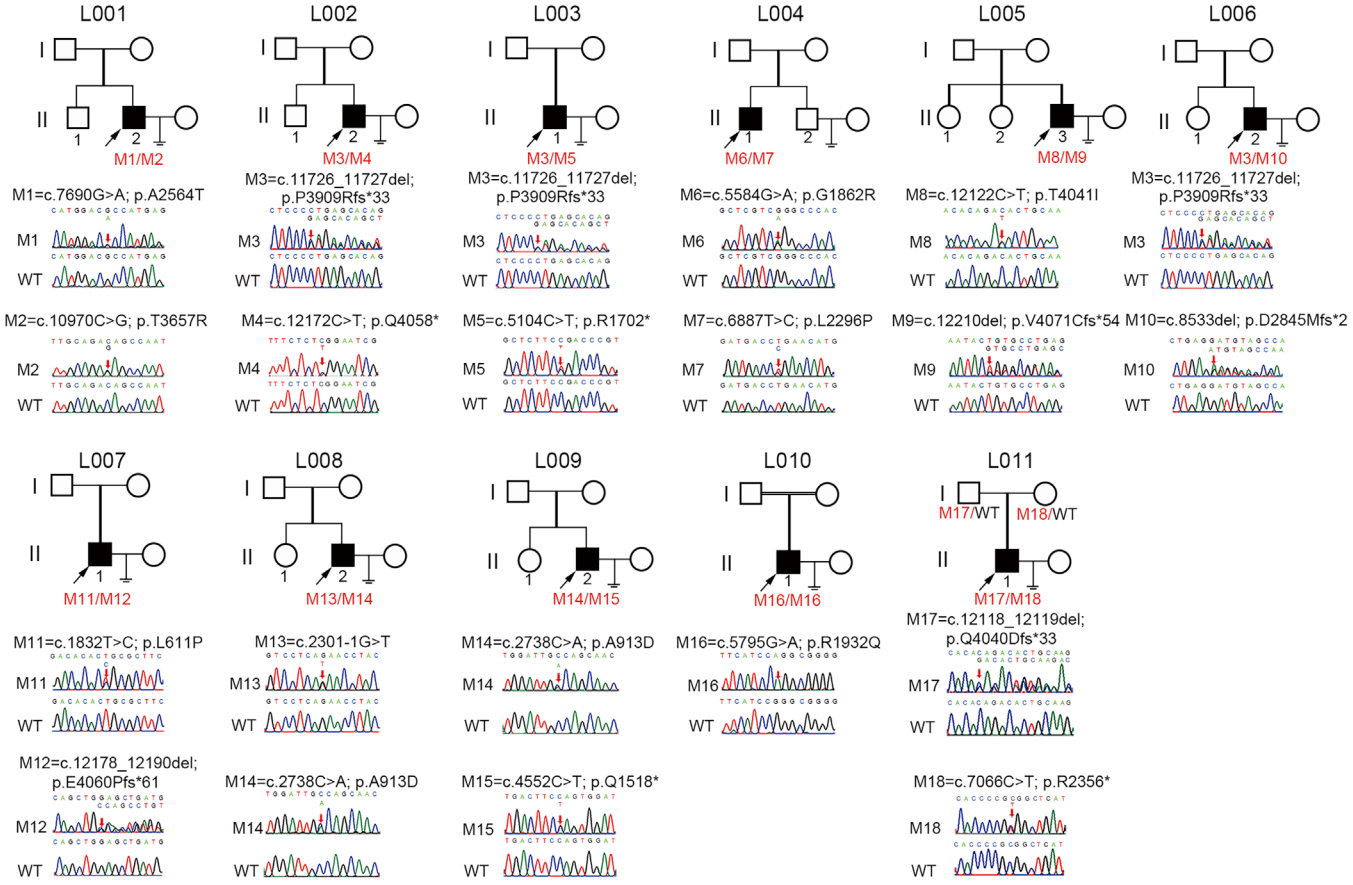

FIGURE 1 Pedigrees of 11 unrelated families affected by *DNAH1* variants identified via whole exome sequencing. Male patients with multiple morphological abnormalities of sperm flagella (MMAF) in these families are indicated with black-filled squares and black arrows. The double lines indicate first-degree consanguinity in L010. Sanger sequencing chromatograms are shown below the pedigrees to show the presence of biallelic *DNAH1* variants in patients. Red arrows indicate the mutant positions. M1–M18: mutation 1–mutation 18; WT: wild-type.

the highest frequency in 31 unrelated males with MMAF, and exon 76 presented as a candidate mutant hotspot consisting of 12 different variants. The other altered amino acids were distributed along the full-length DNAH1 protein.

3.3 | Asthenoteratozoospermia phenotypes in males harboring biallelic *DNAH1* variants

A detailed description of physical characteristics and semen parameters of the males harboring biallelic *DNAH1* variants is presented in Table 2. The 11 probands were aged between 27 and 46 years, and they were affected by primary infertility for 1–16 years. The semen volumes and pH were all within the normal ranges according to the WHO guidelines,[31,32] while oligospermia of different degrees was observed in several probands, such as L003-II-1, L004-II-1, and L009-II-2. Significantly, the progressive motility rate of spermatozoa in the affected individuals decreased dramatically to zero, and the immobility rate was nearly 100%, which was consistent with the previous studies on MMAF.[14,30]

The morphology of spermatozoa was then assessed with Papanicolaou staining and SEM. Spermatozoa from a fertile male showed normal long and smooth flagella, whereas spermatozoa from males harboring biallelic *DNAH1* variants displayed MMAF phenotype, including absent, short, coiled, angulation, and irregular-caliber flagella (Figure 3A). The quantification of different categories of abnormal flagellar morphologies demonstrated the consistent phenotypes of different probands (Figure 3B and Table 2). The main abnormal types were absent, short, and coiled, which accounted for more than 80% of the sperm flagella analyzed. The results of SEM exhibited clearer and more typical MMAF phenotypes of the spermatozoa obtained from males affected by *DNAH1* variants (Figure 3C).

As previously reported, DNAH1 deficiency impaired the structure of IDAs and induced morphological abnormalities of spermatozoa in infertile humans and mice.[9,12] To explore the effect of *DNAH1* variants on the molecular characteristics of spermatozoa, immunofluorescence assays with antibodies against DNAH1, DNALI1 (a light intermediate chain protein of IDA),[36] DNAI1 (an intermediate chain protein of ODA),[37] and α-TUBULIN (the main component of microtubules) were performed. The results showed that biallelic *DNAH1* variants induced the absence of DNAH1 and a decrease in α-TUBULIN in the spermatozoa of males with MMAF (Figure 4A). As expected, the DNAH1 deficiency destroyed the distribution of DNALI1 in the IDAs but did not affect the location of DNAI1 in the ODAs, highlighting the deformation

TABLE 1 A detailed description of the genetic variants of *DNAH1* identified in infertile men with multiple morphological abnormalities of sperm flagella (MMAF).

Subjects	cDNA alteration	Amino acid alteration	Exon/ intron	Mutation zygosity	Allele frequency		In silico bioinformatics prediction				Known/ novel
					gnomAD	gnomAD-EAS	PolyPhen-2	MutationTaster	SIFT	CADD	
L001-II-2	c.7690G>A	p.A2564T	Exon 49	Het	0.0000603	0	Probably_damaging	Disease_causing	Damaging	Damaging	Novel
	c.10970C>G	p.T3657R	Exon 69	Het	0.0003371	0.004305	Possibly_damaging	Disease_causing	Damaging	Damaging	Known
L002-II-2	c.11726_11727delCT	p.P3909Rfs*33	Exon 73	Het	0.0001007	0.001389	NA	Disease_causing	NA	NA	Known
	c.12172C>T	p.Q4058*	Exon 76	Het	ND	ND	NA	Disease_causing	NA	Damaging	Novel
L003-II-1	c.5104C>T	p.R1702*	Exon 32	Het	0.00002809	0.0003894	NA	Disease_causing	NA	Damaging	Known
	c.11726_11727delCT	p.P3909Rfs*33	Exon 73	Het	0.0001007	0.001389	NA	Disease_causing	NA	NA	Known
L004-II-1	c.5584G>A	p.G1862R	Exon 35	Het	0.00001961	0.0001129	Probably_damaging	Disease_causing	Damaging	Damaging	Known
	c.6887T>C	p.L2296P	Exon 44	Het	ND	ND	Probably_damaging	Disease_causing	Damaging	Damaging	Novel
L005-II-3	c.12122C>T	p.T4041I	Exon 76	Het	0.00001343	0.0001144	Probably_damaging	Disease_causing	Damaging	Damaging	Novel
	c.12210del	p.V4071Cfs*54	Exon 76	Het	0.000004015	0.00005566	NA	Disease_causing	NA	NA	Novel
L006-II-1	c.8533del	p.D2845Mfs*2	Exon 54	Het	ND	ND	NA	Disease_causing	NA	NA	Novel
	c.11726_11727delCT	p.P3909Rfs*33	Exon 73	Het	0.0001007	0.001389	NA	Disease_causing	NA	NA	Known
L007-II-1	c.1832T>C	p.L611P	Exon 11	Het	0.000004012	0	Probably_damaging	Disease_causing	Damaging	Damaging	Novel
	c.12178_12190del	p.E4060Pfs*61	Exon 76	Het	ND	ND	NA	Disease_causing	NA	NA	Novel
L008-II-2	c.2301-1G>T		Intron 13	Het	0.000004047	0.00005600	NA	NA	NA	NA	Novel
	c.2738C>A	p.A913D	Exon 17	Het	0.000004348	0.00005944	Probably_damaging	Disease_causing	Damaging	Damaging	Novel
L009-II-2	c.2738C>A	p.A913D	Exon 17	Het	0.000004348	0.00005944	Probably_damaging	Disease_causing	Damaging	Damaging	Novel
	c.4552C>T	p.Q1518*	Exon 27	Het	0.00001608	0.0001669	NA	Disease_causing	NA	Damaging	Known
L010-II-1	c.5795G>A	p.R1932Q	Exon 37	Hom	ND	ND	Probably_damaging	Disease_causing	Damaging	Damaging	Novel
L011-II-1	c.7066C>T	p.R2356W	Exon 45	Het	0.000008097	0.00005585	Probably_damaging	Disease_causing	Damaging	Damaging	Known
	c.12118_12119delCA	p.Q4040Dfs*33	Exon 76	Het	ND	ND	NA	Disease_causing	NA	NA	Novel

Note: The transcript used in this study was NM_015512.5.

Abbreviations: CADD, Combined Annotation Dependent Depletion; EAS, East Asian; gnomAD, Genome Aggregation Database; Het, heterozygous; Hom, homozygous; NA, not available; ND, not found; PolyPhen-2, Polymorphism Phenotyping version 2; SIFT, Sorting Intolerant from Tolerant.

TABLE 2 Semen characteristics and sperm morphology of the patients harboring biallelic *DNAH1* variants.

Subject	L001-II-2	L002-II-2	L003-II-1	L004-II-1	L005-II-3	L006-II-1	L007-II-1	L008-II-2	L009-II-2	L010-II-1	L011-II-1	Control	Reference value
Age	46	31	30	27	32	28	33	32	29	29	29	26	–
Duration of infertility (years)	16	5	6	7	3	5	6	1	6	2	2	–	–
Mutation site 1	p.A2564T	p.P3909Rfs*33	p.R1702*	p.G1862R	p.T4041I	p.D2845Mfs*2	p.L611P	c.2301-1G>T	p.A913D	p.R1932Q	p.R2356*	–	–
Mutation site 2	p.T3657R	p.Q4058*	p.P3909Rfs*33	p.L2296P	p.V4071Cfs*54	p.P3909Rfs*33	p.E4060Pfs*61	p.A913D	p.Q1518*	p.R1932Q	p.Q4040Dfs*33	–	–
Semen parameters													
Semen volume (mL)	3.7	2.2	4.5	4.2	2.9	4.5	3.9	3.9	1.7	2.2	2.8	2.0	1.5 (1.4–1.7)
Semen pH	7.5	7.4	7.2	7.4	7.4	7.4	7.3	7.3	7.3	7.3	7.3	7.3	≥7.2
Sperm concentration (10^6/mL)	12	16	9	8	18	13	11	15	5	13	25	60	15 (12–16)
Total sperm count (10^6/ejaculate)	44	35	41	34	52	59	43	59	9	29	70	120	39 (33–46)
Motility (PR + NP %)	0 + 1	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 1	57 + 9	40 (38–42)
Immobility (IM %)	99	100	99	100	100	100	100	100	100	100	99	34	–
Normal sperm morphology (%)	NA	NA	NA	1.29	0.93	0.82	0.66	0.86	0.97	0.42	1.06	9.60	4 (3–4)
Morphology of sperm head	NA	NA	NA	4.5	4.28	6.09	2.65	6.05	7.96	7.56	8.46	11.02	–
Morphology of sperm flagella													
Normal flagella (%)	NA	NA	NA	2.25%	4.22%	2.77%	1.89%	3.64%	3.93%	0.58%	4.42%	87.29%	–
Absent flagella (%)	NA	NA	NA	35.14%	36.85%	48.44%	45.95%	24.55%	25.76%	50.72%	48.30%	0.28%	–
Short flagella (%)	NA	NA	NA	30.18%	25.53%	20.59%	31.66%	41.36%	43.67%	26.80%	25.63%	0.55%	–
Coiled flagella (%)	NA	NA	NA	22.97%	25.14%	22.49%	15.30%	15.45%	15.28%	14.41%	15.465	5.80%	–
Angulation (%)	NA	NA	NA	7.21%	6.53%	2.60%	2.10%	8.86%	8.30%	2.88%	3.39%	4.14%	–
Irregular caliber (%)	NA	NA	NA	2.25%	1.73%	3.11%	3.10%	6.14%	3.06%	4.61%	2.80%	1.93%	–

Note: The normal values of semen parameters were determined according to the World Health Organization (2010) manual criteria. Symbol "–" denotes no reference range.
Abbreviations: IM, immobility; NA, not available; NP, non-progressive motility; PR, progressive motility.

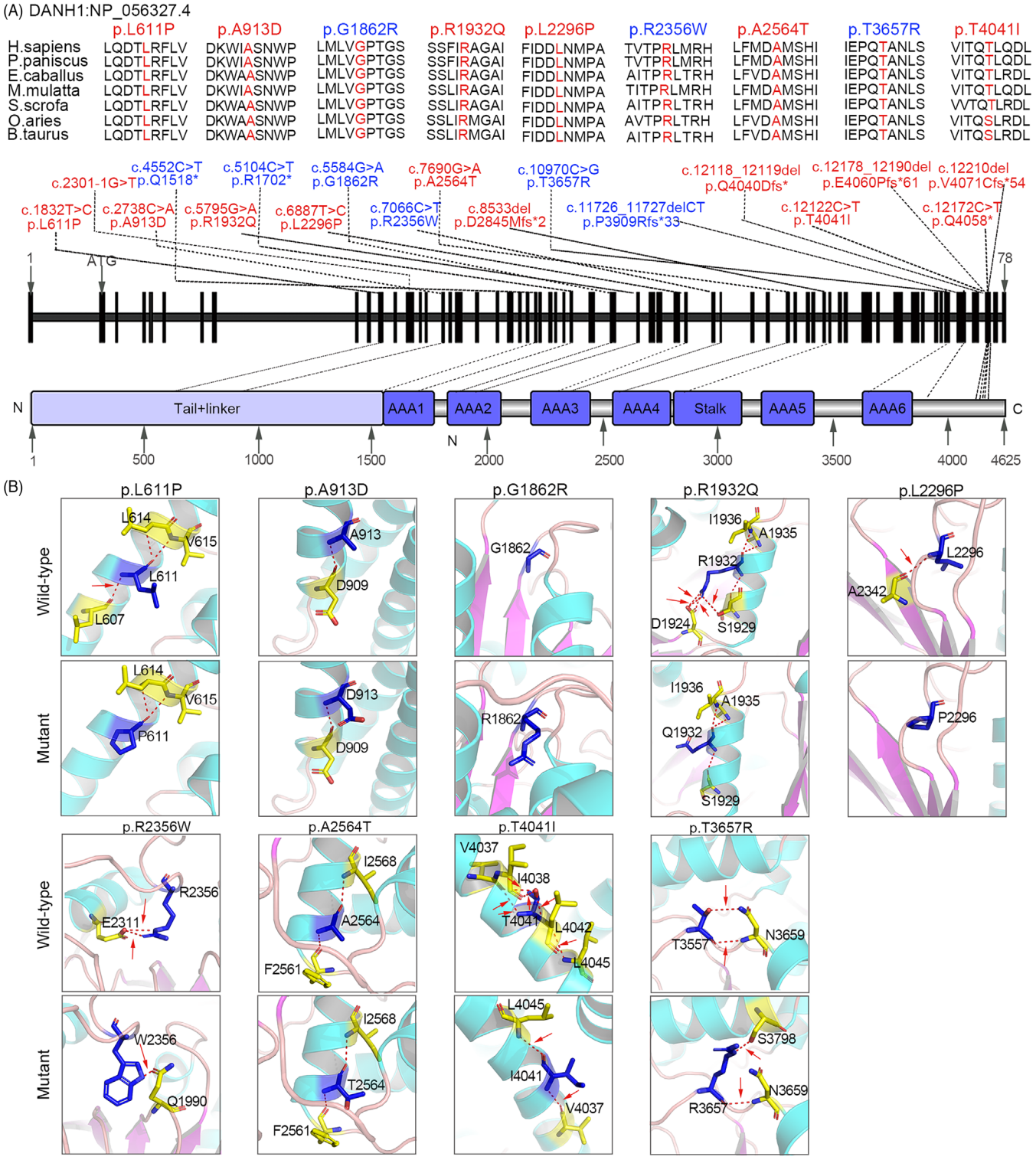

FIGURE 2 Bioinformatic analysis of dynein axonemal heavy chain 1 (DNAH1) variants. (A) Locations and conservation analysis of the detected variants in DNAH1 protein. The positions of all variants are indicated in the genomic and protein structure of DNAH1, and the conservation characteristics of nine missense variants (p.L611P, p.A913D, p.G1862R, p.R1932Q, p.L2296P, p.R2356W, p.A2564T, p.T3657R, and p.T4041I) are indicated. The red font represents 12 novel variants, and the blue font represents six previously reported variants. AAA1–AAA6: six known domains of ATPase associated with diverse cellular activities (AAA); tail + linker: amino terminal tail structure and linker domain; stalk: coiled-coil stalk domain. (B) Protein conformation predictions of *DNAH1* variants. The dashed lines represent hydrogen bonds, and the arrows indicate hydrogen bond changes.

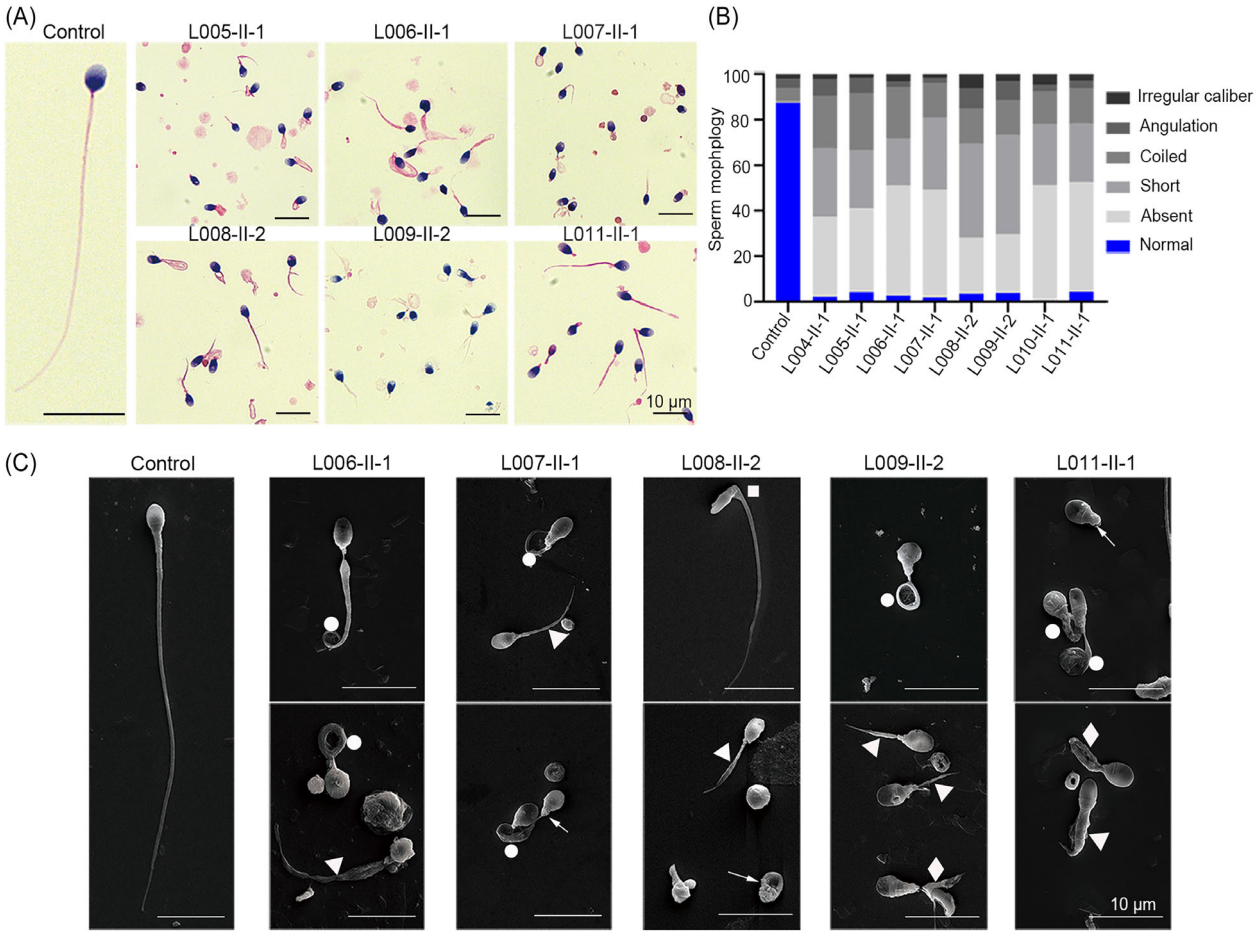

FIGURE 3 Multiple morphological abnormalities of sperm flagella (MMAF) phenotype of the spermatozoa affected by biallelic *DNAH1* variants. (A) Papanicolaou staining of spermatozoa from a fertile male and infertile males with biallelic *DNAH1* variants. The control spermatozoa showed normal long and smooth flagella, whereas the dynein axonemal heavy chain 1 (DNAH1)-deficient spermatozoa displayed the typical characteristics of MMAF (including absent, short, coiled, angulation, and irregular-caliber flagella). Scale bars: 10 μm. (B) Quantification of different categories of flagellar morphologies. (C) Scanning electron microscopy (SEM) analysis of the control spermatozoa and the DNAH1-deficient spermatozoa demonstrated clearer and more typical MMAF phenotypes, including absent (arrow), short (triangle), coiled (circle), angulation (square), and irregular flagella (diamond). Scale bars: 10 μm.

of IDAs (Figure 4B,C). Thus, we speculated that biallelic *DNAH1* variants destroyed the structure of IDAs instead of ODAs, which resulted in the MMAF phenotype and asthenoteratozoospermia of the affected males.

3.4 | DNAH1-deficient spermatozoa presented with axoneme ultrastructural defects

To further investigate the deformations of flagella ultrastructure in patients with MMAF, sperm samples from control subjects and DNAH1-deficient patients were observed through TEM in vertical and cross-sections. As shown in Figure 5A1–A4, the normal control spermatozoa consist of an oval head with a condensed nucleus half surrounded by an acrosome, and a long smooth flagellum with central axoneme of "9 + 2" structure. The central axoneme was surrounded by mitochondria sheath, outer dense fibers (ODF), and/or fibrous sheath in the different segments of flagella, while the IDAs and ODAs were attached to the peripheral MTD normally to maintain the mobility of sperm flagella. As shown by Figure 5B1–G1 and B2–G2, a large cytoplasmic bag with unassembled axonemal and peri-axonemal components and various head deformations such as small, tapering, or pyriform shapes, were present in the DNAH1-deficient spermatozoa. In addition, several types of disorganized axoneme were observed in the cross-sections (Figure 5B3–G3 and B4–G4), including (i) the most frequent deformation of the absence of CP; (ii) absence of IDAs; (iii) displacement of ODF and/or MTD; and (iv) some unorganized axoneme clusters from several flagellar pieces wrapped by a single piece. Together, these observations suggested that the biallelic *DNAH1* variants are associated with changes in flagella ultrastructure and subsequently cause primary male infertility.

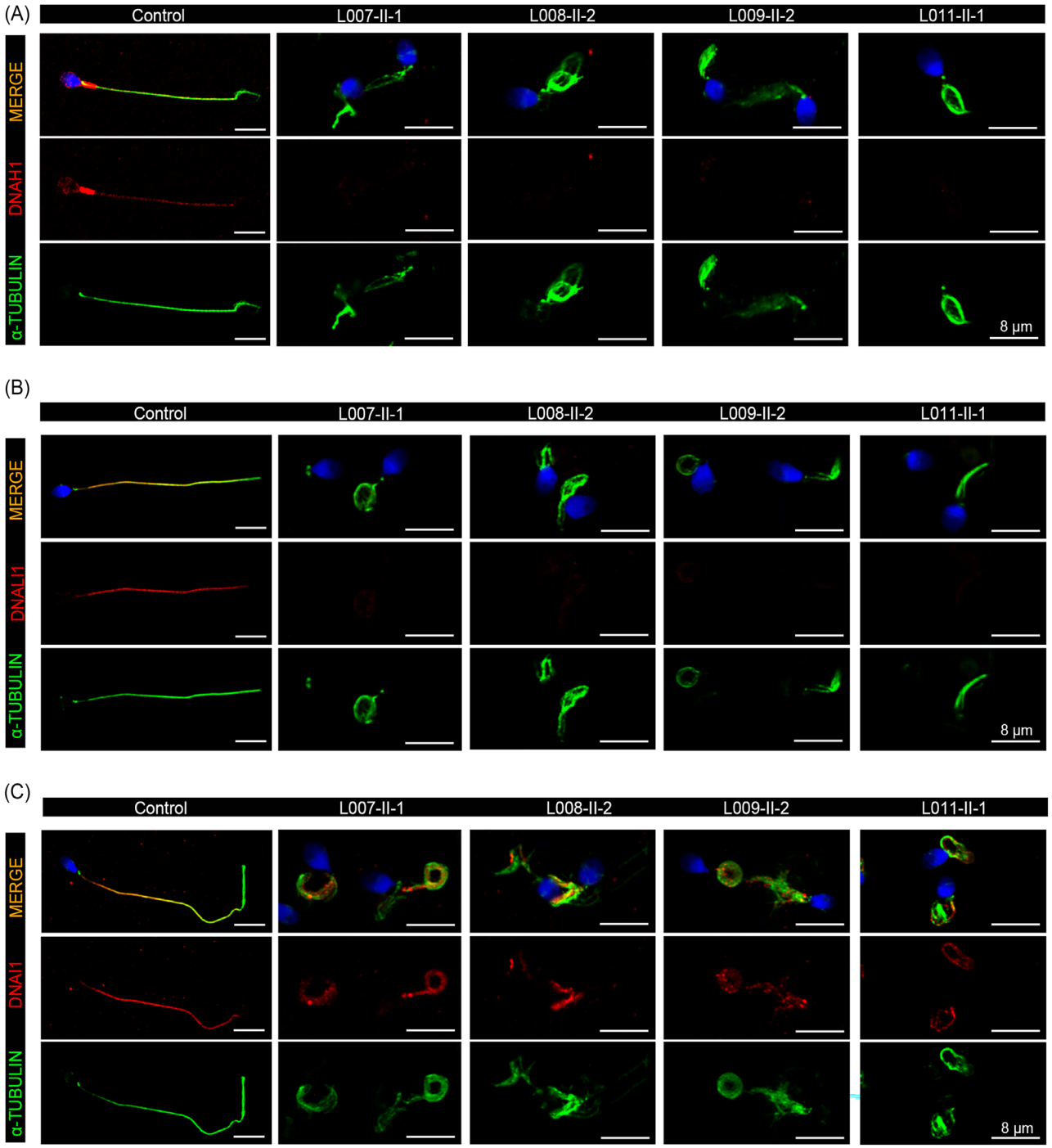

FIGURE 4 Biallelic *DNAHI* variants destroyed the structure of inner dynein arms (IDAs) instead of outer dynein arms (ODAs). (A) Immunostaining of spermatozoa with dynein axonemal heavy chain 1 (DNAH1) and α-TUBULIN antibodies showed the location of DNAH1 along the normal sperm flagella and the absence of DNAH1 and decreased a-TUBULIN in the spermatozoa affected by *DNAHI* variants. Scale bars: 8 μm. (B and C) Immunostaining of spermatozoa with DNALI1/DNAI1 and α-TUBULIN antibodies showed the destruction of IDAs but not ODAs in the DNAH1-deficient spermatozoa. The nuclei were stained with DAPI. Scale bars: 8 μm.

3.5 | Outcomes of ICSI cycles in the DNAH1-deficient patients

ICSI cycling was attempted for seven probands who signed an informed consent form for the ICSI procedure. A detailed description of physical characteristics of the female partners and the clinical outcomes of ICSI cycles is presented in Table 3. The female partners were aged between 25 and 30 years, and their basal hormone levels were all within normal ranges. Following the ovarian stimulation with standardized protocols based on their ovarian reserve, a total of 78 oocytes were

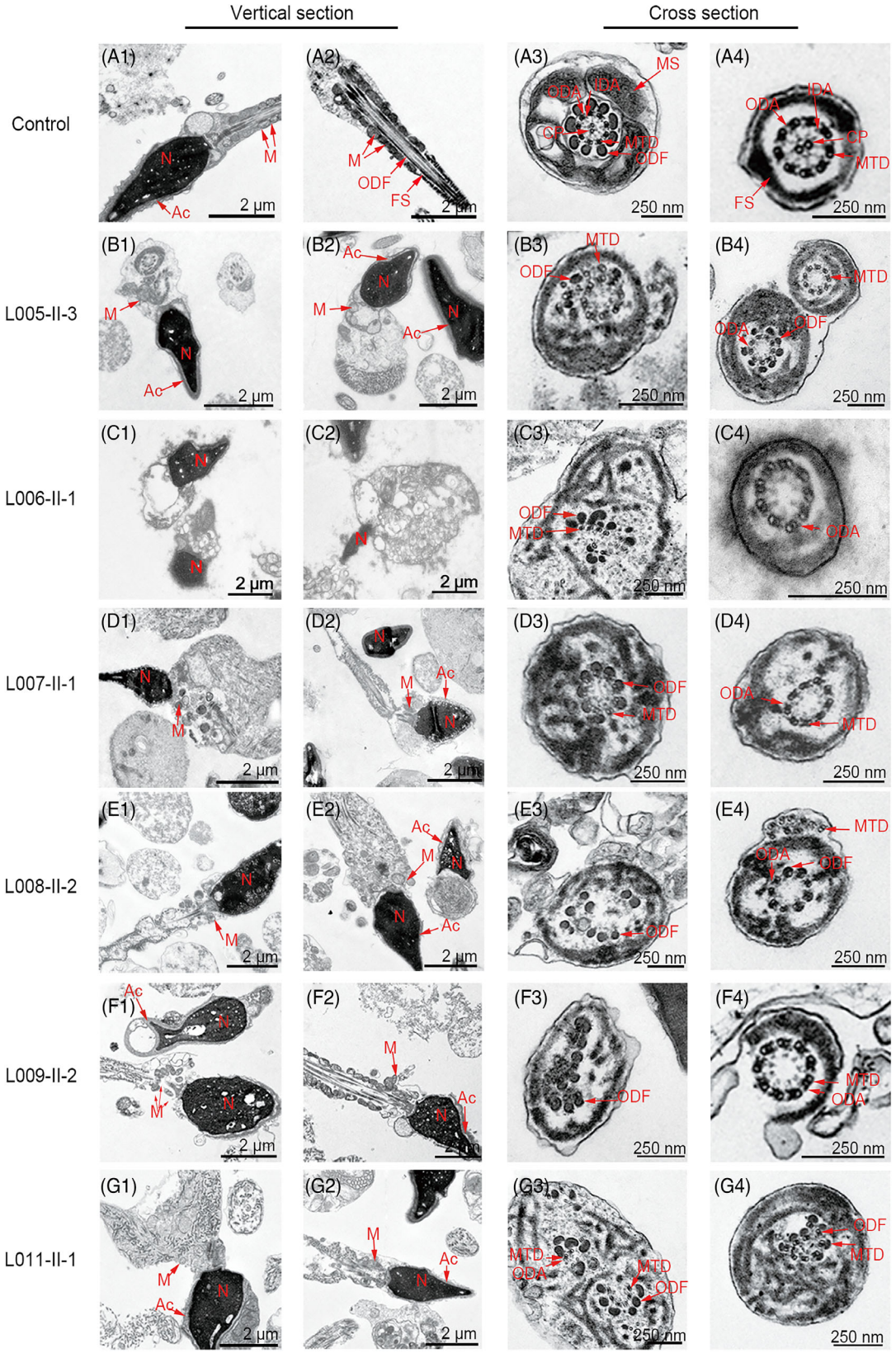

FIGURE 5 The ultrastructural defects observed in dynein axonemal heavy chain 1 (DNAH1)-deficient spermatozoa. (A1–A4) Vertical and cross sections of the spermatozoa obtained from a fertile male showed the organizational structure of flagella axoneme with inner dynein arms (IDAs) and outer dynein arms (ODAs). (B1–G1 and B2–G2) Vertical sections of the DNAH1-deficient spermatozoa. A large cytoplasmic bag with unassembled axonemal and peri-axonemal components was observed. Ac: acrosome; FS: fibrous sheath; M: mitochondria; N: nucleus. Scale bars: 2 μm. (B3–G3 and B4–G4) Cross-sections of the DNAH1-deficient spermatozoa. Several types of disorganized axoneme without IDAs, including the loss of central pair (CP) and displacement of outer dense fiber (ODF) and/or microtubule doublets (MTDs), were observed. Scale bars: 250 nm.

TABLE 3 The clinical outcomes of assisted reproductive technology with intracytoplasmic sperm injection (ICSI) in the patients affected by biallelic *DNAH1* variants.

Subject	L001-II-2	L002-II-2	L004-II-1	L005-II-3	L006-II-1	L008-II-2	L011-II-1	Reference/ average value
Basal information								
Female age (years)	25	27	27	30	27	29	28	–
Body mass index	18.44	21.08	31.39	19.07	19.43	21.95	29.97	–
FSH (mIU/mL)	5.97	6.29	6.69	4.44	6.87	7.04	5.19	3.03–8.08[b]
LH (mIU/mL)	7.46	2.12	2.73	3.65	3.69	13.57	3.58	1.80–11.78[b]
E2 (pg/mL)	34.78	32.04	27.03	30.00	51.00	45.00	31.41	21.00–251.00[b]
PRL (ng/mL)	12.27	14.77	14.88	10.00	12.52	13.73	21.60	5.18–26.53
Prog (ng/mL)	0.3	0.1	0.3	0.1	0.3	0.2	0.3	<0.1–0.3[b]
AMH (ng/mL)	4.66	1.49	0.64	2.17	4.06	2.07	8.24	0.24–11.78
COS cycle								
Protocol	GnRH-antagonist protocol	Short-acting GnRH-agonist long protocol	Mild-stimulation protocol	Short-acting GnRH-agonist long protocol	Short-acting GnRH-agonist long protocol	Short-acting GnRH-agonist long protocol	GnRH-antagonist protocol	–
E2 level on the trigger day (pg/mL)	2532	1979	532	2675	5000	1089	5000	–
No. of follicles ≥14 mm on the trigger day	15	4	2	10	18	7	14	56
No. of follicles ≥18 mm on the trigger day	5	3	2	4	13	4	4	31
ICSI progress								
No. of oocytes retrieved	10	4	2	14	17	9	22	78
No. of MII oocytes (%)	4/10 (40)	3/4 (75)	2/2 (100)	10/14 (71.4)	16/17 (94.1)	8/9 (88.9)	20/22 (90.9)	63/78 (80.8)
No. of 2PN (%)	3/4 (75)	3/3 (100)	2/2 (100)	9/10 (90)	13/16 (81.3)	8/8 (100)	18/20 (90)	56/63 (88.9)
No. of usable embryos (%)	2/3 (66.7)	3/3 (100)	2/2 (100)	6/9 (66.7)	8/13 (61.5)	2/8 (25)	17/18 (94.4)	40/56 (71.4)
No. of blastocysts (%)	–	–	–	5/6 (83.3)	6/8 (75)	–	14/17 (82.3)	25/31 (80.6)
No. of frozen embryos	–	1	2	5	5	–	11	24

(Continues)

TABLE 3 (Continued)

Subject	L001-II-2	L002-II-2	L004-II-1	L005-II-3	L006-II-1	L008-II-2	L011-II-1	Reference/ average value
Clinical outcome								
Transfer cycles	1 (ET)	1 (ET)	1 (FET)	2 (FET)	2 (FET)	1 (ET)	–	8
Transferred embryos	2	2	2	1 + 2	2 + 2	2	–	15
No. of gestational sacs (%)	2	2	2	1	1 + 1	0	–	9/15 (60)
No. of clinical pregnancies (%)	1	2	2	1	1 + 1	–	–	8/9 (88.9)
No. of newborns (%)	Abortion[a]	2	2	1	Miscarriage + ongoing	–	–	5

Note: Symbol "–" denotes no reference range.

Abbreviations: AMH, anti-Mullerian hormone; COS, controlled ovarian stimulation; E2, Estradiol; ET, fresh embryo transfer; FET, frozen–thawed embryo transfer; FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone; LH, luteinizing hormone; MII, metaphase II; 2PN, two pronucleus; PRL, prolactin.

[a]The abortion was conducted because of fetal factor of cardiac dysplasia.

[b]These data refer to the value in follicular phase.

retrieved during follicular puncture, 63 (63/78, 80.8%) of which were in metaphase II (MII). After ICSI of the spermatozoa obtained from corresponding probands, 88.9% (56/63) of the MII oocytes achieved successful fertilization with two pronucleus. As shown in Table 3, 71.4% (40/56) of the fertilized embryos developed into usable embryos, and three couples chose blastocyst culture of 31 embryos with the formation of 25 blastocysts (25/31, 80.6%). So far, six couples have implemented eight cycles of fresh or frozen–thawed embryo transfer. Of the 15 transferred embryos, a clinical pregnancy was obtained in six cycles of nine embryos, which led to five deliveries in three couples. One embryo natural reduction and one abortion caused by fetal factor of cardiac dysplasia were observed in L001-II-2. One miscarriage and one ongoing pregnancy were described for L006-II-1. Thus, we speculated that patients with MMAF induced by biallelic *DNAH1* variants have a good prognosis for fertility following ICSI treatment with a fertilization rate of 88.9%, a usable embryo formation rate of 71.4%, and a blastocyst formation rate of 80.6%.

4 | DISCUSSION

In this study, we recruited 45 unrelated Han Chinese families with infertile males exhibiting MMAF and identified 18 variants in *DNAH1* from 11 irrelevant individuals. A total of 66.7% (12/18) of the identified variants were novel and had not been previously reported in patients with MMAF. We systematically described the effect of biallelic *DNAH1* variants on the sperm motility, morphology, and flagella ultrastructure. These findings expanded the variant spectrum of *DNAH1* gene and provided more evidence for the gene–disease relationship between *DNAH1* and male infertility with MMAF. Moreover, the favorable fertility outcomes of ART with ICSI for these patients will facilitate the genetic counseling of infertile males with MMAF in the future.

To date, *DNAH1* has been mostly identified in males with MMAF with a frequency of 4.8%–57.1%, which is 24.4% (11/45) in this study. We summarized and analyzed all *DNAH1* variants reported previously and in the present study, and then linked 111 variants of *DNAH1* to 125 males with MMAF globally (Table S2). The number of loss-of-function variants, including frameshift ($n = 19$), nonsense ($n = 19$), and splice-site loss variants ($n = 18$), was approximately equivalent to that of the missense variants ($n = 52$). Among them, 27 variants were found in more than one MMAF patient, presenting as p.P3909Rfs*33 ($n = 31$), p.Q1518* ($n = 5$), p.W870* ($n = 4$), p.R2356W ($n = 4$), p.R4096L ($n = 3$), p.R1702* ($n = 3$), p.W1955* ($n = 3$), p.R2622* ($n = 3$), p.L2071R ($n = 3$), p.4067_4068del ($n = 2$), p.R4096H ($n = 2$), p.R4133C ($n = 2$), p.I710Hfs*4 ($n = 2$), p.R868* ($n = 2$), p.A913D ($n = 2$), p.E2086Gfs*8 ($n = 2$), p.Q2511Sfs*27 ($n = 2$), p.P1582_G1584del ($n = 2$), p.R971H ($n = 2$), p.K1279R ($n = 2$), p.T1372M ($n = 2$), p.W2385R ($n = 2$), p.R3169G ($n = 2$), p.R3229C ($n = 2$), p.E3284K ($n = 2$), p.W1036* ($n = 2$), and p.A1876P ($n = 2$). Surprisingly, p.P3909Rfs*33, p.Q1518*, p.R2356W, p.R1702*, p.R4133C, p.A913D, p.R971H, p.K1279R, p.E3284K, and p.A1876P have significantly higher allele frequencies in East Asia according to the gnomAD database, and 85.2% (23/27) of these candidate mutant hotspots were identified in Han Chinese males,

suggesting that these loci might have a founder effect in East Asian population.

The axonemal dynein motors, IDAs and ODAs, are large multi-subunit complexes distributed regularly along the axoneme and can convert the chemical energy from adenosine triphosphate (ATP) into mechanical energy for sperm movement.[38] Functionally, the ODAs are organized at 24-nm intervals and regulate the amplitude and frequency of flagella oscillation, and the IDAs are arranged within 96-nm repeats to control the beat waveform.[39] The highly heterogeneous IDAs are organized into seven dynein structures (dynein a, b, c, d, e, f/I1, and g) and are arranged in 3-2-2 groups corresponding to three subtypes (IDA1–IDA3).[38] *DNAH1* encodes an axonemal inner arm dynein heavy chain of the IDA3, especially IDA d, which is directly connected to the stalk of RS3 through an arc-like structure.[9] Moreover, DNALI1 as a light intermediate chain of IDAs was reported to interact with the C-terminus of DNAH1 in IDA3.[9,40] Therefore, the severe axonemal disorganization observed in DNAH1-deficient patients may occur because of the absence of RS3 anchoring site resulting in the loss of CP, which weakens all the axonemal organization and makes the flagella susceptible to mechanical stress during spermiogenesis and transport. In addition, different from our observations in *CFAP43*-deficient patients in which heterogeneity was observed between different patients in morphological abnormalities and sperm motility,[30] the phenotypes of males affected by *DNAH1* variants were generally consistent, which might reinforce the irreplaceability of DNAH1 in sperm flagella axoneme.

The sperm malformation and immobility caused by genetic factors cannot be improved with drugs. Currently, ICSI is reported to be the practicable way to assist patients with MMAF in obtaining their own genetic offspring.[25] Of the 114 previously reported patients with *DNAH1* variants, 37 couples successfully obtained transferable embryos through ICSI, and 20 clinical pregnancies and eight deliveries were reported.[13,19,24,25,41,42] In our study, seven of the 11 identified probands accepted ART treatment with ICSI and all obtained transferrable embryos through one oocyte retrieval. Six of them achieved clinical pregnancy successfully and three couples gave birth to five healthy babies. Despite the decreased ovarian reserve function of the partner of patient L004-II-1 when the anti-Mullerian hormone level was as low as 0.64 ng/mL, the two mature oocytes fertilized and developed into usable embryos and were delivered successfully. Statistically, the rates of fertilization, usable embryos and blastocyst formation for patients with *DNAH1* variants following ICSI were 88.9%, 71.4%, and 80.6%, respectively. Thus, we suggested that the infertile couples affected by *DNAH1* variants undergo ICSI therapy to obtain their genetic progenies. During genetic counseling, couples should be informed that their newborn will be at risk of inheriting a heterozygote, so genetic screening should be recommended for their partners.

5 | CONCLUSIONS

These findings further expand the variant spectrum of *DNAH1* gene related to multiple morphological abnormalities of sperm flagella and male infertility in humans, thus providing a theoretical basis for the molecular diagnosis of infertile males with multiple morphological abnormalities of sperm flagella. Genetic screening and counseling are essential for the personalized diagnosis and treatment of males with multiple morphological abnormalities of sperm flagella, and the intracytoplasmic sperm injection treatment is highly suggested for patients with *DNAH1* variants to achieve fertilization and conception.

AUTHOR CONTRIBUTIONS

Tingting Lin, Wenhong Lu, and Guoning Huang designed and supervised the study experiments. Jing Ma, Haibing Yu, and Tingwenyi Hu collected data and conducted the clinical assessments. Shunhua Long and Longlong Fu performed whole exome sequencing and analyzed its data. Shunhua Long and Xiangrong Tang carried out the immunofluorescence staining. Weiwei Liu, Wei Han, Haiyuan Liao, and Tao Fu conducted the assisted reproductive technology cycle. Shunhua Long and Longlong Fu drafted the manuscript. Tingting Lin and Wenhong Lu critically commented on and edited the manuscript. All authors revised and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Validation of the relationship between rapid eye movement sleep and sleep-related erections in healthy adults by a feasible instrument Fitbit Charge2

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Abstract

Background: Sleep, particularly rapid eye movement sleep, has been found to be associated with sleep-related erections. While RigiScan is currently a more accurate method for monitoring nocturnal erectile events, the Fitbit, a smart wearable device, shows great potential for sleep monitoring.

Objectives: To analyze the relationship between sleep-related erections and sleep by recruiting sexually active, healthy men for simultaneous monitoring of sleep and nocturnal penile tumescence and rigidity.

Patients and methods: Using Fitbit Charge2 and RigiScan, we simultaneously monitored nocturnal sleep and erections in 43 healthy male volunteers, and analyzed the relationship between sleep periods and erectile events with the Statistical Package for Social Sciences.

Results: Among all erectile events, 89.8% were related to rapid eye movement, and 79.2% of all rapid eye movement periods were associated with erectile events. Moreover, a statistical correlation was shown between the duration of rapid eye movement and the time of total erectile events (first night: $\rho = 0.316$, $p = 0.039$; second night: $\rho = 0.370$, $p = 0.015$).

Discussion and conclusion: Our study shows a potential link between sleep-related erections and rapid eye movement sleep, which has implications for the current examination of sleep-related erections and further research into the mechanisms of erectile function. Meanwhile, the wearable device Fitbit has shown a potential promise for sleep monitoring in patients with erectile dysfunction. The results provide an alternative approach for further research on the relationship between erectile function and sleep with large sample sizes in the future.

KEYWORDS
Fitbit, rapid eye movement sleep, sleep-related erections, smart wearable device

Guodong Liu, Yuyang Zhang, Wei Zhang, and Xu Wu contributed equally to this work.

1 | INTRODUCTION

The process of achieving an erection is commonly understood to involve the evaluation and integration of various stimuli, such as touch, sight, smell, and imagination. These stimuli result in signals that are ultimately transmitted through neural pathways to the penis and somatic pathways to the perineal muscles.[1] Despite being a seemingly straightforward hemodynamic process, achieving a regular erection requires a high level of coordinated control, as it involves complex changes in blood flow, intracavernosal pressure, and penile volume.[2] There are many transmitters and transmission systems involved in the central regulation of penile erection, the details of which are still not fully understood.[1]

Sleep-related erections (SREs), which were first discovered in the Middle Ages, were reported by Ohlmeyer and Hüllstrung in 1944.[3] SREs can partially reflect the erectile function of the penis and are a valuable method for assessing erectile function.[4] SREs are also widely known as nocturnal penile tumescence (NPT), a term coined by Karacan, who attempted to monitor SREs as early as the last century. Karacan used a silicone elastic ring filled with mercury to monitor penile erections behind the glans.[5] In 1985, Bradley et al. invented a new detection instrument, the RigiScan device, which uses a mercury strain gauge to monitor nocturnal penile tumescence and rigidity (NPTR).[6] Until now, the RigiScan device has been gradually upgraded and NPTR monitoring has been adopted in the European Association of Urology (EAU)[7] and American Urological Association (AUA)[8] guidelines, which are widely used for erectile function examination, especially in differentiating psychogenic erectile dysfunction (ED) from organic ED. However, the exact physiological significance of SREs remains substantially unexplored. It has been suggested that SREs can improve the physiological process of cavernous smooth muscle oxygenation, which contributes to the maintenance of a normal erectile function.[9] It may provide an explanation for ED in patients with obstructive sleep apnea, where low oxygen levels lead to reduced nitrogen-oxygen synthesis in the cavernous tissue, consequently contributing to ED.[10,11]

Despite the close relationship between SREs and erectile function, the neurophysiology underlying SREs remains poorly understood. Sachs suggests that various brain areas may contribute to SREs occurrence under different circumstances.[12] Montorsi and Oettel's research shows that NPT occurs naturally in healthy men from infancy to old age, with the majority occurring during rapid eye movement (REM) sleep, unrelated to dream content.[13] However, SREs can be influenced by many factors: a healthy man having dreams causing anxiety, aggression, and other negative content, depressed individuals, and first-night effects, and so forth, but this still cannot deny that SREs is an area worth to be examined.[14] As early as the last century, polysomnography (PSG) examination and monitoring of NPTR have been performed simultaneously.[15] Hirshkowitz and Schmidt have suggested that combined PSG would be meaningful for NPTR examination after summarizing the clinical perspectives and neural mechanisms of SRE.[4] Although PSG is considered the gold standard for sleep monitoring, it has some drawbacks: it is an expensive examination, requires a hospital visit, and is not widely available in all hospitals or departments. Furthermore, PSG can limit examiner mobility, change sleep environments, and can only be performed for a limited time, all of which can affect the examination results.

The use of wearable devices that monitor wrist behavior, combined with proprietary algorithms for sleep assessment, is becoming increasingly popular. Initially, these devices, which rely on motion-based algorithms, were not highly accurate in assessing sleep stages.[16] However, with advancements in technology, Fitbit has emerged as one of the most popular wearable devices capable of estimating not only sleep parameters and stages but also heart rate during waking and sleeping hours.[17] According to Fitbit's official website, it had more than 28 million active users in over 110 countries as of November 1, 2019. In addition, it is the most used wearable device when performing biomedical research.[18] In 2019, the United States National Institutes of Health declared the enrollment of Fitbit technology in the All of Us Research Program. However, despite its widespread use, no studies have yet linked Fitbit to NPTR examination. Therefore, we conducted this study to analyze the relationship between SREs and sleep by recruiting normal healthy men to undergo both Fitbit sleep monitoring and NPTR examination.

2 | METHODS

2.1 | Study population

The study was conducted between August 2020 and May 2022 at the Department of Urology, the First Affiliated Hospital of Anhui Medical University, in accordance with the Declaration of Helsinki (revised in 2013). A total of 43 healthy, sexually active male participants aged 18–60 years with a body mass index (BMI) between 18 and 28 were recruited for the study. The International Index of Erectile Function-5 (IIEF-5) questionnaire was used to measure their erectile function, with all participants scoring more than 21, indicating normal erectile function. To ensure the validity of the study, exclusion criteria included patients with sleep quality-affecting diseases, such as obstructive sleep apnea syndrome (OSAS), chronic obstructive pulmonary disease, depression, and heart failure, as well as those with cardiovascular system diseases, respiratory diseases, metabolic syndrome, inflammatory diseases, and diabetes mellitus. Participants who had taken any medication affecting sleep or erection in the previous 3 months or had a history of alcohol or substance abuse were also excluded. All participants showed normal SREs in terms of duration of ideal erectile episodes and erectile rigidity, which met the inclusion criteria. The study was authorized by our hospital's Ethics Committee after all participants completed an informed consent form detailing the study's goal and conduct (Quick-PJ 2022-07-61).

2.2 | Study procedure

Appropriate questionnaires were used to gather the general information of the participants, including age, height, weight, frequency of

late-night activities, Epworth Sleepiness Scale, Stop-Bang Scale, and Insomnia Severity Index. The NPTR examination and nocturnal sleep testing were carried out concurrently, with each participant spending two consecutive nights in a dedicated single male screening laboratory. Prior to the test, they were required to maintain a regular schedule for a week and avoid any activities that may interfere with sleep on the day of the test, such as alcohol, coffee, sleeping drugs, and excessive exercise.

The Fitbit Charge2 (a smart wearable band, hereafter referred to as Fitbit) was used to track the sleep of attendees. Before nocturnal monitoring, subjects wore the band on their non-dominant wrist, adjusting the appropriate bandwidth until the end of the check the next morning. The device records wrist activity using an accelerometer and pulses through optical plethysmography. It generates different types of sleep data depending on whether it meets some criteria during data collection, including sufficient battery power, sleep duration more than 3 h, and adequate skin contact with the optical plethysmography pulse wave sensor.[19] All data signals were sent via Bluetooth to the Fitbit app on the mobile device. The NPTR examination was performed by measuring nocturnal tumescence (assessed by circumference) and erectile hardness with the RigiScan device. Moreover, the tumescence and erectile rigidity at the tip and base of the penis were recorded continuously throughout the night.[6] All data including the onset time, duration, and average rigidity of erectile events were visualized in the RigiScan software.

2.3 | Data extraction for sleep and SREs

Sleep parameters were meticulously documented, including total sleep time (TST), sleep onset latency (SOL), wake time after sleep onset (WASO), REM sleep time, light sleep (N1 + N2) time, and deep sleep (N3 + N4) time, along with the onset and duration of each sleep period. According to the criteria proposed by Sohn et al.,[20] a valid erectile event was defined as a lasting time of at least 3 min, a tumescence of the penile base more than 1 cm, and a rigidity value of at least 20%. The change in tumescence at the tip of the penis was practically parallel to the process at the base of the penis, as the study was conducted in healthy subjects with normal erectile capacity. The RigiScan software ultimately exhibited the penile tumescence and stiffness readings as a graph. The timings were determined as the commencement and end periods of the erectile event when tumescence was above and below the threshold of 1 cm above baseline.

2.4 | Statistical analysis

Categorical variables were defined by frequencies and percentages for questionnaire information, while continuous variables were characterized by means and standard deviations (mean ± SD). Furthermore, for each of the two nights, we computed the mean and SD of data related to sleep and erectile events, including total sleep time (TST), sleep onset latency (SOL), wake time after sleep onset (WASO), REM period sleep time, light sleep time, and deep sleep time mean values, counts, and durations of erectile events. Comparisons between groups on both nights were calculated using a two-sample *t*-test in groups. The occurrence of temporal overlap between erectile events and REM periods was considered relevant, and the mean ± SD deviation of the time gap between the two events was evaluated (onset time of erectile events minus onset time of REM periods). In addition, we calculated the corresponding power by R studio software. For the analysis between each scale of sleep and REM sleep, the Spearman analysis (Epworth Sleepiness Scale and Insomnia Severity Index) and the one-way ANOVA (late nights and Stop-Bang Questionnaire) were performed. In addition, we evaluated the temporal correlation between the duration of REM and erectile events on each of the two nights by calculating the Spearman correlation coefficient through correlation analysis. Statistical analyses were performed with the SPSS statistical package (SPSS, Chicago, Ill., USA). Two-tailed *p*-values less than 0.05 were considered statistically significant.

TABLE 1 Demographic and clinical characteristics of samples.

	Mean ± SD
Age	32.02 ± 5.45
BMI	23.04 ± 1.79
Late nights	Never (14%), occasionally (55.8%), frequently (30.2%)
Epworth Sleepiness Scale	7.3 ± 1.86
Stop-Bang Questionnaire	1 (30.2%), 2 (51.2%), 3 (18.6%)
Insomnia Severity Index, ISI	6.51 ± 1.72

3 | RESULTS

In the study, 43 healthy individuals were recruited according to the inclusion and exclusion criteria. Basic information was collected from all participants, including age, BMI, frequency of late-night, Epworth Sleepiness Scale, Stop-Bang Scale, and Insomnia Severity Index, as shown in Table 1. We further performed a statistical analysis of all the data on sleep and erection for both nights. An example of a participant's nocturnal penile erection and corresponding sleep status is presented in Figure 1, which clearly demonstrates a potential relationship between SREs and REM. Throughout the study, the average count of erections on the first night was 4.14 and the average time of erections was 58.72 min; the average count of REM sleep was 4.79 and the average time was 78.55 min. Among all erectile events, 87.6% were related to REM episodes and 75.7% of all REM events were related to erectile events. The analysis of the second night showed an average of 4.35 erectile events (69.09 min) and 4.93 REM sleep events (85.83 min). Of all erection events, 89.8% were associated with REM periods, and 79.2% of all REM periods were related to erection events.

The results of paired samples *t*-test analysis showed that sleep onset latency ($p = 0.001$), time of REM ($p = 0.009$), slow wave sleep

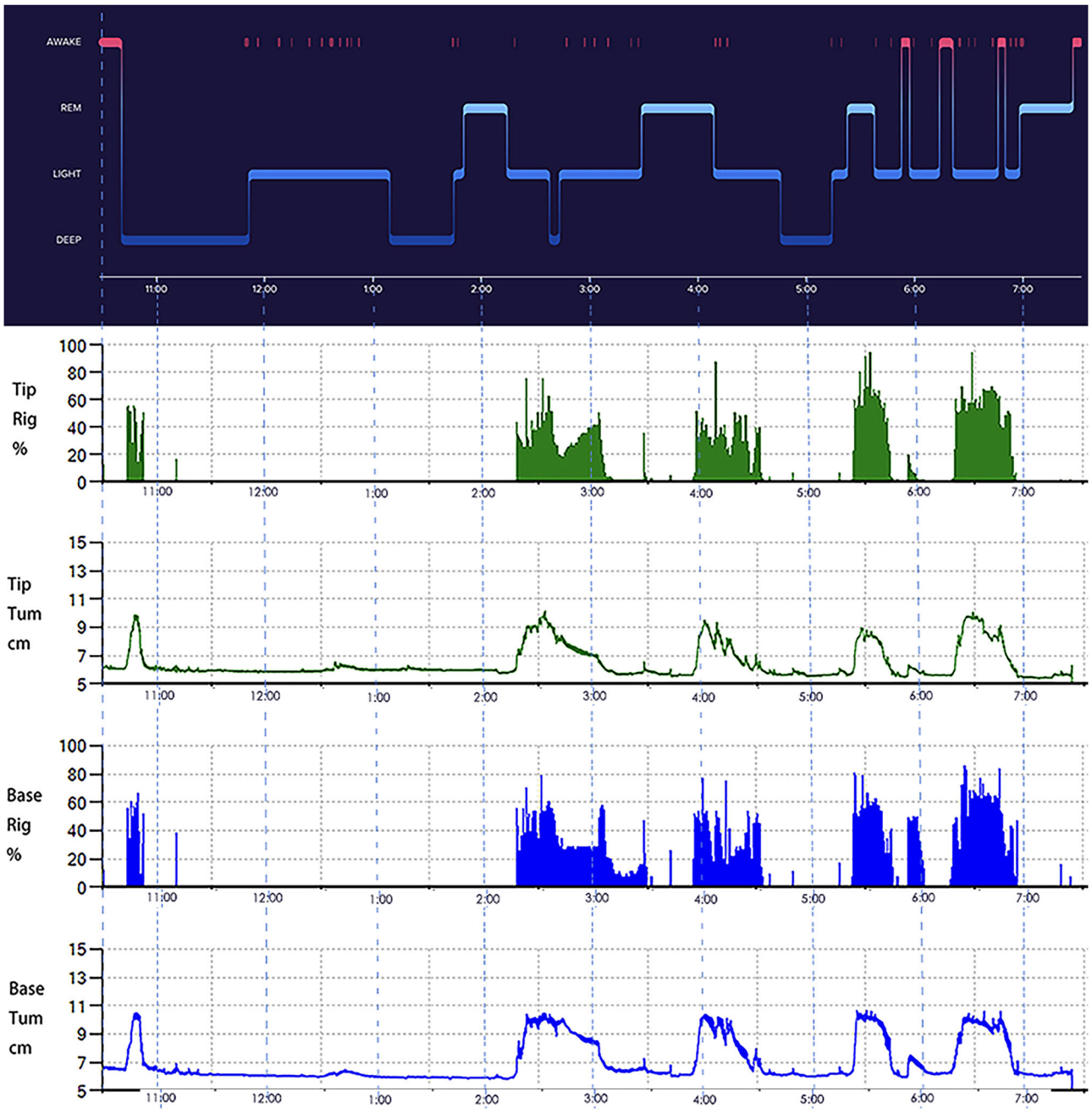

FIGURE 1 A graph combining the process of nocturnal penile erection and the corresponding sleep state of a healthy man.

($p = 0.002$), erection time at the tip ($p < 0.001$), and count of erections at the base ($p = 0.006$) were significantly different between the two nights. We further calculated the corresponding power, and the results showed that most of them were optimal (>0.8). Detailed data are shown in Table 2. The correlation between the duration of nocturnal REM and the erection was further analyzed using Spearman analysis, which showed a moderate correlation between REM duration and total erectile events in the two nights (night 1: $\rho = 0.316$, $p = 0.039$, night 2: $\rho = 0.370$, $p = 0.015$), as detailed in Table 3. We represented this more visually by the corresponding histogram (Figure 2) and scatter plot (Figure 3).

4 | DISCUSSIONS

Our study provides evidence of a potential association between SRE and REM sleep in healthy men with normal erectile function, through the combined smart wearable device Fitbit. In a large follow-up study in Massachusetts, the crude prevalence of ED was 26/1000 man-years.[21] Furthermore, this number is projected to increase rapidly with the growing number of related comorbidities, affecting an estimated 320 million men by 2025.[22] With the rise in incidence of ED, there has been a heightened interest in understanding the mechanisms underlying erectile function. Although the understanding of

TABLE 2 Comparison of sleep episodes and erection events between the two nights.

	Night 1	Night 2	*p*-Value	Power
Total sleep time, min	436.5 ± 53.82	442.62 ± 40.43	0.411	0.09
Sleep onset latency, min	11.92 ± 5.08	9.43 ± 3.7	**0.001**	**0.73**
WASO, min	45.37 ± 12.8	43.02 ± 10.55	0.179	0.15
Time of REM, min	78.55 ± 18.62	85.83 ± 13.63	**0.009**	0.53
Counts of REM	4.79 ± 1.17	4.93 ± 0.99	0.509	0.09
Light sleep	248.88 ± 33.2	243.66 ± 23.58	0.264	0.13
Slow wave sleep	51.73 ± 14.42	60.65 ± 14.52	**0.002**	**0.81**
Counts of erection (tip)	4.14 ± 1.01	4.35 ± 0.92	0.304	0.17
Erection time (tip)	58.72 ± 11.00	69.09 ± 11.23	<**0.001**	**0.99**
Counts of erection (base)	4.12 ± 0.96	4.37 ± 0.93	**0.006**	0.23
Erection time (base)	63.15 ± 12.28	71.55 ± 13.66	0.195	**0.84**
Relate times (tip)	3.63 ± 0.9	3.88 ± 0.7	0.102	0.30

Note: *p*-values were calculated by paired samples *t*-test. The power was calculated by R studio.
Abbreviations: REM, rapid eye movement period; WASO, wake time after sleep onset.

TABLE 3 Correlation analysis of the time of the REM episodes and erection events during nocturnal sleep.

		Night 1 Erection time (tip)	Night 2 Erection time (tip)
Night 1	Time of REM	0.316[a] $p = 0.039$	0.261
Night 2	Time of REM	0.070	0.370[a] $p = 0.015$

[a]In the Spearman analysis, the significance (two-tailed) was less than 0.05.

erection mechanisms at the peripheral level has improved considerably in recent years, the control of erection at the supraspinal level remains poorly understood.[23,24]

It has been reported that sleep is the foundation of health, and insufficient sleep may lead to serious adverse consequences, including obesity, neurocognitive dysfunction, mood disorders, cardiovascular disease, diabetes, and even mortality.[25] Therefore, accurate and effective objective sleep measurement is crucial for predicting and analyzing sleep and its related diseases. According to the Association for the Psychophysiological Study Sleep of America, sleep is mainly divided into REM sleep and non-REM sleep periods, with non-REM periods further divided into light sleep (S1, S2) and slow wave sleep (S3, S4), based on specific EEG rhythms, muscle, and eye activity information.[26–29] Presently, PSG is accepted as the gold standard for sleep assessment, as it provides an objective and accurate measurement of sleep. However, the fact is that the PSG examination is not suitable for widespread use among all people owing to the price and the complexity of the examination instrument. Wrist behaviorology has been widely used as a novel technique for sleep monitoring, which is more practical than PSG and more suitable for recording sleep characteristics over time in a non-laboratory situation.[30] The current generation of wearable devices has adopted a multisensory approach to sleep assessment. These devices can accurately identify sleep stages by using various information (such as heart rate and heart rate variability) as well as motion. Experimental evidence suggests that these devices, such as Fitbit, show promise in detecting sleep–wake states and sleep stages, particularly in REM sleep measurement.[31–33]

REM and non-rapid eye movement (NREM) sleep are different states of the brain during sleep, and are associated with different psychological experiences and functional effects.[34] The electroencephalogram (EEG) is dominated by synchronized large-amplitude slow-wave activity during NREM sleep, but shows a wakeful-like desynchronized EEG and complicated spontaneous neurological features during REM sleep.[35,36] Research on REM sleep can be divided into the following main areas: (i) identifying the neurons, pathways, and neurotransmitter effector systems responsible for REM sleep; (ii) determining the mechanisms controlling the timing and duration of REM sleep; (iii) exploring the relationship between REM sleep and learning, memory, and other cognitive processes.[34] Although the exact mechanisms underlying REM sleep are not yet fully understood, several scholars have discovered that the pontine reticular formation (subcoeruleus, SubC) region located ventral to the locus coeruleus is closely associated with the generation of REM through pharmacological injuries and unit recording techniques in several animal species.[37]

Through lesion experiments on rats, Schmidt et al. found that selective disruption of the lateral preoptic area (LPOA) in the forebrain will undermine the REM-related SRE while leaving erections unchanged during wakefulness, suggesting that the LPOA may perform a key role in the generation of SRE.[38] However, the mechanisms underlying the regulation of REM generation by LPOA from the brainstem and the neurotransmitters involved remain to be further explored. There are currently recognized two output pathways originating from the LPOA.[4,39] The first pathway involves the LPOA projecting to 5-hydroxytryptamine paracellular neurons in the ventral medulla. These neurons excite sympathetic preganglionic neurons in the thoracolumbar (T11–L2) region that innervate the penis. The second pathway involves the LPOA projecting through the paraventricular nucleus of the thalamus, which projects oxytocin to sympathetic preganglionic neurons in the sacrococcygeal (S2–S4) spinal cord. These pathways

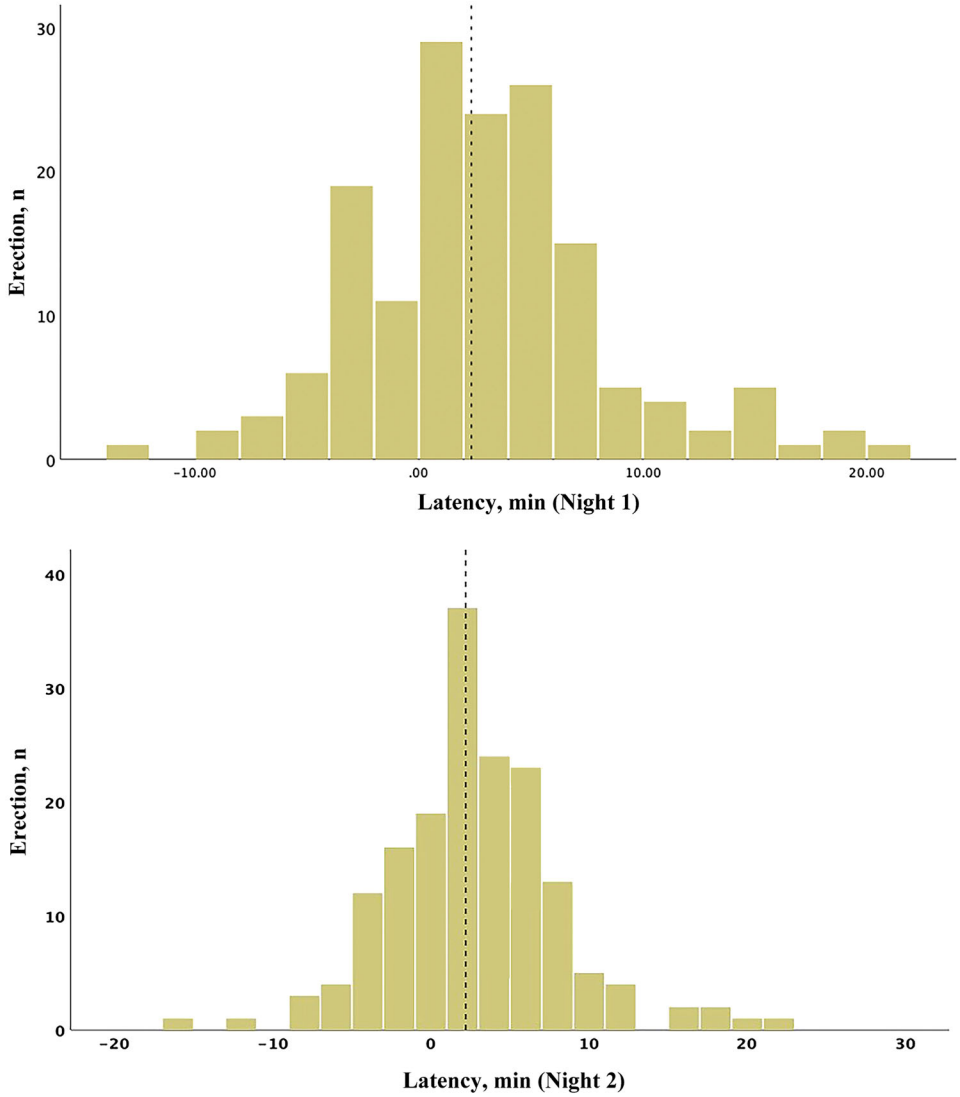

FIGURE 2 Histogram of the latency between rapid eye movement (REM) sleep and corresponding erection events over two nights for 43 subjects.

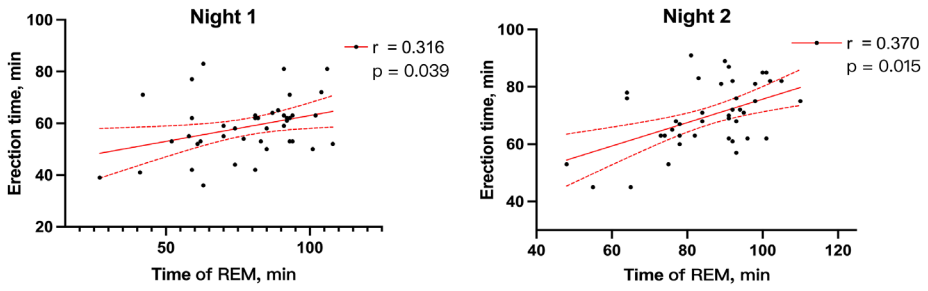

FIGURE 3 Scatter plot and regression line between the total effective time of the erectile events and the total duration of rapid eye movement sleep.

may contribute to the regulation of REM generation. In addition, some scholars have revealed a relationship between REM sleep and some gonadal-related hormones, such as prolactin,[40] testosterone,[41] and estradiol,[42] which contribute to the regulation of REM.

Furthermore, SREs have been used to distinguish psychogenic ED from organic ED as early as the last century, yet decades later, the physiological role of SREs is still poorly understood.[4] Several authors have found that testosterone may play a critical role in the regulation of SREs. As early as the last century, hypogonadism caused by hyperprolactinemia was found to be associated with a decrease in SREs.[43–45] Interestingly, there is a paradoxical relationship between hormones that affect both REM sleep and SREs, where the effects on both are

opposite (e.g., prolactin promotes REM, but diminishes SREs). There are currently no plausible explanations for that because the relationship between REM or SREs and these hormones is still in its early stages, the underlying mechanisms are still unknown, and there are no studies that connect all of them. Therefore, the exact connections remain to be revealed by further and better experiments. And we intend to add the study of related gonadal hormones in our future experiments.

Several studies have demonstrated that REM and SREs are not entirely dependent on each other. Steiger et al. discovered that the impact of psychotropic drugs on sleep rhythm and NPT appears to be separate. They applied the selective reversible MAO-A inhibitors moclobemide and brofaromine in healthy subjects, resulting in the inhibition of REM sleep, while NPT was almost unchanged.[46,47] Besides, various scholars have observed that NPT is significantly reduced in depressed patients, although this is not significantly linked to sleep.[48,49] Ware et al. revealed that there was a significant increase with time to NPT in normal subjects after the use of the antidepressant trazodone, even though REM was inhibited.[50] In the above-mentioned study, sleep and NPT did not show a significant correlation.

In our study, there was a significant difference between some of the monitoring results of the first and second nights (sleep onset latency, time of REM, slow wave sleep, erection time, and counts of erection), with most of the parameters being somewhat better on the second night than on the first night. This is consistent with the first-night effect proposed by some authors, where most NPTR parameters are better on the second night than on the first night.[51] Interestingly, the first-night effect also exists in sleep monitoring.[52] And many studies have shown that monitoring by two nights can effectively avoid false abnormal results because of the first-night effect.[53]

It is now widely accepted by scholars that the etiology of ED is usually multifaceted.[54] Moreover, with more effective drug therapy available today, distinguishing between organic and psychogenic ED is no longer a necessary issue that must be addressed. Besides, the use of the NPT exam today remains somewhat controversial: SREs are mediated by sympathetic, psychogenic pathways, whereas sexual-induced erections are mediated by parasympathetic, reflex pathways.[24] Therefore, a normal nocturnal erection is not necessarily diagnostic of psychogenic ED. On the other hand, abnormal nocturnal erection results do not exclude normal erectile function. For example, the results of the test are affected by interfering events, such as sleep disturbances, the influence of gonad-related hormones, negative dreams,[55] and so forth.

In addition, some organismal factors like hypertension, diabetes, and sleep disorders can alter NPTR results in patients with psychogenic ED, suggesting that it may not always be reliable in distinguishing between psychogenic and organic ED.[24] Nonetheless, NPTR is still the best diagnostic modality available for a detailed examination of erectile function, with high sensitivity and specificity,[56] and recommended by recent guidelines.[7,8] The abnormal results of NPTR may at least indicate the presence of some kind of organic abnormality, possibly in the erectile pathway itself, or in the part of the REM that is linked to the SRE, which is a point worth exploring in scientific research.

We obtained evidence for a potential association between SREs and REM sleep through time-related data analysis by NPTR examination in combination with the smart wearable device Fitbit. Although sleep, especially REM sleep, has been shown to be associated with SREs, studies on the relationship between them have not been conducted on a large scale because of the limitations in equipment and technology. Until now, the primary purpose of measuring SREs has been to distinguish between organic ED, where SREs are reduced or absent, and psychogenic ED, where findings are typically normal. However, our study suggests a potential link between SREs and sleep-related pathways, indicating that a more thorough analysis of SREs is warranted. It is potentially of great interest both for the current examination of SREs and for further research regarding the mechanisms of erectile function. Our results may also provide a potential option and research direction for research in ED. Sleep function, especially REM sleep, is closely related to erection. It suggests that impaired sleep is likely to be involved in the development of ED as well. Moreover, the use of smart wearable devices like Fitbit offers a convenient method of monitoring sleep, which could be leveraged to identify ED patients with sleep impairment and to explore the role of sleep in the pathogenesis of ED through further detailed studies.

There are several limitations to our study that should be considered. First, our sample size is still not large enough, resulting in low test power for some of the results, and thus affecting the reliability of the results. Moreover, the statistical test power of some data was less than 0.8, indicating that the reliability of the statistical conclusions has to be verified by larger experiments. Besides, it is important to note that individual differences may affect the accuracy of the results, particularly in regard to NPTR and sleep monitoring. Some uncontrollable factors can cause the test results to be affected, for example, the weather, the subject's mood, and adaptation to a new environment. Therefore, while our study provides valuable insights, it is important to acknowledge these limitations and consider them when interpreting the findings.

5 | CONCLUSION

Our study, which combined the novel wrist-based behavioral sleep monitoring devices Fitbit and nocturnal penile tumescence and rigidity, showed a potential association between sleep-related erections and rapid eye movement sleep in healthy individuals with normal erectile function. Furthermore, the total duration of nocturnal sleep rapid eye movement was also linearly correlated with the total time of erectile events, suggesting that there may be some possible connection between sleep-related erections and rapid eye movement. In conclusion, Fitbit, a modern smart wristband with a sleep monitoring function, enabled us to collect sleep data and investigate the possible correlation with sleep-related erections in healthy men. The results lay a foundation for future large sample-size studies on the relationship between erectile function and sleep.

AUTHOR CONTRIBUTIONS

Guodong Liu: conception and design. Guodong Liu, Yuyang Zhang, and Wei Zhang: draft and revision of the article content. Guodong Liu,

Yuyang Zhang, Wei Zhang, and Xu Wu: acquisition of data. Guodong Liu, Yuyang Zhang, and Wei Zhang: analysis and interpretation of data. Hui Jiang and Xiansheng Zhang: review and editing. Xiansheng Zhang: final approval of the completed article.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting our conclusion could be obtained from the corresponding authors upon reasonable request.

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ORIGINAL ARTICLE

Comparison of the gut microbiome composition between men with erectile dysfunction and a matched cohort: a pilot study

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Abstract

Background: To-date there have been minimal studies to investigate an association between the gut microbiome and erectile dysfunction. There have been many inflammatory diseases linked to gut microbiome dysbiosis; such as cardiovascular disease and metabolic syndrome. These same inflammatory diseases have been heavily linked to erectile dysfunction. Given the correlations between both conditions and cardiovascular disease and the metabolic syndrome, we believe that it is worthwhile to investigate a link between the two.

Objective: To investigate the potential association between the gut microbiome and erectile dysfunction.

Methods: Stool samples were collected from 28 participants with erectile dysfunction and 32 age-matched controls. Metatranscriptome sequencing was used to analyze the samples.

Results: No significant differences were found in the gut microbiome characteristics, including Kyoto Encyclopedia of Genes and Genomes richness ($p = 0.117$), Kyoto Encyclopedia of Genes and Genomes diversity ($p = 0.323$), species richness ($p = 0.364$), and species diversity ($p = 0.300$), between the erectile dysfunction and control groups.

Discussion: The association of gut microbiome dysbiosis and pro-inflammatory conditions has been well studied and further literature continues to add to this evidence. Our main limitation for this study was our small-sample size due to recruitment issues. We believe that a study with a larger population size may find an association between the gut microbiome and erectile dysfunction.

Conclusions: The results of this study do not support a significant association between the gut microbiome and erectile dysfunction. Further research is needed to fully understand the relationship between these two conditions.

KEYWORDS
erectile dysfunction, gut dysbiosis, gut microbiome, men's sexual health

1 | INTRODUCTION

The gut microbiome (GM) is the composition of microbes, including bacteria, viruses, and fungi that make up the gastrointestinal (GI) tract of their host organism.[1] The GM plays an important role in numerous vital bodily functions, ranging from supplying essential nutrients, synthesizing vitamin K, to promoting angiogenesis and enteric nerve function.[2] GM dysbiosis is characterized as a disruption to the microbiota homeostasis caused by an imbalance in the microflora, changes in their functional composition and metabolic activities, or a shift in their local distribution.[3] Dysbiosis has been associated with a variety of chronic conditions including cardiovascular disease (CVD) and the metabolic syndrome (MetS), among other diseases.[3] Over the past few decades, the shift on GM research has been on attempting to establish fixed patterns of dysbiosis with certain diseased states, with the hopes that the GM can be used as a preventative, diagnostic, and therapeutic tool in the management of diseases.[4]

Erectile dysfunction (ED) is the inability of a man to attain and/or maintain an erection sufficient for satisfying sexual activity.[5] Men with ED have a significantly reduced quality of life and may experience depressive symptoms and anxiety related to sexual performance which can lead to avoiding engaging in sexual relations altogether.[6] ED is a very common condition with an estimated combined prevalence of mild to moderate ED of 52% in men aged 40–70 years.[5] Due to its relatively high prevalence, it comes as no surprise that it imposes a significant economic burden on the society. One study in the United Kingdom (UK) estimated £53 million total cost on UK society per year.[7] An important aspect in the management of ED is its prevention with most of the focus being on lifestyle changes. It has been shown to be strongly associated with CVD and MetS, both of which can at times be alleviated with lifestyle modifications such as regular exercise, proper diet, and healthy weight loss.[8] However, due to the continued high prevalence of ED and its economic burden, adding new preventive measures and treatments would be extremely beneficial. Current treatment options include, oral therapy with phosphodiesterase 5 inhibitors (PDE5-i), intracavernosal injections, and vacuum device among others and the gold standard treatment to refractory ED is with the use of a penile prosthetic.[9]

To the best of our knowledge, there has been only one human study to date that has attempted to establish an association with the GM and erectile function and one animal-model study that has attempted to establish an association between the GM and the ED.[10,11] The aforementioned human study used 16S ribosomal RNA (rRNA) gene sequencing in order to identify species of prokaryotes. Another recent study has shown that idiopathic human germ cell aplasia is associated with an increased signaling of retinoic acid (RA) in Sertoli cells and a decreased signaling of vitamin K in Leydig cells, which contributes to the modified composition of the basal membrane of the seminiferous tubules and testicular extracellular matrix.[12] Idiopathic human germ cell aplasia is a condition in which only Sertoli cells line the seminiferous tubules, resulting in a lack of the sperm production. The study suggests that this deregulation occurs independently of impaired systemic levels of vitamin K and RA, and highlights the importance of the testis microenvironment in the pathogenesis of germ cell aplasia. Finally, both ED and GM dysbiosis have been seen in patients with CVD and MetS.[3,8] As such, we hypothesize that there is a potential relationship between the GM and ED. The goal of our study is to better characterize any potential relationships.

2 | MATERIALS AND METHODS

2.1 | Study population

All subjects were consented under Institutional Review Board (IRB) 2017–3746. Participants were enrolled in either an ED group or age matched control group (age matched controls were within ± 10 years) to compare their GM compositions. All participants with ED had an International Index of Erectile Function (IIEF-5) score of 21 or below and all controls had an IIEF-5 score of 22 or above.[13] Participants from the ED group were enrolled at a Men's Health Clinic and were formally diagnosed with vasculogenic ED after performing diagnostic penile Doppler ultrasound. Healthy controls were selected directly from a large database provided by Viome Inc. Inclusion criteria were male sex assigned at birth, 30 years or older, body mass index (BMI) of 35 or lower, and sexually active within the past 4 weeks with a partner. Exclusion criteria were known current or past history of malignancy, currently taking testosterone or hormone replacement therapy, recent use of steroids or other immunosuppressive therapy within the past 4 weeks, recent antibiotic use within the past 4 weeks, previous history of scrotal/testicular/penile surgery, and the presence of pro-inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, chronic prostatitis, CVD, DM, peripheral vascular disease, etc. Participants were also administered a Patient Health Questionnaire-9 (PHQ-9) and a Generalized Anxiety Disorder-7 (GAD-7) in order to measure depression and anxiety in each cohort.[14,15]

2.2 | Sample collection

Stool samples were collected using the commercial Gut Intelligence kit (Viome Life Sciences, Inc.) by each study participant at their own residences. The kit included a sample collection tube with an integrated scoop, a proprietary RNA preservative, and sterile glass beads. A pea-sized stool sample was collected and placed inside the tube and vigorously shaken to homogenize the sample, exposing it to the RNA preservative. The sample was then shipped at ambient temperature using a common courier to a lab for analysis. All samples remained at ambient temperature for no longer than 28 days, to meet the requirements established by the clinical validation.[16]

2.3 | Metatranscriptomic analysis of stool samples

16S rRNA is a small subunit ribosomal RNA molecule that is present in prokaryotes and is essential for protein synthesis. It is widely used to identify and classify bacteria species by comparing the sequence of the 16S rRNA gene. This process is called 16S rRNA gene sequencing. The 16S rRNA gene is highly conserved in bacteria, and its sequence can be used to distinguish different bacterial species, but generally not strains. For example, the 16S rRNA gene can be used to compare the genome of a pathogenic bacterium to the genome of a non-pathogenic strain to identify any differences in the sequence of the 16S rRNA gene. In metatranscriptome analysis, all RNA is analyzed, not just the 16S rRNA. Metatranscriptome analysis involves sequencing the messenger RNA (mRNA) molecules that are present in a microbial community to determine which genes are being expressed and which microorganisms are present. Importantly, metatranscriptomic analyses allow for the quanitification of all microorganisms, not just prokaryotes, and can provide valuable insights into microbial gene expression profiles.

We, therefore used, the unbiased metatranscriptomic (RNA sequencing) analysis following the clinically validated methods that were previously described.[16] Briefly, stool samples were lysed by bead beating in the RNA preservation buffer (RPB). The following steps were automatically performed using 96-channel liquid handlers. RNA was extracted using silica beads and a series of washes, followed by elution in molecular biology grade water. DNA was degraded using RNase-free DNase. Prokaryotic rRNAs were removed using a subtractive hybridization method, where biotinylated DNA probes with sequences complementary to microbial rRNAs were added to total RNA, the mixture was heated and cooled, and the probe-rRNA complexes were removed using magnetic streptavidin beads. The remaining RNAs were converted to directional sequencing libraries with unique dual-barcoded adapters and ultrapure reagents. Libraries were pooled and quality controlled with dsDNA Qubit (Thermo Fisher Scientific) and Fragment Analyzer (Advanced Analytical) methods. Library pools were sequenced on Illumina NovaSeq instruments using 300 cycle kits.

2.4 | Bioinformatic analysis

Viome's bioinformatics methods include quality control, taxonomic classification (at strain, species, and genus ranks), and microbial quantitative gene expression analysis. The quality control includes per-sample and per-batch metrics, such as the level of barcode hopping, batch contamination, positive and negative process controls, DNase efficacy, and number of reads obtained per sample. Following the quality control, the paired-end reads are aligned to a catalog containing rRNA and 53,660 genomes spanning archea, bacteria, fungi, protozoa, and viruses. Reads that map to rRNA are discarded. Strain-level relative activities were computed from mapped reads via the expectation–maximization (EM) algorithm.[17] Relative activities for the biological functions were computed by mapping paired-end reads to a catalog of 52,324,420 genes, quantifying gene-level relative activity with the EM algorithm and then aggregating gene-level activity by Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog (KO) annotation.[18] The identified and quantified active microbial species and KOs for each sample were then used for downstream analysis.

2.5 | Data analysis

Multiplicative replacement method[19] is employed to deal with zero entries and the data are transformed with centered log-ratio (CLR).[20] CLR transformation is commonly done to reduce false discoveries due to the compositional nature of sequencing data. Statistical analyses were performed in Python. All samples that were included in the study passed quality acceptance criteria and received at least 5,000 reads aligning to microbial species, at least 200 KOs detected, and at least 50 species detected. FDR differential expression of microbes and microbial functions were compared between groups. We used the one-way ANOVA test to evaluate the differential expression and χ^2-test to evaluate the differential prevalence. False discovery rate (Benjamini/Hochberg) was used for multiple hypothesis testing.

3 | RESULTS

Initially, 82 participants were enrolled into the ED group for the study. Of these 82, 48 participants collected and sent their stool samples to the lab for analysis. The participants that did not collect and send their stool samples stated that they either did not understand instructions to submit their samples or had ultimately decided they no longer wanted to participate any further in the study. Twenty-eight of the 48 samples that were collected and shipped, passed quality control and were included in this study. Quality control measures include: insufficient stool collection, incorrect collection method, or damage during shipping. 32 years age matched controls were collected to be compared to our ED group. The average age of the ED group was 54.57 ± 11.37 years and the average age of the control group was 49.78 ± 9.46 years ($p = 0.085$). The average BMI of the ED group was 27.30 ± 3.51 years compared to the control group's BMI of 24.37 ± 2.85 years ($p < 0.001$). 5/28 from the ED group reported a past diagnosis of hypertension compared to 0/32 in the control group ($p = 0.013$). The average IIEF-5 score in the ED group was 12.81 ± 6.54 compared to the control group's score of 24.31 ± 0.95 ($p < 0.001$). The average PHQ-9 score in the ED group was 3.69 ± 2.96 compared to the control group's score of 1.25 ± 1.58 ($p < 0.001$). The average GAD-7 score in the ED group was 3.46 ± 4.15 compared to the control group's score of 1.28 ± 1.96 ($p = 0.012$) (Table 1).

No significant differences were found between the ED group and control group for microbial taxonomy or for microbially expressed functions. That is, there were no significant differences observed between groups in KEGG richness ($p = 0.117$), KEGG diversity ($p = 0.323$), species richness ($p = 0.364$), or species diversity ($p = 0.300$) (Figure 1).

TABLE 1 Comparison of demographics.

	Erectile dysfunction group	Control group	*p*-Value
Age (years)	54.57 ± 11.37	49.78 ± 9.46	0.085
Body mass index (kg/m^2)	27.30 ± 3.51	24.37 ± 2.85	<0.001
IIEF-5[a]	12.81 ± 6.54	24.31 ± 0.95	<0.001
Hypertension	5/28 participants	0/32 participants	0.013
PHQ-9[b]	3.69 ± 2.96	1.25 ± 1.58	<0.001
GAD-7[c]	3.46 ± 4.15	1.28 ± 1.96	0.012

[a]International Index of Erectile Function 5.
[b]Patient Health Questionnaire 9.
[c]Generalized Anxiety Disorder 7.
*Comparison of means two-sample *t*-test was performed for age, body mass index, IIEF-5, PHQ-9, and GAD-7.
**Comparison of proportions was performed for hypertension.

4 | DISCUSSION

Our results suggest that there are no significant differences between KEGG richness, KEGG diversity, species richness, or species diversity between our ED and control group. We did a thorough review of the medical literature and to the best of our knowledge, there is currently one human paper and one animal model study associating ED with the GM.[10,11]

Li et al. investigated the relationship between the GM and a type 2 diabetic with ED model in Sprague–Dawley rats.[11] Thirty-five Sprague–Dawley rats were randomly divided into a control group (n = 15) with normal diet and an experimental group (n = 20) which is a construction of a type 2 diabetic ED model. Stool samples were collected at the second week when the type 2 diabetic model was established and again at the eighth week after establishment of the type 2 diabetic ED model. The authors found that the relative abundance of opportunistic pathogens such as *Enterococcus*, *Corynebacterium*, *Aerococcus*, *Facklamia* were increased and that of beneficial bacteria such as *Allobaculum*, *Bifidobacterium*, *Eubacterium*, *Anaerotruncus* were decreased in the type 2 diabetic ED model at the eighth week when compared to the type 2 diabetic model established at the second week.

Okamoto et al. performed a cross-sectional study that surveyed 192 men who participated in the Iwaki Health Promotion Project in 2015 in Hirosaki, Japan.[10] The GM was assessed by sequencing 16S rRNA, which was extracted from stool samples. They evaluated erectile function with IIEF-5, and the men were divided into two groups: low-IIEF-5 (≤ 16) and high-IIEF-5 (> 16). They controlled for differences

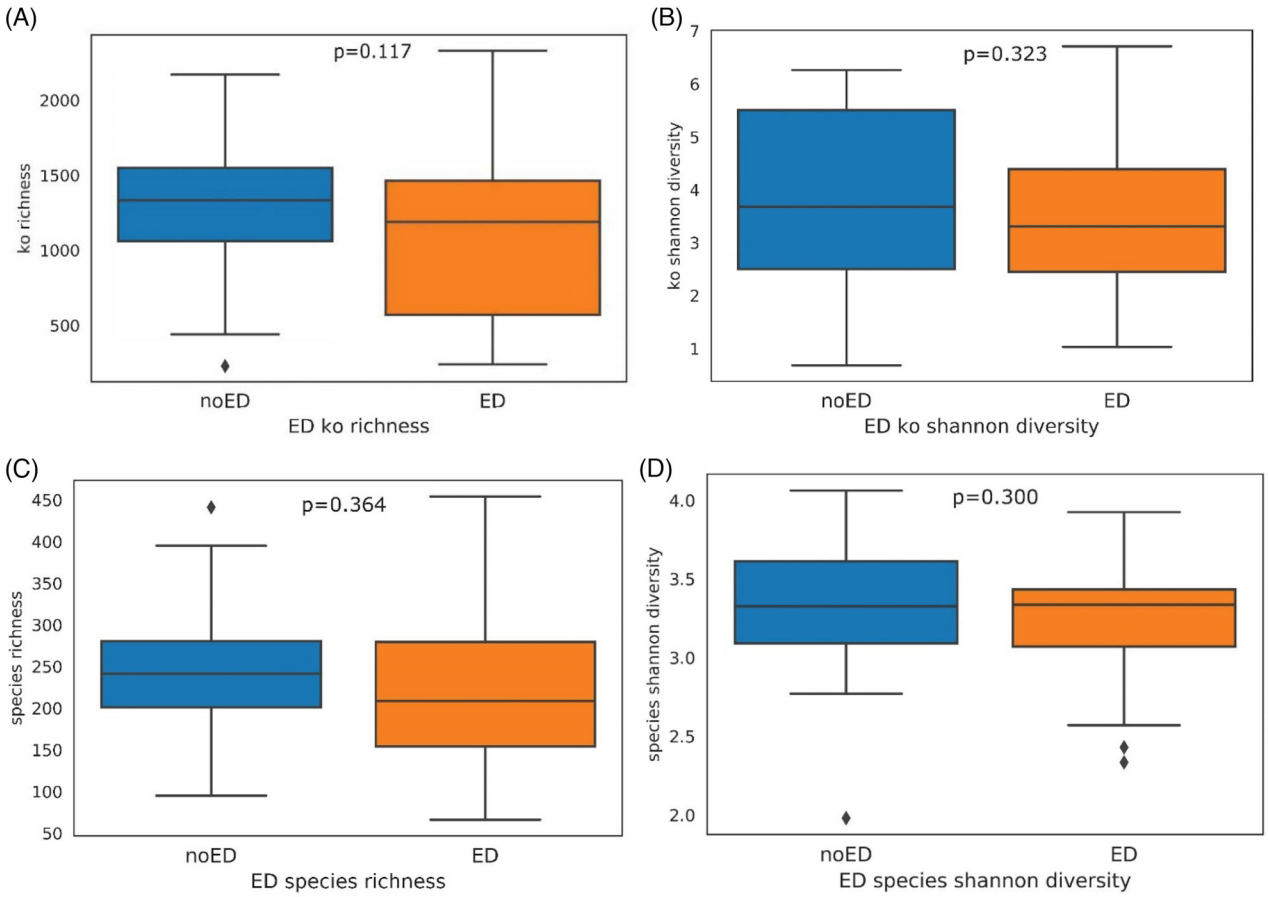

FIGURE 1 Comparison of gut microbiome richness and diversity.

in past medical history of hypertension, diabetes mellitus, chronic kidney disease, and CVD between the groups. On multivariate analysis, they reported that the relative abundance of *Alistipes* was significantly lower and *Clostridium XVIII* was significantly higher in the low-IIEF-5 group (Odds ratio (OR), 2.06; 95% Confidence interval (CI), 1.20−3.55, $p = 0.009$) and (OR, 0.81; 95% CI, 0.66−0.99, $p = 0.040$), respectively. *Alistipes* is a genus of bacteria hypothesized to be involved in decreasing inflammation and vascular endothelial dysfunction. *Clostridium XVIII* is significantly more abundant in diabetic patients with diarrhea and constipation and is increased in patients with irritable bowel syndrome (IBS).[21] On the other hand, *Clostridium XVIII* is also believed to be a commensal bacterium that has been reported to induce differentiation of regulatory T-cells (Tregs).[22] Tregs are important in maintaining GM homeostasis and are beneficial in suppressing immune-related diseases. These contradictory findings suggest that *Clostridium XVIII's* role in the human GM is yet to be ascertained and must be further studied. A weakness of this study is the method used to analyze the individual stool samples. 16S rRNA sequencing only allows for analysis of prokaryotes and only describes their taxonomy. Furthermore, 16S rRNA sequencing does not analyze the KO of the microbes, making it difficult to ascertain their metabolic and genomic impact on the host's overall health. Finally, it would be beneficial to include a control group with IIEF-5 > 21 to further stratify grouping.

The basis of our study is that we had hypothesized that there could potentially be a correlation between ED and GM dysbiosis due to the fact that both are associated with inflammatory conditions; such as DM and MetS.[3,8] Trimethylamine N-oxide (TMAO) is one hypothesized pro-inflammatory molecule generated via GM metabolism and is thought to have a direct correlation with increased risk of CVD. Several animal and human models have shown that TMAO levels are directly associated with atherosclerosis.[23] The activation of nuclear factor kappa B (NF-κB) is another proposed mechanism of the development of atherosclerotic plaques originating from inflammatory cascades in the GM.[24] To-date several studies have been completed that suggest that patients diagnosed with ED are at a significantly increased risk of experiencing a cardiovascular event when compared to a relatively healthy matched control.[8] Furthermore, it has been proposed that men with more severe CVD are at a higher risk of developing ED, suggesting a bidirectional relationship between CVD and ED.[25] The strong associations between the GM and ED with CVD suggest that there may be associations to be found among the two. Additonally, the GM has been found to have a significant impact on the levels of androgens and the regulation of sex hormones.[26] Gender differences also play a role in the composition of gut microbiota, with men having less variety than women. Prevotella, which is more abundant in men, has a positive correlation with testosterone, while females have a higher abundance of Bacteroides. Moreover, GM is involved in regulating the production and metabolic processes of androgens, and specific bacteria have been identified as being able to transform and utilize sex steroids. Research suggests that the microbiota may both positively and negatively regulate sex hormone levels depending on host physiology and other factors. Further research is needed to better understand the mechanisms by which GM regulates androgens and steroid.

The overproduction of metabolites by *Bacteroidetes*, which is seen in higher abundance in individuals with MetS, stimulates production of inflammatory cytokines.[27] MetS has been shown to have an association with changes in GM composition. One thorough study showing this association is The Metabolic Syndrome in Men (METSIM) study which was published in 2017. The authors studied 10,197 Finnish men and the results showed that *Methanobrevibacter* and *Peptococcaceae* were correlated with reduced triglyceride levels, *Tenericutes* and *Christensenellaceae* were strongly associated with lower BMI and triglyceride levels and higher HDL levels, and TMAO was directly associated with the abundance of *Peptococcaceae* and *Prevotella* and negatively associated with the abundance of *Faecalibacterium prausnitzii*.[28]

Several studies have shown that MetS is associated with ED in a bidirectional manner as well.[8] That is, patients who have ED are more likely than not to have a higher BMI, higher blood glucose levels, elevated triglycerides, lower HDL cholesterol, and elevated blood pressure when compared to relatively healthy matched controls. The strong associations between the GM and ED with MetS suggest that there may be associations to be found among the two.

The average PHQ-9 score in the ED group was 3.69 ± 2.96 compared to our control group's score of 1.25 ± 1.58 ($p < 0.001$). The average GAD-7 score in the ED group was 3.46 ± 4.15 compared to our control group's score of 1.28 ± 1.96 ($p = 0.012$). It is important to mention that a score of 1–4 on the PHQ-9 and a score of 0–4 on the GAD-7 correspond to minimal depression and anxiety, respectively. On average, both groups report minimal depression and anxiety however the statistical significance is still of importance. This increased severity of depression and anxiety in men who suffer from ED exemplifies the importance of developing further diagnostic and therapeutic treatment options for the disease.

The main limitation of our study is the small-sample size which may have not been sufficiently powered to show differences. We had initially planned to continue enrolling patients in both groups of the study but due to the COVID pandemic, recruitment was significantly halted and a decision was made to analyze the data that we had already collected. We believe that if larger sample sizes were collected then we would have potentially found significant differences between our groups. The main strength of this study is that unlike the aforementioned studies which analyzed all samples solely by 16S rRNA gene sequencing, in our study all samples were analyzed using metatranscriptome analysis which allows us to study the taxonomy of all microbes as well as their KOs. This more thorough and detailed analysis increases chances of finding true associations with the GM.

5 | CONCLUSIONS

In this first of its kind study, there were no significant differences observed in KEGG richness, KEGG diversity, species richness, and species diversity between our ED and control group. In the future, studies with larger sample sizes and multi-institutional studies are needed to better characterize any associations and to help uncover potential targets in the GM for early treatment or prevention of ED.

AUTHOR CONTRIBUTIONS

Mohamad M. Osman made contributions to the research design, helped with the acquisition, analysis, and interpretation of the data, and drafted and revised the paper critically. Muhammed A. Hammad and David W. Barham revised the paper critically. Ryan Toma, Guruduth Banavar, Ying Cai, Pedro Moura, Nan Shen, and Momchilo Vuyisich contributed to analysis and interpretation of the data. Farouk M. El-Khatib, Sharmin Dianatnejad, Jeanie Nguyen, Maxwell Towe, Edward Choi, and Qiaqia Wu contributed with the acquisition of the data. Natalie R. Yafi and Faysal A. Yafi contributed to the research design and revised the paper critically.

CONFLICT OF INTEREST STATEMENT

Dr. Yafi reports associations with Acerus Pharmaceuticals as speaker, Halozyme as advisory board and speaker, Coloplast as advisory board and speaker, Clarus as speaker, Cynosure as consultant, Promescent as advisory board, and Viome as clinical trial primary investigator. All other authors declare that they have no conflict of interest.

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DATA AVAILABILITY STATEMENT

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

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ORIGINAL ARTICLE

Single men's attitudes towards posthumous use of their sperm cryopreserved due to illness in Israel

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Abstract

Background: Banking of frozen spermatozoa by single men opens the possibility of procreation long after their death. Requests for posthumous reproduction by the families of the deceased are growing, raising an ethical debate, especially when written instructions were not left by the patients and in cases of unplanned perimortem collection. The issue of the progenitors' intention to procreate after death is the key to ethically based decision-making in these cases.

Objectives: To evaluate the attitude of single men cryopreserving spermatozoa before life-threatening medical situations towards post-mortem usage of their cryopreserved spermatozoa.

Materials & methods: Adult single men prior to sperm cryopreservation before cytotoxic therapy were asked to sign a structured form declaring their will and instructions for the usage of their cryopreserved spermatozoa in case of their demise.

Results: Four hundred fifty-two men of diverse ethnicity, religious and cultural backgrounds signed the form providing instructions for the use of their cryopreserved spermatozoa in case of mortality. Their age was 27.4 ± 8.06 years. Seven (1.5%) patients willed their spermatozoa for posthumous reproduction to a sibling, 22 (4.9%) to parents, and 26 (5.7%) to their informal female partners. The significant majority (n = 397; 87.8 %) of the single men were ordered to destroy their cryopreserved spermatozoa in case of their expiry. Note that, 26–39 years old men were less likely (81.8% vs. >90% in other ages) to order sperm destruction, as well as men with a poorer prognosis (83% vs. 90%).

Discussion: In this study group, most single men cryopreserving spermatozoa in the face of future life-threatening morbidity do so for their own future live parenthood, and are not interested in posthumous reproduction.

Conclusion: Our results doubt the claim that single men who had an unplanned perimortem sperm collection can be universally presumed to have wished to father a child

Anat Stein and Eran Altman contributed equally and should be considered joined first authors.

posthumously. Any claimed assumed consent in these cases should be considered for each case individually based on its specific circumstances.

KEYWORDS
cancer patients, posthumous reproduction, sperm cryopreservation

1 | INTRODUCTION

Male fertility preservation might be either planned or unplanned. In the first scenario, spermatozoa are frozen after consultation before life-threatening conditions with an anticipated negative impact on male fertility, and written documentation of the patients' instruction in the case of mortality is obtained. In the latter scenarios, spermatozoa are retrieved after an unexpected event leading to brain or cardiac death without any documentation of the patients' consent or wish.[1] The frozen-thawed specimens may be used for various modes of assisted reproduction depending on the quantity and quality of the cryopreserved spermatozoa and the female partner's parameters.

In the case of death, the motivation for posthumous reproduction may be shared by both the deceased men and surviving parties or may originate from the surviving parties alone, especially when sperm cryopreservation was unplanned.[2] In such a case society is faced with increasing legal, cultural, ethical, social, and religious dilemmas, especially when young men's life is lost under tragic circumstances and their will for procreation remains obscure, despite the fact that it is considered a fundamental right within various societies, traditions, and religions.[3] The informed consent and directive orders in cases of planned sperm cryopreservation are the ethical and legal basis for the use of spermatozoa after death. However, in unplanned cases of perimortem sperm preservation is performed under an emergent approval without any knowledge of the deceased person's will or intentions. It is in such cases that the use of the cryopreserved spermatozoa might be under debate between relatives and the state or society because explicit instructions were not left by the deceased. Requests for posthumous use of the frozen spermatozoa with a woman unknown to the deceased sometimes rise on behalf of parents (posthumous grandparenthood). Their argument is usually based on the assumption that the wish of the deceased person was to father a child and become a parent. An ethical and legal dilemma exists in such cases between the compassion for those who lost a young son under tragic circumstances on one hand, and the insult to the autonomy of the deceased person by procreating him without his explicit consent, a mandatory request for ethical posthumous reproduction,[4] on the other. One approach to resolving this dilemma was introduced in Israel as Attorney General guidelines in the lack of specific legislation. These guidelines determined that in the absence of written instructions, posthumous reproduction with frozen spermatozoa is permitted only to the female partner of the deceased, presuming that if the deceased man had the wish to procreate it was with her. The use of the spermatozoa in such cases is permitted only after a minimum period of 1 year of death, in order to make sure that the female partner's will was free from emotional and other stress.[5] These guidelines do not recognize any parental (or other family relatives') rights to use the spermatozoa of the deceased person and have withstood attempts to challenge them in court.[6] Nevertheless, requests have been submitted to courts over the years by parents seeking permission to use their deceased son's spermatozoa to create genetic grandchildren,[5,7] but in most cases were refused.

Another ethical approach to this issue claims that the use of cryopreserved spermatozoa from a deceased identifiable man, even by a woman not known to him in life, is ethically preferable to anonymous donor spermatozoa from the perspective of the child. The potential advantages are genealogical certainty, information about the identity, life narrative, and ancestry of the genetic progenitor as well as a close relationship with extended family members.[8] Nevertheless it is difficult to claim that such a possible benefit to a potential child is superior to the autonomy of the deceased man whose spermatozoa was cryopreserved perimortem without his explicit consent.

In the face of the increasing demand from the parents of young men who deceased suddenly for posthumous grandparenthood for familial continuity, we sought to study the attitude of single men cryopreserving spermatozoa in a planned manner in a scenario where near death is a realistic possibility, towards posthumous reproduction. We believe that their positions and instructions will represent this age group and are also relevant to cases of sudden death and unplanned collection, which raise an ongoing debate between the right to individual autonomy and the family's wish for continuity.

2 | MATERIALS AND METHODS

Data was retrieved from the written instructions left by single adult men that cryopreserved spermatozoa in our bank between 2000 and 2020, for the usage of their spermatozoa in case of future mortality. Prior to freezing, all men facing cytotoxic or radiotherapy for the treatment of various malignancies were asked to sign a structured form, declaring their consent or refusal for usage of their cryopreserved spermatozoa in case of future mortality. The men were asked to choose one of three options:

1. To write a separate will, in which written instructions for what should be done with the frozen spermatozoa in case of death are given.
2. To bequest the frozen spermatozoa to a specific person for posthumous reproduction.
3. To discard the frozen spermatozoa sample.

The data was collected anonymously under our Institutional Review Board approval (RMC-19-0697) which waived the requirement for informed consent for the anonymous retrospective collection.

The Chi-Square test was used to evaluate for association between pairs of categorical variables. The differences were considered to be statistically significant at $p < 0.05$ at a confidence interval of 95%.

3 | RESULTS

During a period of 20 years, 2731 oncological male patients facing cytotoxic/radiotherapy possibly impairing spermatogenesis were referred to our bank to cryopreserve sperm samples for future use. Out of them, 602 (22%) were single men. One hundred forty-six were minors under 18 and the other 456 men were adults. None of them had children. Their age was 27.4 ± 8.06 years and the average number of vials cryopreserved was 7.9. Full data, including specific diagnosis, was available for 452 out of 456 adult oncological patients with cryopreserving spermatozoa.

Out of the single adult men group, 26 (5.7%) patients willed their spermatozoa for posthumous reproduction to informal female partners; seven (1.5%) to a sibling, and 22 (4.9%) to their parent/s. The significant rest 397 (87.8%) ordered to destroy their cryopreserved spermatozoa in case of their expiry.

Two hundred twelve (212) out of 230 (92.2%) patients aged 18–25, 148/181 (81.8%) of patients 26–39, and 37/41 (90.2%) of patients 40 or older, ordered sperm destruction in case of their demise ($p = 0.005$).

The patients were also divided into good ($n = 310$) and poor ($n = 142$) prognosis groups based on the diagnosis prior to sperm cryopreservation. 279/310 (90%) of the good prognosis and 118/142 (83%) of the poor prognosis patients ordered their spermatozoa to be discarded in case of their expiry ($p = 0.037$).

Four hundred sixteen of the men were of Jewish origin, and 36 were Arab Muslims. Only one of the Arab Muslim men willed his spermatozoa to a sibling and the rest of them ordered discarding it in case of death ($p = 0.07$).

4 | DISCUSSION

The possibility to start gestation with a dead man's spermatozoa, especially in the absence of documented permission from him, creates legal, cultural, ethical, social, and religious debates. Family continuity, feelings of responsibility, and the need to compensate the families for their loss on one hand, and the superiority of individual autonomy on the other, create different conceptualizations of posthumous reproduction.

ESHRE and the ASRM consider the conception of a child using a deceased man's cryopreserved spermatozoa ethically sound provided explicit consent has been made during his life, (thereby protecting his autonomy), and that the surviving partner received proper counseling after a period of grief.[9,10] Cultural, ethical, and social positions concerning this dilemma differ from country to country. In France, Germany, Hungary, Slovenia, and Sweden, posthumous reproduction with a deceased man's spermatozoa is forbidden. In the Czech Republic, Estonia, Greece, Netherlands, and the UK, posthumous usage of spermatozoa is allowed subject to written consent.[11] In Australia posthumous use of gametes is acceptable subject to consent and counseling after an appropriate period of grief is allowed.[12] In Malaysia, the most populated Muslim nation, the use of gametes or embryos harvested from cadavers in ART is prohibited.[13] In Israel, in the absence of written instructions, posthumous reproduction with frozen spermatozoa is permitted only to the female partner of the deceased after a minimal grief period of one year.[5] The rights of other family members to use the spermatozoa of the deceased person are not recognized.[6]

When death is unanticipated, explicit consent is not available, thereby preventing posthumous spermatozoa procurement according to most guidelines and ethical opinions.[4] On the other hand, others claim that the autonomy of a deceased person is not necessarily superior to the welfare of the living relatives (widow, parents, or prospective children) especially if posthumous conception can bring significant advantages to the family of the deceased, like a continuation of his 'bloodline', allowing a widow's wishes for a child, and his parents wished for a grandchild to be satisfied.[4] The debate between these two concepts often produces conflicts in the legal and even public media arenas,[2,6,11] where the accepted approach is often challenged by relatives of young men who lost their lives in tragic unanticipated circumstances. Another aspect of this debate is concerns that having been conceived from a dead father, and the economic and social hardship of being raised by a single parent in a planned orphancy set-up, may negatively affect the welfare of the child. The Human Fertility and Embryology Authority (HFEA Act 1990) insisted that the welfare of the child including the "need of that child for a father", as well as the aspect of "planned orphans" must be taken into consideration.[14] Little is known regarding the psychosocial development of children born to parents deceased before their birth. Some authors have cautioned that the knowledge and possible stigma of being born to a dead parent could subject children conceived posthumously to undue psychological stress or harm.[9] On the other hand, others ponder whether it is better for children conceived posthumously to be born to a dead genetic parent, rather than from donor insemination,[8] or even not be born at all.[2]

In this study we tried to shed some light on the posthumous reproductive wishes of young single men prior to cytotoxic therapy for malignancies, presuming that their positions on this issue will be representative of other men whose spermatozoa were preserved perimortem in an unplanned manner. Our patient group was heterogeneous in terms of ethnicity and religious belief and included single men who cryopreserved their spermatozoa for a substantial period of time. All the men in the group were Israeli citizens. Biological parenthood and familial continuity are extensively sought in all major ethnic groups composing the Israeli population.[15] Assisted reproduction is generously financed by the public Israeli healthcare system, and the number of assisted reproduction cycles per capita in Israel is the highest worldwide.[16] The issue of continuity of young men who lose their lives during military service is of special sensitivity in the Israeli public agenda.[17] Despite the pronatalist social context in Israel, our results

revealed that the significant majority (87.4%) of the single men cryopreserved the spermatozoa for their own potential future personal use only, and ordered them to destroy it in case of their expiry. Less than 5% willed their spermatozoa to their blood relatives for posthumous procreation. Although these findings may be limited to Israel, if the vast majority of single men cryopreserving spermatozoa in the face of illness in such a pronatalist society oppose posthumous reproduction, this finding may be of wider relevance to other societies as well.

The percentage of men ordering sperm destruction in case of expiry was even higher than 90% when the oncological prognosis was relatively good or if they were younger than 26 or older than 39. Despite the statistically significant differences between the patient subgroups, the most important observation is that no matter what more than 80% of the men have decided against posthumous reproduction in their case. We hypothesize that the men in the good prognosis group were less likely to seriously contemplate their death and that men without children who are very young or above forty are even less likely to desire parenthood in unusual circumstances.

Most studies addressing the position of men cryopreserving spermatozoa in the face of diseases were performed in couples, an ethically less problematic population in the aspect of posthumous reproduction.[18,19] Pastuzak et al. argued that men with malignancy, and perhaps even a prolonged life-threatening disease, are likely to consent to post-mortem sperm use, depending on age, paternity, and relationship status.[19] A correlation between the relationship status and consent for posthumous reproduction was reported by Nakhuda et al. Note that, 77.8% of individuals in a relationship permitted their spouse to use their spermatozoa for procreation in case of death.[20]

On the other hand, the vast majority of the single men in our cohort opposed posthumous reproduction with their cryopreserved spermatozoa. We presume that these single men preserving their fertility in the face of disease do have procreation wish in life, but not in case of their death without playing an active role in upbringing or having any relationship with the child.[5,21–23] Additionally, they might not want to have a child with a woman not in a significant relationship with them, or have their own positions on the controversial issue of "need of that child for a father", as well as creating "planned orphans".[14] Despite the values of continuity and parenthood, it seems that most of them are not interested in posthumous reproduction if they do not survive. We believe that these definite findings should project also on the management of cases of unplanned perimortem sperm collection. If in a certain society > 80% of young single men choose against posthumous reproduction in the face of life-threatening morbidity, then the default management in cases of unplanned collection should be not to use the spermatozoa unless solid evidence exists that the deceased person wanted to. Any claim for implicit consent in such cases should be individually examined based on its specific circumstances.

AUTHOR CONTRIBUTIONS

Anat Stein – Data collection, quality control, and writing of the first manuscript draft. Eran Altman – Clinical consultation and data collection. Mali Rotlevi – Sperm cryopreservation. Donia Seh – Data Curation. Avital Wertheimer – Review of the manuscript. Avi Ben-Haroush – Critical review of the data and manuscript. Yoel Shufaro – Study design, data analysis, critical review, and finalization of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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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.

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ORIGINAL ARTICLE

A 10-year experience in testicular tissue cryopreservation for boys under 18 years of age: What can be learned from 350 cases?

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Abstract

Background: A growing number of centers worldwide are preserving testicular tissue (TT) of young boys at risk of fertility loss to preserve their fertility. Data in this regard are scarce and experience sharing is essential to the optimization of the process.

Objectives: This report of our 10-year activity of pediatric fertility preservation (FP) has the objective to (1) improve knowledge regarding the feasibility, acceptability, safety, and potential usefulness of the procedure; (2) analyze the impact of chemotherapy on spermatogonia in the cryopreserved TT.

Materials and methods: For this retrospective study of data prospectively recorded, we included all boys under 18 years of age referred to the FP consultation of our academic network between October 2009 and December 2019. Characteristics of patients and cryopreservation of testicular tissue (CTT) were extracted from the clinical database. Univariate and multivariate analyses were used to assess factors associated with the risk of absence of spermatogonia in the TT.

Results: Three hundred and sixty-nine patients (7.2 years; 0.5–17.0) were referred to the FP consultation for malignant (70%) or non-malignant (30%) disease, of whom

88% were candidates for CTT, after a previous chemotherapy exposure (78%). The rate of recorded immediate adverse events was 3.5%, with painful episodes dominating. Spermatogonia were detected in the majority of TTs: 91.1% of those exposed to chemotherapy and 92.3% of those not exposed ($p = 0.962$). In multivariate analysis, the risk of absence of spermatogonia was almost three-fold higher in boys > 10 years of age ([OR] 2.74, 95% CI 1.09–7.26, $p = 0.035$) and four-fold higher in boys exposed to alkylating agents prior to CTT ([OR] 4.09, 95% CI 1.32–17.94, $p = 0.028$).

Discussion/conclusion: This large series of pediatric FP shows that this procedure is well accepted, feasible, and safe in the short term, strengthening its place in the clinical care pathway of young patients requiring a highly gonadotoxic treatment. Our results demonstrate that CTT post-chemotherapy does not impair the chance to preserve spermatogonia in the TT except when the treatment includes alkylating agents. More data on post-CTT follow-up are still required to ensure the long-term safety and usefulness of the procedure.

KEYWORDS
alkylating agent, boys, chemotherapy, cryopreservation, fertility preservation, pediatric oncology, prepubertal, testicular tissue

1 | INTRODUCTION

A growing number of childhood cancer survivors enter adulthood and are at risk of adverse treatment-related effects, such as infertility,[1] which underscores the challenge of fertility preservation (FP) in pediatric patients. Fertility loss can be also induced by genetic disorders, such as Klinefelter syndrome or Fanconi anemia. In boys, who do not yet produce spermatozoa, cryopreservation of testicular tissue (CTT) has been used for 20 years to preserve spermatogonia.[2,3] Proof-of-concept studies have recently been conducted in non-human primates, with the use of cryopreserved testicular tissue (TT) restoring fertility and resulting in healthy progeny.[4,5] However, no use of cryopreserved tissue in childhood has been reported for procreative purposes in humans.

Despite its experimental designation, CTT is becoming widespread in clinical practice. After the first two cases published in 2000,[2] a number of clinics around the world have reported their experience through series including up to 189 patients (Table 1). A recent survey has identified 1033 cases around the world,[6] and national and international guidelines have been issued.[7–9] Experience sharing in this experimental field is essential as questions remain concerning the optimization of the process. In this context, we report our 10-year experience of CTT in boys under 18 years of age from a single academic collaborative network. Our objectives were to (1) report the characteristics of our population; (2) analyze the indications, acceptability, feasibility, and safety of the FP procedure; (3) analyze the impact of chemotherapy on the pool of spermatogonia in the cryopreserved TT; and (4) describe the changes in our activity during the study period.

2 | PATIENTS AND METHODS

2.1 | Study design and participants

This retrospective study of data prospectively recorded reports the FP of a single collaborative network, including seven departments of pediatric hematology and oncology (I: Department of Hematology, Adolescents and Young Adults Unit, Saint-Louis Hospital, Paris; II: Department of Pediatric Immunology and Hematology, Robert Debré Hospital, Paris; III: SIREDO Center [Care, innovation, research in pediatric, adolescent and young adult oncology], Curie Institute, Paris; IV: Department of Immuno-Hematology and Pediatric Rheumatology. Necker-Enfant Malades Hospital, Paris; V: Department of Pediatric Oncology, Gustave Roussy Institute, Villejuif; VI: Department of Pediatric Onco-Hematology, Armand Trousseau Hospital, Paris; VII: Pediatric Department Sickle Cell Referral Center, Intercommunal Hospital of Créteil, Créteil), four departments of pediatric surgery (Department of Pediatric Surgery and Urology Robert Debré Hospital, Paris; Department of Visceral and Urological Pediatric Surgery, Necker Hospital, Paris; Department of Pediatric Surgery, Armand Trousseau Hospital, Paris; Department of Visceral, Urological and Traumatological Surgery, Intercommunal Hospital of Créteil, Créteil), and one consultation of FP (Department of Hematology, Adolescents and Young Adults Unit, Saint-Louis Hospital, Paris) between October 2009 and December 2019. The eligible population consisted of all boys under 18 years of age who underwent a highly gonadotoxic treatment for malignant or non-malignant disease, who had a disease associated with a risk of premature fertility loss, or who were pubescent but for whom sperm

TABLE 1 Published series of more than 30 cases of testicular tissue cryopreservation (for the same team, the most recent series was chosen).

Authors (y)	Country	Biopsied patients	Age (y) mean	Age (y) range	Study period	Gonadotoxic treatment before FP	Malignant disease/non-malignant disease (%)	Most frequent malignant disease (%)	Most frequent non-malignant disease (%)	Postoperative complications, *n* (%)
Ginsberg et al. (2014)[17]	USA (Pennsylvania)	48	NA	NA	January 2008–April 2013	Exclusion	88/12	Neuroblastoma (19)	Aplastic anemia (25) Immunodeficiencies (25)	1/48 (2%)
Sadri-Ardekani et al. (2016)[27]	USA (North Carolina)	33	5.8	0.6–16.0	July 2014–July 2015	NA	43/57	Acute leukemia (6) Rhabdomyosarcoma (6) Neuroblastoma (6)	Bilateral undescended testes (58)	0/33 (0%)
Ho et al. (2017)[19]	Australia	44	7.9	0.3–16.8	1987–2015	Inclusion	70/30	Acute leukemia (14) Sarcoma (22)	Aplastic anemia (10)	1/44 (2.3%)
Uijldert et al. (2017)[18]	Netherlands	64	8.3	0.5–15.5	March 2011–February 2017	Exclusion	100/0	Solid tumor (53) Central nervous system tumor (34)	–	3/64 (4.6%)
Stukenborg et al. (2018)[16]	Sweden	32	6.1	0.6–13.1	2014–2017	Inclusion	56/44	Leukemia (37)	Sickle cell disease (19)	NA
Valli-Pulaski et al. (2019)[12]	USA (coordinating center: Pennsylvania)	189	7.9	0.4–34.0	January 2011–November 2018	Inclusion	61.5/38.5	Malignant hematological diseases (23) Bone and muscle malignancies (20) Central nervous system tumor (16)	Hemoglobinopathy (16)	6/189 (2.6%)
Braye et al. (2019)[15]	Belgium	112	NA	0.6–18.0	2002–2018	Inclusion	35/65	Cancer (35)	Sickle cell disease (20) Klinefelter syndrome (30)	0/112 (0.0%)
Goossens et al. (2020)[6,a]	Centers in Europe, the USA, Israel, and Jordan	1033	NA	0.25–18.0	NA–2019	NA	62/38[b]	Malignant hematological diseases (47) Central nervous system tumor (19%)	Sickle cell disease (35) Bone marrow failure/aplastic anemia (23) Immunodeficiencies (21)	NA
Kanbar et al. (2021)[11]	Belgium	139	7.5	0.5–16.1	May 2005–May 2020	Inclusion	85/15	Acute leukemia (22) Sarcoma (25)	Sickle cell disease (7)	3/139 (2.1%)
Joshi et al. (2021)[28]	USA (Minnesota)	37	10.0[c]	0.9–17.0	2016–2020	Inclusion	80/20	Malignant hematological diseases (46) Bone and muscle malignancies (30)	Gender dysphoria (11) Non-malignant hematologic condition (3)	3/37 (8.1%)
Present study	France	350	7.1	0.6–17.1	October 2009–December 2019	Inclusion	70/30	Acute leukemia (32) Neuroblastoma (15) Central nervous system tumor (10)	Hemoglobinopathy (16)	10/283[d] (3.5%)

Abbreviation: NA, not available.

[a]At least 522 patients in this series (50.5%) were described in the publications cited above in the table.

[b]Missing data ($n = 18$).

[c] Median.

[d]Missing data ($n = 66$).

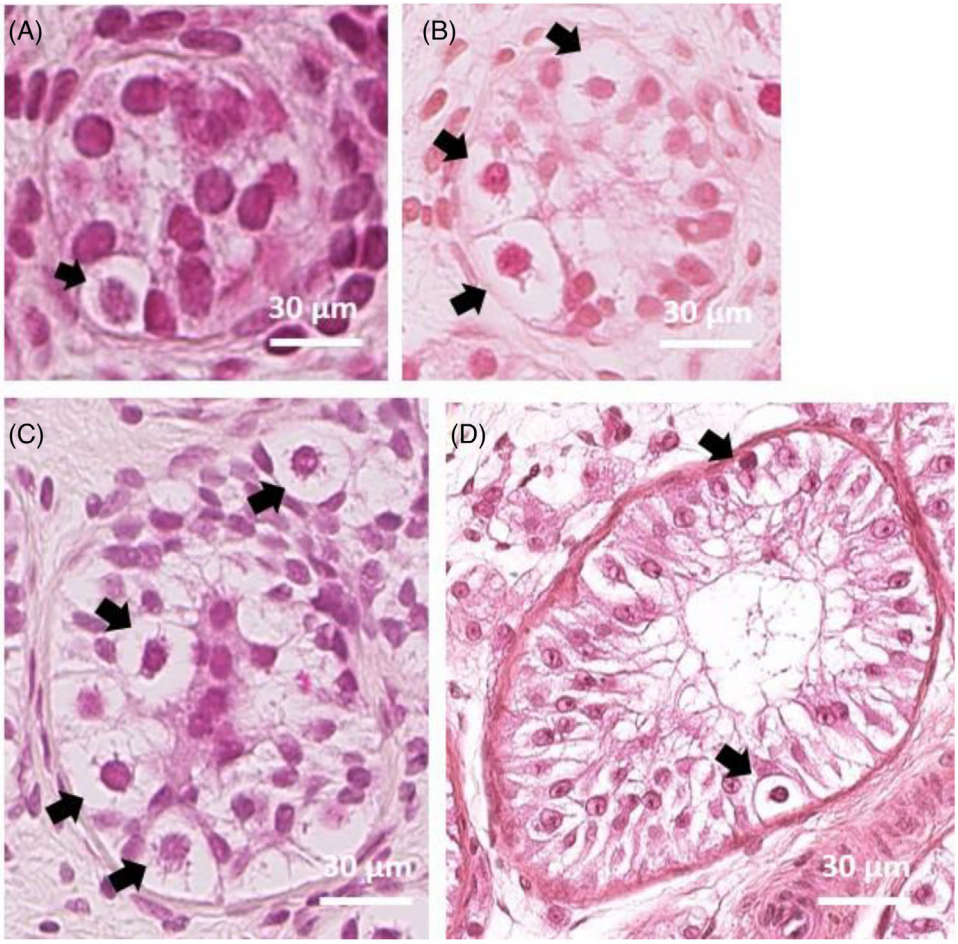

FIGURE 1 Hematoxylin and eosin staining of immature testicular tissue of (A) 1-year-old; (B) 6-year-old; (C) 10-year-old; and (D) 15-year-old patients. The pictures present seminiferous tubule sections containing a majority of Sertoli cells and a few spermatogonia (black arrow).

freezing was not possible. These comprised highly gonadotoxic conditioning regimens before allogeneic or autologous hematopoietic stem cell transplantation, with or without total body irradiation, treatments including an alkylating agent with a cyclophosphamide equivalent dose ≥ 7500 mg/m^2, and testicular irradiation at a dose ≥ 6 Gy.[9]

Ethics approval was granted by the Cochin University Hospital Ethical Review Committee for publications, Paris, France (reference AAA-2020-08041).

2.2 | Procedures

Patients identified by pediatricians as being at high risk of premature fertility loss, as stated above, were referred to the FP consultation of our network. Patients were seen by FP specialists (CaP, LS, and BS). During FP consultation, after recording the medical data, the principle of TT cryopreservation was explained to the patient when mature enough and their parents in readily understandable terms through informative and illustrative documents (personal tool developed by Ariane Bonnefont, psychopathologist in the mobile support and palliative care team, Robert Debré hospital). Potential fertility restoration solutions using TT were explained, as was the experimental nature of the procedure, since no birth involving cryopreserved TT has been reported to date in humans. Parents or legal guardians provided their signed informed consent for the FP procedure, as did the patients when appropriate. According to French bioethics law, patients will consent to cryobanking when they will reach the age of majority. The costs for the procedure and the storage were covered by dedicated public funds.

The testicular surgery was performed under general anesthesia, at the same time as central line placement, bone marrow biopsy, or tumor surgery when possible. In the operating room, after a horizontal scrotal incision, the tunica albuginea was opened, and the testis exteriorized in order to take a corresponding third of the tissue in case of unilateral retrieval and a corresponding quarter of the tissue in case of bilateral retrieval. After specimen retrieval, the tunica albugina was closed using a running absorbable suture followed by tunica vaginalis and skin layers. The retrieval was performed on one testis or both depending on the surgeon's practices. The carrying out of a bilateral retrieval could be justified because of a possible heterogeneity of spermatogonial distribution between the two testicles and/or in order not to create asymmetry in testicular size following

the surgery. The TT sample, immersed in transport medium (FertiCult™ Flushing medium, FertiPro), was immediately transported on ice to the laboratory, where it was cut into calibrated pieces and placed individually in cryovials containing cryoprotective medium (5% DMSO, Sigma-Aldrich; 0.05 mmol/L sucrose, Sigma-Aldrich; 5% human serum albumin solution™, Vitrolife; diluted in culture medium, FertiCult™ medium). Subsequently, the samples were frozen in a programmable freezer (Planer, Planer Limited, until 2015; Freezal or Nanodigicool, Air Liquide, afterward) according to a controlled slow freezing protocol without (start at 5°C, 2°C/min to −9°C, 7 min of soaking, 0.3°C/min to −40°C, 10°C/min to −140°C) or with automatic seeding, adapted from Keros et al.[3] The cryovials were stored at the temperature of liquid nitrogen.

For each patient, a TT fragment underwent histological analysis by a specialized pathologist to search for the presence of tumor cells and to identify spermatogonia. It was fixed in 4% formaldehyde, embedded in paraffin, cut into sections (3.5 μm-thick), and then stained with hematoxylin and eosin. In the case of bilateral retrieval, the examination was conducted on both samples. Spermatogonia were identified according to their location within the seminiferous tubule resting on the basal membrane. The nuclei of these cells are spherical or ovoid exhibiting homogeneous and finely granular pale or dark chromatin according to the criteria of Clermont (Figure 1).[10] Evaluation of the presence or absence of spermatogonia was performed on all seminiferous tubules present in the histological section. Spermatogonial depletion was defined as the absence of spermatogonia in the entire histological section analyzed. The FP data were recorded in a clinical database (General Data Protection Regulation no. 20190131145955). The information extracted from the database comprised the patient characteristics (age, accompanying relatives at the FP consultation, puberty, disease, treatment history including exposure to alkylating agents, and indication of FP) and the CTT procedure characteristics (patient age at CTT, operator, biopsy laterality, presence of spermatogonia/tumor cells in TT, and post-surgery adverse events). The data were analyzed overall and within two periods to analyze the change in practices over time: the first 5 years (2010–2014) and the last 5 years (2015–2019). The influence of age, laterality of the retrieval, and exposure to previous chemotherapy, including alkylating agents, on the presence of spermatogonia in the TT was assessed.

2.3 | Statistical analysis

The association between chemotherapy and the presence of spermatogonia was assessed with a Chi-square test or Fisher test as appropriate. Among patients exposed to chemotherapy before CTT, univariate and multivariate logistic regressions were used to assess factors associated with the risk of absence of spermatogonia. In univariate analysis, factors with a p-value ≤ 0.20 were introduced in the multivariate model. Comparisons of variables between the two periods of activity were performed using Student's unpaired t-test or chi-square test as appropriate. A p-value ≤ 0.05 was considered statistically significant. The analyses were performed with R software version 4.1.1.

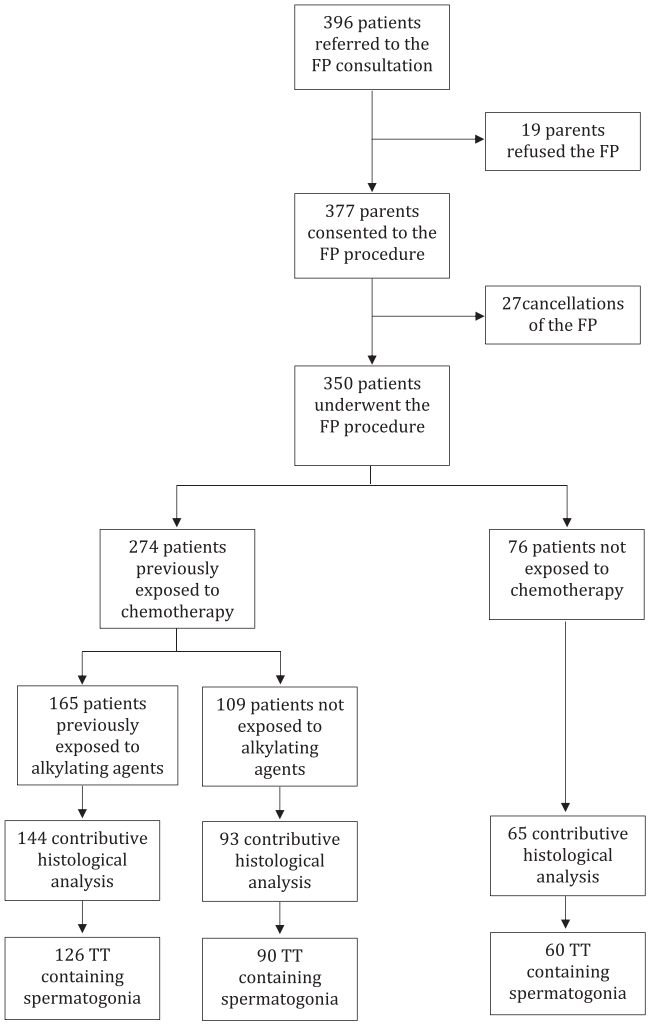

FIGURE 2 Study flowchart of the fertility preservation activity from October 2009 to December 2019.

3 | RESULTS

In the study period, 396 patients under 18 years of age were referred to the FP consultation of our network (Figure 2), coming from one of the seven departments of pediatric hematology and oncology (I to VII): I, $n = 192$ (48.5%); II, $n = 59$ (14.9%); III, $n = 27$ (6.8%); IV, $n = 67$ (16.9%); V, $n = 22$ (5.6%); VI, $n = 10$ (2.5%); VII, $n = 15$ (3.8%); and four isolated patients (1%). The patient was present at the consultation in 77.0% of cases (305/396), accompanied by both parents in 45.0% of cases (137/305), by their mother in 36.7% of cases (112/305), by their father in 14.4% of cases (44/305), or by others in the remaining 2.6% of cases (8/305). In 1.3% of cases, the hospitalized patient was seen alone initially and the parent afterward (4/305). The median age of patients at consultation was 7.2 years (range: 0.5−17.0, 95% CI 6.5−7.9). Thirty-two percent of patients (126/396) were < 5 years of age and 72% (91/126) of them were present at the consultation. As shown in Figure 3, 70% of patients suffered from malignant disease (276/396), and hemoglobinopathies (17%) were the main non-malignant disease (66/396). The indications for FP were (1)

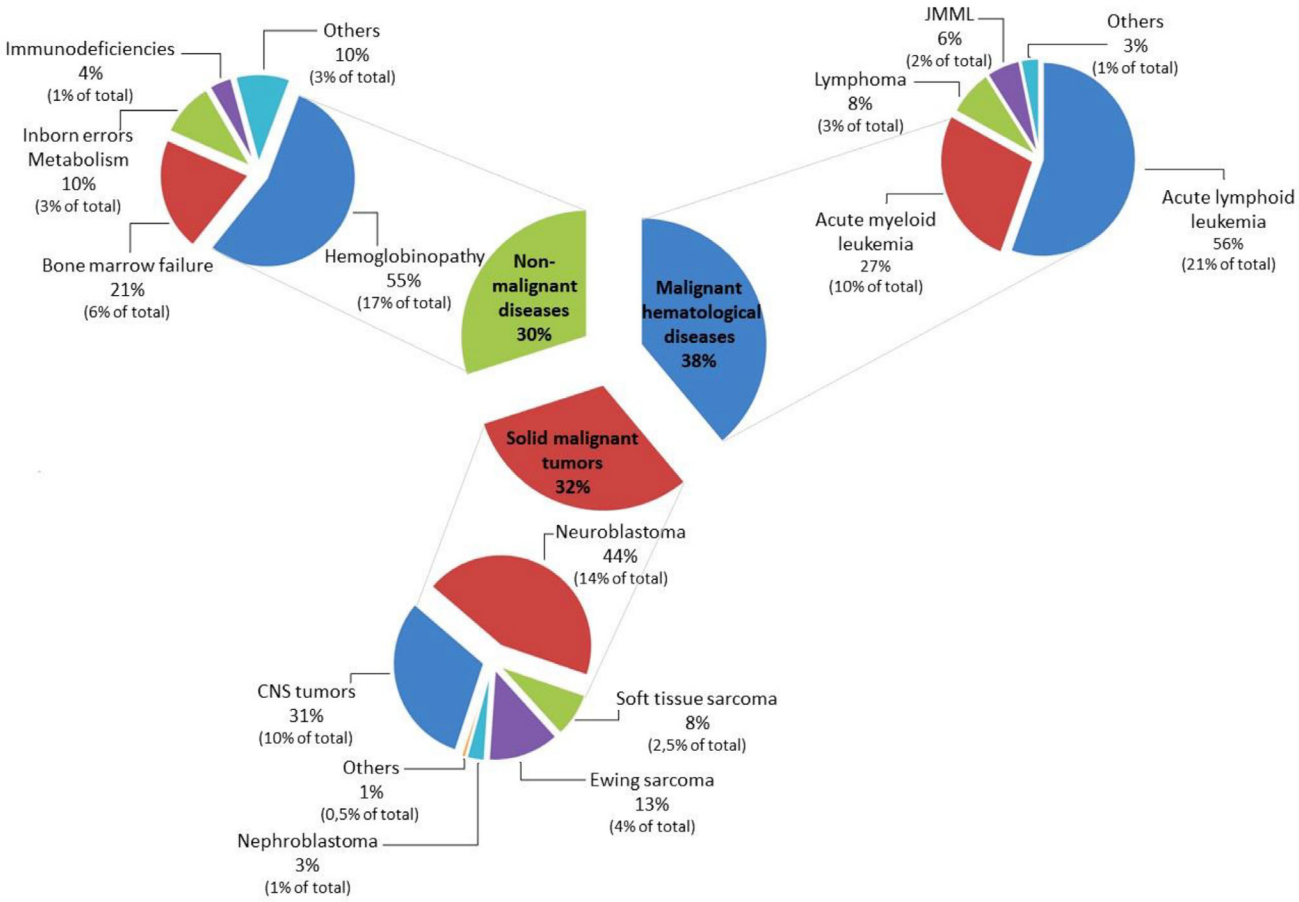

FIGURE 3 Disease classification of boys under 18 years of age referred to the fertility preservation consultation. Malignant hematological diseases ($n = 151$, 38%), solid malignant tumors ($n = 125$, 32%), and non-malignant diseases ($n = 120$, 30%). Abbreviation: JMML, juvenile myelomonocytic leukemia; CNS, central nervous system.

conditioning treatment before hematopoietic stem cell transplantation, allogeneic in 66% of cases (261/396) or autologous in 18% of cases (73/396), (2) chemotherapy intensification in 13% of cases (51/396) and in 3% of cases (11/396), for other situations, such as pelvic radiotherapy, orchidectomy, and Klinefelter syndrome (for azoospermia or semen collection refusal).

Of the 396 families, 95% consented to the CTT procedure (377/396) (Figure 2). Ninety-three percent of them underwent testicular surgery for CTT (350/377) (Figure 2). The surgery was canceled due to poor health status or a hematological disorder such as thrombopenia. Characteristics of the patients who underwent CTT are presented in Tables 2 and 3. The median patient age at surgery was 7.1 years (range 0.6–17.1, 95% CI 6.4–7.9). The proportion of boys who were offered TT cryopreservation who were pubescents was 15.4% (54/350). Ten were not mature enough for semen collection; six refused and two failed the semen collection; six had started chemotherapy before the FP procedure, which was a reason to not store spermatozoa; 11 had altered sperm quality (azoospermia, oligozoospermia, or necrozoospermia); one had an orchidectomy for oncological reasons; for 18, data were unavailable (Table 4). Prior to the CTT, 78% of the patients had been exposed to a sequential multidrug therapy (274/350), including alkylating agents in 47% of cases (165/350). The median time between the consultation and the surgery was 13 days in the case of malignant hematological disease (range 0–103), 12 days in the case of solid malignant tumor (range 1–216), and 20 days in the case of non-malignant disease (range 1–631). When the start of the chemotherapy was urgent, the consultation and the biopsy were performed in a very timely manner, that is, within 24–48 h (31 cases). The longer times corresponded to cases of hematopoietic stem cell transplantation for hemoglobinopathy. The tissue retrieval was unilateral in 63.5% of cases (222/350) and bilateral in 36.5% (128/350), depending on the surgery center and/or surgeon practices. Among the 54 pubertal patients, intratesticular sperm retrieval was attempted for 21 and was positive for six of them. A median number of 12 TT fragments were cryopreserved per patient, ranging from 2 to 175. Postoperative information was available for 283 patients (283/350, 80.8%), and adverse events were recorded in 3.5% of cases (10/283): six painful episodes, one local infection, and three hematomas. Nine of the complications occurred after a unilateral biopsy and one after a bilateral, which then did not appear to be associated with a higher risk of complication.

The screening of neoplasia by histological analysis was negative for all patients with malignant disease. Histological identification of spermatogonia was performed for 302 patients (302/350, 86.3%): 237 for boys exposed to chemotherapy before cryopreservation (237/274,

TABLE 2 Characteristics and distribution of patients under 18 years of age undergoing testicular tissue cryopreservation according to the disease.

	Patients *n* (%)	Median age at cryopreservation years [range]	History of chemotherapy *n* (%)	History of alkylating agent *n* (%)
Malignant hematological diseases				
Acute lymphoblastic leukemia	75 (21.5)	10.1 [0.9–17.1]	75 (100)	66 (88)
Acute myeloblastic leukemia	36 (10)	6.3 [0.8–16.4]	36 (100)	2 (5.5)
Lymphoma	10 (3)	15.1 [5.9–17.0]	9 (90)	7 (70)
Juvenile myelomonocytic leukemia	5 (1.5)	3.5 [1.1–8.3]	4 (80)	1 (20)
Others	5 (1.5)	5.6 [3.8–4.4]	3 (60)	0 (0)
Total	**131 (37.5)**	**9.0 [0.8–17.1]**	**127 (97)**	**76 (58)**
Solid malignant tumors				
Neuroblastoma	52 (15)	3.5 [1.0–15.2]	52 (100)	52 (100)
Central nervous system tumors	35 (10)	5.3 [0.6–13.4]	35 (100)	10 (29)
Ewing sarcoma	15 (4)	11.4 [0.8–15.1]	14 (93)	14 (93)
Soft tissue sarcoma	10 (3)	5.0 [0.7–14.9]	10 (100)	9 (90)
Nephroblastoma	4 (1)	6.4 [2.6–7.5]	4 (100)	3 (75)
Others	1 (0.5)	3.2 [–]	1 (100)	0 (0)
Total	**117 (33.5)**	**4.6 [0.6–152]**	**116 (99)**	**88 (75)**
Non-malignant diseases				
Hemoglobinopathy	58 (16)	8.0 [2.1–15.1]	29 (50)	0 (0)
Aplastic anemia	20 (6)	7.0 [1.0–13.5]	1 (5)	0 (0)
Inborn errors metabolism	11 (3)	6.9 [0.9–11.5]	0 (0)	0 (0)
Immunodeficiencies	5 (1.5)	6.6 [1.8–15.2]	0 (0)	0 (0)
Others	8 (2.5)	13.8 [4.2–16.7]	1 (12)	1 (12)
Total	**102 (29)**	**8.0 [0.9–16.7]**	**31 (30)**	**1 (1)**
Global population	**350 (100)**	**7.1 [0.6–17.1]**	**274/350 (78)**	**165/350 (47)**

86.5%) and 65 for boys not exposed to chemotherapy (65/76, 85.5%) (Figure 2). The histological analysis was unsuccessful for one patient because the sample was a fragment of albuginae and not TT. For the other 47, the presence (or absence) of spermatogonia was omitted in the analysis conclusion. The 48 patients without histological analysis were significantly younger than the other 302 (5.1 [0.8–15.0] vs. 7.4 [0.6–17.1], $p < 0.001$). They were otherwise comparable (data not shown). Overall, spermatogonia were detected for 91.4% of patients (276/302), which was not different between boys exposed to chemotherapy and boys not exposed to chemotherapy (91.1% [216/237] vs. 92.3% [60/65], $p = 0.962$). Univariate and multivariate logistic regression for the risk of absence of spermatogonia in TT of boys exposed to chemotherapy is presented in Table 5. The risk of spermatogonia-depleted TT was significantly higher in boys ≥ 10 years of age ([OR] 2.88, 95% CI 1.14–7.24, $p = 0.025$) and in boys previously exposed to alkylating agent ([OR] 4.28, 95% CI 1.22–14.98, $p = 0.023$). In multivariate logistic regression, age ≥ 10 years old exposed patients to an almost three-fold higher risk of spermatogonial depletion ([OR] 2.74, 95% CI 1.09–7.26, $p = 0.035$) and previous exposure to alkylating agents to a four-fold higher risk of spermatogonial depletion ([OR] 4.09, 95% CI 1.32–17.94, $p = 0.028$).

At the time of the data analysis, 301 (86%) were alive, with a median age of 12.8 years (ranging from 2.3 to 27.4, 95% CI 12.2–13.4).

Concerning the change in the FP activity, the consultations increased by 1.6-fold between the first and the second period, with no change in the number of collaborating departments in the network. The activity volume progressed until 2017, with a plateau at approximately 60 consultations per year. We observed no difference regarding the accompanying relatives. The median age of patients at consultation was not different (7.4 [0.7–16.6] vs. 6.9 [0.5–17.0], respectively, $p = 0.51$, 95% CI: −0.61 to 1.22). Two-thirds (12/19) of the refusals of the CTT were recorded during the first period of activity. The main reasons were the experimental and invasive nature of the procedure, as well as religious considerations. During the two study periods, patients suffering from malignant hematological diseases decreased progressively from 45% to 34% of cases, with a reciprocal increase in malignant solid diseases, which represented 24% of cases during the first period and 36% during the second period. The proportion of non-malignant diseases remained unchanged at 30%. Indications for CTT were stable during the 10 years. The proportion of patients previously exposed to chemotherapy, including alkylating agents, was not different. Along with the increase in consultation activity, the CTT increased by 1.8-fold.

TABLE 3 Clinical and histological characteristics of patients under 18 years of age undergoing a testicular tissue cryopreservation.

	Global population	Malignant hematological diseases	Solid malignant tumors	Non-malignant diseases
Patients, *n* (%)	350 (100.0)	131 (37.5)	117 (33.5)	102 (29.0)
Relatives, *n* (%)				
Father, *n* (%)	40 (11.5)	10 (7.5)	12 (10.0)	18 (17.5)
Mother, *n* (%)	100 (28.5)	31 (23.5)	39 (33.5)	30 (29.5)
Both parents, *n* (%)	110 (31.5)	35 (27.0)	42 (36.0)	33 (32.5)
Other, *n* (%)	100 (28.5)	55 (42.0)	24 (20.5)	21 (20.5)
History of chemotherapy, *n* (%)	274 (78.0)	127 (97.0)	116 (99.0)	31 (30.0)
History of alkylating agent, *n* (%)	165 (47.0)	76 (58.0)	88 (75.0)	1 (1.0)
Median age at cryopreservation, years [range]	7.1 [0.6–17.1]	9.0 [0.8–17.1]	4.6 [0.6–152]	8.0 [0.9–16.7]
Median delay to surgery, days [range]	13 [0–631]	13 [0–103]	12 [1–216]	20 [1–631]
Biopsy laterality, one versus two sides, *n* (%)	222 (63.5)/128 (36.5)	124 (94.5)/7 (5.5)	43 (37.0)/74 (63.0)	55 (54.0)/47 (46.0)
Number of TT fragments cryopreserved, median [range]	12 [2–175]	11 [2–175]	10 [3–52]	13 [4–90]
Post-surgery adverse events				
Pain	6	4	1	1
Hematoma	3	2	1	–
Infection	1	–	–	1
Presence of spermatogonia in TT, *n* (%)	276/302[a] (91.4)	107/122[a] (87.7)	88/95[a] (92.6)	81/85[a] (95.3)

Abbreviation: TT, testicular tissue.
[a]Total contributive histological analysis.

TABLE 4 Indication for cryopreservation of immature testicular tissue in pubertal patients ($n = 54$).

Indications for CTT in prepubertal patients	Patients (*n*)
Immature sexuality	10
Sperm collection refusal	6
Sperm collection failure	2
Chemotherapy starting before sperm collection	6
Altered sperm parameters	11
Orchidectomy for oncological reason	1
Unavailable data	18

There was no significant difference in age at surgery (7.5 [0.7–16.0] vs. 6.6 [0.5–17.0], respectively, $p = 0.29$, 95% CI −0.45 to 1.246). No statistical difference was recorded concerning the retrieval laterality (60% [75/125] vs. 65% [146/224] and 40% [50/152] vs. 35% [78/224] for unilateral and bilateral testicular retrievals, respectively, $p = 0.3$). Adverse events were equally distributed over the two periods.

4 | DISCUSSION

Our report shows that FP in boys under 18 years of age is well accepted and feasible at several sites coordinated in a network and is a low-risk procedure. Most importantly, it highlights the presence of spermatogonia in most TT samples irrespective of previous chemotherapy exposure.

This study reports the largest series of CTT FP in boys under 18 years of age from a single clinical network. Despite the uncertainty of the future benefits, FP for young boys appears to be of great interest to parents and patients. Indeed, both parents were present at the consultation in half of the cases, and they accepted the procedure in 95% of cases, even for children who were just a few months old. The progress of fertility restoration using TT recorded in non-human primate[4] allowed practitioners to more confidently and more positively offer this procedure. In consequence, a minority of refusal was recorded during the second period of activity. The steady rise in the number of referred patients with a consistent number of corresponding centers also highlights the increased awareness of pediatric healthcare providers over time. This is in line with recent observations from an international survey that reported a four-fold increase in CTT between 2012 and 2019 worldwide.[6] However, this global evaluation could mask discrepancies between centers and countries, since Kanbar et al. reported a decreased number of referred patients between 2013 and 2019 compared to 2005–2012.[11]

Patients had malignant diseases in the majority of cases, with differences, however, between series (62%–100%, Table 1). Malignant hematological diseases and tumors of the central nervous system (CNS) were the most frequent,[6] which was also the case in the present study, in which neuroblastoma slightly outnumbered tumors of the CNS. The increased percentage of solid malignant tumors observed

TABLE 5 Univariate and multivariate logistic regression for the risk of absence of spermatogonial stem cell in testicular tissue (TT) for the subgroup of boys exposed to chemotherapy before cryopreservation ($n = 237$).

	Univariate analysis		*Multivariate analysis*	
	Odds ratio [95% CI]	***p*-value**	**Odds ratio [95% CI]**	***p*-value**
Age at cryopreservation (years)				
Age < 10	1	0.025	2.74 [1.09–7.26]	0.035
≥ 10	2.88 [1.14–7.24]			
Puberty				
No	1	0.542[c]	–	–
Yes	1.54 [0.52–4.56]			
Disease				
Malignant	1	0.14[b]	–	–
Non-malignant	NE[b]		–	–
Alkylating agent[a]				
No	1	0.023	4.09 [1.32–17.94]	0.028
Yes	4.28 [1.22–14.98]			
Biopsy laterality				
Unilateral	1	0.546	–	–
Bilateral	0.64 [0.04–0.67]		–	–

[a]Chemotherapy comprising alkylating agent.
[b]Chi-2, presence of spermatogonial stem cell in TT of all non-malignant patients.
[c]Fisher's test.

in our series is explained by the deferred integration of the oncopediatrician in the network, starting at the end of the first period. An obvious particularity of our experience is that TT from patients with hemoglobinopathies was cryopreserved in the same proportion throughout the study period. While Goossens et al. highlighted that most coordinating sites included in their survey had started an FP program for oncological patients and had stored TT for the non-malignant disease at a later stage.[6]

In our experience and thanks to strengthened coordination, FP was undertaken in a very timely manner according to the start of the treatment, and the TT processing and freezing arising from different collection sites were possible. This strategy was also reported by a US-coordinated network of eight centers[12] and previously validated for ovarian cortex cryopreservation.[13]

In our series, there was a low postoperative complication rate of 3.5%, which is comparable to the figures previously published (0.0%–8.1%) (Table 1). Painful episodes were the most frequent event and often required administration of opioid analgesics. Aside from this, testicular retrieval remains a low-risk procedure. Its long-term safety on gonadal function should be thoroughly investigated. Follow-up data are still scarce but the first results support the notion that removing a small part of the TT during childhood does not clearly induce more deleterious effects on reproductive health in adulthood than the cancer treatment itself.[11,14]

The scheduling of FP procedures differs between centers. Some recruit patients exposed to a high risk of fertility loss; most of the time after an initial first-line chemotherapy at a low risk of gonadotoxicity.[15–18] Although others advocate obtaining the testicular sample at the time of diagnosis before initiation of the treatment even in case of moderate risk.[11,19] Since surgical removal of TT is an invasive procedure and the methods to restore fertility are still experimental, we chose the first option with a cohort of patients mostly exposed to chemotherapy before the CTT. In these cases, we did not observe a decreased number of spermatogonia in the TT of patients previously exposed to chemotherapy. Spermatogonia were present in TT in 91% of cases compared to 92% in other publications where patients were referred before starting treatments.[11] Based on a large series, our study supports the notion that previous exposure to low-risk chemotherapy[20] should not be considered to be a limitation for CTT since it does not impair the chance of finding spermatogonia. By contrast, in cancer patients, chemotherapy prior to CTT could be an advantage since it potentially decreases the risk of malignant cell contamination of the TT.[21] These findings must nonetheless be qualified, as the risk of spermatogonia-depleted TT was significantly increased when the first-line therapies contained alkylating agents. Prepubertal boys exposed to alkylating agents have been shown to have reduced spermatogonial counts compared to healthy controls.[16,22,23] Altogether, these results are consistent with data from the Childhood Cancer Survivor Study cohort showing a correlation between an increasing cumulative exposure dose of alkylating agents and the prevalence of azoospermia.[1]

In the peripubertal period, spermatogonia leave their quiescent state and actively divide to support the physiological expansion observed at that time.[24] This mitotic activity probably renders

spermatogonia highly sensitive to the cytotoxic effect of chemotherapy and could explain the higher risk of spermatogonia-depleted TT observed in boys ≥ 10 years of age.

TT retrieval was performed unilaterally or bilaterally, depending on surgeon and/or surgical center practices, with the same chance of finding spermatogonia. This study did not reveal any superiority of one strategy over the other.

Autograft of cryopreserved TT is currently the most promising fertility restoration option, as the birth of a healthy offspring has been reported in a non-human primate.[4] Contamination of the graft with cancer cells may be at risk of malignant disease relapse once transplantation takes place, as shown by TT contaminated with leukemic cells in graft experiments in an animal model.[25] The risk is high in leukemic patients in whom malignant cells may be present in the bloodstream and in the testes, which are considered a sanctuary site for leukemic cells. In our experience, there is no evidence of malignant cell infiltration in the cryopreserved TT retrieved from cancer patients in remission. However, histology and immunohistochemistry used on germinal tissue have been shown to not be accurate enough to formally eliminate the presence of malignant cells.[21] Prior to any clinical use, a comprehensive tissue evaluation for malignant contamination should be performed using customized technologies, such as molecular biology analysis, which can provide much better sensitivity, as previously described for the ovary[21] and preliminary tested for the prepubertal TT.[26] Fifty-seven patients had reached the age of majority at the time of data collection and the oldest patient of our series is now 27 years old. We anticipate that patients will soon return and ask for the option to restore their fertility. Despite the promising progress already obtained, continued clinical and basic science research is required in humans to tackle the challenge of creating healthy live births in the near future thanks to cryopreserved TT.

The study has limitations that warrant being pointed out. The data on the safety of the procedure should be considered carefully because the patients were followed for a short time after CTT; long-term follow-up data are missing. In the case of previous exposure to an alkylating agent, the risk of spermatogonia depletion was not associated with the cyclophosphamide equivalent dose. Additionally, as no use back of the cryopreserved TT has been performed yet, we cannot definitively conclude on its usefulness.

5 | CONCLUSION

Pediatric CTT for FP is well-accepted, feasible, and safe. This report supports its place in the clinical care pathway of children and adolescents undergoing a highly gonadotoxic treatment. In addition, it provides new information regarding the usefulness of this procedure in cancer patients previously exposed to chemotherapy including alkylating agents for which the percentage of spermatogonia-depleted TT remains low. Long-term follow-up of patients who received gonadotoxic treatment and underwent CTT must absolutely be carried out to evaluate their reproductive health in adulthood and who will need to rely on the use of the cryopreserved TT.

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AUTHOR CONTRIBUTIONS

V.B.-L. and Ca.P. performed the conceptualization of the study. V.B.-L., C.J., and A.-S.G. performed the data collection, N.B. and A.V. analyzed the data. N.B., V.B.-L., A.-S.G., and Ca.P. interpreted the data. V.B.-L. and Ca.P. were in charge of the preparation, drafting, and editing of the manuscript. Ca.P., L.S., and B.S. performed the fertility preservation consultations. K.Y., M.F., F.D., L.L., C.D., C.R., G.L., M.-D.T., and Co.P. referred the patients to the fertility preservation network. A.P.-J., M.P., C.C., S.S., P.P.-C., S.I., H.L., G.L., M.S., E.C., and I.B. participated in the fertility preservation network. All of the authors contributed to the critical review of the manuscript and agreed to the publication of the data.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

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.

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ORIGINAL ARTICLE

Sperm DNA methylation dynamics after chemotherapy: a longitudinal study of a patient with testicular germ cell tumor treatment

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Abstract

Background: An important issue for young men affected by testicular germ cell tumor (TGCT) is how TGCT and its treatment will affect, transiently or permanently, their future reproductive health. Previous studies have reported that xenobiotics can induce changes on human sperm epigenome and have the potential to promote epigenetic alterations in the offspring.

Objectives: Here, we report the first longitudinal DNA methylation profiling of frozen sperm from a TGCT patient before and up to 2 years after a bleomycin, etoposide, and cisplatin (BEP) chemotherapy.

Materials and methods: A TGCT was diagnosed in a 30-year-old patient. A cryopreservation of spermatozoa was proposed before adjuvant BEP treatment. Semen samples were collected before and after chemotherapy at 6, 9, 12, and 24 months. The DNA methylation status was determined by RRBS to detect DNA differentially methylated regions (DMRs).

Results: The analysis revealed that among the 74 DMRs showing modified methylation status 6 months after therapy, 17 remained altered 24 months after treatment. We next associated DMRs with differentially methylated genes (DMGs), which were subsequently intersected with loci known to be important or expressed during early development.

Discussion and conclusion: The consequences of the cancer treatment on the sperm epigenome during the recovery periods are topical issues of increasing significance as epigenetic modifications to the paternal genome may have deleterious effects on the offspring. The altered methylated status of these DMGs important for early development might modify their expression pattern and thus affect their function

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during key stages of embryogenesis, potentially leading to developmental disorders or miscarriages.

KEYWORDS
bisulfite sequencing, chemotherapy, epigenome, methylation, sperm, testis cancer

1 | INTRODUCTION

Over the past quarter century and particularly in the last decade, the incidence of testicular cancer (TC) has been rising rapidly.[1,2] Although it is a proportionately rare disease which accounts for approximately 1% of all new male cancer cases, TC remains the most frequent cancer in male adolescents and young adults from 15 to 35 years in industrialized countries.[3–5] The TC incidence rate ranges between 5.7 and 13.4 per 100,000 in Europe.[5,6] In France, no national cancer registry exists, thus estimations of TC incidence are based on six specific cancer registries. Consequently, hypotheses on TC incidence tendencies are defined by a single standardized sperm bank network, the Centre d'Etude et de Conservation des Oeufs et du Sperme humain (CECOS), which provides precious data to indirectly estimate global TC incidence. This unique public network of sperm banks was established in 1973 and covers the whole country through 33 affiliated regional sperm banks. In the last decade, prognoses and survival rates have improved very markedly.[7,8] An important issue for these young men is how TC and its treatment will affect, transiently or permanently, their future reproductive health.[9,10] Semen banking is thus recommended before any treatment.[11,12]

Several late adverse effects of chemotherapy or radiotherapy have been described.[13] Post-treatment gonadic function could notably be drastically affected, and the intensity of spermatogenesis alterations is related to the type of drugs used, the cumulative doses of the drug(s), the radiation doses received by the testis, and the time between the last treatment and sperm analysis.[14,15] Other studies have also examined the impact on sperm DNA fragmentation and genome integrity[16–18] and DNA methylation alterations[19,20] after cancer treatment. Apart from the GAMATOX prospective study published by Rives and colleagues,[21] most of these debated studies are retrospective and consequently the dynamics of recovery are not precisely described. The consequences of cancer treatment on the sperm epigenome during the recovery periods are topical issues of significance as epigenetic modifications to the paternal genome may have deleterious effects on the pregnancy or the offspring.[22–24] One case report showed a drop on the methylation level in one patient following treatment by temozolomide for an anaplastic oligodendroglioma.[20] The other study reported modifications on DNA methylation regions in 18 sarcoma patients treated by cisplatin ± ifosfamide but the cross-sectional design of this study limited results interpretation.[19]

Here, we report a case study aiming at longitudinally evaluating the temporal evolution of the sperm DNA methylation status of a testicular germ cell tumor (TGCT) patient before and 6, 9, 12, and 24 months after a bleomycin, etoposide, and cisplatin (BEP) chemotherapy.

2 | MATERIALS AND METHODS

2.1 | Patient samples and ethical considerations

A TGCT was diagnosed in a 30-year-old patient. A preservation of fertility was proposed before treatment. Chemotherapy consisted of four cycles of BEP protocol: (i) etoposide (100 mg/m^2/day) from days 1 to 5; (ii) cisplatine (20 mg/m^2/day) from days 1 to 5; and, (iii) bleomycin (30 mg in 30 mn) at days 1, 8, and 15. After a local ethic committee approval with inclusion in GERMETHEQUE National Biobank (BB-0033-00081) and written informed consent (GERM1015012), sperm analyses were performed before and after his adjuvant treatment. Two semen samples were obtained before treatment (samples were named PreCT_1 and PreCT_2), while a single sample was obtained at each of four time points after treatment, that is, at 6, 9, 12, and 24 months (PostCT_6 m, PostCT_9 m, Post_CT12m, PostCT_24 m). Analysis of semen was performed in our laboratory according to standard World Health Organisation criteria (WHO, 2010). Sperm was collected in a sterile container by masturbation and analyzed after liquefaction according to WHO criteria. The parameters assessed included volume of ejaculate, sperm concentration, motility (forward movement), vitality, and morphology (David's criteria). The total sperm count was obtained by multiplying the sperm concentration by the volume of ejaculate. Throughout the study, the patient confirmed that he did not make any lifestyle changes (non-smoking, no alcohol or other drug consumption, no change in family or work situation) that could impact the sperm methylome.

2.2 | Sperm DNA extraction and purification

Sperm freezing straws were thawed and subjected to density gradient centrifugation (30 min at 830 g, 25°C) on a two-layer 80%/40% Percoll. Pellet (corresponding to the "motile" fraction) was washed and centrifuged twice for 15 min with 10 mL of Wash Buffer [NaCl 150 mM, Ethylenediaminetetraacetic acid (EDTA) 10 mM, pH 8] at 1600 g. Only the motile fraction was considered for the analysis. Cell lysis was performed in a low-binding tube and incubated in a thermomixer (Eppendorf) overnight at 55°C with 500 μL of Digestion Buffer (Tris

50 mM pH 8, NaCl 100 mM, EDTA 10 mM, SDS 1%) to which were added 21 μL DTT 1 M, 20 μL PK 20 mg/mL, and 2.5 μL Triton X-100.

Cell debris was pelleted down by centrifugation at 14,000×*g*, and the supernatant was subjected to a 10 mg/mL RNAse A digest for 30 min at 37°C in a thermomixer. DNA was extracted with an equal volume of phenol:chlorophorm:isoamyl alcohol followed by a second extraction with chloroform:isoamyl alcohol. The aquaeus phase containing DNA was purified by a standard precipitation with NaCl 300 mM/2.5 V alcohol 100% and 1 μL GlycoBlue (Thermo Fisher Scientific). The final DNA concentration was measured using DsDNA QuantiFluor Dye System (Promega).

2.3 | Reduced representation bisulfite sequencing libraries

The reduced representation bisulfite sequencing (RRBS) method[25] was performed using the Premium RRBS Kit according to the manufacturer's instructions (Diagenode). Briefly, 100 ng of DNA was digested using the methylation-sensitive restriction enzyme, MspI, followed by end-repair and A-tailing. Adaptor ligation was followed by a size selection (40–220 bp) with the AMPure XP Beads (Beckman Coulter). Quantification prior to sample pooling was evaluated by a q-PCR analysis. Then, DNA samples with adjusted quantity underwent bisulfite conversion followed by desulphonation and on-column purification. The converted DNA was amplified by polymerase chain reaction (PCR) and cleaned-up with the AMPure XP Beads. Finally, the quality of the RRBS libraries was evaluated using an Agilent 2100 bioanalyzer. The RRBS libraries were sequenced on an Illumina Hiseq 4000 sequencer as single-end 50 base reads following Illumina's instructions. Image analysis and base calling were performed with RTA 2.7.3 and bcl2fastq. Following the sequencing, quality controls were performed for each sample with FastQC. Briefly, we ensured that a percentage higher than 95% of bases above Q30 (always ≥Q27) was obtained for each sample. The proportion of each nucleotide along the read was verified, to ensure that reads start with CGG due to Msp1 digestion between cytosines at CCGG sites. Following a general tendency across samples, the nucleotide proportions on the first base of reads converge to a 50–50 ratio between cytosines and thymines (slightly higher for Cs). The cytosines correspond to bisulfite unconverted methylated cytosines, while thymines represent bisulfite converted unmethylated cytosines. This observation, paired with the high %G (≈95%) on the second and third bases of the reads, retranscribe MspI cutting sites.

2.4 | Data preprocessing and analysis

A four-step workflow was designed to preprocess RRBS data, to analyze the CpG methylation data and, finally to identify and annotate differentially methylated regions (DMRs) (Figure 1).

2.4.1 | Read mapping

Reads were mapped on the human genome (hg38) with the Bismark tool (release v0.17.0).[26] After data quality control, methylation calls were extracted by Bismark and a CpG coverage file (.cov file) containing the average methylation level of each covered CpG site was generated for each sample.

2.4.2 | CpG detection and methylation level

The *clusterSites* function implemented in BiSeq (v1.19.0) was used to identify CpG islands for each sample.[27] This function takes into account spatial correlation of the methylation of nearby CpG sites, constraining the analysis on CpG sites among CpG clusters. CpG islands were defined as regions containing at least 20 frequently covered CpG sites—that is, corresponding to those covered in 100% of the samples—with a maximum distance of 100 bp from each other. This is important to note that the frequently covered CpG sites were considered to define the CpG cluster boundaries only. For downstream analysis, all CpG methylation data within these CpG clusters were used. Since methylation levels show strong spatial correlation,[28] we smoothed the raw methylation to reduce the required sequencing coverage data, as previously suggested.[29,30] In addition, DMR detection methods without smoothing data often discard CpG sites of low coverage from further analyses. The smoothing of the methylation data was performed with the *predictMeth* function implemented in BiSeq (with a bandwidth of $h = 80$ bp). Within each CpG cluster and for each sample, a smoothing function is modeled on the local raw methylation data. The resulting methylation levels range from 0 to 1.

2.4.3 | Detection of differentially methylated regions

The detection of DMRs corresponds to the identification of genomic regions holding smoothed CpG methylation level differences between two experimental conditions. The *compareTwoSample* function implemented in BiSeq was used to identify DMRs for every pairwise comparison between samples. DMRs are assembled with CpGs closer than a maximum distance of 100 base pairs that have a methylation difference greater than 30%. DMRs extension is stopped if one of these rules is broken, establishing the DMR boundaries and thereby can be constituted of only one differentially methylated CpG. It is worthwhile to remember that one of the earliest studies of DNA methylation impacting transcription factor binding considered a single 6 bp region and the impact of one methylated cytosine within that region.[31] The resulting nonredundant DMRs are then aggregated together in a single DMR file. Each detected DMR is then quantified in each sample by calculating the median smoothed methylation level of all CpG sites within this genomic region.

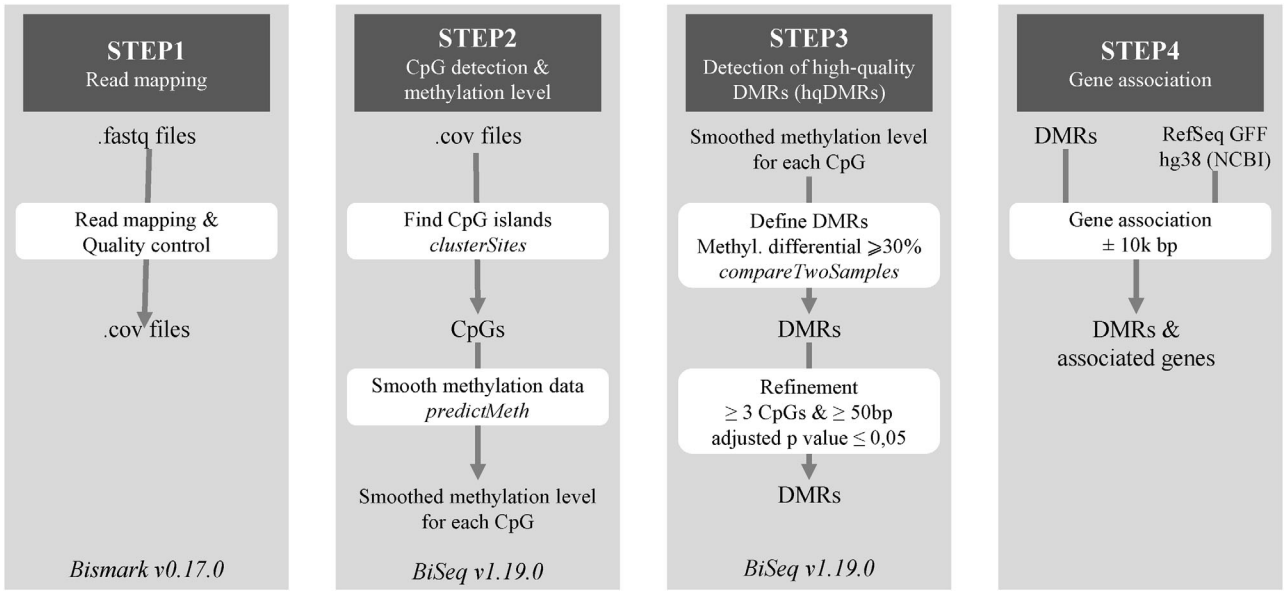

FIGURE 1 RRBS data preprocessing and analysis. The workflow includes four distinct steps: read mapping on the human genome and quality control (STEP1), CpG detection and methylation level measurement (STEP2), detection of differentially methylated regions (DMRs) (STEP3), and gene association (STEP4).

To further characterize chemotherapy-associated differential methylation of sperm DNA, we focused our analysis on a subset of DMRs (with a minimum length of 50 bp and composed of at least three CpG sites) showing changes between the two PreCT samples (PreCT_1 & PreCT_2) and each PostCT sample (i.e., at month 6, 9, 12, or 24 after treatment) (Figure S1, panels A–D; Table 3). We first applied a methylation difference cutoff of at least 30% for each of the eight pairwise comparisons (PreCT_1 vs. each of the four PostCT samples and PreCT_2 vs. each of the four PostCT samples). We next used the linear models for microarray data (LIMMA) package and a *p*-value cutoff of 0.05 adjusted with the Benjamini and Hochberg method[32] to compare in a single statistical test the two PreCT samples to each of the four PostCT samples.

2.4.4 | Gene association

We first assembled a unique set of human reference transcripts. To address this issue, Ensembl[33] and RefSeq[34,35] transcript annotations from the hg38 release of the human genome were downloaded from the University of California Santa Cruz (UCSC) genome browser website[36] on November 11, 2017. Both transcript annotation files (GTF and GFF formats) were subsequently merged into a combined set of nonredundant human reference transcripts with Cuffcompare.[37] Based on this reference transcriptome, we further associated DMRs with their proximal adjacent human genes, that is, less than 10 kbp away. MethGO was also used to analyze the RRBS data at gene-centric levels based on the features described in the human reference transcriptome (promoters, genes, exons, introns, and intergenic regions).[38]

2.5 | Functional analysis

The enrichment analysis module implemented in the AMEN suite[39] was used to identify gene ontology terms significantly over-represented in each gene group by calculating Fisher's exact probability using the Gaussian hypergeometric function (*p*-value ≤ 0.01 adjusted by the false discovery rate (FDR) method, number of genes in a given group associated with a given annotation term ≥ 5).

2.6 | Genes of interest

2.6.1 | Genes expressed during early embryo development

To identify genes expressed during the early embryo development process, we integrated microarray data (Affymetrix Human Genome U133 Plus 2.0 Array) published by Xie et al.[40] available on the NCBI Gene Expression Omnibus (GEO) repository under the accession number GSE18290. This dataset includes the transcriptome of 6 embryonic stages (18 samples): 1-, 2-, 4-, and 8-cell stages as well as morula and blastocyst. Raw data (cel files) were normalized and background corrected with the RMA method based on the Brainarray custom CDF environment for directly mapping Affymetrix gene to Entrez gene identifiers (hta20_Hs_ENTREZG version 22.0.0).[41] Briefly, genes showing a signal ≥background cutoff (median of the normalized dataset, cutoff 5.04) and a fold-change ≥ 3 in at least one pairwise comparison were selected. To define the set of 4,264 differentially expressed genes displaying significant statistical changes across

early embryo development stages, the LIMMA package was used (FDR-adjusted *F*-value ≤ 0.01)[42] implemented in AMEN.[39]

2.6.2 | Genes associated with embryo development

A list of 969 human genes known to be associated with the embryo development process were selected based on their association with the "embryo development" Gene Ontology term (GO:0009790) according to the 'gene2go' file downloaded from the NCBI website.

2.6.3 | Imprinted genes

A list of 91 human maternally and paternally imprinted genes has been gathered from the Geneimprint web-based portal (http://www.geneimprint.com/site/genes-by-species.Homo+sapiens).[43]

3 | RESULTS

3.1 | Patient and sperm collection

Sperm characteristics of the chemotherapy-exposed patient with TGCT including time of semen collection, volume, and sperm quality are presented in Table 1. After cancer diagnosis and one month prior to chemotherapy the sperm concentration ranged from 110 to 140 million spz/mL, while it droped to 10 million spz/mL 6 months after the treatment and progressively increased up to 70 million spz/mL at month 24. Therefore, there was a general reduction in sperm count after the chemotherapy treatment followed by a steady increase in the 2 years following the treatment, as previously described in other patients.[10]

3.2 | Low but genome wide demethylation of the sperm chromatin after chemotherapeutic treatment

We used RRBS to profile the methylation status of the CT patient's sperm DNA at the single cytosine level and investigate the potential epigenomic impact of the chemotherapeutic treatment. Following sequencing and quality controls, a total of 67–106 million reads per sample (Figure 2A; Table 2) were next mapped on the converted human genome (mapping efficiency ranging from 60.8%–64.9%), enabling the analysis of 483–779 million of cytosines, among which 83–125 million (16.0%–17.7%) corresponded to CpGs (Figure 2B). We limited the coverage of CpGs to the 90% quantile for further analysis to reduce biases due to unusually high coverages. The median coverage of those CpGs ranged from 12–17 reads across the six samples (Figure 2C). Finally, we defined a set of 69,675 CpG islands corresponding to 1,058,776 spatially correlated CpGs for which smoothed methylation status could be compared between all samples.

TABLE 1 Semen characteristics before and after chemotherapy according to the time of follow-up.

Sperm samples	Date	Volumes (mL)	Sperm[C] (10^6/mL)	Mobility *a*+*b* or *P* (%)	Mobility *c* or NP (%)	Mobility *d* or *I* (%)	Typical forms	MAI	*n*° total (10^6)/ sperm straw
PreCT_1	12-02-2015	4.6	140	49	1	50	53	1.36	8.4
PreCT_2	17-02-2015	4.4	110	58	2	40			6.6
PostCT_6 m	24-09-2015	3.52	10	50	4	50	60	1.4	0.6
PostCT_9 m	21-12-2015	6.32	30	37	3	60	44	1.29	1.8
PostCT_12 m	01-04-2016	4.1	55	74	1	25	55	1.67	3.3
PostCT_24 m	28-03-2017	3.69	70	60	5	35			4.2

Note: P, progressive motility; NP, non-progressive motility; I: immotile; MAI, multiple anomalies index. PreCT, pre-chemiotherapy semen samples; PostCT, post-chemiotherapy semen samples. _6 m, _9 m, _12 m, and _24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemiotherapy, respectively.
Abbreviation: NA, Not available.

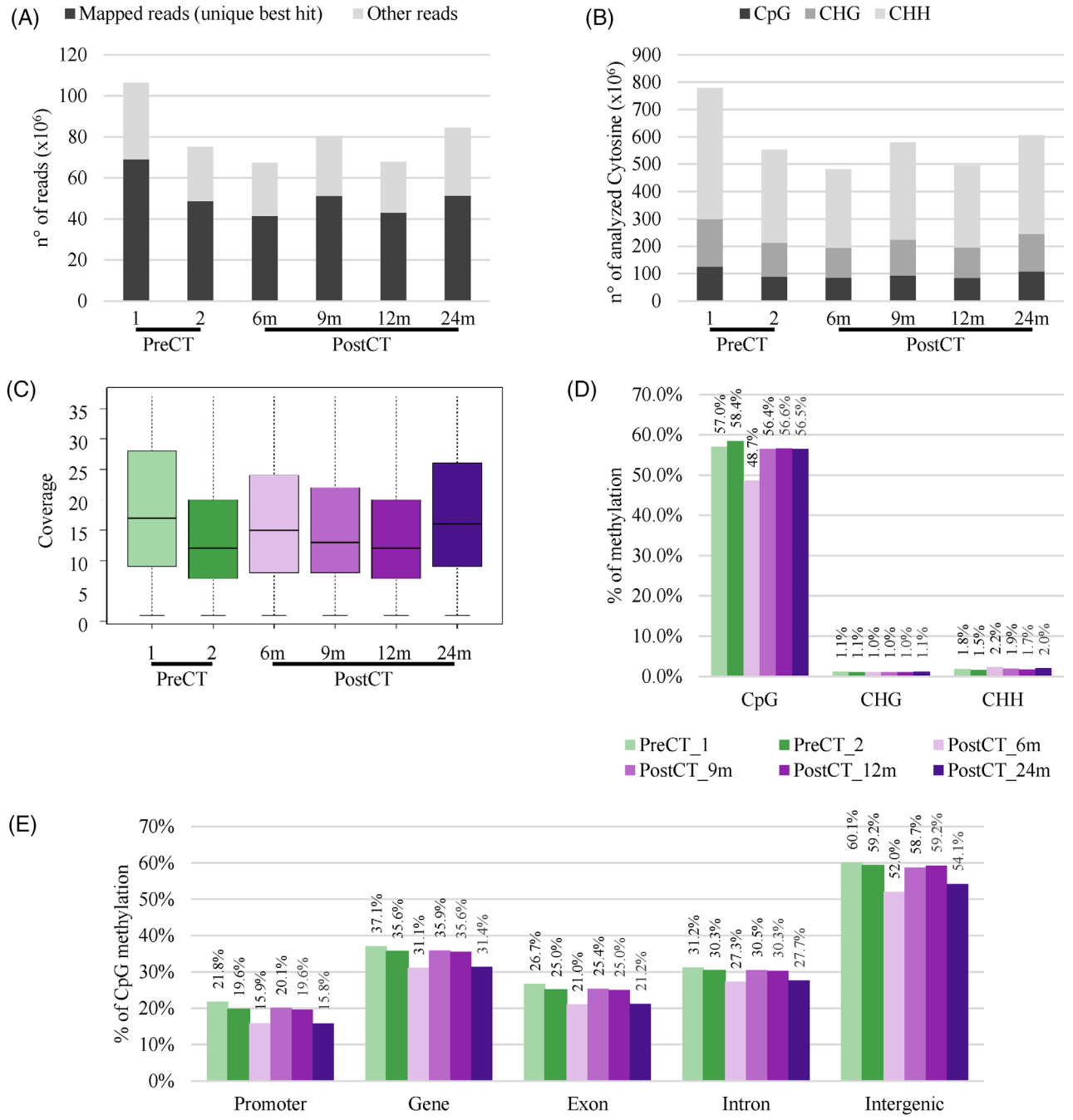

FIGURE 2 Read mapping and methylation statistics. (A) Read mapping statistics for each individual sample. (B) Sample-wise numbers of CpG (or CG), CHG and CHH (where H corresponds to A, T or C). (C) Sample-wise coverage distribution of CpG sites. Distribution of coverage across samples after coverage limitation (90% quantile). (D) Proportion of methylated CpG, CHG, and CHH for each sample. (E) Distribution of methylated CpGs among genomic features. A minimum depth of four reads is required for each CpG. CpG methylation levels are averaged by feature to produce feature-associated methylation levels. PreCT, pre-chemiotherapy semen samples (colored in green in panels C–E); PostCT, post-chemiotherapy semen samples (colored in purple in panels C–E). _6 m, _9 m, _12 m, and _24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemiotherapy, respectively.

It is important to note that only 1.1% of CHG and 1.5%–1.8% of CHH (where H corresponds to A, T, or C) were methylated, whereas 57.0% and 58.4% of all CpGs were methylated before a chemotherapeutic treatment (PreCT_1 and PreCT_2), respectively (Figure 2D). The global methylation level of CpGs decreased 6 months after the chemotherapy (48.7%) but returned close to the PreCT levels from 9 months onward (56.4%–56.6%) (Figure 2D). As the CpG methylation status of the human genome has become increasingly investigated, it has emerged that differential methylation is not only restricted to the CpG islands, but also extends to CpG regions, for example, at enhancers or across gene bodies.[44,45] We therefore executed the MethGO tool[38] to perform methylation calls for each type of genomic features, that is,

TABLE 2 Overview of read numbers and mapping statistics of the sperm samples.

Sample	Total read numbers	Alignement w/ unique best hit	Mapping efficiency	C analyzed	C (CpG) meth	C (CHG) meth	C (CHH) meth	C (CpG) unmeth	C (CHG) unmeth	C (CHH) unmeth
PreCT_1	106436954	69110632	64.90%	778705427	71413272	1903803	8479740	53875724	172542424	470490464
PreCT_2	75158930	48618089	64.70%	552997295	51832601	1308423	5258670	36857678	122796167	334943756
PostCT_6 m	67406100	41488376	61.50%	482585530	41350774	1144074	6476325	43595438	108378866	281640053
PostCT_9 m	80582196	51202989	63.50%	580087194	52524632	1358030	6677337	40526864	129292525	349707806
PostCT_12 m	67730924	43078958	63.60%	495981405	47051533	1179854	5009548	36070643	111305601	295364226
PostCT_24 m	84423184	51330408	60.80%	605277808	60471251	1500762	7229269	46628671	136892428	352555427

Note: PreCT, pre-chemiotherapy semen samples; PostCT, post-chemiotherapy semen samples. _6 m, _9 m, _12 m, and _24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemiotherapy, respectively.

for promoters, genes, exons, introns, and intergenic regions. A low but genome-wide hypomethylation of CpGs was observed at 6 months for all genomic features (Figure 2E).

3.3 | One hundred and seventy-nine genomic regions are associated with altered sperm DNA methylation profiles following chemotherapy

By performing all pairwise comparisons in BiSeq, we first identified 554 DMRs consisting of at least three CpG sites and covering at least 50 bp. Hierarchical clustering and principal component analysis based on these 554 DMRs revealed the proximity between the two PreCT samples and the PostCT_24 m and their clear difference with the PostCT_6 m, while PostCT_9 m and PostCT_12 m appeared in between (Figure 3). This would be suggestive of important methylation changes that occur early after the treatment followed by a progressive recovery toward a near normal methylation profile.

To further characterize chemotherapy-associated differential methylation of sperm DNA, we focused our analysis on the comparisons of PreCT samples with PostCT samples at each time point, that is, at month 6, 9, 12, or 24 after treatment (Figure S1A–D; Table 3). Altogether, these comparisons allowed us to identify 179 DMRs (FDR-adjusted p-value ≤ 0.05) associated with 102 differentially methylated genes (DMGs) following TGCT chemotherapy (Figure S1E; Table 3). Consistent with the above hypothesis, the number of DMRs was highest at 6 months. Importantly, while the number of DMRs also decreased from 9 months onward, 43 DMRs could be identified 24 months post-chemotherapy treatment. The functional analysis of the 102 DMGs did not reveal any enriched GO terms.

3.4 | Differential DNA methylation of thirteen genes in sperm persists up 24 months after chemotherapy

To capture the most direct and longitudinal effects of the chemotherapy on DNA methylation, we further focused on the DNA methylation changes occurring 6 months after chemotherapy (PreCT vs. PostCT_6 m samples). Among the 179 DMRs, 74 showed a differential methylation status between the PreCT and PostCT_6 m samples (16 hyper- and 58 hypo-methylated) associated with 49 DMGs (15 hyper- and 34 hypo-methylated) (Figure S1A; Table 3). We further sub-clustered these 74 DMRs according to their methylation profiles during the entire longitudinal study: six profiles could be identified, including three that were associated with hyper-methylation (P1-3) and three with hypo-methylation (P4-6) (Figure 4). Briefly, patterns P1 (5 DMRs; 3 DMGs) and P4 (40 DMRs; 21 DMGs) included DMRs that quickly went back to their pre-treatment methylation status just after 9th months onward. Patterns P2 (4 DMRs; 5 DMGs) and P5 (8 DMRs; 7 DMGs) corresponded to DMRs that slowly returned to their initial methylation status after 12/24 months. Finally, patterns P3 (7 DMRs; 7 DMGs including LRCOL1, PTPRS, MAP1S, PTPN23/SCAP,

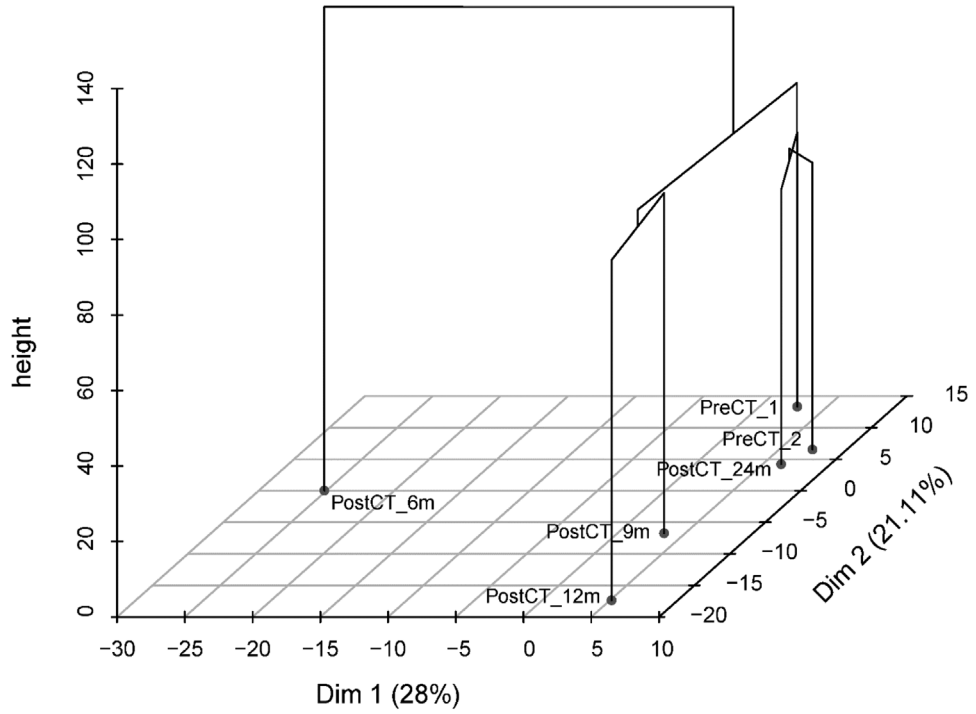

FIGURE 3 Hierarchical clustering analysis on principal components of the samples. This graph shows a combined plot of hierarchical clustering and principal component analysis (PCA) in a three-dimensional view. The two first axes of the PCA are based on the 554 DMRs. PreCT = pre-chemiotherapy semen samples; PostCT = post-chemiotherapy semen samples. _6 m, _9 m, _12 m, and _24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemiotherapy, respectively.

AFAP1-AS1/AFAP1) and P6 (10 DMRs; 6 DMGs including ALG10B, BCR/FBXW4P1, PDZD2, ELFN1/ELFN1-AS1) composed of hypermethyled (P3) and hypomethylated (P6) DMRs persisting up to 24 months post-chemotherapy.

3.5 | The altered sperm DNA methylation of two genes important for embryogenesis persists up to 24 months post-chemotherapy

To investigate whether DMRs and their related DMGs were associated with specific biological processes, we first performed a Gene Ontology term analysis. However, no significant enrichment was found. In a second step, we wondered whether some of the 15 hyper- and 34 hypo-methylated DMGs showing altered methylation status 6 months after chemotherapy could play roles during embryogenesis. To address this issue, we first filtered a list of 4,264 genes differentially expressed during the early development.[40] We also assembled a set of 969 genes known to be associated with embryo development based on their associated GO terms. By intersecting those two gene lists with the set of 49 selected DMGs, we were able to identify two genes differentially expressed during early embryogenesis (KMT2C and SCAP) (Figure S2) and five genes known to be associated with embryo development (BCR, GBX2, PTK2, SATB2, and SLC39A3) (Figure S3; Table 3). Among these genes, one DMR that mapped to one of the SCAP gene exons and one DMR that mapped to one of the BCR gene introns (into the core of the FBXW4P1 pseudogene) remained hypomethylated 24 months after chemotherapy (Figure 5A,B).

4 DISCUSSION

Enhancement of early detection and improved cancer treatment protocols over the last 20 years has increased the number of men surviving TC. As progress in adjuvant TGCT treatments in combination with surgery have made TGCT one of the most treatable of all cancers, the 5-year survival rate is above 90% today. However, projection into the future and the anticipation of adverse effects after treatment has become a major issue for these patients. Indeed, post-treatment quality of life is one of the most important criteria in the management of TC in young men as fertility is often impaired after chemotherapy and radiation therapy. Cryopreservation of sperm before cancer treatment is currently the only method to preserve male fertility. Discussion about fertility preservation before starting treatment is therefore needed. Male cancer survivors are less likely to father a child compared to the background population. This is influenced by cancer site, age of onset, and parity status at diagnosis.[46] Still, sperm parameters typically recover after treatment, even if this recovery is low. The evaluation of post-treatment paternity in testicular tumor survivors is indeed regularly described in the literature. For instance, a retrospective and multicenter study between 1980 and 1994 in Norway involved 1814 men, regardless of treatment.[47] In this survey, 71% of patients

TABLE 3 Overview of differentially methylated regions (DMRs) and associated differentially methylated genes (DMGs).

Contrasts	PreCT vs PostCT (all)	PreCT vs PostCT_6 m		PreCT vs PostCT_9 m		PreCT vs PostCT_12 m		PreCT vs PostCT_24 m	
Methylation status	All	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo
n° DMRs (n° non-overlapping DMRs)	179 (104)	16 (13)	58 (34)	18 (14)	37 (20)	31 (16)	20 (16)	31 (15)	12 (9)
n° DMGs	102	15	34	13	21	13	26	15	10
Expr. in early embryo dev. (4264 genes)	3	SCAP	KMT2C	None	SH2D4B	None	SH2D4B	SCAP	None
Associated with embryo dev. (968 genes)	6	GBX2, PTK2	BCR, SATB2, SLC39A3	GBX2	SATB2	None	HPN, BCR	None	None
Imprinted genes (91)	None	None	None	None	None	None	None	None	None

Note: PreCT = pre-chemiotherapy semen samples; PostCT = post-chemiotherapy semen samples. _6 m, _9 m, _12 m, and _24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemiotherapy, respectively.

procreated without the use of sperm straws, while only 6.3% of patients used sperm straws. Furthermore, among men who procreated without the use of sperm straws, 22% used assisted reproductive technology (ART). In another study by Huyghe et al.,[48] 67% of patients with TC became fathers after treatment without the use of ART.

Here, we investigated the impact of a BEP therapy on the sperm epigenome of a TGCT patient. Our longitudinal study included the use of pre-treatment samples and their comparisons to those recovered from the same patient up to 24 months after the treatment. As compared with most retrospective studies using sperm from fertile men as a control group, our experimental design should lower the possibility to identify methylation changes related to the cancer status rather than from the treatment itself. Still, it is important to remember that the DNA methylation pattern of samples before chemotherapy cannot be considered per se as the 'normal' physiological DNA methylation status since the testicular tumor itself can affect sperm DNA methylation. Furthermore, it cannot be completely ruled out that some of the changes we observed, especially those showing a persistent methylation alteration 2 years after chemotherapy, could be the result of an evolution of the cancer, or of a subsequent disruption of the gonadal environment of the patient. It was indeed demonstrated that TC patients treated with 2–4 cycles of BEP displayed increased follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels for at least 24 months.[16] Epigenetic changes related to the environment are well known in animal models. Environmental factors can indeed induce transgenerational inheritable DNA methylation changes in mammalian germline.[49,50] As the patient made no major changes in his personal way of life, we assumed that most sperm parameter variations, including methylation changes, could be attributable to chemotherapy. Following four cycles of BEP, the patient's sperm count drastically decreased at month 6 and progressively increased from 9 months onward, albeit at lower levels than before treatment. This is consistent with a previous study on 129 volunteer TGCT patients and a control group of 257 fertile men that showed a decrease of sperm characteristics after BEP treatment, with lowest values at 3 and 6 months.[10] In the latter study, sperm count indeed recovered to pre-treatment values at 12 months after treatment after two or fewer BEP cycles, but not after radiotherapy or more than two BEP cycles.

Sperm cells are particular cells able to transmit information from one generation to the next. Any alteration of the epigenome will be transmitted to the zygote and then to the embryonic cells. Hence, gamete exposure has consequences on epigenetic markers and may interfere with the inheritance of specific features to the offspring. Several players implicated in this non-genetic inheritance have been described so far: DNA and RNA methylation, histone modifications, non-coding RNAs and extracellular vesicles.[51] DNA methylation regulates gene expression and genome activity without modification of the coding sequence. DNA methylation is technically easy to investigate and therefore the most documented epigenetic mark. DNA methylation involves methyl groups that are attached to a DNA molecule. While BEP has been reported to affect sperm chromosomes and to alter DNA status,[10,15,21,54] to the best of our knowledge no study specifically investigated its impact on DNA methylation. In the

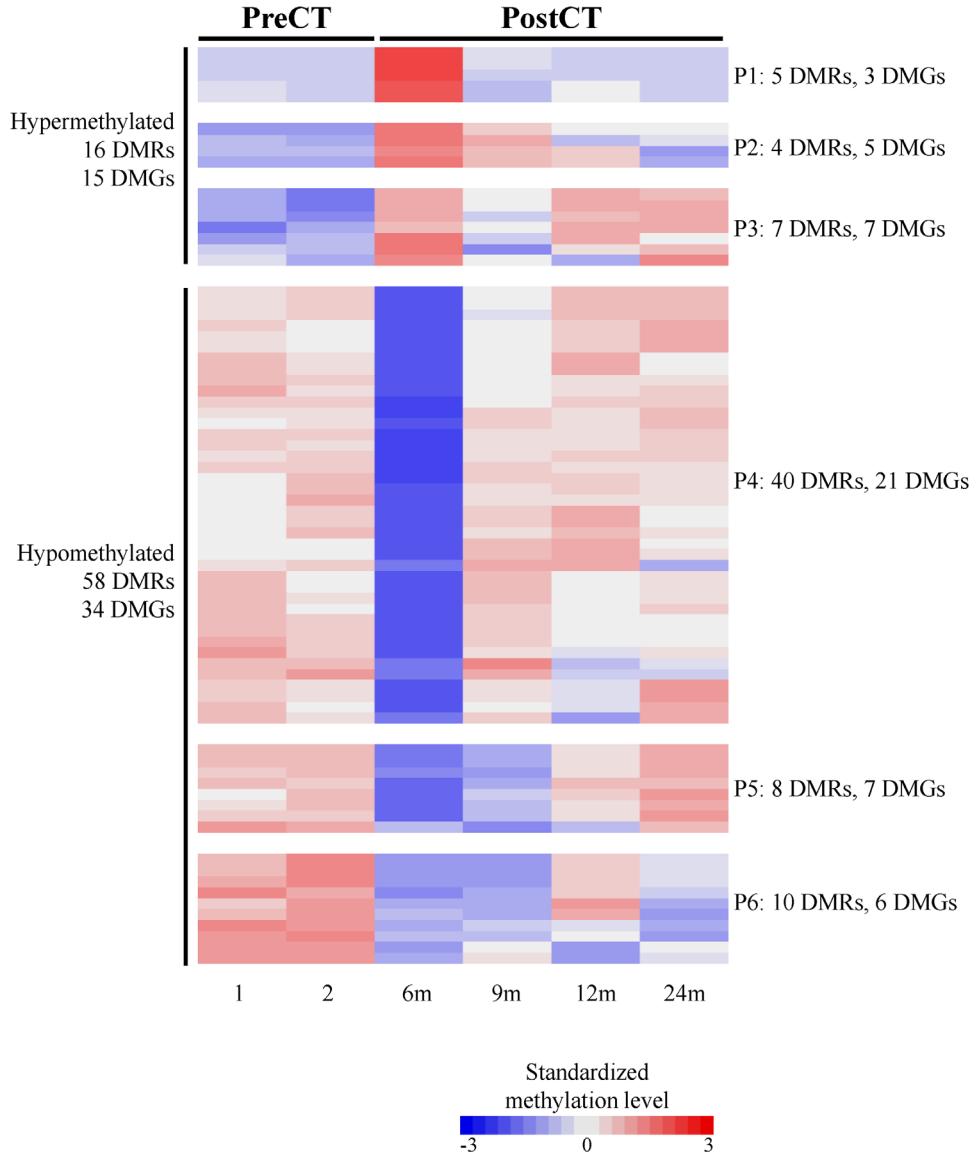

FIGURE 4 Methylation profiles of DMRs showing altered methylation 6 months after chemotheray. A false-color heatmap shows the standardized methylation level of 74 DMRs organized in six methylation patterns (P1–P6). Each line corresponds to a DMRs and each column to a sample. The number of DMRs and related DMGs are given for each of the six resulting methylation patterns on the right. Standardized methylation levels are displayed according to a color code ranging from blue (hypomethylation) to red (hypermethylation). PreCT, pre-chemiotherapy semen samples; PostCT, post-chemiotherapy semen samples. 6 m, 9 m, 12_m, and 24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemiotherapy, respectively.

current study, the most important methylation changes were observed 6 months after chemotherapy: these were mainly associated with a demethylation of CpG islands which returned to normal after 24 months for most of them. Among the 49 DMGs associated with the 74 DMRs at 6 months, we identified two genes expressed during early embryonic stages (KMT2C and SCAP) and five genes known to be involved in the embryo development (BCR, GBX2, PTK2, SATB2, SLC39A3). Altering the methylation status of such genes might modify their expression pattern and thus affect key stages of embryogenesis and lead to potential developmental disorders or miscarriages.[52–54] We also highlighted 13 DMGs associated with 17 DMRs that show a persistent methylation alteration even 24 months after chemotherapy. Although these genes are not necessarily involved in early development their altered methylation patterns could be passed on to the offspring and eventually have an impact at a later stage.

One of the two DMGs expressed during early embryonic stages is KMT2C (Lysine Methyltransferase 2C), a member of the mixed-lineage leukemia (MLL) family encoding a histone methyltransferase that regulates gene transcription by modifying chromatin structure. It is interesting to note that its loss decouples enhancer H3K4 monomethylation, H3K27 acetylation, and gene activation during embryonic stem cell differentiation.[55] KMT2C mediates mono and tri-methylation of histone H3 at lysine 4. It has been shown that loss of KMT2C causes defects in lung maturation in KO mice, leading to immediate death

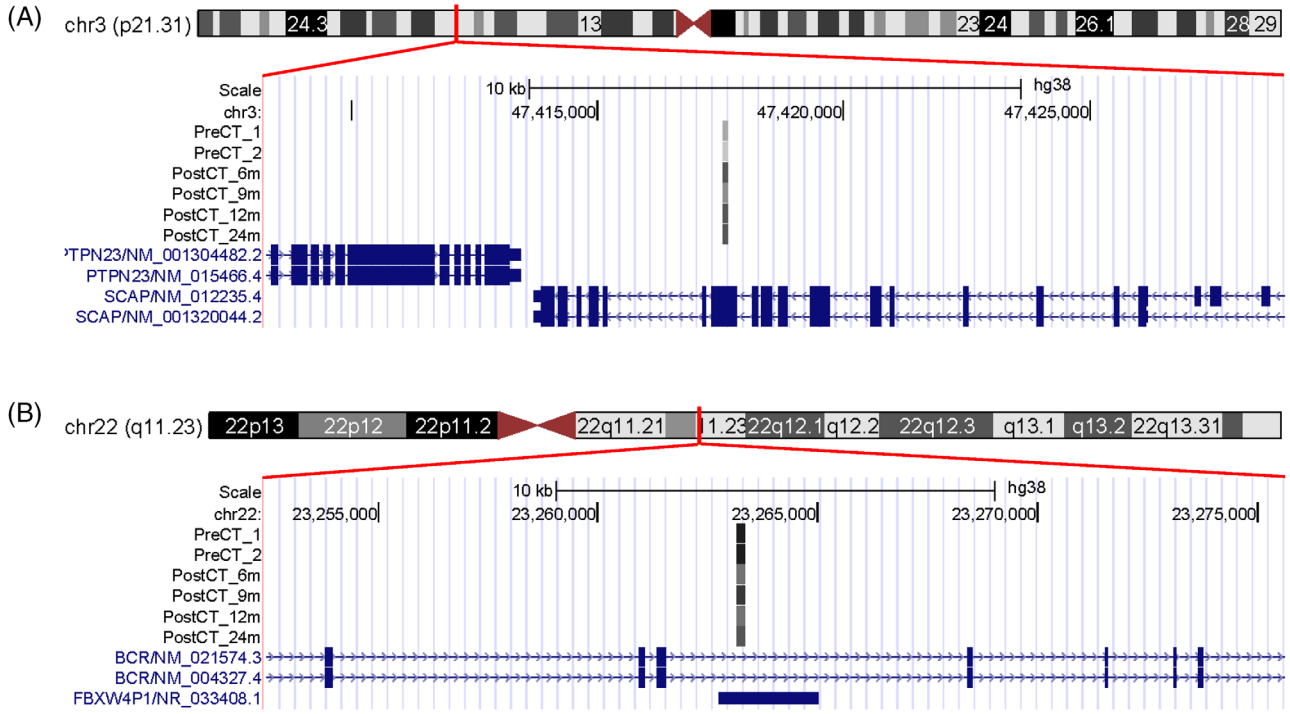

FIGURE 5 Sustained methylation alterations of the DMRs associated with SCAP and BCR. A screen capture of the UCSC genome viewer showing the methylation level of DMRs associated with SCAP (panel A, hypermethylated) and BCR (panel B, hypomethylated) in each sample, based on a color code ranging from white (0% of methylation) to black (100% of methylation). Corresponding transcript structures, including exons and introns, are shown at the bottom in blue. PreCT, pre-chemiotherapy semen samples; PostCT, post-chemiotherapy semen samples. _6 m, _9 m, _12 m, and _24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemiotherapy, respectively.

after birth.[56] The encoded methyltransferase is also implicated in early embryogenesis and spermatogenesis by setting methylation marks in nucleosomes.[57] Male mice lacking a fully functional enzyme showed reduced fertility.[58] Genome-wide association study (GWAS) linked non-synonymous single-nucleotide polymorphisms (SNPs) in KMT2C gene to fertility variation between fertile and infertile men.[59] The second DMG expressed during early embryonic stages is SCAP (SREBF chaperone) which encodes a Sterol regulatory element-binding protein (SREBP) cleavage-activating protein. In cholesterol-depleted cells, SCAP binds to SREBPs and mediates their transport from the endoplasmic reticulum to the Golgi apparatus. SREBPs are then proteolytically cleaved and stimulate sterol biosynthesis. It is well documented that impaired sterol synthesis results in developmental defects in humans and animals. Lipid homeostasis should be critical for normal development, because over regulation of cholesterol and phospholipid synthesis is required to satisfy the demand for membrane biogenesis during periods of rapid cell proliferation.[60] Interestingly, long-term dysregulation of SCAP/SREBP signaling was reported as a common feature in mice born from assisted reproductive technologies.[61]

Among DMGs involved in the embryo development, the Breakpoint Cluster Region protein (BCR) is one of the two genes in the BCR–ABL complex, which is associated with the Philadelphia chromosome. While the BCR gene product harbors a serine/threonine kinase activity, its function is not clear. The literature is rich on the association between BCR–ABL and oncohematological pathologies, but little is known about its role during embryonic development. BCR contributes to cell division and may play a role during early embryonic development or spermatogenesis as it is expressed by preimplantatory embryos in bovine[62] and is differently methylated in sperm of subfertile bulls leading to altered gene expression.[63] BCR–ABL gene product appears to have a lethal effect during this period of development by introducing the BCR gene into mouse embryos.[64] Gastrulation Brain Homeobox 2 (GBX2) is a homeobox gene involved in the normal development of rhombomeres 1–3 which is the mid/hindbrain region. This gene is a dosage-dependent transcription factor involved in the regulation of proper expression of other genes. GBX2 expression occurs during gastrulation and continues to be expressed in the late stages of embryogenesis.[65] GBX2 inhibits the expression of LHX9, which is a LIM/homeobox protein and plays a role in the gonadal development. Moreover, GBX2 is involved in pluripotency and may be an actor of iPSC.[66] Methylation of GBX2 mediates the effect of the lifestyle intervention in pregnant women with obesity on lean mass in the offspring and early growth.[67] The PTK2 gene encodes a cytosolic protein tyrosine kinase, that is, found concentrated in the focal adhesions that form among cells attaching to extracellular matrix constituents. The encoded protein is a member of the FAK family which plays a regulatory role in modulation of cellular proliferation, protection from apoptosis, adhesion, spreading and migration and is important for the development of the placenta, as well as of several organ systems, like the musculoskeletal, nervous, cardiovascular, genitourinary, and respiratory organ systems.[68] Additionally, FAK has been shown to be implicated in the pathophysiology of pregnancy-related

disorders and congenital neonatal diseases and defects.[69] The special AT-rich sequence-binding protein 2 (SATB2) encodes a DNA-binding protein that specifically binds nuclear matrix attachment regions and is involved in transcriptional regulation and chromatin remodeling. Defects in human SATB2 cause cognitive deficits, craniofacial dysmorphism, behavioral changes, and osteoporosis.[70] Finally, the SLC39A3 gene encodes a Zinc transporter ZIP3 protein for which physiological functions remain unknown. A study has reported that embryos developed abnormally in ZIP1, ZIP3 double-knockout mice if dietary zinc was limited during pregnancy.[71] SLC39A3 may be involved in psychiatric phenotypes and has been associated with bipolar disorders in humans.[72]

It is important to notice that our analysis did not find any methylation changes on the genes subjected to parental imprint (Table 3). Any failure to establish correct germline-specific DNA methylation patterns will have serious consequences for post-fertilization development, primarily due to the necessity for epigenetic marking of genomic imprints. Genomic imprinting refers to the epigenetic mechanism that results in the parent-of-origin monoallelic expression of autosomal genes.[73] It is a particularly unique epigenetic mechanism, since an individual somatic cell has both active and repressed alleles of the same gene. Imprinted DMRs are established in the male and female gametes. It was reported that the promoters of many key genes involved in the early embryonic development are hypomethylated in mature sperm.[74] These observations support the hypothesis that paternal genome methylation pattern could influence embryonic development through regulation of developmental gene expression.[75] Together with the fact that most of the methylation alterations regained the initial state of pre-chemotherapy, our results are rather reassuring for any project of children for the current patient. It would be necessary however to confirm our observations with other patients. Notably, our data are insufficient to conclude on whether the sperm quality of patients treated for TC is similar to that of untreated men and further studies are also needed to investigate the impact of such epigenetic modifications on embryogenesis. A statistically significant but modest increase in the risk of major congenital abnormalities among offspring of males with a history of cancer, independent of the mode of conception has been observed.[76] The increase in prevalence of birth anomalies among children of fathers with malignancy might be due to cancer per se or a common underlying paternal factor, for example, genomic instability.[77] On the other hand, no additional increased risk of congenital malformations was observed in children of men with testicular germ-cell cancer treated with radio- or chemotherapy, reassuring concerned patients.[78] However, a close follow-up of children could be important in the long term since too few studies have sought to identify the genetic and/or malformation risks of children born using the spermatozoa of cancer survivors. In this context, patients should receive information on the potentially increased risk of sperm DNA damage before and after gonadotoxic therapy while the development of less gonadotoxic protocols in the management of cancers should be studied to improve the post-cancer quality of life of patients.

AUTHOR CONTRIBUTIONS

Frédéric Chalmel and Célia Ravel supervised the research and designed the study. Anne-Sophie Neyroud, Célia Ravel and Frédéric Chalmel wrote the manuscript. Bertrand Evrard prepared the samples. Frédéric Chalmel, Antoine D. Rolland, and Nathan Alary prepared, analyzed, and interpreted data. Anne-Sophie Neyroud, Louis Bujan and Célia Ravel interpreted data. Nathalie Dejucq-Rainsford, Louis Bujan, Nathan Alary, and Antoine D. Rolland contributed to the manuscript. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in ReproGenomics Viewer at https://rgv.genouest.org/.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Metabolic characterization of human sperm cells using the Seahorse metabolic flux analyzer

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Abstract

Background: The concerning trend on male infertility global prevalence, together with the unexplainable causes in half of those cases, highlights that there are still aspects of this disease to be understood and solved. To address this issue, one should not only be aware of the limitations of the implemented diagnostic tools, but also understand the sperm cell in depth, structurally, biochemically, molecularly in order to develop reliable and ready-to-be new/improved diagnostic tools. In this sense, the sperm cells metabolism, highly related to its functionality, seems to be a promising aspect to explore. Though there is much information on the human sperm metabolism, there is still a lack of a quick integrated and comprehensive analysis that may be introduced with the potential to reveal innovative clinically relevant information.

Objectives: Find metabolic details on human sperm that can be accessed easily, in real time and using few cells, relying on the bivalent potential of the Seahorse flux analyzer (SFA).

Results: We have obtained standard records on human sperm cells' oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), that together with the metabolic metrics provided information on sperm cells' oxidative and glycolytic metabolism. Furthermore, a metabolic interindividual variation was observed.

Discussion and conclusion: Although the comparison with other species or cell types is not linear and warrant further studies, the metabolic profile of human sperm cells seems to be similar to that of other species. Altogether our results corroborate the value of SFA for metabolic human sperm cell analysis, warranting new studies, and anticipating several applications in the male infertility field.

KEYWORDS
human sperm cell, infertility, metabolism, Seahorse metabolic flux analyzer

1 | INTRODUCTION

Recent estimates point to a global incidence of infertility of 8%—12% among aged-reproductive couples,[1–4] a scenario that is likely to worsen in the upcoming years. Male infertility alone accounts for 20%—30% of this panorama, while when associated with the female factor, this contribution increases to 50%.[5]

Although male infertility causes have been extensively studied,[6,7] in nearly 50% of the cases no apparent cause can be identified, the so-called male infertility of unknown origin.[3,5–7]

In terms of diagnosis, when a couple seeks medical help to conceive, several exams are performed to assess the possible existence of problems that might justify their infertility state.[8–11] On the male side, this usually involves a workup starting by the medical history followed by a general physical examination, endocrine characterization, and seminal analysis. The latter is performed to assess sperm quality,[12–15] according to strict guidelines, that established that a sample may only be considered normal (normozoospermic) if several reference limits are met.[16] Even though this analysis is globally a reference for assessing male fertility status, being easy and cost-effective, there are growing concerns regarding its value on the prediction of the exact cause of infertility and pregnancy likelihood.[6,14,17] In fact, sperm cells' function and fertilization potential depend on several other features that are not frequently assessed in the routine seminal analysis.

The limitations of the seminal analysis emphasize the need to undertake a broader and more integrated analysis that will ideally include the evaluation of other sperm parameters, based on more reliable and essential functional aspects using new/improved methodological approaches that enable their proper assessment.

This approach may elucidate and shed light on still unknown molecular mechanisms that might help to explain unknown origin male infertility.

One important aspect of sperm function that could be more factored in terms of possible analysis/diagnostics is metabolism. Metabolically speaking, the sperm is a very versatile cell, and despite the debate on which is the preferred pathway to energy attainment [glycolysis vs. Oxidative phosphorylation (OXPHOS)][18,19]; greatly fomented by the compartmentalization of the two main adenosine triphosphate (ATP) - producing pathways in the mid and principal piece of the sperm cells,[19–21] the most recent studies support a wide versatility of these cells at a metabolic level.

In fact, alternative and unexpected metabolic pathways were recently described in human sperm, as is the case of fatty acids oxidation, glycerol, and ketone bodies catabolism.[19] Making a (very) long story short, the sperm cell is able to use the most convenient fuels and metabolic pathways depending on the available substrates determined by the circumstances and surrounding environment, namely the one in the female reproductive tract.[18,22–24]

There are several methods to infer on the metabolic state of a cell, ranging from the evaluation of the activity of specific metabolic enzymes, or the quantification of medium and intracellular metabolites, to the use of fluorescent probes directed to a specific mitochondrial or metabolic trait. Regarding metabolic enzymes, usually the approaches include the evaluation of their activity or levels, by spectrophotometry[25] immunocytochemistry,[26] Western blot or proteomic approaches,[27,28] while the metabolites levels are frequently assessed by nuclear magnetic resonance (NMR)[23,29] or by spectrophotometry, as is the case for reactive oxygen metabolites quantification in serum.[30] Among the fluorescent probes, *Mito Tracker green* (MTG[31]) and *JC-1*,[31] that provide information on mitochondrial content and membrane potential (MMP[32]), respectively, or dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) and *Mitosox Red*, that measure reactive oxygen species (ROS) production, intimately related to oxidative stress and indirectly to infertility,[33] are commonly used. However, although these methods are very useful, they do not provide integrated information on cellular metabolism. More recently, some promising technologies have been developed with the specific aim of evaluating different aspects of metabolic function, based on dynamic and real-time analysis and relying on minimal amount of samples. For example, *Oroboros*, which allows real-time measurement of mitochondrial respiratory kinetics in sperm using an improved version of the former Clark oxygen electrodes,[34] yet providing no relevant information regarding other metabolic processes.[35] On the other hand, *Myoxsys*, specifically designed for male infertility assessment in a clinical setting, is an apparently robust tool that, assessing the balance between oxidants and antioxidants, provides a measure of oxidative stress, that might be related to the men's infertility status. Yet, besides the fact of being performed on seminal fluid (and not on sperm cells), it provides no relevant metabolic information.[36] Finally, the recent *Seahorse Flux Analyzer* (SFA), a high throughput technology was designed to study energy metabolism, by measuring the immediate surrounding environment of the sampled cells in real time. SFA specifically measures the oxygen consumption rate (OCR) and/or extracellular acidification rate (ECAR), indirect readouts of the two main metabolic pathways for energy metabolism and ATP production (mitochondrial respiration and glycolysis, respectively), essentially relying on drug injection strategies at specific stages and allowing to assess how the cells' metabolism responds. The measurement of OCR also provides information on basal respiration, ATP-linked respiration, proton leak, maximal respiration capacity, and non-mitochondrial respiration of the cells. On the other hand, ECAR provides indirect information on glycolysis, due to the acidic nature of the glycolytic resulting products (pyruvate or lactate), also allowing to collect information regarding the glycolytic reserve capacity and non-glycolytic acidification. Due to its versatility and considering the low amount of sperm sample needed, that will be crucial in a clinical setting, together with the amount of information provided, SFA seems to be an ideal candidate to analyze human sperm energy metabolism. Yet, although this technology has been described in mouse sperm,[37,38] there were no studies in humans until March 2022, when a publication came out, but yet only focusing on the OCR and using a reduced number of samples,[39] warranting further studies. It is thus our objective, resorting to SFA, to sistematically chacacterize human sperm metabolism establishing a method that may eventually be of use to improve diagnosis and treatments in an infertility context.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Unless stated otherwise, all chemicals and reagents were from Sigma–Aldrich.

2.2 | Human biological material

Human semen samples were provided by the Reproductive Medicine Unit of the University Hospitals of Coimbra (CHUC; Coimbra, Portugal) from healthy men who were undergoing routine semen analysis for fertility treatment in which the male factor was excluded.

All the donors signed informed consents and the biological material was used in accordance with the proper ethical and Internal Review Board (IRB) guidelines provided by CHUC.

Semen samples were obtained after a period of 3–5 days of sexual abstinence by masturbation. Seminal analysis was performed according to the World Health Organization (WHO) guidelines[16] and normozoospermic samples for concentration, motility, and viability were used, with no visible leukocytes or other round cells.

2.3 | Samples preparation

After liquefaction, sperm cells were isolated by density-gradient centrifugation (SupraSperm; Medicult-Origio) and allowed to capacitate in sperm preparation medium (SPM; Medicult-Origio) for at least 3 h at 37°C and 5% CO_2 before initial experiments were performed, as previously described.[40] Sperm samples were then assessed for concentration, motility, and viability.

2.4 | Concentration, motility, and viability evaluation

These parameters were assessed according to WHO (2010) recommendations, and examined by bright field optical microscopy for the former and phase-contrast optical microscopy for the latter two (Leica DB400B; Leica microsystems). Concentration of each sample was assessed after spermatozoa immobilization by osmotic shock with a Neubauer chamber. Motility was rated in three different categories, progressive, non-progressive, and immotile and the results were expressed as the percentage of motile spermatozoa, that entails progressive plus non-progressive motility. Finally, viability was evaluated using the eosin exclusion assay, in which 5 μL of sample was mixed with 5 μL of 0.5% (w/v) Eosin Y in a microscopic slide. This assay is based on the membrane integrity which means that eosin will only enter sperm cells that have compromised membranes, that are non-viable cells, and will, therefore, present pink-reddish heads in contrast with the viable cells that will present white heads. For motility and viability, a total of 100 spermatozoa were evaluated in different fields.[40]

2.5 | Seahorse flux analyzer set up

The Seahorse XF24 Analyzer (Agilent) was used to monitor OCR and ECAR, to infer on the mitochondrial bioenergetic status and glycolytic function, respectively. Once concentration was determined, the needed volume of sperm cells was calculated. The adequate cell amount for optimal response was established as 4×10^6 cell/well for an SFA model XFe24. Sperm samples were divided into two aliquots and centrifuged at 300×*g* for 5 min. The resulting pellets were then resuspended in the two different assay media: (1) **OXPHOS+BSA medium- OBm** (PBS, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5.0 mM D-glucose, 1.0 mM sodium pyruvate, 10 mM sodium lactate, 3 g/dm^3 BSA, and 2 mM L-glutamine, pH 7.2) for OCR assay, and (2) **ECAR medium- ECm** (PBS, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 2 mM L-glutamine, pH 7.2) for ECAR assay.

Samples were then rinsed twice (300 g, 5 min) using the respective medium. For the assay, 450 μL of the suspensions were plated per well in a Seahorse 24-well culture microplate (Agilent), coated with 0.5 mg/mL Concanavalin A (ConA) in either (1) **OBm** for OCR assay, and (2) **ECm** for ECAR assay. To ensure that the wells were evenly and thinly coated, the plate was only incubated with ConA for 30 min and the excess polymer per well was discarded prior to the addition of the sperm samples. After, the loaded plate was centrifuged at 200×*g* for 1 min, rotated 180°, centrifuged again and then incubated for 1 h at 37°C in a non-CO_2 incubator. This procedure allows the sperm heads to be firmly attached to the bottom of the well, while the tails are freely moving. For every sample, after centrifugation, each well was observed under the microscope to confirm that there were no sperm cells detached and that they were uniformly attached to the bottom of the wells. In this way, we can be positive that the redout was exactly from 4 million cells, excluding the need of further counting and additional enzymatic procedures.

Prior to the assay working stocks were prepared as follows: **ECAR**: 50 mM glucose, 10 μM oligomycin, and 100 mM 2DG; **OCR**: 10 μM oligomycin, 5 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), 10 μM Antimycin A, and 10 μM Rotenone. To measure glycolytic function, glucose (5 mM), oligomycin (1 μM), and 2-deoxyglucose (2DG; 100 mM) were injected after measurements 3, 6, and 9, respectively. To assess the mitochondrial function, oligomycin (1 μM), FCCP (0.5 μM) and rotenone + antimycin A (1 μM each) were injected after measurements 3, 6, and 9, respectively. Blank ConA-coated wells in the four corner positions of the plate, incubated with media without cells, were used for blank control measurements, as suggested by the manufacturer, to account for the plate position-dependant variation.

OCR and ECAR were then monitored in real time, according to the manufacturer's standard protocol with slight modifications.

2.6 | Data analysis and metrics calculation

2.6.1 | Wave Software settings

SFA assay was performed using 'WAVE' software [39] with slight modifications (shown in Table 1) to allow an optimal response. Furthermore, the option 'Equilibrate' was deselected, as this step of the protocol implies 12 min of homogenization in the well (3 loops of 2 min each of mixing and waiting), a tribulation that resulted in a lot of detaching cells with broken tails, as we observed in our microscopic evaluation.

TABLE 1 Adjustments made to wave standard protocol for sperm samples.

Stage	Standard (min)	Adapted (min)
Mix	3	1,5
Wait	2	0.5
Measure	3	3

After the final records were obtained, the results were normalized for 1 million cells.

2.6.2 | Metabolic metrics

The measurement of OCR allows us to further calculate several useful metrics such as the basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity (also shown as fold-increase), non-mitochondrial respiration and coupling efficiency (OCR-derived metrics; Table 2; https://shorturl.at/ijkpN).

Similar to the OCR, the measurement of ECAR provides indirect information on glycolysis, due to the acidic nature of the glycolytic resulting products (pyruvate or lactate), also allowing to calculate other functionally-relevant metrics (Table 2) such as on glycolysis itself, glycolytic capacity, the glycolytic reserve capacity (and also shown as fold increase—the ratio and finally non-glycolytic acidification https://shorturl.at/ijkpN. All the metrics were calculated using the average of the three time-point measurements taken after/before the injection of the appropriate substances.

2.7 | Statistical analysis

Statistical analysis was performed using the SPSS Software for Windows version 27.0 (SPSS, Inc.). All the variables were evaluated for

TABLE 2 OCR and ECAR-derived metabolic metrics (for details: Https://shorturl.at/ijkpN).

OCR metrics	Calculation	Bioenergetic meaning
Basal respiration	Measurement prior to oligomycin addition—non mitochondrial respiration	Reveals the minimal rate of metabolism needed to support basic cellular functions
Proton leak	Measurement after oligomycin injection through measurement prior to FCCP—non mitochondrial respiration	Mirrors the natural leakage of protons across the mitochondrial inner membrane, in favor of gradient, independently of complex V activity
ATP production	Basal OCR—proton leak	Provides an indication/prevision on the respiration that was being used to drive ATP production under basal conditions
Maximal respiration	Measurement after oligomycin through measurement prior to rotenone and antimycin	Corresponds to the highest rate that mitochondria can work
Spare respiratory capacity	Maximal respiration—Basal respiration	Important indicator on the reserve capacity of the organelle to deal with a scenario of energetic crisis
Spare respiratory capacity (as fold increase)	Maximal respiration/basal respiration	Expresses how close to its maximal capacity is the electron transport chain working during basal respiration
Non-mitochondrial respiration	Measurement after addition of rotenone and oligomycin through the end of the assay	Refers to the oxygen consumption that is not dependent of the mitochondria, but instead of other cytoplasmatic enzymes that also consume oxygen (e.g., membrane NAPDH oxidase)
Coupling efficiency	ATP production/basal respiration *100	The coupling efficiency provides information on the proportion of basal respiration that is used to drive ATP Synthesis. Being therefore a readout on oxidative phosphorylation efficiency.
ECAR metrics	**Calculation**	**Bioenergetic meaning**
Glycolysis	Measurement after glucose injection through measurement prior to oligomycin injection—measurement prior to glucose injection	Indication of the contribution of the breakdown of glucose to pyruvate to the ECAR increase
Glycolytic capacity	First measurement after oligomycin injection through measurement prior to 2-deoxyglucose injection-measurement prior to glucose injection	Measurement of how cells respond to metabolic demand after the inhibition of mitochondrial ATP synthesis.
Glycolytic reserve capacity	Glycolytic capacity—glycolysis	Mirrors the needed increase in glycolysis to meet cellular energetic requirements without mitochondrial ATP production. Indicative of the reserve capacity
Glycolytic reserve capacity (as fold increase)	Glycolytic capacity/ glycolysis	Indicative of the reserve capacity
Non-glycolytic acidification	Measurement prior to glucose injection	A measurement in ECAR increase not due to glucose metabolism.

Note: ECAR, extracellular acidification rate; OCR, oxygen consumption rate.

TABLE 3 Human sperm samples characterization according to WHO guidelines.

	Assay			Motility (%)			Viability (%)		Morphology (%)	
Sample	OCR	ECAR	Concentration (cells/mL)	Progressive	In situ	Immotile	Live	Dead	Normal	Abnormal
N1	X		80×10^6	62	19	19	84	16	5	95
N2	X		59×10^6	66	11	23	83	17	4	96
N3	X	X	255×10^6	75	14	11	86	14	1	99
N4	X	X	102.5×10^6	67	18	15	92	8	7	93
N5	X	X	122.5×10^6	34	36	30	77	23	3	97
N6	X		137.5×10^6	73	8	19	81	19	0	100
N7	X		49.5×10^6	68	4	28	82	18	4	96
N8	X	X	123×10^6	68	2	30	85	15	5	95
N9	X	X	97×10^6	43	1	56	45	55	0	100
N10		X	30×10^6	77	8	15	87	14	3	97
N11		X	137.5×10^6	59	6	35	80	20	3	97
N12		X	26×10^6	63	10	27	78	22	2	98
N13		X	107×10^6	87	3	10	81	11	5	95
N14		X	157.5×10^6	85	5	10	89	11	4	96
N15	X		82×10^6	75	11	14	82	18	0	100
N16	X		149×10^6	66	2	32	72	28	3	97

normal distribution by the Shapiro–Wilk test and the homogeneity of variances was assessed by Levene's test. Accordingly, correlations were obtained through the Spearman test.

Statistical comparisons between the created subgroups were performed using a *t*-test or a related Mann–Whitney *U*-test. Values of $P \leq 0.05$ were considered significant. Results are presented as means ± standard error mean (SEM) and the number of experiments is indicated. The interindividual variation was assessed by the coefficient of variation test (https://real-statistics.com/students-t-distribution/coefficient-of-variation-testing/; Accessed on 16 May 2023).

3 | Results

3.1 | Human sperm samples characterization

As previously mentioned, to ensure consistency in terms of standardizing the assay, only normozoospermic samples were used in this study, with all the parameters measured above the reference values established by the WHO, except for morphology, where nine samples did not meet the normality[16] (Table 3).

3.2 | OCR assay

To assess the oxidative metabolism of the human sperm cells, several drugs, namely oligomycin, FCCP, Antimycin A, and Rotenone, were injected. These drugs, injected by that order, have resulted in typical responses as follows: oligomycin by inhibiting the ATP synthase, reduced oxygen consumption by halting oxidative phosphorylation while FCCP as an uncoupler of oxidative phosphorylation, increased OCR to a maximal value. Finally, Antimycin A and Rotenone by inhibiting electron transport chain (ETC) complex III and I, respectively, and blocking ETC workflow resulted in a compromised OCR (Figures 1A and 2A).

Although some interindividual variation was detected, typical of human sperm samples (Figure 2A–I, Table 4), the records were still consistent and reliable.

The calculated OCR metrics of our cells indicate that they are bioenergetically healthy and efficient as the maximal respiration indicates that the cells can respond well to energetic challenges. Yet, the proton leak is unexpectedly higher when compared to other cells,[62–65] a situation also reflected in the predicted ATP production and coupling efficiency (Figures 1B,C,2B–I, Table 2).

3.3 | ECAR assay

During ECAR assay, designed to assess the glycolytic metabolism, we have obtained typical responses, namely an increase in ECAR after the injection of glucose, due to the production of pyruvate/lactate. Also, following the injection of oligomycin and due to the metabolic pressure upon glycolysis to compensate for the lack of mitochondria's ATP production, a further increase in ECAR was observed. Interestingly, the human sperm response to oligomycin was observed to be not very expressive. Finally, the injection of 2DG, halted the glycolysis pathway as it blocks hexokinase enzyme (first enzyme of glycolysis pathway), resulting in substantial decrease of ECAR (Figures 3A and 4A).

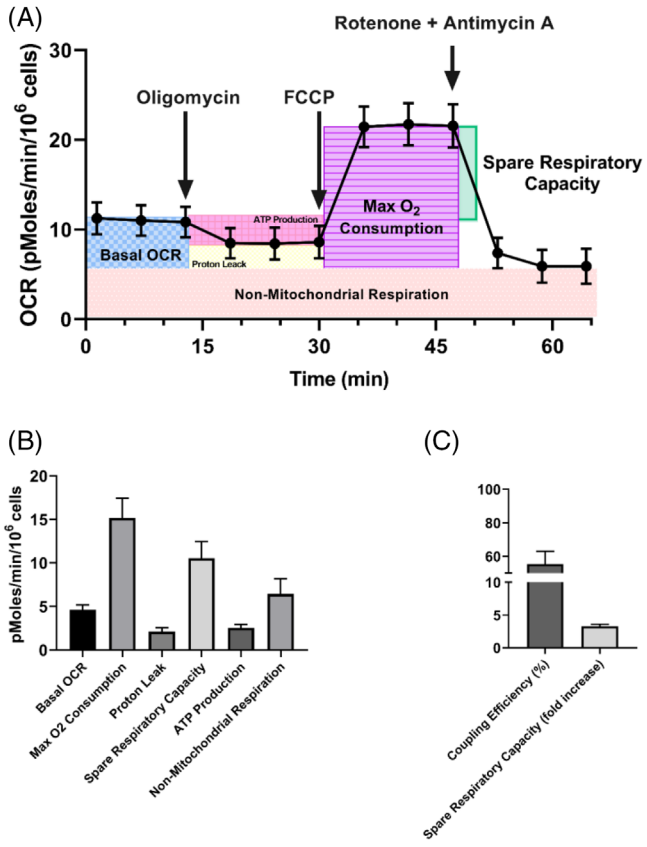

FIGURE 1 Human sperm oxidative metabolism assessed through OCR monitoring using the Seahorse metabolic flux analyzer. (A) OCR profile of the analyzed samples (mean values ± SEM; $n = 11$) and representation of the calculated metrics; (B,C) OCR-derived metrics (mean values ± SEM; $n = 11$). OCR, oxygen consumption rate.

The interindividual variation in the human sperm samples was also observed in terms of ECAR (Figure 4A–F, Table 4). Regarding the ECAR metrics (Table 2), overall, they indicate that our cells are glycolytic active despite the not so high glycolytic capacity and reserve (Figures 3B,C and 4B–F).

3.4 | Correlations

After analyzing the obtained records for OCR and ECAR, we wondered if there might exist any correlation between OCR and ECAR metrics and important seminal parameters, namely, motility and viability. We were able to identify statistically significant correlations between viability and both non-mitochondrial respiration ($\rho = -0.683^*$) and glycolytic reserve capacity ($\rho = 0.673^*$) (Table S1).

We then subdivided our samples into two groups according to their motility and viability: group higher*M* (total motility > 75; $n = 9$); group lower*M* (total motility < 75; $n = 7$); group higherV (viability > 85; $n = 5$) and lower*V* (viability < 85; $n = 11$). As expected, there were no significant differences between each group regarding the analyzed metrics. However, we were able to find other significant correlations.

In group higher*M*, we found correlations between (a) total motility and both coupling efficiency ($\rho = 0.812^*$) and spare capacity as fold increase ($\rho = -0.899^*$) and (b) progressive motility and spare capacity as fold increase ($\rho = -0.928^*$).

In group lower*M*, we found correlation between (a) total motility and coupling efficiency ($\rho = -0.975^*$) and spare capacity as fold increase ($\rho = 0.975^*$) and (b) between viability and non-mitochondrial respiration ($\rho = -0.900^*$), spare capacity as fold increase ($\rho = 0.900^*$) and glycolytic reserve capacity ($\rho = 0.900^*$).

Regarding the division according to sperm viability, group higher*V* presented correlations between (a) total motility and basal OCR, ATP turnover and non-mitochondrial respiration ($\rho = 1.000^*$), (b) between progressive motility and maximal O_2 consumption ($\rho = 1.000^*$) and spare capacity as fold increase ($\rho = -1.000^*$) and (c) between viability and coupling efficiency ($\rho = 1.000^*$), spare capacity and proton leak ($\rho = -1.000^*$).

Group lower*V* presented significant correlations between (a) total motility and non-glycolytic acidification ($\rho = -1.000^*$) and (b) between viability and glycolytic reserve capacity ($\rho = 0.900^*$).

4 | DISCUSSION

The limitations in the male fertility assessment, including the available diagnostic tools, seem to be the key factor for the existence of a high percentage of cases in which the infertility cause cannot be identified. In this sense, the development and implementation of new tools capable of providing additional and more reliable information on the sperm function, seem to be an emergent need. However, not only is the human sperm cell itself complex, but also the choice of the best parameters as redouts of the cell function, as well as the establishment of methodologies and thresholds that can be used all over the world in andrology labs in a simply, quick, and easy fashion. Having in consideration the existent knowledge on sperm cell, their metabolism and well-known connection with their function,[19,42] this seems to be an aspect in which it is worth to invest.

In fact, sperm cells have a very particular metabolism and can apparently choose different metabolic pathways according to substrates' availability.[19–21] Additionally, one of the main challenges with these cells is that besides being different from the other cells in the organism, particularly by the fact of being motile, there are also species-specific differences, making it difficult to make comparisons and drawn conclusions with the metabolic outcomes obtained on other cellular types[43–53] but also on spermatozoa from different species.[37,38,54–56]

Previously, the SFA has been used in sperm cells from rodents to study metabolic alterations related to important processes such as sperm capacitation[38,57,58]; or alternatively to study metabolic details, such as the importance of certain metabolites (e.g., beatine) for male fertility.[59] On the other hand, in species such as the bull, the SFA has confirmed the oxidative nature of sperm metabolism in this specie.[34,54,55] These studies were certainly the lever for further studies on the potential of this tool for fertility diagnosis. Nonetheless, in

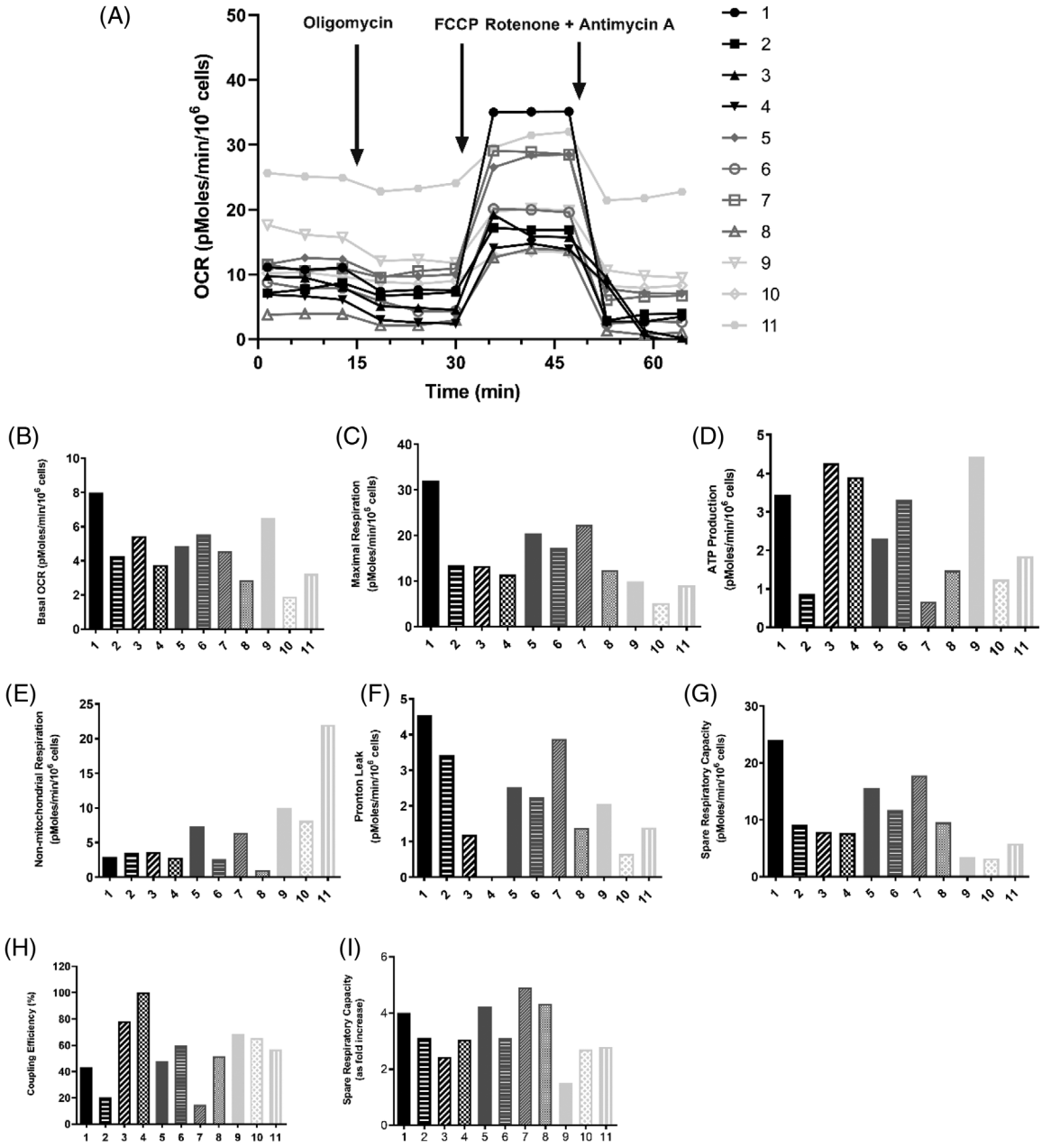

FIGURE 2 Individual sperm samples' oxidative metabolism assessed throught the OCR monitoring using the Seahorse flux analyzer. (A) OCR profile of the 11 analyzed samples; (B–I) OCR-derived metrics of the 11 analyzed samples, namely: basal OCR, maximal respiration, ATP production, non-mitochondrial respiration, proton leak, spare respiratory capacity, coupling efficiency and spare respiratory capacity (fold increase). OCR, oxygen consumption rate.

humans, to our knowledge, this methodology has only been described in one study, though only a few samples were used, and the report only focused on the OCR, under an experimental setting of different storage conditions.[39]

In the present work, we have not only performed a complete characterization of human sperm cells but have also taken advantage of the bipotential of SFA, obtaining information on oxidative and glycolytic metabolism through the obtained standard records for OCR and ECAR, further proved to be correlated with sperm seminal quality.

Indeed, we have clearly identified some particularities of human sperm, that completely fit what has been described so far on their metabolism. First, the previously reported interindividual variation in terms of samples,[60,61] was clearly mirrored in metabolic terms. Second, the proton leak in our samples was observed to be higher than expected, when comparing to other cell types, which is also mirrored in a lower coupling efficiency.[62–65] Finally, our ECAR records have highlighted a particularity not evident in other reports; in fact, although the sperm cells have responded to oligomycin in the OCR assay, that did not happen always/or so expressively in the ECAR assay. We believe

TABLE 4 Interindividual variation in human sperm samples regarding the OCR and ECAR metrics.

OCR metrics	Coefficient of variance (*V*)	p-Value
Basal respiration	0.373	0.001
Proton leak	0.658	0.001
ATP production	0.553	0.001
Maximal respiration	0.496	0.001
Spare respiratory capacity	0.607	0.001
Spare respiratory capacity (as fold increase)	0.298	0.001
Non-mitochondrial respiration	0.914	0.001
Coupling efficiency	0.453	0.001
ECAR metrics	**Coefficient of variance (*V*)**	***p*-Value**
Glycolysis	0.603	0.002
Glycolytic capacity	0.603	0.002
Glycolytic reserve capacity	0.910	0.002
Glycolytic reserve capacity (as fold increase)	0.241	0.002

Note: The results of the coefficient variation test were considered significant when $p < 0.05$.

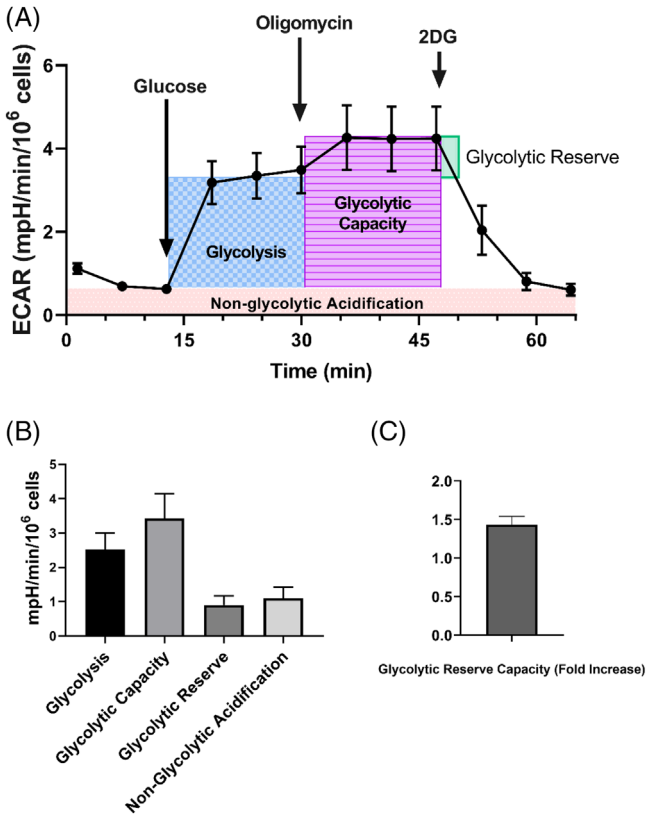

FIGURE 3 Sperm glycolitic metabolism assessed trough the ECAR monitoring using the Seahorse flux analyzer. (A) ECAR profile of the analyzed samples (mean values ± SEM; $n = 10$) and representation of the calculated metrics; (B,C) ECAR-derived metrics (mean values ± SEM; $n = 10$), namely glycolysis, glycolytic capacity, glycolytic reserve, non-glycolytic acidification, and glycolytic reserve capacity (fold increase). ECAR, extracellular acidification rate.

that this is a singular metabolic trait of the human sperm cells, that are probably running glycolysis already at a higher rate, warranting further studies in the topic to completely confirm this indication. Moreover, it is important to mention, that in order to assess the glycolytic pathway, sperm cells were incubated without energy subtracts for 15 min, this starvation period may be altering this metabolic pathway and it would be interesting to use the glycolytic rate assay in future studies to address this issue. Furthermore, in general we have concluded that the metabolic profile of our cells was not much different from that of other species such as the mouse or the bull, with typical responses after the several drugs injections.[37,38,54,55] This is not unexpected as, similarly to the human sperm cells, and despite controversies on the preferred/main pathway for energy attainment,[37,38,54,55] mouse and bull's sperm cells, are capable of using both OXPHOS and glycolysis.[37,38,54,55]

Nevertheless, this kind of analysis will always provide the important information. As an example of its potential, it has been show, by SFA, that in the rodent genus *Mus*, the relative relevance of the OXPHOS and glycolysis is species-specific and that the oxidative pathway was favored in species with higher sperm competition with the suggestion that, in this competitive scenario, adaptations in sperm metabolism will be promoted favoring the use of OXPHOS in relation to glycolysis, as this pathway will assure the energetic fulfilments associated with the need to move more and faster.[37,57,58] Nevertheless, in other species, such as the bull, the OXPHOS seems to be the main energy source for sperm in better quality samples.[34,54,55] In either case, one should not forget that the oviduct environment has a crucial role in the final choice of the pathway for energy generation and that the quantity and how quickly the ATP is needed might also have a role on the preferred metabolic pathway choice.[19] These data definitely open the road for several future studies on humans.

Furthermore, when comparing the ECAR and OCR rates of 10^6 spermatozoa to the ones from 10^6 cells of other human cell types, though direct comparisons with other cell types are not linear, it seems that sperm cells have overall lower metabolic rates than most cellular types. Indeed, sperm cells appear to have lower metabolic rates than not only undifferentiated and highly proliferative cells, such as stem cells or cancer cells,[43,44,46–48] but also differentiated somatic cells, such as neuronal or cardiac cells.[49–52] Interestingly, their metabolic metrics seem more similar to the ones from quiescent cells.[45,53] These observations might be explained by the absolute size of the sperm cells, in which the midpieces containing only approximately 50–75 mitochondria and the glycolytic machinery limited to the tail, will result in a comparatively smaller metabolic machinery than many somatic cell types.[66,67] Yet, only a normalization of the metabolic parameters according to the cellular volumes/mass will allow to clarify these issues. Further work should be developed to drawn more definitive conclusions, but it is still important to stress the need of normalizing the presentation of these results.

In this work, we have also shown that the SFA is a promising tool to evaluate sperm metabolic aspects and that those are well

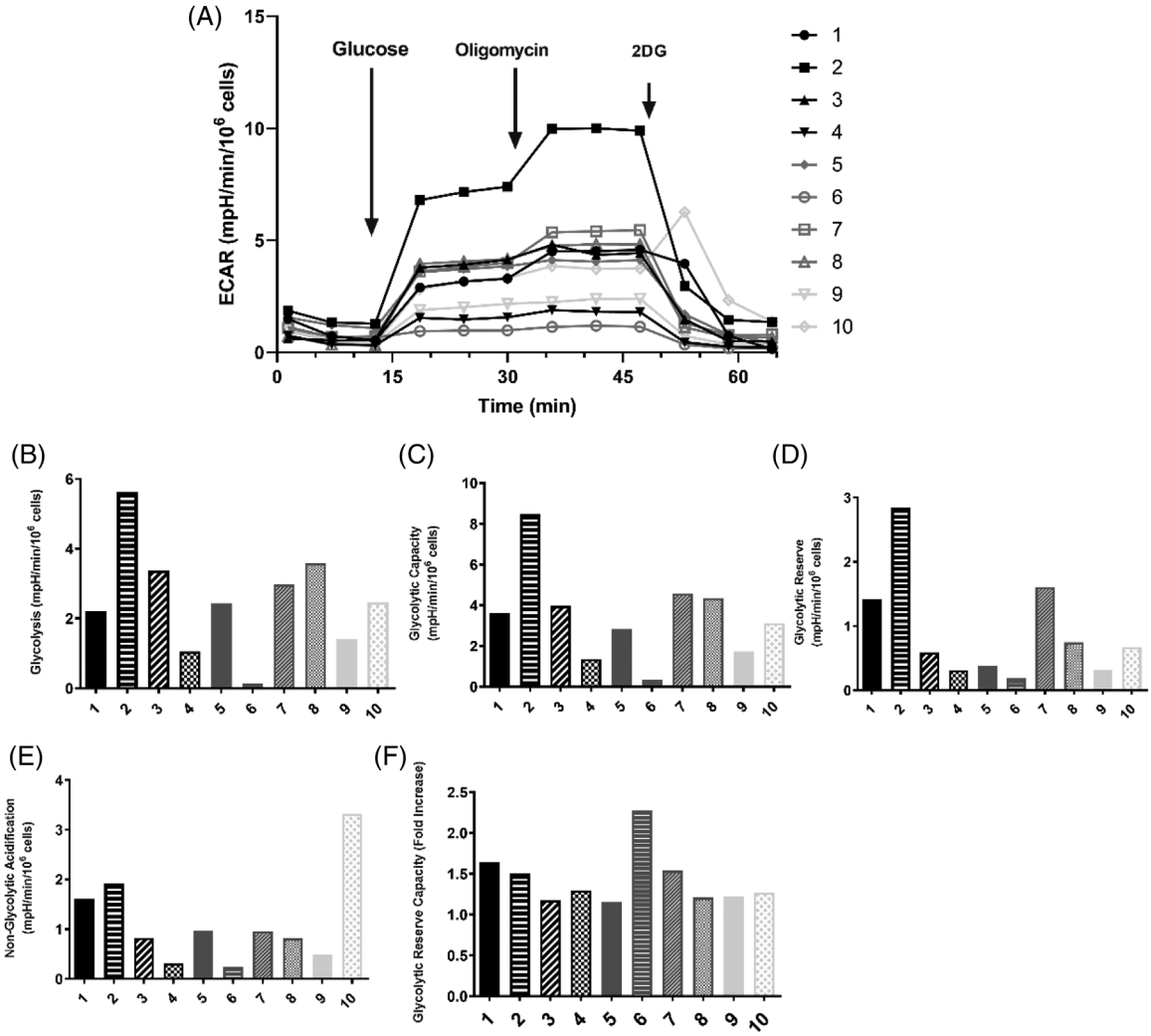

FIGURE 4 Individual sperm samples' glycolitic metabolism assessed through the ECAR monitoring using the Seahorse flux analyzer. (A) ECAR profile of the 10 analyzed samples; (B–F) ECAR-derived metrics of the 10 analyzed samples, namely glycolysis, glycolytic capacity, glycolytic reserve, non-glycolytic acidification, and glycolytic reserve capacity (fold increase). ECAR, extracellular acidification rate.

correlated with the sperm function, thus constituting a promising additional tool to the routine seminal analysis. In fact, the observed correlations among viability with non-mitochondrial respiration (negative) and with glycolytic reserve capacity (positive) suggest that this crucial parameter is significantly dependent on both pathways of the ATP production. Our results are in accordance with those obtained in bull sperm by Madeja and colleagues in which they have shown a positive correlation between mitochondrial oxygen consumption and sperm viability and motility, indicative of an increase in the mitochondria activity.[54] Furthermore, after analyzing our samples according to their seminal quality, artificially dividing them according to their motility (one group with total motility lower than 75, and other with total motility higher than 75) and viability (one group with viability lower than 85, and other with viability higher than 85), we found that the sperm quality is likely to be mirrored in terms of metabolism, with our results suggesting that higher quality samples are more associated with oxidative metabolism, stressing the relevance of analyzing samples with different qualities in this context.

According to what has been discussed so far, in the future it will be interesting to compare the magnitude of the OCR and ECAR responses, as well as the OCR and ECAR-related metrics, among species or even cellular types, aiming to identify metabolic particularities and differences that might justify some functional differences. This exercise, however, will imply to analyze the same type of results and the same metrics (especially regarding normalization/cell number, as unfortunately the existent literature is not homogeneous in this regard), that will be more easily obtained in an integrated comparative study with that specific purpose.

Furthermore, in the human side, it will be particularly relevant to conduct these experiments in samples with different seminal qualities, especially on asthenozoospermic samples, as motility is a feature highly energy-dependent, or in samples from fertile versus infertile patients, a topic in which we are now focusing.

In conclusion, this work could potentially open road to the implementation of a more systematic assessment of sperm metabolic aspects on patients undergoing fertility treatments or with diseases

that might affect their fertility, or even apply the SFA to human sperm cells in several other contexts (e.g., exposition to environmental contaminants). Finally, this approach will certainly help to further characterize and understand the molecular mechanisms underlying infertility of unknown origin, working on the way to develop new therapies, further promoting reproductive health, and better fertilization rates worldwide.

AUTHOR CONTRIBUTIONS

SA and MIS conceived and designed the study. MIC, AFM, and MIS performed the experiments. SA and MIS analyzed and interpreted the data. SA, MIS, MIC, AFM, and JRS contributed to the critical discussion of the manuscript. SA, MIS, MIC, and JRS revised the final draft of the manuscript, and all authors approved the final version. SA was in charge of the supervision and funding acquisition.

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CONFLICT OF INTEREST STATEMENT

All the authors declare there is not any identifiable conflict of interests, neither of personal nature nor financial.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Financial decision analysis based on "willingness to pay" for surgical sperm retrieval approaches among men with non-obstructive azoospermia in the United States

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Abstract

Objective: To determine the most financially optimal surgical approach for testicular sperm retrieval for men with non-obstructive azoospermia.

Design: A decision tree was created examining five potential surgical approaches for men with non-obstructive azoospermia pursuing one cycle of intracytoplasmic sperm injection. An expected financial net loss was determined for each surgical option based on couples' willingness to pay for one cycle of intracytoplasmic sperm injection resulting in pregnancy. The branch with the lowest expected net loss was defined as the most optimal financial decision (minimizing loss to a couple). Fresh testicular sperm extraction implied testicular sperm extraction was performed in conjunction with programmed ovulation induction. Frozen testicular sperm extraction implied testicular sperm extraction was performed initially, and ovulation induction/intracytoplasmic sperm injection was canceled if sperm retrieval failed. The surgical options included fresh conventional testicular sperm extraction, with and without "back-up" sperm cryopreservation, fresh microsurgical testicular sperm extraction, with and without "back-up" sperm cryopreservation, and frozen microsurgical testicular sperm extraction. Success was defined as pregnancy after one intracytoplasmic sperm injection cycle.

Materials and methods: Probabilities of successful sperm retrieval with conventional testicular sperm extraction/microsurgical testicular sperm extraction, post-thaw sperm cellular loss following frozen microsurgical testicular sperm extraction, ovulation induction/intracytoplasmic sperm injection cycle out-of-pocket costs, intracytoplasmic sperm injection pregnancy rates for men with non-obstructive azoospermia, standard conventional testicular sperm extraction cost and average willingness to pay for intracytoplasmic sperm injection cycle were gathered from the systematic literature review. Costs were in USD and adjusted to inflation (as of April 2020). Two-way sensitivity analysis was performed on varying couples' willingness to pay for one

cycle of intracytoplasmic sperm injection and varying microsurgical testicular sperm extraction out-of-pocket costs.

Results: According to our decision tree analysis (assuming minimum microsurgical testicular sperm extraction cost of $1,000 and willingness to pay of $8,000), the expected net loss for each branch was as follows: -$17,545 for fresh conventional testicular sperm extraction, -$17,523 for fresh microsurgical testicular sperm extraction, -$9,624 for frozen microsurgical testicular sperm extraction, -$17,991 for fresh conventional testicular sperm extraction with "backup", and -$18,210 for fresh microsurgical testicular sperm extraction with "backup". Two-way sensitivity analysis with a variable willingness to pay values and microsurgical testicular sperm extraction and in-vitro fertilization costs confirmed that frozen microsurgical testicular sperm extraction consistently presented the lowest net loss compared to other options. Interestingly, when directly comparing fresh microsurgical testicular sperm extraction and conventional testicular sperm extraction with "back-up", scenarios with decreasing willingness to pay and lower microsurgical testicular sperm extraction costs demonstrated fresh conventional testicular sperm extraction with "back-up" as more optimal than fresh microsurgical testicular sperm extraction with "back-up".

Conclusions: For those couples who must pay out of pocket, our study suggests that frozen microsurgical testicular sperm extraction is the most financially optimal decision for the surgical management of non-obstructive azoospermia, regardless of microsurgical testicular sperm extraction cost and the couple's willingness to pay.

KEYWORDS

azoospermia, cost-effective, micro-TESE, testicular sperm extraction

1 | INTRODUCTION

Testicular sperm extraction (TESE) remains the only viable option for men with non-obstructive azoospermia (NOA) to conceive a biological child. However, there are several ancillary decisions pertaining to the surgical approach that affected couples must make in order to conceive. Microsurgical TESE (micro-TESE) is considered the gold standard for the management of NOA as it has a superior surgical sperm retrieval rate when compared to conventional TESE (c-TESE).[1] However, micro-TESE is relatively more time intensive, costly, requires microsurgical expertise, and ready access to a surgical microscope. Given such limitations, c-TESE remains a viable, albeit less favorable, surgical option. Couples must also choose between fresh TESE—performed in conjunction with programmed ovulation induction and oocyte retrieval—or frozen TESE which is performed electively with a plan to use thawed testicular sperm for use with intracytoplasmic sperm injection (ICSI) at a future date.

In the absence of insurance coverage for fertility procedures, these decisions may have significant financial implications for couples. Presently, only 13 states mandate insurance coverage for in-vitro fertilization (IVF).[2] Even with insurance coverage for IVF, couples are subject to variable insurance deductibles which may impact their decision-making calculus.[3] Other patients without insurance coverage for IVF are left to navigate out-of-pocket (OOP) costs for surgical sperm retrieval and sperm cryopreservation processing and storage.

The aim of this study is to determine the most financially optimal approach for surgical sperm retrieval and sperm processing for men with NOA. We seek to provide clinicians and couples with meaningful financial planning information that can be tailored for families depending on their individualized financial situation and willingness to pay (WTP) for a successful pregnancy. This calculus accounts for surgical sperm retrieval rates, OOP costs for various surgical approaches, the risk of post-thaw cellular loss following sperm cryopreservation, and sperm processing and storage costs.

2 | MATERIALS AND METHODS

A decision tree was created comparing five potential surgical approaches for men with NOA pursuing one cycle of ICSI with the goal to conceive a child biologically related to both partners. The surgical options included fresh conventional TESE (c-TESE, with and without "back-up" sperm cryopreservation for later usage, respectively), fresh microsurgical TESE (m-TESE, with and without "back-up"

TABLE 1 Summary of costs and probabilities.

	Probability	Cost (2020 USD)	Reference
Successful sperm retrieval with fresh c-TESE	35%		Bernie et al., 2015[1]
Successful sperm retrieval with fresh m-TESE	52%		Bernie et al, 2015[1]
Sperm presence post-thaw after frozen m-TESE	94%		Kathrins et al., 2017[5]
Pregnancy after ICSI using fresh sperm	29%		Ohlander et al., 2014[6]
Pregnancy after ICSI using frozen-thawed sperm	28%		Ohlander et al., 2014[6]
Live birth after ICSI using fresh sperm	23.5%		Yu et al., 2015[9]
Live birth after ICSI using frozen-thawed spermatozoa	20.5%		Yu et al., 2015[9]
Base mTESE cost		$1000	Established as a base cost for analysis
Base cTESE cost		$788	Established as base cost (at our institution) for analysis
1 cycle of IVF/ICSI		$17,891	Wu et al. 2014[7]
Sperm cryopreservation processing and 1-year storage		$1352	Established as an averaged base cost for analysis
Base WTP cost		$8000	Settumba et al. 2019[10]

sperm cryopreservation, respectively), and frozen m-TESE. Fresh TESE implied TESE was performed concurrently in conjunction with programmed ovulation induction and oocyte retrieval. Frozen TESE implied TESE was performed initially, and ovulation induction/oocyte retrieval/ICSI was deferred if sperm retrieval failed. Frozen c-TESE was not examined given the expected relative rarity of that surgical approach. Success was defined as pregnancy after one ICSI cycle. Our model assumed a lack of insurance coverage for assisted reproductive therapies and that costs are defrayed by patients' OOP. We accounted for the low risk of sperm post-thaw complete cellular loss such that previously cryopreserved sperm are either absent or unusable for ICSI upon thaw. The decision tree model was constructed using TreeAge version 2.07.

Probabilities of successful sperm retrieval for c-TESE and m-TESE, post-thaw sperm cellular loss rate following frozen surgical sperm retrieval, ovulation induction/ICSI cycle OOP costs, ICSI pregnancy rates for men with NOA, standard m-TESE and c-TESE costs and average WTP for one ICSI cycle were gathered from a systematic literature review (Table 1). Base mTESE OOP cost started at $1000 as a reference standard. Variable mTESE costs ranged from $1000 to $9000 in this analysis. This range was comparable to quoted prices from the authors' home institution and published literature, with most U.S. providers offering mTESE $2500–$4999.[4] The probability of successful surgical sperm retrieval with fresh c-TESE and fresh m-TESE were 35% and 52%, respectively.[1] The probability of sperm present post-thaw after frozen m-TESE was 94%.[5] The probabilities of pregnancy after ICSI using fresh and frozen-thawed sperm were 29% and 28%, respectively, which were not statistically different values in a published meta-analysis.[6] (Figure 1)

Surgically retrieved sperm banking costs included the cost of cryopreservation and 1-year storage fee based on averaged laboratory pricing elicited from academic IVF centers in Massachusetts, Pennsylvania, New Jersey, and Ohio and a private center in Massachusetts. (Personal communication) Total IVF cost included the cost of ICSI based on published values.[7] For each successful pregnancy branch, a couple's WTP was included as a positive value in order to measure a favorable outcome. This is in contrast to costs which were represented as negative values. Settumba et al. determined that the mean WTP for 1 cycle of IVF/ICSI given a 20% probability of success was $11,750 AUD in 2006 (converted to $8720.33 in 2020 USD).[8] As such, we chose a base WTP for pregnancy after one cycle of IVF/ICSI of $8000 USD for our analysis. All costs were in USD and adjusted to inflation (as of April 2020). Two-way sensitivity analyses were performed for varying couples' WTP for one cycle of ICSI and varying m-TESE and IVF OOP costs. Variable costs and probabilities are summarized in Table 1.

Further fixed cost assumptions included oocyte freeze-all in case of spermatozoa were unavailable on the day of IVF/ICSI. Derived from Chambers et al, costs subsequent to sperm retrieval included that of ICSI, assisted hatching, and blastocyte culture.[8]

3 | RESULTS

Assuming the base scenario, the expected net loss for each branch was as follows: -$17,545 for fresh c-TESE, -$17,523 for fresh m-TESE, -$9624 for frozen m-TESE, -$17,991 for fresh c-TESE with "back-up", and -$18,210 for fresh m-TESE with "back-up". In all sensitivity analysis scenarios varying WTP and m-TESE cost, frozen m-TESE was always the most financially optimal decision with the lowest expected net loss for a couple. Two-way sensitivity analyses were then performed comparing only fresh c-TESE vs. fresh m-TESE and fresh c-TESE with "back-up" vs. fresh m-TESE with "back-up", respectively. WTP ranged from the base value of $4000 up to $20,000. OOP m-TESE costs ranged from $1000 to $9000. The first analysis demonstrated that m-TESE consistently had a lower expected net loss when compared to c-TESE (Table 2). The second analysis, interestingly, showed that scenarios with decreasing

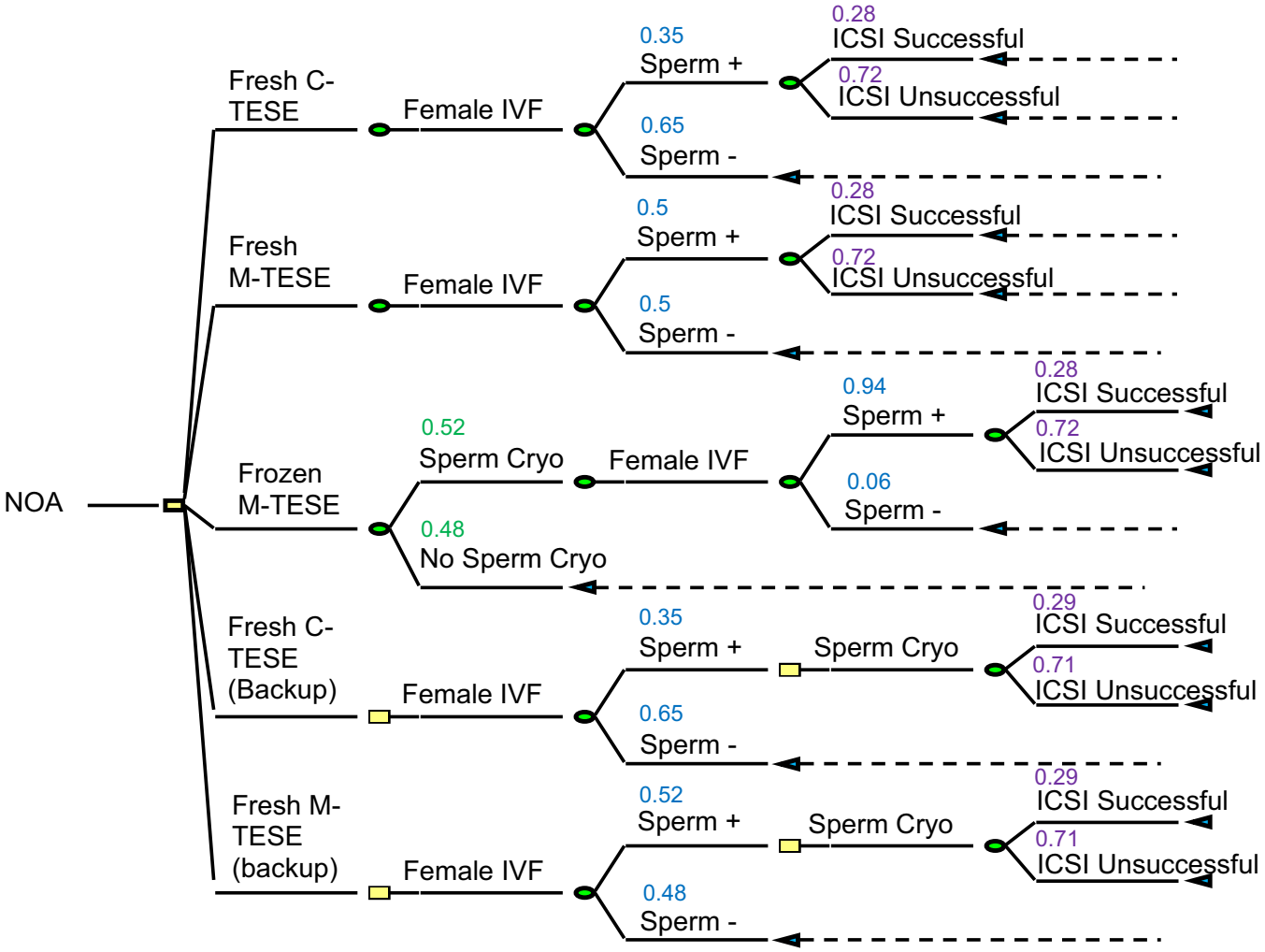

FIGURE 1 Decision algorithm.

TABLE 2 Two-way sensitivity analysis representing the most optimal financial decision between m-TESE vs. c-TESE with varying Willingness to Pay (WTP) m-TESE cost scenarios. m-TESE consistently had a lower net loss compared to c-TESE with the exception of two scenarios.

	WTP($)									
		4000	6000	8000	10,000	12,000	14,000	16,000	18,000	20,000
m-TESE cost ($)	1000	***cTESE***	***cTESE***	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	1200	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	1400	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	1600	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	1800	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	2000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	2200	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	5000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	6000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	8000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	9000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE

Bold indicates financially optimal surgical approach

TABLE 3 Two-way sensitivity analysis representing the most optimal financial decision between m-TESE with back-up vs. c-TESE with back-up with a varying willingness to pay (WTP) m-TESE cost scenarios.

	WTP ($)									
		4000	6000	8000	10,000	12,000	14,000	16,000	18,000	20,000
m-TESE cost ($)	1000	***cTESE***	***cTESE***	***cTESE***	***cTESE***	***cTESE***	mTESE	mTESE	mTESE	mTESE
	1200	***cTESE***	***cTESE***	***cTESE***	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	1400	***cTESE***	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	1600	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	1800	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	2000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	2200	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	5000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	6000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	8000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE
	9000	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE	mTESE

Bold indicates financially optimal surgical approach

TABLE 4 Two-way sensitivity analysis representing the most optimal financial decision between fresh c-TESE and frozen m-TESE without back-up (fmTESE) with a varying willingness to pay (WTP) and IVF cost scenarios (with fixed extraction costs).

	WTP($)									
		4000	6000	8000	10,000	12,000	14,000	16,000	18,000	20,000
IVF cost ($)	5000	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE
	7000	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE
	9000	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE
	10000	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE
	20000	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE
	25000	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE	fmTESE

WTP and lower m-TESE costs favored c-TESE with "back-up" over m-TESE with "back-up" (Table 3). A third analysis exploring variable IVF costs and WTP values (m-TESE cost was fixed at $4000 and c-TESE was fixed at base cost) showed that for all scenarios, frozen m-TESE was favored over fresh c-TESE (Table 4).

4 | DISCUSSION

This study approaches the decision for surgical sperm retrieval method through the perspective of the patient/couple. The economic burden of ART is borne nearly entirely by the couple in the US (with the exception of those states with expansive infertility insurance coverage mandates). Our approach focused on the all too common situation of absent government or insurance subsidization for IVF. One review indicated that the cost of one cycle of IVF was 44% of an individual's disposable income.[8] To put this in perspective, the mean US disposable income per capita was $44,110 in 2018 USD (most recent data from OECD). The practical income available for fertility treatment is likely less than the traditionally defined disposable income, as couples must account for not only taxes but housing and food costs.[9] As such, patients and families need to weigh household affordability along with traditional clinical measures of success. Household affordability is reflected in the economic concept of a WTP. Each WTP is unique and reflects a couple's inherent risk tolerance and financial circumstance. Couples will obviously incur financial losses given the OOP costs, however, the ideal scenario is to minimize the net loss. Furthermore, other clinical factors such as physical examination findings, genetic testing, and hormonal testing may persuade a clinician to recommend one surgical approach over another regardless of the most cost-effective approach.

The results of this analysis conclude that of the five surgical sperm retrieval options, frozen m-TESE was always the most financially optimal decision with the lowest expected net loss. Previous studies have explored the cost-effectiveness of different artificial reproductive technologies in varying clinical scenarios. For example, Heidenreich et al. determined that microsurgical vasectomy reversal was more cost-effective than surgical sperm retrieval/ICSI in terms of live pregnancy rates.[11] Gilbert et al. noted in a meta-analysis that for men who undergo testicular cancer treatment (chemotherapy, radiation therapy, or retroperitoneal lymph node dissection), pre-treatment cryopreservation was more cost-effective than post-treatment surgical sperm

retrieval/IVF.[12] This is the first study of its kind to consider only the financial implications of various surgical sperm retrieval approaches—the probabilities of successful sperm retrieval were held constant and WTP varied. Given that frozen m-TESE dominated all other options, sub-category sensitivity analyses were performed comparing fresh c-TESE and fresh m-TESE alone and with "back-up" sperm cryopreservation, should a couple wish to use frozen sperm for a subsequent ICSI cycle in the future. Fresh m-TESE alone mostly dominated fresh c-TESE alone regardless of varying WTP and m-TESE OOP costs—this was expected given the higher probability of successful sperm retrieval using m-TESE. The second analysis interestingly showed that c-TESE with "back-up" dominated scenarios in which both WTP and m-TESE costs were lower, suggesting that couples who were not able to attribute a high WTP for a successful pregnancy coupled with comparable m-TESE costs (c-TESE is traditionally less expensive than m-TESE), should opt for c-TESE despite the decreased probability of successful sperm retrieval.

Our results raise the question of the economic relevance and utility of the other surgical TESE options. If patients determine that frozen m-TESE is the most optimal approach given they are liable for costs, demand may shift toward that option. One can envision a scenario in which states that lack insurance coverage for TESE will have frozen m-TESE emerge as the prevailing extraction technique. This has interesting economic and staffing implications (e.g., the need for fellowship-trained specialists in male reproductive medicine and microsurgery in those areas). The reverse may be true as well—if more states mandate coverage for ART, less financially optimal techniques such as c-TESE (with or without backup) may have greater than expected utilization. This can be expected because if patients are not directly paying for ART and its components such as testicular sperm extraction and sperm processing/storage, then the driving factor for a particular surgical option rests in the hands of the provider (e.g. surgeon preference, convenience, etc.). As such, there may be a more even split in the utilization of c-TESE vs. m-TESE in these areas. As the scenario for most people in the United States is a lack of IVF coverage, our analysis does not account for situations with insurance coverage for the various components of ART in the setting of NOA. Our base costs are derived from Massachusetts clinical locations and are not representative of other states' fertility practices which may not have infertility and IVF insurance mandates. We have attempted to account for this limitation through broad sensitivity analyses and national averages for testicular sperm processing and storage.

Beyond optimizing financial decision-making, there is a lack of consensus on the optimal clinical approach for several aspects of our decision tree. While debate remains over the best approach to surgical sperm retrieval—fresh vs. frozen—meta-analysis data indicates both approaches have equivalent ICSI fertilization and pregnancy rates among men with NOA.[6] Certainly, the post-thaw complete cellular loss may represent a devastating scenario for a couple because a subsequent m-TESE attempt to rectify the situation may ultimately fail to retrieve sperm. Our assessment of post-thaw complete cellular loss risk is based on limited published data and only pertains to the situation of no identifiable ICSI-appropriate sperm identified after thaw, regardless of motility. This scenario is distinct from the finding of only non-motile sperm after thaw which some IVF centers may deem unsuitable for ICSI. However, recently published data indicate that non-motile testicular sperm may still result in a successful ICSI cycle.[13]

Donor sperm is uncommonly used in the setting of an unsuccessful surgical sperm retrieval attempt with a plan to use fresh sperm for ICSI. One study found that only 28% of such couples in that situation actually opt to use donor spermatozoa "backup" for ICSI, otherwise opting to defer the ICSI cycle altogether. However, this study was based on a single, high-volume urban academic center experience and may not be broadly applicable to broader patient populations.[14] While the incidence of missed fresh IVF cycles due to an inability to obtain sperm surgically is low, it must still be considered.[15] Our analysis assumed a desire only to have a child biologically related to both partners and thus did not account for donor sperm use. Furthermore, there are ethical implications for fresh TESE that are not relevant for frozen TESE—namely exposure of the female partner to the medical risks of ovulation induction only to defer ICSI in the setting of unsuccessful retrieval for her partner.[16]

There were several limitations to this study. First, only financial outcomes were considered. This did not include the physical and psychological tolls that many couples face when pursuing ART. By utilizing a WTP we hope to account for these indirect costs and risks in order to attribute some economic value to a desired outcome (e.g., successful pregnancy). A different method of determining WTP would have been to perform a contingent valuation survey for the US population in question. This method was not utilized here given the relatively small percentage of the population with NOA pursuing surgical sperm retrieval, but it would have been theoretically possible and a future direction for study. Our base WTP was based on an Australian cohort, which was deemed representative of the general population there in terms of age, gender, and level of education.[10] Additionally, if probabilities of success were to change (perhaps future innovation could improve the success of sperm retrieval), this may affect financial outcomes. One cost-benefit analysis from Iran demonstrated that the WTP of IUI and IVF were sensitive to treatment success.[17] However, Gardino et al. noted that WTP for ovarian cryopreservation did not have a "certainty premium," such that the WTP for 100% success was disproportionately larger than lower success probabilities. In fact, WTP was fairly consistent across varying success rates.[18] This suggests that perhaps even within the more accurate contingent valuation method, couples may still not assign accurate WTP values as they highly value the possibility of a live birth, independent of the actual probability that it will occur.

We did not account for significant variability in the US IVF insurance mandates and relied on the most burdensome financial scenarios in which the costs are borne by patients out-of-pocket, which is the reality for most Americans. Only successful pregnancy was used as the final clinical outcome, and future studies may incorporate live births as an endpoint in WTP in the setting of NOA. The probabilities of live births after ICSI using fresh and frozen-thawed sperm were 23% and 20.5%, respectively, although these values were not statistically different in a published meta-analysis.[9] This analysis did not account for the cost

of complications or loss of productivity which are critical societal and financial considerations for patients/couples. The authors of this paper also acknowledge that the cost of ART largely contributed to the cost as opposed to the cost of sperm retrieval, which we examined in our final sensitivity analysis with varying IVF costs.

5 | CONCLUSIONS

Our analysis suggests that frozen m-TESE is the most financially optimal decision for the surgical management of NOA, regardless of m-TESE and IVF costs and the couple's WTP when considering OOP costs outside of insurance coverage.

AUTHOR CONTRIBUTIONS

Study conception and design: Tracy X. Han, John Ernandez, Brittany Berk, Ramy A. Ghayda, and Martin Kathrins; Analysis and interpretation of results: TXH, MK, BB, and JE; Draft manuscript preparation: TXH and MK.

All authors reviewed the results and approved the final version of the manuscript.

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 on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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ORIGINAL ARTICLE

Inter-disciplinary provider development of an online, interactive adolescent varicocele decision aid prototype

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Abstract

Background: Decision-making regarding varicocele management can be a complex process for patients and families. However, to date, no studies have presented ways to mitigate the decisional conflict surrounding varicoceles.

Objective: To facilitate a discussion among physicians in order to develop a framework of the decision-making process regarding adolescent varicocele management, which will inform the development of the first online, interactive decision aid.

Materials and methods: Semi-structured interviews with pediatric urologists and interventional radiologists were conducted to discuss their rationale for varicocele decision-making. Interviews were audio recorded, transcribed, and coded. Key themes were identified, grouped, and then qualitatively analyzed using thematic analysis. Utilizing the common themes identified and the Ottawa Decision Support Framework, a decision aid prototype was developed and transformed into a user-friendly website: varicoceledecisionaid.com.

Results: Pediatric urologists ($n = 10$) and interventional radiologists ($n = 2$) were interviewed. Key themes identified included: (1) definition/epidemiology; (2) observation as an appropriate management choice; (3) reasons to recommend repair; (4) types of repair; (5) reasons to recommend one repair over another; (6) shared decision-making; and (7) appropriate counseling. With this insight, a varicocele decision aid prototype was developed that engages patients and parents in the decision-making process.

Discussion and conclusions: This is the first interactive and easily accessible varicocele decision aid prototype developed by inter-disciplinary physicians for patients. This tool aids in decision-making surrounding varicocele surgery. It can be used before or after consultation to help families understand more about varicoceles and their repair, and why intervention may or may not be offered. It also considers a patient and family's personal values. Future studies will incorporate the patient and family perspective into the decision-making aid as well as implement and test the usability of this decision aid prototype in practice and in the wider urologic community.

KEYWORDS
adolescents, decision-making, qualitative research, varicoceles

1 | INTRODUCTION

Decision-making regarding varicocele surgery can be a complex process for patients and parents. There are multiple indications to seek treatment, including testicular hypotrophy, pain, infertility, and appearance.[1] Further complicating the decision-making process are the variety of treatments options used—observation, open, laparoscopic, and embolization. The interventions available, if chosen over observation, are irreversible, and each carries unique risks and benefits that may be difficult to fully understand and appreciate.[2] Additionally, it is unknown whether adolescent varicoceles will lead to adult infertility and benefit from early repair.[3] Due to this, individuals who choose to seek information and treatment for their or their child's varicocele may experience decisional conflict, a state of uncertainty regarding which choice to make when competing options involve risk, regret, or challenge to personal life values.[4,5] A recent study determined that 55% of patients considering varicocele surgery for infertility experienced decisional conflict.[4] However, to date, no studies have identified or presented ways to mitigate this decisional conflict, the basis of this investigation.

The American Urologic Association and the American Academy of Pediatrics endorse shared decision-making to help patients feel more informed, clarify what is important to them, and minimize decisional regret.[6–9] One method of shared decision-making is the utilization of a decision-making aid, a tool designed to provide balanced, unbiased information for patients to conceptualize options, outcomes, and personal values.[10] Randomized trials have shown that patients who receive decision-making aids are more knowledgeable, have realistic expectations, and actively participate in their care. This ultimately improves decision-making quality.[11–13]

In the field of pediatric urology, physician and patient-centric decision aids have proven to be highly useful when making decisions regarding the pursuit of hypospadias repair.[14,15] A web-based decision aid was also successfully developed for parents of children and adolescents with cancer to use surrounding informed decisions regarding fertility preservation.[16] However, to our knowledge, few other pediatric urology decision aids have been published. The objective of this work was to facilitate a discussion among physicians to develop a framework of the decision-making process regarding adolescent varicocele management to inform the development of the first online, interactive decision aid. Building on the success of other decision aids in medicine, our aim is to harness physician knowledge to assist patients and families in making the best decision for themselves given the complexity of adolescent varicocele management.

2 | MATERIALS AND METHODS

Attending pediatric urologists and interventional radiologists with a broad spectrum of experience levels were recruited from seven academic medical centers across the country. Convenience sampling was employed due to physician availability and coordination of interviews. Nevertheless, different hospital types (free-standing vs. a children's hospital within a hospital) and various geographic locations were prioritized. Physicians were contacted via e-mail to discuss study participation. The purpose of the interview was explained immediately prior to study participation.

The design of this study was qualitative in nature and informed by human-centered design methodologies.[17] From May to August 2022, physicians discussed their recommendations for a varicocele decision aid prototype via individual, semi-structured, qualitative interviews conducted over Zoom (zoom.com, version 5.11.1, 2022). During the first two interviews, which were with pediatric urologists, a script of questions was pilot tested and then modified. This script was refined to a list of 16 questions that was uniformly read during each subsequent interview ($n = 10$), followed by an open forum for comments on additional points the interviewee felt were important for patients to know when making an informed decision regarding varicoceles (Supplementary Figure 1). Demographic data were also collected at the end of each interview. A single individual performed all interviews. The interviewer had no prior relationships with the participants and was trained to limit interviewer bias through (1) acknowledging the potential for bias, (2) appreciating unique perspectives, and (3) asking open-ended questions. Interviews were audio recorded, verbatim transcribed, reviewed for accuracy, and then deidentified.

Transcripts were coded in the standard fashion by a single individual, key themes and subthemes were identified, grouped, and then qualitatively analyzed. Interviews were conducted until thematic saturation was reached. Using the Ottawa Decision Support Framework, a decision aid prototype was developed that combined these provider themes with existing evidence regarding adolescent varicocele outcomes and management. The Ottawa Decision Support Framework is a standardized framework that conceptualizes the needs of patients, their families, and practitioners to make difficult decisions when the various options feature different values. It has been successfully used in various other healthcare settings.[15,18,19] The decision aid prototype was transformed into a user-friendly, interactive webpage which can be found at varicoceledecisionaid.com.

After creating an initial varicocele decision aid prototype, cognitive interviews were conducted with 4 of the 12 physician participants to assess its face and content validity. These participants were asked to verbalize their thoughts aloud as they viewed the online decision aid on a desktop computer to obtain initial, unprompted verbal feedback to further inform its modification. This cognitive interview technique is well-established in its effectiveness for evaluating the usability and design of decision aids.[15,20–22] After obtaining this feedback, the varicocele decision aid was appropriately modified and republished.

3 | RESULTS

Pediatric urologists ($n = 10$) and interventional radiologists ($n = 2$) across seven different academic institutions were interviewed. Fifty percent of participants practiced at an institution in the northeast, 33% in the west, and 17% in the southeast. Forty-two percent were employed at free-standing children's hospitals. Participants had a

median of 9.5 years (IQR 2–16.8) in practice post-fellowship. Sixty-seven percent were male and 75% were Caucasian. Each interview lasted a median of 12 min and 28 s (IQR 11:10–14:24); this time includes both the amount of time for the interviewer to ask the questions and the interviewee to respond. Both the pediatric urologists and interventional radiologists were familiar with guidelines set by the American Urological Association and the Practice Committee of the American Society for Reproductive Medicine regarding varicocele treatment and management.[23]

Key themes and subthemes were identified and are outlined below: (1) definition and epidemiology of varicoceles; (2) observation as an appropriate management choice; (3) reasons to recommend varicocele repair (testicular asymmetry, possible future fertility issues, and/or pain); (4) types of varicocele repair (open +/− microscopic, laparoscopic, and embolization); (5) reasons to recommend one repair over another (success rates, complication rates, and provider skill); (6) physicians participating in shared decision-making with families; and (7) need for appropriate patient and family counseling (Table 1).

With this insight, the authors developed a contemporary varicocele decision aid prototype that combines collective physician expertise with evidence-based educational content to engage patients and parents more thoroughly in the decision-making process. It consists of six modules: varicocele basics, treatment options, an interactive decision tool ("help me decide"), frequently asked questions, words of wisdom from physicians, and a contact link. All information provided is at or below an eight grade reading level based on the Gunning Fog index.

The first two modules of the varicocele decision aid aim to give users readable, physician-sourced, evidence-based information on varicoceles, which will subsequently inform decision-making. The varicocele basics module uses pictures and lay terms to discuss the definition and epidemiology of varicoceles. This module offers a rationale for conservative management; in most circumstances, they are harmless, do not cause issues, and are not life-threatening (Figure 1). The problems that varicoceles can cause (small testicular size, fertility issues, discomfort, and unsightly appearance) are detailed in the information to follow. The next module (treatment options) features a statement on how observation is an appropriate option for most patients and that surgery is only recommended when a problem exists. Interventions (open +/− microscopic, laparoscopic, and embolization) are subsequently described, and their unique risks and benefits are listed in an easily navigable format.

The third module ("help me decide") was created using the Ottawa Decision Support Framework and has the goal of providing patients and their families appropriate counseling to help make an informed decision that aligns with their personal values.[18] This interactive module provides users with data on the benefits and harms of each intervention, and guides participants through a series of questions to discern what factors matter most to them. Using a decisional conflict scale, participants are asked to rank each statement on a scale of 0–5, 0 signifying not at all important and 5 signifying very important. For example, users are asked questions, such as "How important is it to avoid a procedure," "How important is it to avoiding fixing something that isn't causing a problem now," and "If you have pain—how important is it to treat the pain if there is no guarantee it will be fixed, and the pain may get worse?" (Figure 2A,B). Users are then prompted by statements to discern how well they have learned the key facts, such as "Do you know the benefits and harms of each option?" and "Are you clear which benefits and harms matter most to you?" If patients are unsure of any of their answers, they are urged to speak with a healthcare provider. They are also provided a list of the next steps to take depending on their decision.

The frequently asked questions section covers general varicocele information, commonly asked questions and their answers, and peri- and post-operative care expectations. These statements were heavily informed by the reported experience of physician participants. The "words of wisdom" module was designed to make users aware of the guidance of a physician when making their decision. Finally, a contact form is provided for users to give feedback on the decision aid and promote future collaboration. Responses are monitored weekly by members of the study team.

4 | DISCUSSION

This is the first project to facilitate a discussion among physicians to develop a framework of the provider and patient/parent decision-making process about adolescent varicoceles to inform the development of an online, interactive, readable decision aid. Based on the key themes and subthemes identified through a qualitative interview process, this decision aid provides physician-vetted evidence-based information. This varicocele decision aid prototype, which can be found at varicoceledecisionaid.com, may help patients and their families make a more informed decision for themselves than if this tool was not readily available.

One of the key themes identified was shared decision-making. As described in Table 1, physician participants noted that certain patients and families often "trust" physician recommendations and "rely" on their discretion for the necessity of intervention. "Comfort level with the surgeon" was also cited as a driving factor. These results are consistent with a previous study on pediatric ureteropelvic junction obstruction which concluded that trust in the surgeon, rather than personal preferences, was the primary factor in driving decision-making.[24] This study hypothesized that in situations where perceived risk is low and parental knowledge is limited, a physician-led approach is advantageous. Similarly, general population knowledge on the multiple indications to seek treatment for varicoceles and the various treatment options is low, and all treatment options are considered to be low risk.[1,25] Therefore, the authors were mindful to make users feel the guidance of a physician when making their decision in the various modules through concepts, such as "words of wisdom."

Two other themes noted were "observation as an appropriate management choice" and the "need for appropriate patient counseling." Quotations from physician participants highlighting these themes include that "most varicoceles are harmless," "not life threatening," and "do not need to be intervened on." Their unknown impact on future

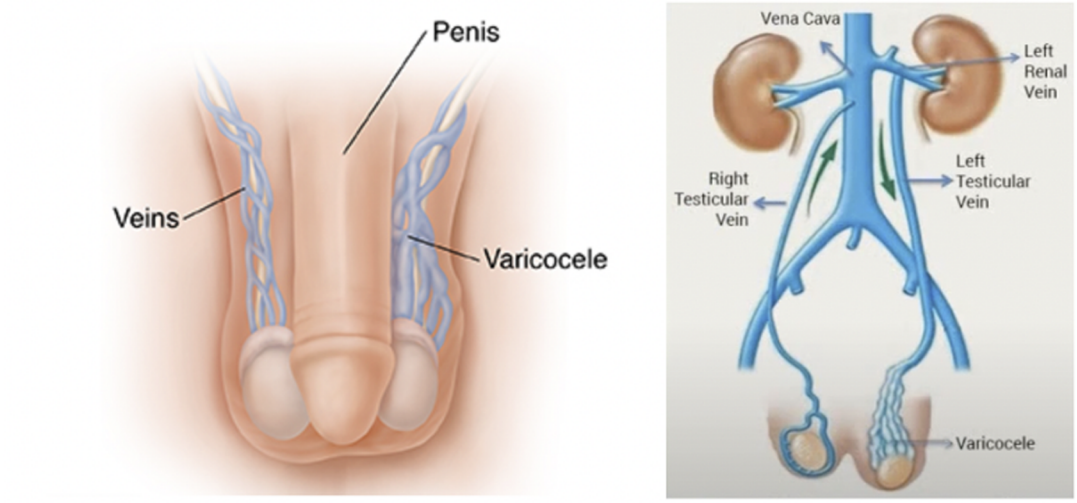
TABLE 1 Quotation examples of themes and subthemes.

Definition and epidemiology of varicoceles	"Varicoceles are like varicose veins in your scrotum. They are big, dilated veins and are often described as feeling like a 'bag of worms.'" "Varicoceles are very, very common. Studies differ but somewhere around 15–20% of young men have a varicocele, usually on the left, and usually asymptomatic."
Observation as an appropriate management choice	"Most varicoceles are harmless, do not cause any problems, and are not life threatening." "By far the majority of varicoceles do not need to be intervened on." "There is an association between varicoceles and problems with fertility… There is also an association between men with bowties and intelligence. But that doesn't always correlate to a cause and effect."
Reasons to recommend varicocele repair - Testicular asymmetry - Possible future fertility issues - Pain - Appearance	"The reasons to have surgery on your varicocele are 1) if the varicocele is very big, the heat can cause poor growth of the testicle… size differential between the testicles is an indication to treat in the pediatric population, 2) if there is trouble with fertility sometimes fixing the varicocele can help with that, but most men with a varicocele will not have trouble with fertility…, 3) you have pain on the side associated with the varicocele, however there should be a discussion with your doctor to see if the varicocele is the true cause of the pain because there are many reason to have testicular pain, and 4) if it is really big and unsightly, and you don't like the way it looks."
Types of varicocele repair - Open +/− microscopic - Laparoscopic - Embolization	"From most invasive to least invasive: an open surgery where you clip the vein in the retroperitoneum… laparoscopic, (sub) inguinal repair/ incision in the groin, or a percutaneous coil placed in the vein (done by an interventional radiologist)." "You can do a laparoscopic approach where you usually transect the vein but sometimes the vein and the artery. You can do an inguinal approach or subinguinal approach with or without a microscope. An interventional radiology can do a sclerosing procedure or coil. The success rates are very high in all of them."
Reasons to recommend one repair over another - Success rates - Complication rates - Provider skill	"The pros to a microsurgical inguinal varicocelectomy are it is less likely to induce a hydrocele than a laparoscopic surgery or coiling. Hydroceles after laparoscopic surgeries are harder to treat." "A laparoscopic varicocelectomy takes a lot less time. A patient who anesthetic risk is higher may find this a more attractive option." "Laparoscopy is safe and effective but provides additional risks because it is done through the abdomen." "With microscopic or laparoscopic surgery there is always a risk of diminishing arterial blood flow." "At high volume centers an endovascular repair is efficacious and can be done reasonably quickly. But at our institution they don't do them very often and the time benefit may not be there." "The complication rate is low in all options. Ask your surgeon which approach they are most comfortable performing."
Physicians participating in shared decision-making with families	"Comfort level with surgeons might drive decision making." "Our patient population has trust in physicians. Patients don't push back on getting a different treatment from what we recommend." "Patients turn to me to tell them if it needs to be fixed or not."
Need for appropriate patient and family counseling	"Reframing that a varicocele is not an abnormality for most people is important. It is an anatomical variant. If there is no problem, it is not worth treating." "Try to get a sense of why your doctor is recommending surgery. What problem are we fixing? Get a sense of what could happen if we do or don't do surgery and back it up with data." "Does treatment help? It is such a gray area. We really need to get more data to see if it's worth treating them or not. This is one of the most ambiguous areas that I take care of." "Patient should be aware of all options—both surgery and interventional—to make a good decision."

• Decision Aid •

VARICOCELES

A WEBSITE MADE BY DOCTORS, FOR PATIENTS AND FAMILIES,
TO HELP WITH DECISIONS SURROUNDING THE DIAGNOSIS OF VARICOCELES



What is a varicocele?

All boys have **veins surrounding their testicles** that bring blood from the testicle (also sometimes called "balls" or "nuts") up to the heart. Sometimes blood drains more slowly and can back up in these veins, causing them to become **swollen** and tortuous. Varicoceles often happen on the left side of the scrotum because of the left veins' longer path back to the heart. When this happens the veins around the testicle are called a varicocele. Some people describe them as a "**bag or worms**" or "**varicose veins in your scrotum**."

Epidemiology

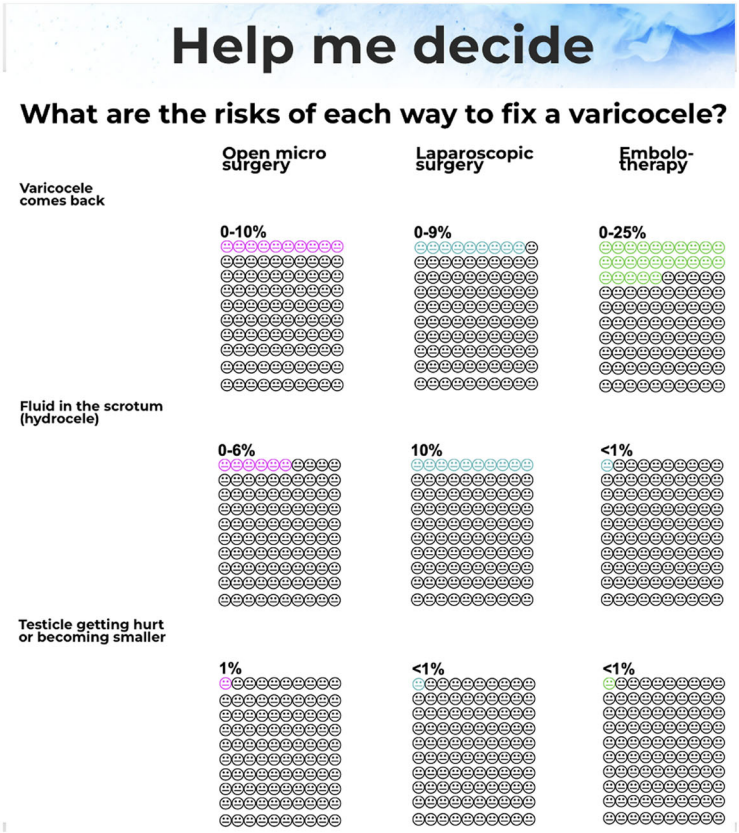
Varicoceles are very common. Doctors and scientists think **15%** of boys and men have varicoceles. They usually develop during puberty, when the testicles grow and need more blood. As boys grow taller, the veins from the testicles to the heart also get longer. This makes it harder for the blood to go all the way back up.

What symptoms do varicoceles cause?

Most boys don't feeling anything from a varicocele. They may not even know they have one until they see a doctor! When a varicocele exists they are never life threatening. Sometimes it hurts a little bit, or feels very heavy and achy at the end of the day. Exercise (running, weight lifting), hot weather, or standing for a while may make the varicocele seem bigger and achier. Laying down sometimes makes it go away. That is because when you lay down it's easier for the blood to go back to the heart. A doctor may use an ultrasound to help see a varicocele that is too small to feel or to look at testicular size. This test that takes 20-30 minutes and does not hurt.

FIGURE 1 Example of content on the "Varicoceles Basics" page.

(A)



(B)

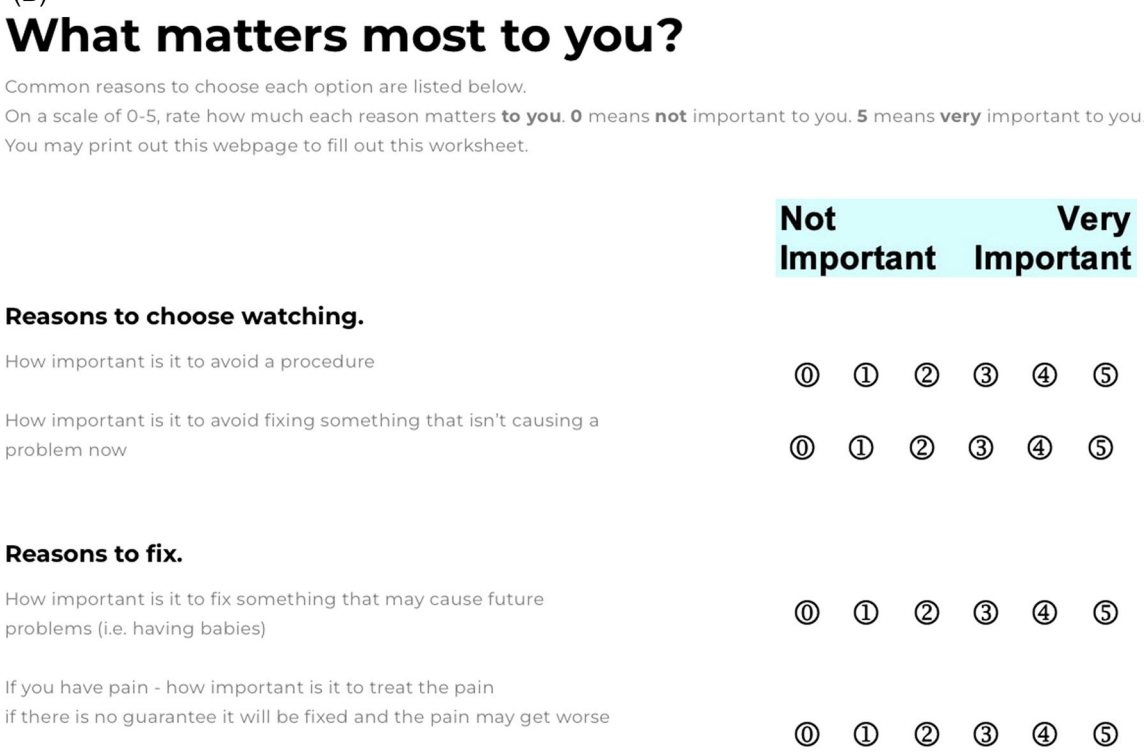


FIGURE 2 Example of content on the "Help me decide" page. (A) Percentages of the benefits and harms of each intervention, (B) excerpt from the varicocele decision aid's interactive questionnaire.

fertility was also described; namely, one physician noted that while there is an association between varicoceles and problems with fertility, the cause and effect is unclear. Furthermore, studies have shown that surgical intervention does not guarantee improved fertility, reduced pain, or prevention of recurrence.[1] Regardless, some patients and families may be more eager than others to find a "fix" to a diagnosis that may or may not cause future problems. This may be especially true for certain populations that are very concerned with future fertility.[26] The authors of this study were mindful of these themes by having users of the decision aid prototype participate in a decisional conflict scale under the "help me decide module." By asking participants to rank each statement, the authors hope that users may realize what factors matter most to them and consider the personal necessity of intervention.

This varicocele decision aid prototype also builds upon previous pediatric urologic decision aids by encompassing important content recommendations identified by parents who made decisions on other potential surgical interventions. Specifically, suggestions cited in the Chan et al. study were incorporated, such as (1) ease of access with online availability; (2) photogenic representation of the disease to accompany text describing the condition; (3) a clear summary of the risks and benefits of each treatment modality; and (4) a review of peri- and post-operative care.[15] Of note, information regarding surgical treatment options, such as open +/− microscopic surgery, laparoscopic surgery, and percutaneous embolization, as well as operative expectations, was derived from physician participant comments (Table 1). By utilizing the knowledge of previous qualitative decision aid research, the authors hope to create a decision aid that better meets the needs of patients and families with a varicocele.

This is the first phase of a multi-phase study. These results solicit physician experiences to create a decision aid prototype. The next steps will focus on gaining additional family and adolescent patient perspectives to identify new themes and enhance this prototype. This will be especially important with regard to the "help me decide" questions. A testimonial portion of the decision aid will be added to include users' experience with the decision-making and the peri- and post-operative process. In the third phase of this study, we plan to conduct a patient-facing clinical trial to assess the level of decisional conflict, satisfaction, and regret in patients adopting versus not adopting this decision aid. In addition to our early results obtained from the collective physician experience, these next steps are important to refine the decision aid with "real-world" information and test its usability. Ultimately, we hope this much-needed resource will contribute to the improvement of quality educational materials provided to families, especially when multiple treatment options are available.

Several limitations are noted. The first phase of this study requested only physician experiences; as discussed above, future studies are warranted to obtain additional information from physicians and patients to refine this prototype. This study also has a small sample size. Findings may not reflect the views of all pediatric varicocele providers, which may limit the generalizability of the varicocele decision aid. This was partially mitigated by prioritizing different hospital types and various geographic locations; however, our sample was majority male and Caucasian. A wider and more balanced sample will ensure biased information is limited and that the decision aid will be well-received by a diverse group of patients. It is also possible that voice intonation and body language was lost in transcription, which could have added meaning to the textual data.

Despite these limitations, this prototype meets the goal of creating a physician-informed theoretical framework to develop a varicocele decision aid prototype. This study addresses a gap in the literature, as there is limited research on decision-making tools for pediatric urology topics. Researching and developing these tools is especially important given the American Urologic Association's and the American Academy of Pediatrics' recent endorsement for the necessity of shared decision-making aids and the need to improve the quality of educational materials provided to families.[6,9]

5 | CONCLUSIONS

This is the first online, interactive varicocele decision aid prototype developed for patients by inter-disciplinary physicians across seven academic institutions from a variety of geographical regions. To promote shared decision-making, it can be used before or after consultation so patients and families may understand more about varicoceles, why an intervention may or may not be offered, and what each specific intervention entails. It also elucidates personal values surrounding this topic. Families are encouraged to bring their results to their doctor visit so any gaps in knowledge may be addressed. Future studies will gain patient and family perspectives, implement, and then test the usability of this decision aid prototype in practice and the wider urologic community.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to the information and material submitted for publication, and all have approved the final manuscript. ARS performed the research, analyzed the data, and wrote the paper. FAF and NRM contributed to the study design and revised the paper. CDAH, MCW, and AJS contributed to revising the paper.

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DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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ANDROLOGY

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ORIGINAL ARTICLE

The impact of acute SARS-CoV-2 on testicular function including insulin-like factor 3 (INSL3) in men with mild COVID-19: A longitudinal study

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Abstract

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may affect the male reproductive system as it uses angiotensin-converting enzyme (ACE)2, which is expressed in testicular tissue, as an entry point into the cell. Few studies have evaluated the long-term effects of mild coronavirus disease 2019 (COVID-19) on testicular function, and insulin-like factor 3 (INSL3) levels have not previously been assessed during acute SARS-CoV-2 infection.

Objectives: The aim of the study was to assess the impact of acute SARS-CoV-2 infection on testicular function including INSL3 and the presence of SARS-CoV-2 RNA in semen in non-hospitalised men with mild COVID-19.

Materials and methods: This longitudinal study included 36 non-hospitalised SARS-CoV-2-positive men (median age 29 years). Inclusion was within seven days following a positive SARS-CoV-2 reverse-transcription polymerase chain reaction test. Reproductive hormone levels, semen parameters, and the presence of SARS-CoV-2 RNA in oropharyngeal and semen samples were assessed during acute SARS-CoV-2 infection (baseline) and at three- and six-month follow-up. Wilcoxon matched-pair signed-rank (two samples) test was used to assess time-related alterations in reproductive hormone levels and semen parameters.

Results: Lower plasma testosterone (T) (total and calculated free (c-fT)) and higher luteinising hormone (LH) concentrations were observed during acute SARS-CoV-2 infection (baseline) compared to three- and six-month follow-up. Consequently, ratios of c-fT/LH were lower at baseline compared to three- and six-month follow-up ($p < 0.001$ and $p = 0.003$, respectively). Concomitantly, lower INSL3 concentrations were observed at baseline compared to three-month follow-up ($p = 0.01$). The total number of motile spermatozoa was also lower at baseline compared to six-month follow-up ($p = 0.02$). The alterations were detected irrespective of whether the men had experienced SARS-CoV-2-related fever episodes or not. No SARS-CoV-2 RNA was detected in semen at any time point.

Discussion and conclusion: This study showed a reduction in testicular function, which was for the first time confirmed by INSL3, in men mildly affected by SARS-CoV-2 infection. The risk of transmission of SARS-CoV-2 RNA via semen seems to be low. Febrile episodes may impact testicular function, but a direct effect of SARS-CoV-2 cannot be excluded.

KEYWORDS
acute SARS-CoV-2, INSL3, insulin-like factor 3, male reproductive function, mild COVID-19, sexual transmission, testicular function

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 was declared a pandemic by the World Health Organization (WHO) on March 11, 2020.[1] More than 767 million COVID-19 cases have been reported globally, and as of July 2023 more than 6.9 million deaths have been linked to COVID-19.[2] Men are at increased risk for severe disease compared to women.[3,4]

Transmission of SARS-CoV-2 is by respiratory and fecal-oral routes.[5] Unlike the Zika virus, which has been detected in semen up to 188 days after infection,[6] SARS-CoV-2 is not believed to be transmitted via semen after the resolution of the illness.[7] However, late 2020 saw many myths related to a potential sexual transmission of SARS-CoV-2.[8] The potential risk of viral transmission affected patients by closing fertility clinics worldwide.[9–11]

In the first wave of the pandemic, a Chinese study of 38 male patients with severe COVID-19 showed that SARS-CoV-2 could be present in the semen of both recovering and acutely ill COVID-19 patients.[12] However, current evidence suggests that the risk of transmission of SARS-CoV-2 to semen is low.[13] Angiotensin-converting enzyme (ACE)2 is used by SARS-CoV-2 as the receptor for viral entry into target cells. ACE2 catalyses the conversion of angiotensin II to the peptide angiotensin-(1–7), which has a range of anti-inflammatory and vasodilatory effects that are mediated by the Mas receptor system.[14] As both ACE2 and Mas are expressed in the testes,[15,16] concerns have been raised that SARS-CoV-2 infection may affect male fertility.[17] Indeed, studies of reproductive function in men recovering from both mild and severe COVID-19 have reported a negative effect on semen parameters, such as sperm count and motility, and reproductive hormone profile.[18,19] Notably, COVID-19 patients had higher levels of luteinising hormone (LH) and lower testosterone (T) concentrations compared to controls.[20,21]

Most of the studies researching the effect of SARS-CoV-2 infection on male reproductive function have recruited men who were hospitalised due to moderate or severe COVID-19. Few studies have assessed testicular function in the active stage of SARS-CoV-2 infection in men with mild disease, and data on the long-term impact of SARS-CoV-2 infection on male reproduction are limited. Therefore, we investigated semen and blood samples from men with mild manifestations of SARS-CoV-2 infection; we investigated the presence of SARS-CoV-2 RNA in semen and evaluated semen parameters and reproductive hormone profiles, including insulin-like factor 3 (INSL3), during the first week of SARS-CoV-2 infection and at three- and six-month follow-up. The peptide hormone INSL3 is produced by the testicular Leydig cells, and serum INSL3 concentrations have previously been shown to be reduced in men with impaired Leydig cell function.[22–24]

2 | MATERIALS AND METHODS

2.1 | Study design

This was a longitudinal cohort study including 36 non-hospitalised men from the general population in the Capital Region of Denmark. All men aged 18–60 years who tested positive for SARS-CoV-2 by routine reverse-transcription polymerase chain reaction (RT-PCR) performed

by a public test provider, either the Danish National Test Centre (TCDK, national screening) or a regional department of clinical microbiology (health care workers, out-patients and individuals having appointments with a healthcare professional) were contacted by secure email and invited to participate in the study. Men with a confirmed SARS-CoV-2 infection who approached the research team were also invited to participate in the study. Participants were included between November 2020 and June 2021. Follow-up appointments were scheduled for three and six months after inclusion. Data collection was completed in December 2021.

2.2 | Participants

Inclusion criteria were male sex, age 18–60 years, first positive SARS-CoV-2 RT-PCR or rapid antigen test within the last seven days, and no severe comorbidity. The duration of acute SARS-CoV-2 infection was defined as seven days in alignment with previous reports.[25,26] SARS-CoV-2 infection was confirmed by a positive RT-PCR test on oropharyngeal swab material at inclusion in 31 participants (86.1%). Five participants (13.9%) had a negative SARS-CoV-2 RT-PCR test at inclusion but had all presented a positive RT-PCR SARS-CoV-2 test within four to seven days before the initial visit. Four of these five men were SARS-CoV-2 immunoglobulin G (IgG) positive at the first visit, whereas the last individual was SARS-CoV-2 antibody negative at inclusion but presented a positive SARS-CoV-2 antibody serology three weeks following inclusion, confirming seroconversion after infection. Participants who had had a vasectomy were eligible to participate in the study but were excluded from the analysis of semen parameters.

Exclusion criteria were the inability to read and understand the Danish language.

Thirty-six men with mild symptoms of COVID-19 i.e., not requiring hospitalisation or advanced medical care, were included in the study. Mild COVID-19 was defined according to the criteria used by the National Institute of Health.[27] Two men were lost to follow-up shortly after inclusion, and one man was unable to attend the six-month follow-up, leaving 33 men for the six-month reproductive hormone analyses. One man had previously undergone vasectomy, and another man underwent vasectomy shortly before the three-month follow-up, leaving 31 men for the six-month evaluation of semen parameters. A questionnaire was answered by all participants, gathering information about the present SARS-CoV-2 infection (onset of symptoms, experienced febrile episodes, and date of the first positive SARS-CoV-2 test), body weight and height, smoking habits, medication, respiratory diseases, reproductive history, previous mumps infection, cryptorchidism, and previous testicular surgery. Due to the infection, all men were isolated at home at the time of inclusion. Thus, the initial visit was conducted at the participants' private address by a member of the research team wearing personal protective equipment. No physical andrological examination was performed. Oropharyngeal and semen swabs for the detection of SARS-CoV-2, semen samples for the assessment of semen quality, and blood samples for the assessment of anti-SARS-CoV-2 IgG antibodies, and hormonal profiles were obtained at inclusion and at follow-up evaluations. An additional blood sample for measurement of anti-SARS-CoV-2 IgG antibodies was drawn three to five weeks after inclusion.

2.3 | SARS-CoV-2 RT-PCR and SARS-CoV-2 serology

Oropharyngeal swabs and semen swabs were collected using either ∑-Virocult (Medical Wire) or eSwab (Copan). The samples were tested for SARS-CoV-2 by RT-PCR applying the E-sarbeco primers and probe[28] and adapted to TaqMan Fast Virus 1-step master mix and LightCycler 480.[29] RT-PCR cycle threshold (ct) values were defined according to the following categories: strongly positive: ct < 25, intermediately positive: ct 25—35, and weakly positive: ct 35—45. Blood samples for anti-SARS-CoV-2 IgG antibodies were collected in 3.5 ml CAT serum separator clot activator Vacuette tubes (Greiner Bio-one, Kremsmünster, Austria), centrifuged for 10 min at 1620 × g and stored at −80°C until analysis. Analyses for anti-SARS-CoV-2 IgG antibodies were performed using the serological immunoassay from YHLO on an iFlash 1800 (YHLO Biotechnology). The serological immunoassay used detected IgG antibodies targeting SARS-CoV-2 nucleocapsid protein and spike protein. IgG antibody values ≥10.0 AU/ml were defined as positive results.

2.4 | Reproductive hormone analyses

Blood samples for hormone analyses were collected in 4 ml Vacuette EDTA tubes, centrifuged as described for the serology samples, and stored at −80°C until analysis. Analyses of LH, FSH, T, sex hormone-binding globulin (SHBG), inhibin B, and INSL3 were performed at the Department of Growth and Reproduction at Rigshospitalet, Copenhagen, Denmark. Plasma concentrations of LH and FSH were measured by chemiluminescence immunoassays using the Atellica instrument (Siemens Healthineers). The limits of detections (LODs) were 0.07 IU/L for LH and 0.3IU/L for FSH, and the corresponding inter-assay coefficients of variation (CVs) were below 15% and 21%, respectively. Plasma T and SHBG were measured by chemiluminescence immunoassays using the Access 2 instrument (Beckman Coulter). The LODs were 0.35 nmol/L for T and 0.33 nmol/Lfor SHBG, and the corresponding CVs were below 4% and 8%, respectively. Plasma inhibin B was measured by an enzyme-linked immunosorbent assay (Beckman Coulter Inhibin B Gen II ELISA; Beckman Coulter) with a LOD of 3 pg/ml and a CV below 6%. Plasma INSL3 was quantified by liquid chromatography-tandem mass spectrometry. The LOD was 0.03 μg/L, and the inter-assay coefficient of variation (CV) was below 9%. The assay was developed for serum INSL3 as previously described.[30] In a study of eight healthy men, plasma INSL3 concentrations were observed to be approximately 20% lower but otherwise highly correlated with serum INSL3 ($R2 = 0.87$, unpublished results). All hormone analyses were accredited by The Danish Accreditation Fund according to the international standard DS/EN 15189 standard.

2.5 | Semen samples

Semen samples were produced by masturbation in the privacy of the participant's home. Participants were given careful instructions about hygiene measures, including hand hygiene, to prevent contamination of the sample. All participants were instructed to wear a face mask when producing the semen sample. The abstinence period was calculated from the self-reported time of previous ejaculation to the time of delivery at the laboratory. No standardised sexual abstinence period was required for the participants. The semen samples were collected into noncytotoxic sterile containers provided by the study center. Following the collection of semen swabs for the detection of SARS-CoV-2, semen samples were transported to the laboratory at Cryos International by a member of the research team while being kept at body temperature. The sample was analysed immediately upon arrival in the laboratory with a maximum of one hour from the time of production to analysis. Semen volume was assessed by weighing the container with the sample provided. If the sample had not already liquified, it was left for approximately 30 min before its viscosity was recorded. A drop of the semen sample was placed in a counting chamber and sperm concentration and motility were assessed by computer-assisted semen analysis (Sperm Class Analyzer 6.6.15 SCA Human Edition). Motility was recorded as total motility and progressive motility was expressed as percentages.

2.6 | Outcomes

The primary study outcomes were to assess the impact of SARS-CoV-2 infection on semen parameters and reproductive hormone profiles and the presence of SARS-CoV-2 RNA in semen. The secondary outcomes were the assessments of the humoral immune response to SARS-CoV-2 and the distribution of SARS-CoV-2 RT-PCR ct values.

2.7 | Statistical analyses

Statistical analyses were performed using the IBM Statistical Package for the Social Sciences (SPSS) 25.0 software for Windows (IBM Corporation). The medians (interquartile range, IQR) for reproductive hormone levels and semen parameters were calculated. Free T (c-fT) was calculated according to the equation suggested by Vermeulen based on measured serum levels of total T and SHBG and assuming a fixed albumin level.[31] The ratios of inhibin B to FSH, c-fT to LH, and total T to LH were calculated. As the distribution of data was skewed, we opted for non-parametric tests where no assumptions of normality were made. Wilcoxon matched-pair signed-rank (two samples) test was used to assess the alterations in reproductive hormone levels and semen parameters from baseline to three- and six-month follow-up. Time-related alterations in reproductive hormone levels or semen parameters were compared between groups (fever vs. no fever) using the independent samples Mann-Whitney U test. A p value less than 0.05 was considered statistically significant.

TABLE 1 Baseline characteristics of the study population ($n = 36$). Data are listed as n (%) or median (interquartile range).

Age (years)	29 (27–44)
BMI (kg/m^2)	24.7 (22.2–27.0)
Time between positive oropharyngeal swab and semen collection (days)	4.0 (3.0–6.0)
COVID-19 vaccination prior to inclusion	5 (13.9)
Influenza vaccination prior to inclusion	3 (8.3)
International travel prior to inclusion	16 (44.4)
Smoking status	
Current	7 (19.4)
Former	15 (41.7)
Never	14 (38.9)
Lung disease	
Asthma	5 (13.9)
Chronic obstructive lung disease	0 (0)
Previous testicular surgery[a]	2 (5.6)
Previous parotitis	4 (11.1)
Previous conception	16 (44.4)
Fertility treatment	4 (11.1)
COVID-19 symptoms	
Any symptom	27 (75.0)
Pain when swallowing	4 (11.1)
Headache	12 (33.3)
Fever	14 (38.9)
Dry cough	15 (41.7)
Musculoskeletal pain	13 (36.1)
Fatigue	12 (33.3)
Reduced taste/smell	10 (27.8)
Throat pain	1 (2.8)
Abstinence period prior to semen sampling (days)	2.0 (2.0-5.0)

[a]One participant had undergone testicular surgery due to cryptorchidism and another had undergone testicular surgery due to testicular torsion.

2.8 | Ethical approval

The study was approved by the Committee on Health Research Ethics of the Capital Region of Denmark (no. H−20027362) and the Knowledge Centre for Data Protection and Compliance of the Capital Region of Denmark (P-2020-387). All participants gave verbal and written consent prior to inclusion.

3 | RESULTS

3.1 | Study population

Table 1 summarises the baseline characteristics of the 36 men who participated in the study. The median (IQR) age of the participants was 29 (27–44) years, and the BMI was 24.7 (22.2–27.0) kg/m^2. Five

TABLE 2 Plasma concentrations of reproductive hormones in the study population during acute SARS-CoV-2 infection (baseline) and at 3- (3 M) and 6-month (6 M) follow-up. Data are presented as medians (interquartile ranges).

	Baseline ($n = 34$)	3 M ($n = 34$)	*p* value (baseline vs. 3 M)[a]	6 M ($n = 33$)	*p* value (3 M vs. 6 M)[b]	*p* value (baseline vs. 6 M)[c]
FSH (IU/l)	4.6 (3.7–6.7)	5.0 (3.5–6.7)	0.51	4.8 (3.7–6.4)	0.94	0.16
LH (IU/l)	4.3 (3.0–5.7)	4.0 (2.9–5.1)	0.02	4.1 (2.7–4.9)	0.18	0.09
Inhibin B (ng/l)	174 (141–210)	175 (150–216)	0.75	168 (146–203)	0.03	0.23
Total T (nmol/l)	25.3 (20.7–32.8)	30.7 (27.1–35.1)	0.003	30.3 (23.0–34.4)	0.10	0.03
C-fT (pmol/l)	587 (450–714)	737 (571–860)	0.01	673 (551–803)	0.15	0.06
INSL3 (ng/l)	418 (301–1267)	499 (345–1153)	0.01	457 (263–1133)	0.02	0.48
Inhibin B/FSH ratio	36 (23–52)	35 (24–47)	0.51	32 (26–50)	0.13	0.62
Total T/LH ratio	6.2 (4.8–8.7)	8.2 (6.0–12.4)	<0.001	7.1 (5.4–10.1)	0.046	0.001
C-fT/LH ratio	0.13 (0.10–0.20)	0.18 (0.15–0.28)	<0.001	0.17 (0.13–0.21)	0.04	0.003

Abbreviations: c-fT, calculated free T; FSH, follicle-stimulating hormone; INSL3, insulin-like factor 3; LH, luteinising hormone; T, testosterone.
[a]*p* value indicates within-subjects change over time from baseline to three-month follow-up (Wilcoxon matched-pair signed-rank (two samples)).
[b]*p* value indicates within-subjects change over time from three- to six-month follow-up.
[c]*p* value indicates within-subjects change over time from baseline to six-month follow-up.

participants (13.9%) had been diagnosed with asthma, however, only three received daily medication by inhaled corticosteroids or beta-agonists. All other participants were previously healthy, and none of the participants were hospitalised during the SARS-CoV-2 infection. One man had undergone testicular surgery in childhood due to cryptorchidism, and another man had been operated on for testicular torsion. None of these two men had previously undergone fertility treatment or contributed to a pregnancy. One man had undergone a vasectomy. Sixteen men (44.4%) had contributed to a pregnancy, including the four individuals who reported previous parotitis. Four men (11.1%) reported previous or current fertility treatment. Five (13.9%) had previously received a COVID-19 vaccine. Of the five men, one man had received the vaccine four months prior to inclusion in the study. The remaining four men had received the vaccine within six to 11 days prior to inclusion. The COVID-19 vaccine is not believed to provide protection at a time point earlier than 12 days after the first dose.[32] The participant who had received the vaccine four months prior to inclusion presented an intermediately positive RT-PCR cycle threshold value at inclusion, thus confirming eligibility.

All men had tested positive for SARS-CoV-2 by RT-PCR within a week before inclusion. The median (IQR) time interval from the first positive oropharyngeal SARS-CoV-2 RT-PCR test to sample collection was 4.0 (3.0–6.0) days. Twenty-seven (75%) of the men had symptoms of COVID-19 at inclusion in the study. None reported symptoms of upper respiratory infections within three months prior to inclusion, thus the probability of including men with SARS-CoV-2 re-infection was low.

3.2 | Reproductive hormone profile

Table 2 shows the reproductive hormone concentrations during acute SARS-CoV-2 infection (baseline) and at three- and six-month follow-up. Median hormone levels were within the normal range at all time points. Concentrations of total T and c-fT and INSL3 were lower during SARS-CoV-2 infection than at follow-up. T and INSL3 concentrations increased from baseline to three months, followed by a decline at six-month which was, however, only statistically significant for INSL3. Both T/LH and c-fT/LH ratios showed a similar pattern of an increase from baseline to three months followed by a lesser decline at the six-month follow-up (e.g., c-fT/LH was 0.13 at baseline, increasing to 0.18 and 0.17 at three- and six-month follow-up ($p = 0.003$), respectively). FSH, inhibin B, and inhibin B/FSH ratios did not differ during the study period.

3.3 | Semen parameters

At baseline, 33 men provided a semen sample. Three men (9.0%) had a sperm concentration <15 million/ml and seven men (21.2%) had a sperm concentration ≥15 million/ml but below 40 million/ml. At three-month follow-up, one participant reported a fever episode during the preceding three months. At six-month follow-up, two men reported fever episodes during the preceding three months.

Table 3 summarises the changes in semen parameters during the study period. The median abstinence period was similar at the three time points. Semen volume increased from baseline to three- and six-month follow-up. Concomitantly, sperm concentration decreased from baseline to the three-month follow-up but increased again at the six-month follow-up. The percentage of progressively motile sperm cells decreased following SARS-CoV-2 infection. Consequently, the total number of motile sperm cells increased from 15 to 26 and 43 million at baseline, three- and six-month follow-up, respectively ($p = 0.02$).

3.4 | Fever vs. no fever

Figures 1 and 2 show the median total sperm count, the percentages of progressively motile sperm cells, LH concentrations, total T/LH, and

TABLE 3 Semen parameters of the study population during acute SARS-CoV-2 infection (baseline) and at three- (3 M) and six-month (6 M) follow-up.

	Baseline ($n = 32$)	3 M ($n = 32$)	*p* value (baseline vs. 3 M)[a]	6 M ($n = 31$)	*p* value (3 M vs. 6 M)[b]	*p* value (baseline vs. 6 M)[c]
Semen volume (ml)	2.3 (1.6–3.5)	3.0 (2.2–4.1)	0.01	2.9 (1.9–4.8)	0.62	0.01
Sperm concentration (million/ml)	67.5 (44.0–122.6)	60.9 (25.9–109.8)	0.04	67.2 (32.7–102.8)	0.11	0.25
Total sperm count (million)	151 (97–294)	170 (90–322)	0.93	158 (110–393)	0.15	0.54
Progressively motile (a+b) (%)	13 (4–22)	20 (8–29)	0.02	21 (10–35)	0.36	0.01
Total motility (a+b) (million)	15 (4–56)	26 (7–63)	0.048	43 (6–102)	0.04	0.02
Abstinence period (days)	2.0 (2.0–5.0)	2.0 (2.0–3.0)	0.10	2.0 (2.0–3.0)	0.50	0.16

Data are presented as medians (interquartile ranges).

[a]*p* value indicates within-subjects change over time from baseline to three-month follow-up (Wilcoxon matched-pair signed-rank (two samples)).

[b]*p* value indicates within-subjects change over time from three- to six-month follow-up.

[c]*p* value indicates within-subjects change over time from baseline to six-month follow-up.

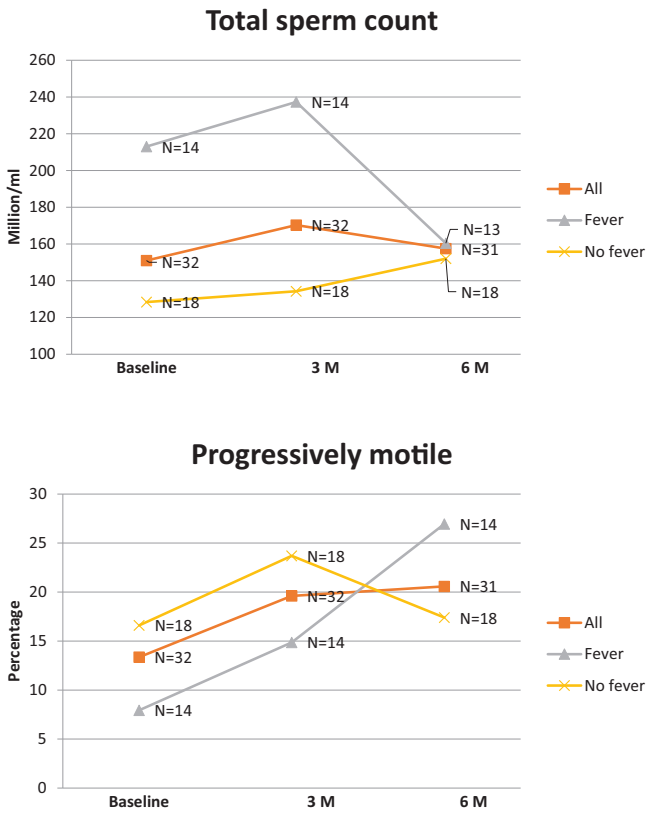

FIGURE 1 Total sperm count and percentages of progressively motile sperm cells during acute SARS-CoV-2 infection (baseline) and at three- (3 M) and six-month (6 M) follow-up in the entire study population and in the group of men with and without fever. Data are presented as medians.

c-fT/LH ratio in the entire study population and in the group of men with ($n = 14$) and without ($n = 18$) fever at inclusion. When restricting the study population to men who did not report fever as a symptom, similar alterations in reproductive hormone levels and semen parameters were observed as in the overall study population (Table S1). Thus, concentrations of LH were significantly higher during the acute SARS-CoV-2 infection, and concentrations of INSL3 and T/LH ratios were lower. Similarly, semen volume and progressively motile sperm cell fraction were lower during the infection relative to three- and six-month follow-up (data not shown).

3.5 | SARS-CoV-2 serology and RT-PCR

No SARS-CoV-2 RNA was detected by RT-PCR in the semen samples of the 36 participants, neither at inclusion nor at the three- and six-month follow-up. None of the 36 participants were concurrently RT-PCR and IgG-positive at the time of inclusion. Serology testing at three weeks showed a 91% seroconversion rate among participants. At the three-month follow-up, 85% had protecting serum levels of IgG, a number that decreased to 54% at the six-month follow-up.

Table 4 illustrates the results of the SARS-CoV-2 RT-PCR analyses. At inclusion eight of the 36 participants (22%) produced oropharyngeal swabs strongly positive for SARS-CoV-2. Twenty-two (61%) were intermediately positive, and one (2.8%) was weakly positive. At the three-month visit, a single participant was still strongly positive for SARS-CoV-2 in the oropharyngeal swab. At the six-month visit, all participants provided a negative oropharyngeal swab (data not shown).

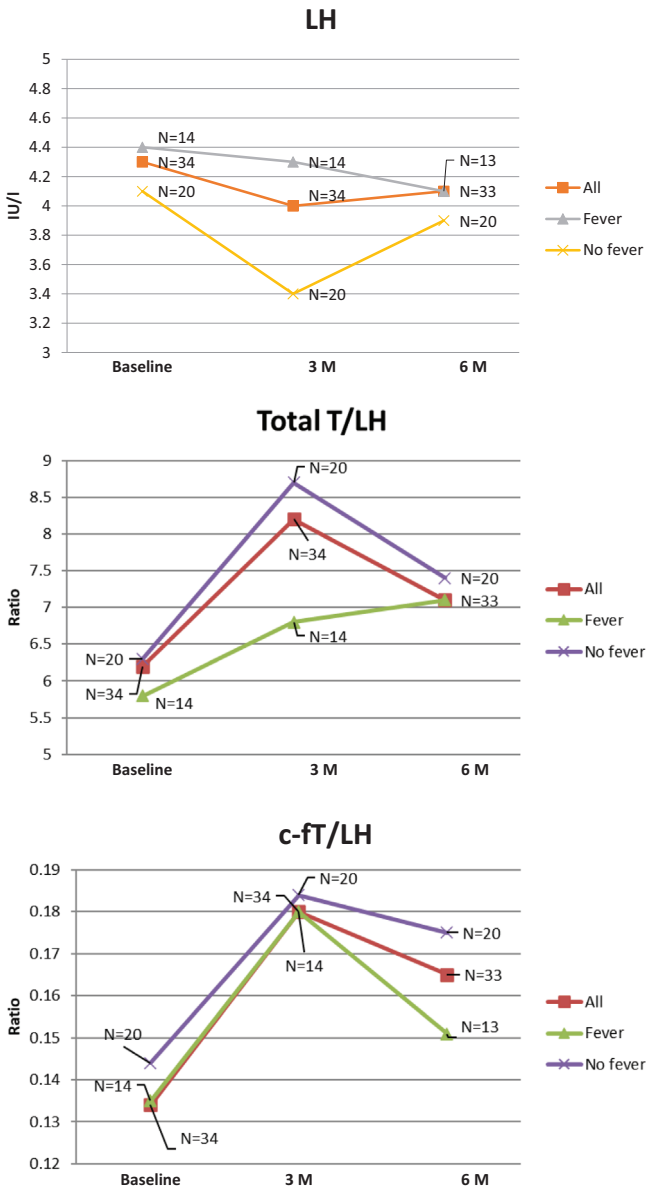

FIGURE 2 Plasma concentrations of luteinising hormone (LH) and total and calculated free (c-f) testosterone (T)/LH ratio during acute SARS-CoV-2 infection (baseline) and at three- (3 M) and six-month (6 M) follow-up in the entire study population and in the group of men with and without fever. Data are presented as medians.

4 | DISCUSSION

In our study of men mildly affected by SARS-CoV-2 infection, the most pronounced negative impact on the testicular function was a reduction in the number of progressively motile spermatozoa during acute infection. A negative impact on reproductive hormone concentrations was also evident. The hormone alterations reversed during the first three months after the infection, whereas the total number of motile spermatozoa was highest at the six-month follow-up. As the study period was limited to six months, we cannot exclude the fact that semen parameters might have continued to improve. Interestingly, the changes were also observed in men who did not report febrile episodes, indicating a direct effect of the virus on testicular function. No SARS-CoV-2 RNA was detected in semen at any time point, indicating a low risk of sexual transmission of SARS-CoV-2.

There are several case reports describing the negative impact of SARS-CoV-2 infection on the male reproductive system.[7,19] Most of the studies during the first wave of the pandemic included hospitalised men with severe COVID-19 and were characterised by wide variations in study methods and limited follow-up.[21,33–36] Later studies tended to focus on men who had recovered from milder manifestations of the disease.[37–41]

Our results may be compared to the findings of a larger study of non-hospitalised SARS-CoV-2-infected men, reporting reduced sperm concentration and reduced total and progressive motility within a month after recovery from the disease.[42] However, the study was limited by the assessment of a single semen sample which was compared to a control population of primarily infertile patients. In a smaller study of men recovering from mild SARS-CoV-2, no changes in semen parameters were observed.[37] Interestingly, our findings are more consistent with the results of studies including more severely SARS-CoV-2-affected men. Thus, in a study of 84 COVID-19 patients who delivered a semen sample 24 hours after hospital discharge and at follow-up visits with 10-day intervals until day 60, significantly lower levels of all semen parameters were observed at all time points compared to controls.[43] Similarly, in a study of 41 COVID-19 patients, assessing semen parameters at a median of 56 days after hospital discharge, 22 men delivered a second semen sample approximately one month later, showing an increase in sperm concentration, total count, and motility which is in line with our observations at the three-month follow-up.[41] Of note, we observed a further increase in total sperm motility at the six-month follow-up, showing that this parameter may be compromised by the infection for at longer duration of time even in mild cases. We further observed a reduced Leydig cell capacity during the SARS-CoV-2 infection. This is best illustrated by the decreased T/LH ratio, pointing to a direct effect of SARS-CoV-2 on the Leydig cells in line with findings from more severely affected men.[21,34] The lowered concentrations of INSL3, a marker of the constitutive Leydig cell capacity,[44,45] during the active SARS-CoV-2 infection further support the direct adverse effect of the virus. INSL3 peaked at the three-month follow-up. Due to the design of our study, we cannot elucidate whether the lower INSL3 concentration detected at the six-month follow-up reflects a more chronic reduction in Leydig cell capacity.

It is well-known that semen quality may be negatively affected by febrile episodes.[46,47] Recovery of motility and morphology parameters may occur approximately 30 days after the normalisation of body temperature, whereas sperm count parameters may need up to 60 days to return to normal, reflecting the duration of the spermatogenic cycle.[48,49] In our sub-analysis of men who did not report fever, we still detected a reduction in the number of progressively motile spermatozoa and a reduced T/LH ratio, indicating a direct detrimental effect of SARS-CoV-2 on testicular function. Comparing our results with other studies investigating the impact of SARS-CoV-2-related febrile episodes on testicular function, it seems that

TABLE 4 Distribution of SARS-CoV-2 reverse-transcription polymerase chain reaction (RT-PCR) cycle threshold (CT) values in oropharyngeal and semen swabs in the study population during acute SARS-CoV-2 infection (baseline) and at three-month (3 M) follow-up.

CT values at baseline	Oropharyngeal swabs	Semen swabs
Strongly positive (<25.0)	8 (22.2%)	0
Intermediately positive (25.0–34.9)	22 (58.3%)	0
Weakly positive (>35.0)	1 (2.8%)	0
Negative	5 (13.9%)	36 (100 %)
CT values at 3 M follow-up		
Strongly positive (<25.0)	1 (3.0%)	0
Intermediately positive (25.0–34.9)	0	0
Weakly positive (>35.0)	0	0
Negative	32 (97.0%)	33 (100%)

Data are *n* (%).

fever may play a role, but that a direct effect of the virus also exists.[39,42,50]

Early reports of SARS-CoV-2 particles detected in semen raised concern about potential sexual transmission of the virus.[12] Indeed, sexual transmission of other single-stranded RNA viruses such as Zika virus and human immunodeficiency virus (HIV) has been observed.[6,51] To date, 26 studies including 861 SARS-CoV-2-positive men have detected the virus in semen samples of a total of 12 men (1.4%) (Table S2). Most of the reported cases were observed in men who were suffering from severe COVID-19, implying a higher systemic viral load and thus a higher risk of alterations of the blood-testis barrier.[12,33,52] Some of the reports were characterised by methodological limitations implying a risk of contamination of semen samples.[18] We did not detect any viral transmission to semen in our study which is consistent with recent research,[13] and this indicates that the risk of sexual transmission of SARS-CoV-2 from men with mild manifestations of COVID-19 is low or even negligible. The strength of this study is the inclusion of all participants in the acute phase of a mild SARS-CoV-2 infection and the serial evaluation of semen and reproductive hormone parameters. To our knowledge this is the first study including an assessment of the impact of SARS-CoV-2-infection on INSL3 concentrations, providing new data on Leydig cell function during mild COVID-19. Furthermore, all reproductive hormone samples were quantified by the same laboratory using highly sensitive analytical methods. This study is limited by the sample size, but nevertheless, alterations in important markers of testicular function were shown. Pre-COVID-19 data on semen quality and hormone profiles were unfortunately not available. Therefore, we cannot determine whether the testicular function of the participants had fully recovered six months after the SARS-CoV-2 infection. Febrile episodes were not in all cases verified by a body temperature measurement which may influence the interpretation of our findings. Furthermore, due to COVID-19 measures semen samples were evaluated using an automated analysis which did not comply with the classical WHO guideline.[53] Thus, the reported semen parameters should be interpreted with caution and cannot accurately predict the fertility of the participants of the study.

5 | CONCLUSIONS

This study shows a reduction in testicular function in men mildly affected by SARS-CoV-2 infection. During the six months following infection, semen parameters improved; however, our study could not determine whether complete testicular function had returned. During the first three months following SARS-CoV-2 infection, Leydig cell function also improved; however, decreased INSL3 concentrations may indicate that the underlying Leydig cell capacity had decreased. It remains to be explained if changes in semen parameters and male reproductive hormones are a consequence of a direct viral invasion of the testes, or an accompanying fever and inflammatory response to SARS-CoV-2. Further studies are needed to explore the long-term effect of SARS-CoV-2 infection on human testicular function.

AUTHOR CONTRIBUTIONS

Mette Petri Lauritsen, Uffe Vest Schneider, Lærke Priskorn, Niels Jørgensen, Anne-Bine Skytte, Henrik Westh, Nina la Cour Freiesleben, and Henriette Svarre Nielsen conceptualised and designed the study. Mette Petri Lauritsen, Thomas Leineweber Kristensen, Christine Bo Hansen, Nina la Cour Freiesleben, and Anna Lando Talbot were responsible for the sample and data collection. Uffe Vest Schneider, Thomas Leineweber Kristensen, and Henrik Westh were responsible for RT-PCR and serology analyses. Anne-Bine Skytte was responsible for the analyses of semen parameters. Trine Holm Johannsen, Lærke Priskorn, Niels Jørgensen, Anders Juul, and Jakob Albrethsen were responsible for the hormone analyses. Mette Petri Lauritsen, Thomas Leineweber Kristensen, Anne Zedeler, Nina la Cour Freiesleben, and Lærke Priskorn accessed and verified the data. Mette Petri Lauritsen, Thomas Leineweber Kristensen, Jørgen Holm Petersen, and Lærke Priskorn performed the data analyses. Jørgen Holm Petersen provided statistical advice. Mette Petri Lauritsen, Thomas Leineweber Kristensen, Christine Bo Hansen, and Lærke Priskorn drafted the figures and tables. Henriette Svarre Nielsen acquired funding. Mette Petri Lauritsen wrote the initial draft of the manuscript. The corresponding author had full access to all the data in the study and had

the final responsibility for the decision to submit the paper for publication. All authors have critically reviewed the manuscript and approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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This study was supported by Cryos International Denmark. The funder of the study was responsible for the analysis of the semen samples of all participants but had no role in the study design, data collection, RT-PCR, SARS-CoV-2 antibody and hormone analyses, interpretation of data, writing of the report or decision to submit the paper for publication.

DATA AVAILABILITY STATEMENT

An anonymised, de-identified version of the dataset can be made available upon request.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

BMP2 restores erectile dysfunction through neurovascular regeneration and fibrosis reduction in diabetic mice

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Abstract

Background: The odds of erectile dysfunction are three times more prevalent in diabetes. Severe peripheral vascular and neural damage in diabetic patients responds poorly to phosphodiesterase-5 (PDE5) inhibitors. However, bone morphogenetic protein 2 is known to be involved in angiogenesis.

Objectives: To assess the efficacy of bone morphogenetic protein 2 in stimulating angiogenesis and augmenting nerve regeneration in a mouse model of diabetic-induced erectile dysfunction.

Materials and methods: The induction of diabetes mellitus was performed by streptozotocin (50 mg/kg daily) administered intraperitoneally for 5 successive days to male C57BL/6 mice that were 8 weeks old. Eight weeks post-inductions, animals were allocated to one of five groups: a control group, a streptozotocin-induced diabetic mouse group receiving two intracavernous 20 μL phosphate-buffered saline injections, or one of three bone morphogenetic protein 2 groups administered two injections of bone morphogenetic protein 2 protein (1, 5, or 10 μg) diluted in 20 μL of phosphate-buffered saline within a 3-day interval between the first and second injections. The erectile functions were assessed 2 weeks after phosphate-buffered saline or bone morphogenetic protein 2 protein injections by recording the intracavernous pressure through cavernous nerve electrical stimulation. Angiogenic activities and nerve regenerating effects of bone morphogenetic protein 2 were determined in penile tissues, aorta, vena cava, the main pelvic ganglions, the dorsal roots, and from the primary cultured mouse cavernous endothelial cells. Moreover, fibrosis-related factor protein expressions were evaluated by western blotting.

Results: Erectile function recovery to 81% of the control value in diabetic mice was found with intracavernous bone morphogenetic protein 2 injection (5 μg/20 μL). Pericytes and endothelial cells were extensively restored. It was confirmed that angiogenesis was promoted in the corpus cavernosum of diabetic mice treated with bone morphogenetic protein 2 through increased ex vivo sprouting of aortic rings, vena cava and penile tissues, and migration and tube formation of mouse cavernous endothelial

Mi-Hye Kwon and Beom Yong Rho made an equal contribution to this study.

cells. Bone morphogenetic protein 2 protein enhanced cell proliferation and reduced apoptosis in mouse cavernous endothelial cells and penile tissues, and promoted neurite outgrowth in major pelvic ganglia and dorsal root ganglia under high-glucose conditions. Furthermore, bone morphogenetic protein 2 suppressed fibrosis by reducing mouse cavernous endothelial cell fibronectin, collagen 1, and collagen 4 levels under high-glucose conditions.

Conclusion: Bone morphogenetic protein 2 modulates neurovascular regeneration and inhibits fibrosis to revive the mouse erection function in diabetic conditions. Our findings propose that the bone morphogenetic protein 2 protein represents a novel and promising approach to treating diabetes-related erectile dysfunction.

KEYWORDS
BMP2, diabetes, erectile dysfunction

1 | INTRODUCTION

A harmonious connection between the neurovascular system, hormonal system, and endothelial, mural, and nerve cells is necessary for a penile erection to occur.[1,2] A chronic failure or incapability to produce and sustain sufficient erection intended for proper intercourse is defined as erectile dysfunction (ED).[2] This is a significant contributor to a deteriorating standard of living. A substantial correlation has been shown between diabetes mellitus (DM) and an increased likelihood of experiencing ED.[3] Diabetes puts men at a threefold amplified risk for ED than healthy males without diabetes.[4] Furthermore, ED and DM are considered to be organically related, and ED may be the first symptom exhibited by DM patients.[5]

The initial treatment for ED in DM (EDDM) is PDE5 inhibitors (PDE5i), also known as phosphodiesterase type 5 inhibitor, which is a medication that prevents the deprivation of cyclic guanosine monophosphate, with the relaxation of cavernous smooth muscle occurring to intensify erectile capability.[6] However, it has been reported that phosphodiesterase-5 (PDE5i) is less effective among DM patients than in the general population,[7] primarily because of severely impaired vascular and neurological functions and in part because of the relationship between the pharmacological efficacy of PDE5i and endogenous nitric oxide levels, which are reduced in DM because of low endogenous nitric oxide synthase activity.[8] If oral medication is unsuccessful, intracavernous injections with vasoactive drugs and penile implants are used.[9] Nonetheless, many patients are reluctant to accept injection therapy or penile surgery. Therefore, a new approach to treating diabetic ED that focuses on improving the condition of the penile neurovascular system is required.

For a long time, researchers have acknowledged the relevance of the bone morphogenetic protein (BMP) pathway in new vascular formation. BMP, which belongs to the transforming growth factor (TGF) superfamily, is required for the development and preservation of homeostasis in a vast array of different tissues, including blood vessels and the nervous system.[10] Over 12 different kinds of BMPs have been found, and BMP2, 4, and 6 are known to be related to angiogenesis.[11] Within the scope of this investigation, our goal was to ascertain whether or not BMP2 is responsible for promoting angiogenesis and nerve regeneration in an EDDM mouse model. Streptozotocin (STZ)-induced diabetic mice with ED were utilized to examine how BMP2 affects in vivo erectile function recovery and in primary grown in vitro mouse cavernous endothelial cells. In this study, we used cultured aortic rings, vena cava, penile tissues, major pelvic ganglia (MPGs), and dorsal root ganglia (DRGs) ex vivo.

2 | MATERIALS AND METHODS

2.1 | Animals and treatments

Male C57BL/6 mice aged 8 weeks old, age-matched, and clear of any pathogens were used in this study. The Medical School of Inha University's INHA Institutional Animal Care and Use Committee (INHA IACUC) gave their consent to the experimental technique that was to be used in this research in advance (IACUC No. INHA 200910-719). Injections of STZ (50 mg/kg of body weight) given peritoneally for 5 days in a row caused diabetes to develop in the test subjects.[12] A non-fasting glucose level of more than 300 mg/dL was deemed diabetic in animals.

Eight weeks post-induction of diabetes, the mice remained placed on a temperature-controlled surgical table supine and administered a dose of xylazine (5 mg/kg) and ketamine (100 mg/kg) intraperitoneally (i.p.) to sedate the mice. In each case, the penis was sterilized before being exposed.[12,13] Mice were then allocated into five groups ($N = 5$ per group): a treatment-naïve control group, an STZ-induced diabetic mouse group administered two intracavernous phosphate-buffered saline (PBS) injections (3 days before and on the day of 8 weeks post-induction of diabetes; 20 μL), and three STZ-induced BMP2 groups (administered two intracavernous BMP2 injections at 1, 5, or 10 μg in 20 μL of PBS, 3 days before and on the day of 8 weeks post-induction of diabetes). PBS or BMP2 protein in PBS was injected into the corpus cavernosum midportion using a 30-gauge insulin syringe. Electrical

cavernous nerve stimulation was used to assess erectile function 2 weeks following BMP2 protein administration. Different animals were used for biochemical and histologic studies.

2.2 | Erectile function measurement

Two weeks after PBS or BMP2 treatment, a combination of xylazine (5 mg/kg) and ketamine (100 mg/kg) was injected into the mice's abdominal cavities to induce anesthesia. Erectile function was evaluated using the method outlined before.[12] After making an incision through the medial of the abdomen to expose the prostate and the bladder as well as locating cavernous nerve. Bipolar electrodes made of platinum wire and were used to stimulate the nerves. After removing the penile skin, a 26-gauge needle was used to inject 250 U/mL into a part of the corpus cavernosum located on one of its sides. Intracavernous pressure (ICP) was gauged using a pressure system Statham P23 sensor interfaced to the student lab program (Biopac Systems, Goleta, CA, USA) for data collection and processing.[12] The stimulation settings were 5 V at 1 min duration, 1 ms pulse width, and 12 Hz. The ICP peak was determined during the tumescence period. The total ICPs were determined as areas under curves calculated starting from the beginning of the nerve stimulation began and 20 s after the stimulus ceased. A non-intrusive mouse tail blood pressure measurement (Visitech Systems, Apex, NC, USA) was used to determine the blood pressure. We determined mean systolic blood pressure (MSBP) through the average value of tail blood pressure without anesthesia in two consecutive sets of 10 times each. Briefly, the mouse from each group of animals was placed in a special holder that can block light and movement. The tip of the tail was fixed with an adhesive bandage and placed on the sensor. Before measuring one set, pre-test was performed three times, and blood pressure was measured 10 times. This step was repeated twice for each mouse. Usually, during the pre-test, the mouse becomes stable, and we can obtain relatively constant blood pressure values. We measured systemic blood pressure before measuring ICP. The ratio of the maximum ICP or total ICP to MSBP was calculated to determine the fluctuations in systemic blood pressure.[12]

2.3 | Histologic examinations

Penile tissues, MCECs, DRGs, and MPGs were all preserved in paraformaldehyde at a concentration of 4% for fixation. Frozen tissue sections with a thickness of 12 μm and ex vivo samples were treated with antibodies overnight against NG2 (1:100, AB5320; Millipore, Temecula, CA, USA), CD31 (1:100, MAB1398Z; Millipore), smooth muscle actin (SMA) (1:100, F3777; Sigma, St Louis, MO, USA), PH3 (1:200, 06-507; Millipore), or neurofilament (1:50, N-5389; Sigma–Aldrich) at 4°C. After being rinsed thoroughly with PBS, the sections were kept at ambient temperature throughout the incubation process with secondary antibodies linked with fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate (Zymed Laboratories, South San Francisco, CA, USA). For nuclei staining, we mounted the samples in a solution that contains the 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, CA, USA) before being examined. Confocal fluorescence microscopes were used to capture digital photographs of the signals (K1-Fluo; Nanoscope Systems, Inc., Daejeon, Korea). Utilizing ImageJ (National Institutes of Health), immunofluorescence pictures were examined.

2.4 | Mouse cavernous endothelial cell culture

Primary endothelial cultivation was performed according to the procedures that were described before.[14,15] After harvesting and washing penile tissues in Hank's balanced salt solution buffer (HBSS, Gibco, Carlsbad, CA, USA), samples were transferred to PBS and rinsed numerous times. The glans penis, dorsal part of the neurovascular bundle, and urethra were removed, while the remainder was used for the endothelial primary cell culture. After being cut into thin slices, tissue from the corpus cavernosum was topped with Matrigel (Becton Dickinson, Mountain View, CA, USA). Gel polymerization was accomplished by incubating it for 5 min at 37°C. Once this was performed, the culture plates were then filled with Gibco culture media 199 (3 mL), which included 20% of fetal bovine serum, 20% of basic fibroblast growth factor, 1% of streptomycin/penicillin antibiotic, and 0.5% of heparin. After 2–3 weeks, the cells that were confluent and growing over the bottoms of the plates were sub-cultured by planting them on a plate covered with gelatin 0.2%. Experiments were conducted on cells from passages 2—3.

2.5 | Establishment of in vitro and ex vivo experimental models mimicking diabetic ED

After being deprived of serum for 24 h, the cells were then subjected to either normal glucose (NG; 5 mmol; Sigma-Aldrich) or high glucose (HG; 30 mmol; G7021; Sigma–Aldrich) for 2 days to mimic diabetes-like neuropathy and angiopathy in vivo and ex vivo, respectively, before finally being treated with BMP2 protein (200 ng/mL) or PBS.

2.6 | Aorta, vena cava, and penile tissue sprouting assays

The aorta, vena cava, and penile tissues were removed from C57BL/6 wild-type mice that were 8 weeks old and then put in an eight-well Chamber Slide System (Nunc Lab-Tek, Sigma–Aldrich); 50 μL Matrigel was placed on top of the cell suspension, which was then sealed and cultivated in complete medium 199. A phase-contrast digital microscope was used to capture images of sprouting cells.

2.7 | MCEC migration assay

The scar block system (SPL Life Sciences, Pocheon-si, Gyeonggi-do, Korea) was used to perform migration tests on 60-mm culture plates.

MCECs that had been treated with BMP2 were put into three-well blocks with >95% confluence in either HG or HG with BMP2 added. Five hours after seeding, blocks were taken out, and cells were subsequently cultured in M199 media for an additional 24 h. A phase-contrast microscope was used to capture still images, and the cells that migrated within the bounds line of the detached blocks were calculated with ImageJ.

2.8 | Tube formation assay

Analyses of angiogenesis were carried out using tube formation analysis in either NG or HG conditions[16] or HG containing BMP2 (200 ng/mL). Briefly, an initial 96-well culture plate was filled with Matrigel with reduced growth factor (354234; Becton Dickinson, Mountain View, CA, USA) that had been kept at 4°C and allowed to gel for 10 min at 37°C. The pretreated 2×10^4 cells/well of MCECs were planted into wells that contained 200 μL of M199 culture media. Images were captured with phase-contrast microscope after 12 h. ImageJ was used to count the master junctions with the 40× screen magnification.

2.9 | BrdU proliferation assay

MCECs were grown above the coverslips placed in 12-well plates for 48 h under either NG or HG conditions before being treated with BMP2 for 24 h and then incubated in media containing BrdU (Invitrogen, Carlsbad, CA, USA) for another 24 h. Then, it was washed twice with PBS, fixed in a 2:1 solution of acetic acid and ethanol for 10 min at −20°C, and washed again with PBS. In order to parse the DNA of BrdU-positive cells, those cells were subjected to a 1-h treatment at room temperature with 1 N hydrochloric acid. Sodium borate 0.1 M at pH 8.5 was used to counterbalance the cells incubated at room temperature for 30 min before three rinses with PBS. It was blocked for an hour with Antibody Dilution Buffer at room temperature. The slides were then incubated overnight with anti-BrdU antibody (1:500; AbD Serotec, Kidlington, UK) at 4°C. It was rinsed with PBS twice and then incubated with secondary antibody for 2 h with anti-rat fluorescein-labeled antibody (1:500; Jackson ImmunoResearch Laboratories Inc.) at room temperature. After counterstaining with DAPI, the number of cells that were actively proliferating was determined with ImageJ.

2.10 | TUNEL assay

TUNEL assays were performed in order to determine the degree of cell death, the test stand for nick end labeling using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate was carried out with fluorescein in situ apoptosis detection kit (ApopTag-S7160; Chemicon, Temecula, CA, USA). A confocal fluorescent microscope was used to count the quantity of apoptotic cells, while the screen magnification was set to 200×. The results are shown as the proportion of TUNEL-positive cells found in each high-power field.

2.11 | Ex vivo neurite sprouting assay

The assays were proceeded according to the prior description, with a few minor adjustments.[16] Under a microscope, MPG and DRG tissues were extracted and moved to sterile HBSS-containing tubes and then washed with PBS twice. Following that, the explants were cut into smaller fragments and then plated on 12-well dishes precoated with poly-D-lysine hydrobromide (Sigma–Aldrich). These plates were then covered with Matrigel filled with 1 mL of complete Neurobasal medium (Gibco) supplemented with 0.5 nM GlutaMAX-I (Gibco) and 2% serum-free B-27 (Gibco) and placed in the 5% CO_2 atmosphere incubator at 37°C. Neurite outgrowth was evaluated after incubation for 7 days.

2.12 | Western blot analysis

In each line, 40 μg of the same amount of protein was electrophoresed on 8%–15% sodium dodecylsulfate-polyacrylamide gel. Following the separation of the proteins, they were subsequently loaded onto polyvinylidene difluoride membranes, which were subsequently cut into appropriately sized strips and probed with antibodies against BMP2 (1:500; ab14933; Abcam, Cambridge, MA, USA), fibronectin (1:500; ab23750; Abcam), collagen I (1:500; ab34710; Abcam), collagen IV (1:500; ab6586; Abcam), and GAPDH (1:5000; WH168846; AB clonal, Woburn, MA, USA). Densitometry was used to perform the analysis, with a sample size of $N = 4$ for each group.

2.13 | Statistical analysis

A *t*-test or one-way ANOVA in GraphPad Prism version 8 (Graph Pad Software, Inc., San Diego, CA, USA) was used to carry out intergroup comparisons based on parametric data. The results are expressed as means ± SEs, and *p*-values ≤0.05 were regarded as statistically significant. ImageJ was used to analyze digital pictures and densitometry findings.

3 | RESULTS

3.1 | Metabolic and physiological variables

At 2 weeks after receiving either PBS or BMP2 therapy, the STZ-induced diabetic mice had a substantially lower mean body weight than the control group. In addition, the STZ-induced diabetic mice group had markedly higher observed fasting and after meal blood glucose concentrations. Both the PBS-treated and the BMP2-treated diabetic groups had comparable mean body weights as well as glucose levels after treatment (Table 1).

TABLE 1 Metabolic variables.

		STZ-induced diabetic mice			
	Control	PBS	BMP2 (1 μg)	BMP2 (5 μg)	BMP2 (10 μg)
Body weight (g)	30.2 ± 0.8	22.1 ± 0.3*	23.9 ± 0.4*	24.2 ± 0.5*	25.4 ± 0.9*
Fasting glucose (mg/dL)	125.0 ± 5.1	433.2.0 ± 30.1*	395.6 ± 46.1*	331.3 ± 54.4*	330.2 ± 51.5*
Postprandial glucose (mg/dL)	170.6 ± 14.8	567.0 ± 15.2*	532.4 ± 28.4*	537.8 ± 21.9*	519.6 ± 32.1*
Blood pressure (mmHg), MSBP	104.8 ± 2.5	108.5 ± 2.6	108.2 ± 0.6	104.7 ± 2.2	103.2 ± 0.8

Note: Values are the mean ± SE from $N = 5$ animals per group.
Abbreviations: BMP2, bone morphogenetic protein 2; MSBP, mean systolic blood pressure; PBS, phosphate-buffered saline; STZ, streptozotocin.
*$p < 0.01$ versus control group.

3.2 | BMP2 protein treatment regained erectile function in diabetic mice

As a result of western blot analysis to confirm BMP2 expression under diabetic conditions, BMP2 was decreased under HG condition compared to NG in MCECs. And BMP2 in penile tissue was decreased in diabetic mice compared to the control group (Figure 1A–D). The efficiency of BMP2 was evaluated in diabetic mice by electrically stimulating the cavernous nerves after 2 weeks of PBS or BMP2 treatment (1, 5, or 10 μg in 20 μL of PBS, 3 days before and on the day of 8 weeks post-induction of diabetes). ICP tracings of stimulated cavernous nerve are shown in Figure 1E. When diabetic mice were given PBS as treatment, the erectile function of the animals was significantly impaired than in the other groups. BMP2 at 5 μg/20 μL significantly regained erectile function to 81% of the control level as determined by max ICP to the MSBP ratio and 83% by total ICP to MSBP ratio (Figure 1F,G). Max ICP and total ICP, not normalized to MSBP, are shown in Figure 1H,I.

3.3 | In diabetic mice, BMP2 therapy increased cavernous endothelial cell and pericyte content

Double immunostaining was performed on cavernous tissue 2 weeks after BMP2 therapy in diabetic mice with antibodies against NG2 (a pericyte marker), SMA (a smooth muscle cell marker), and CD31 (an endothelial cell marker) to investigate the impact of the BMP2 protein pertaining to the proliferation of new cavernous endothelial cells and pericytes. PBS-treated diabetics had considerably fewer cavernous endothelial cells and pericytes than the control group, and BMP2 injection markedly increased the numbers of both cell types (Figure 2).

3.4 | Angiogenesis was induced by BMP2 protein in vitro and ex vivo

Angiogenic effects of BMP2 were evaluated using ex vivo mouse vascular tissue sprouting and an in vitro migration and tube formation experiment. BMP2 (200 ng/mL) enhanced the sprouting of endothelial cells from aortic rings and vena cava, and penile tissues and the migration and capillary tube formation by MCECs under HG conditions (Figure 3).

3.5 | BMP2 protein promoted cell proliferation and inhibited apoptosis under diabetic conditions

To determine whether cavernous endothelial cells under the HG condition or in diabetic mice were preserved by cell proliferation, we stained MCECs with BrdU or phosphohistone H3 (cell proliferation markers) and stained penile tissue with phosphohistone H3. BMP2 treatment of MCEC exposed to HG conditions significantly increased the number of BrdU- and phosphohistone H3-positive endothelial cells in the corpus cavernosum of diabetic mice (Figure 4A,B,F,G). The number of endothelial cells stained with PH3 was also increased in the BMP2-treated diabetic mice as compared with PBS-treated diabetic mice (Figure 4D,I). TUNEL assays revealed a greater number of apoptotic cells in MCECs and penile tissue under diabetic conditions than under non-diabetic conditions. BMP2 was shown to drastically lower the number of cells that underwent apoptosis under diabetic conditions (Figure 4C,E,H,J).

3.6 | Neural regeneration was induced by BMP2 protein in nerve tissues

To evaluate the potential neural regenerative effect of BMP2 protein, DRG and MPG were grown in the HG condition, and then subjected to treatment with BMP2 at a dose of 200 ng/mL. Immunohistochemical labeling of DRG and MPG tissues with neurofilament demonstrated that the levels of neurite outgrowth were substantially reduced in the HG group compared to the NG group. Treatment of DRG and MPG tissues with BMP2 completely restored neurite outgrowth under HG condition (Figure 5A,C,D). Immunofluorescent staining with neurofilament was performed to confirm the neuronal cell content in mouse corpus cavernosum tissue. BMP2 significantly restored the cavernous expression of neurofilaments in the diabetic mice (Figure 5B,E).

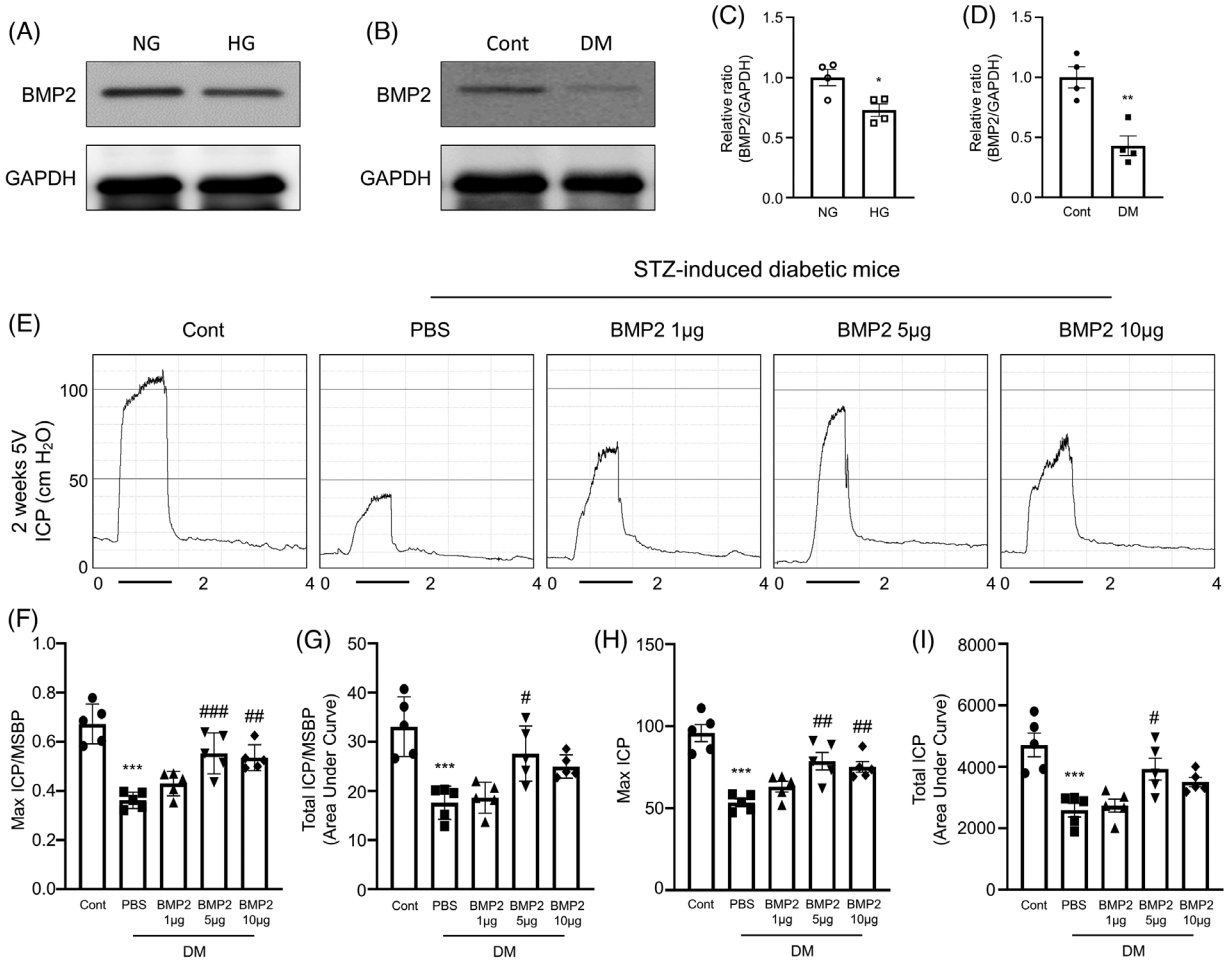

FIGURE 1 Bone morphogenetic protein 2 (BMP2) protein restored erectile function in diabetic mice. (A and B) Representative western blots confirming the expression of BMP2 in mouse cavernous endothelial cells (MCECs) exposed to normal and high glucose (HG) conditions or in penile tissues of control and diabetic mice. (C and D) The band showing the expression of BMP2 was measured using ImageJ and normalized. Bars represent the respective mean and standard error ($N = 4$). Relative ratios were calculated with respect to the normal glucose (NG) or control group. $^{*}p < 0.05$ versus the NG group. $^{**}p < 0.01$ versus control group. (E) Representative intracavernosal responses from age-matched control mice and streptozotocin (STZ)-induced diabetic animals tested after treatment with either phosphate-buffered saline (PBS) or BMP2 protein (1, 5, or 10 μg/20 μL; days −3 and 0). The solid line denotes duration of the stimulus period. (F and G) Proportions of total intracavernous pressure (ICP) or mean maximal ICP to mean systolic blood pressure (MSBP) (referred to as the "area under the curve") in the study groups. (H and I) Mean maximal ICP or total ICP, not normalized by MSBP. Bars represent means (±SEs) of $N = 5$ animals per group. $^{***}p < 0.001$ versus control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus the PBS-treated diabetes mellitus (DM) group.

3.7 | BMP2 protein reduced extracellular matrix production in MCECs under the HG condition

The anti-fibrotic impact of BMP2 was investigated using western blotting on MCECs that had been treated with either PBS or BMP2 and then subjected to HG. We found that BMP2 significantly reduced the expression of fibrosis-related factors, viz. fibronectin, collagen I, and collagen IV in MCECs (Figure 6).

4 | DISCUSSION

In this work, we studied the mechanism through which BMP2 protein protects diabetic mice from developing ED as a result of exposure to STZ. According to previous study, the BMP pathway is involved in the formation of new blood vessels. Previous research has reported that BMP2 promotes angiogenesis. Langenfeld et al.[17] reported that 98% of lung cancer patients exhibit aberrant BMP2 expression and showed that BMP2 affects tumor growth through angiogenesis in an in vivo lung cancer model. Sato et al.[18] found that BMP2 and 4 were involved in vascular diseases, including atherosclerosis, and suggested that these two proteins be considered novel therapeutic targets in atherosclerosis. These findings provide a justification for the clinical use of BMP2 for the treatment of a variety of vascular ailments. ED is a type of vascular disease, but it has not been determined whether the BMP pathway influences ED.

We first tested the outcome of BMP2 in diabetic mice with ED. Two successive injections of 5 μg BMP2 protein into corpus cavernosum

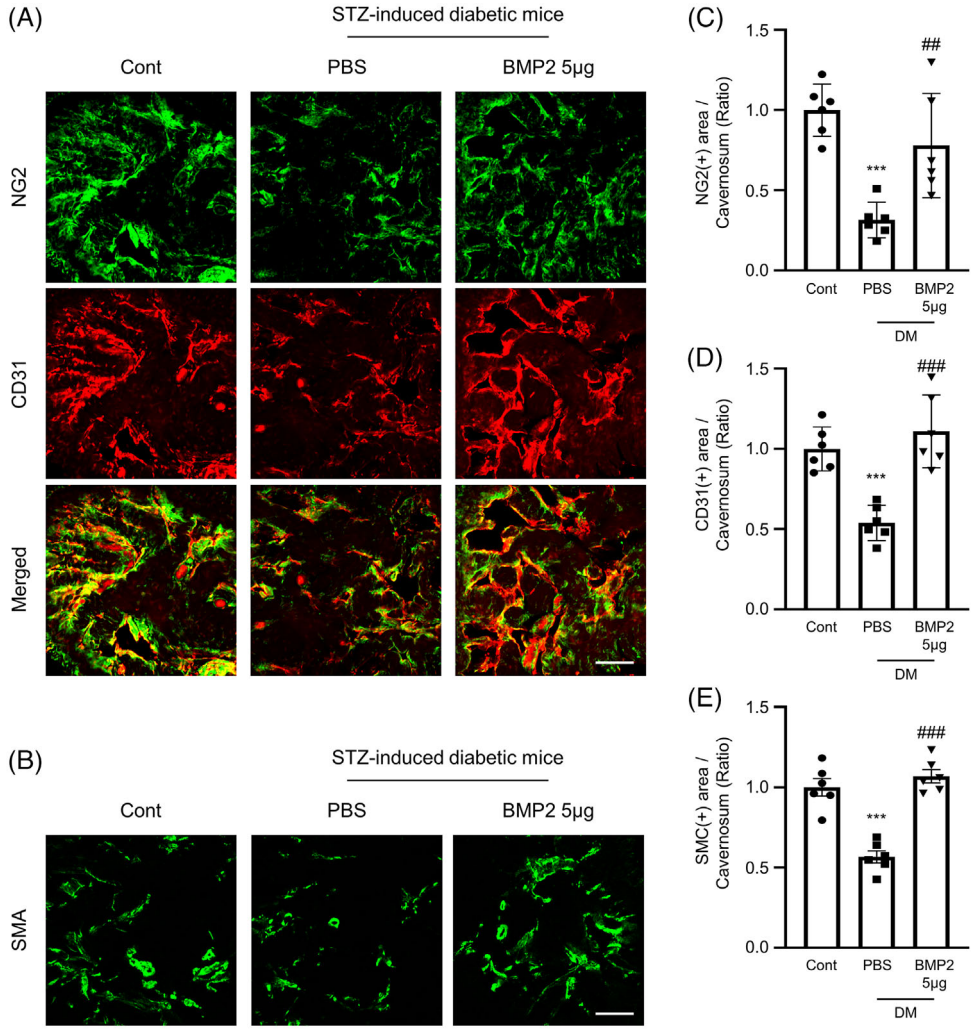

FIGURE 2 Bone morphogenetic protein 2 (BMP2) protein treatment preserved cavernous endothelial cells, pericytes, and smooth muscle cells in diabetic mice. (A and B) Double staining for CD31 (red) and NG2 (green) and single staining for SMA (green) in cavernous tissue from age-matched controls and streptozotocin (STZ)-induced diabetic mice 2 weeks post-treatment with either injection of phosphate-buffered saline (PBS) or BMP2 protein (5 μg/20 μL). Scale bar = 100 μm. (C–E) ImageJ was used to evaluate the contents of cavernous endothelial cells, pericytes, and smooth muscle cells. Bars represent means (±SEs) of $N = 6$ animals per group. Relative ratios were calculated with respect to control group. $^{***}p < 0.001$ versus control group. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus the PBS-treated diabetes mellitus (DM) group.

improved erectile function as measured by ICP test. When we investigated the mechanism responsible for this effect, we found that it was because of enhanced penile vascular regeneration. To confirm these observations, aortic rings, vena cava, and penile tissue were cultivated ex vivo under HG conditions, and it was observed that BMP2 protein enhanced angiogenesis, which showed that BMP2 protein effectively promoted vascular regeneration in EDDM.

In addition to this, we examined the neurotrophic effects of the BMP2 protein in MPG tissues grown in vitro under HG conditions. Treatment with BMP2 protein under HG conditions increased neurite sprouting in MPG tissues, which had poor neurite outgrowth. Previous studies have investigated the role of BMP2 in nerve regeneration. According to Yan et al.,[19] BMP2 enhances the differentiation of neural stem cells into dopaminergic neurons through miR-145-mediated Nurr1 overexpression, and BMP2 is linked to enteric nervous system development in Hirschsprung's disease, according to Huang et al.[20] In addition, Wang et al.[21] A new research suggests that BMP2 might function as a neurotrophic factor while also playing a role in the process of nerve regeneration in the peripheral nervous system.

We previously found that TGF-β1 expression and extracellular matrix protein synthesis were increased in diabetic mouse cavernous tissues compared to normal controls.[22] Under diabetic circumstances, lower levels of fibronectin, collagen I, and collagen IV production are indicators of successful BMP2 protein in preventing fibrosis. Previous research has shown that the BMP pathway has anti-fibrogenic actions in a variety of organs.[23,24] In particular, according to Gao et al., BMP2 inhibits the TGF-β-induced pancreatic stellate cell activation, an important finding considering that TGF-β is the principal cytokine in the pancreas that is responsible for fibrosis and that BMP2 has anti-fibrotic effects,[25] which concurs with our results. Because of their structural

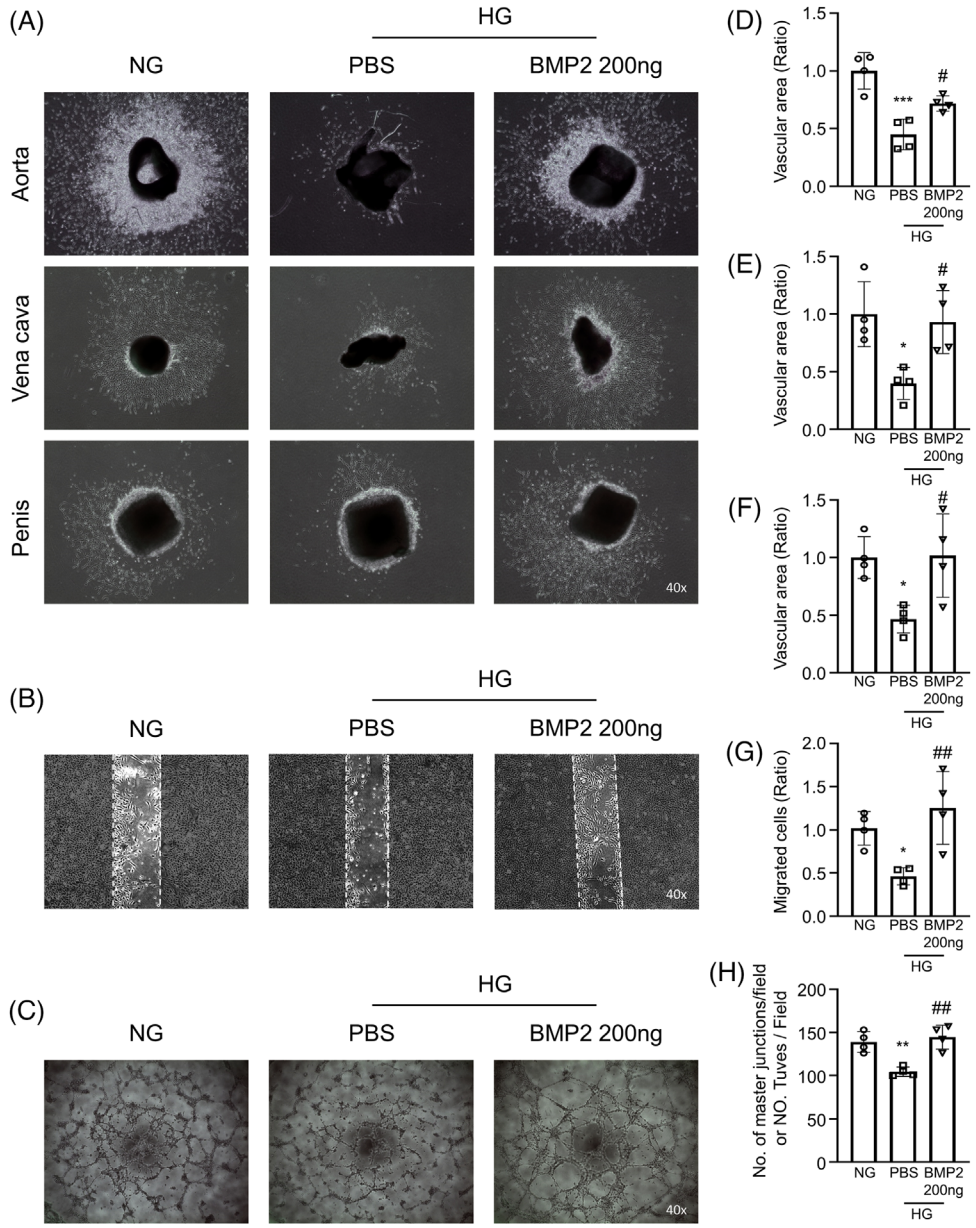

FIGURE 3 Bone morphogenetic protein 2 (BMP2) protein enhanced migration, tube formation, and sprouting angiogenesis in tissues under a high-glucose (HG) condition. (A) Ex vivo microvascular sprouting assay on aortic rings, vena cava and penile tissues exposed to phosphate-buffered saline (PBS) or BMP2 protein (200 ng/mL) under a HG condition (original magnification 40×). (B) Migration test was performed on mouse cavernous endothelial cells (MCECs) that had been treated with either PBS or BMP2 protein (200 ng/mL) under a HG condition (original magnification 40×). (C) Tube formation assay for examination of the capacity of MCECs to form tubes after being subjected to HG conditions in the presence of either PBS or BMP2 (200 ng/mL) (original magnification 40×). (D–F) Areas of outgrowing micro vessels from aorta abdominals and vena cava also from penile tissues ($N = 4$). Relative ratios were calculated with respect to the normal glucose (NG) group. $^{*}p < 0.05$, $^{***}p < 0.001$ versus the NG group; $^{\#}p < 0.01$ versus the HG + PBS group. (G) The number of migrated cells was quantified using ImageJ ($N = 4$). Relative ratios were calculated with respect to the NG group. $^{*}p < 0.05$ versus the NG group; $^{\#\#}p < 0.01$ versus the HG + PBS group. (H) Number of tubes per high-powered field ($N = 4$). $^{**}p < 0.01$ versus the NG group; $^{\#\#}p < 0.01$ versus the HG + PBS group.

similarities and shared signal transductions, there is an interaction between signaling pathways of TGF-β and BMP, and these interactions are implicated in a variety of biological processes and illnesses.[26,27] Moreover, their interaction may lead to the downregulation of TGF-β1R and SMAD2, which are responsible for fibrosis.[27,28] In a previous study, we found that TGF-β1 increased TGF-β1-related fibrotic changes in EDDM.[22] Therefore, in-depth research into the TGF-β and BMP pathways could provide some hints about the treatment of ED.

We report for the first time on the effects of BMP2 protein in diabetic ED. However, the research, on the other hand, has certain drawbacks. First, time-dependent erectile function experiments on BMP2 protein were not performed. Therefore, in order to evaluate

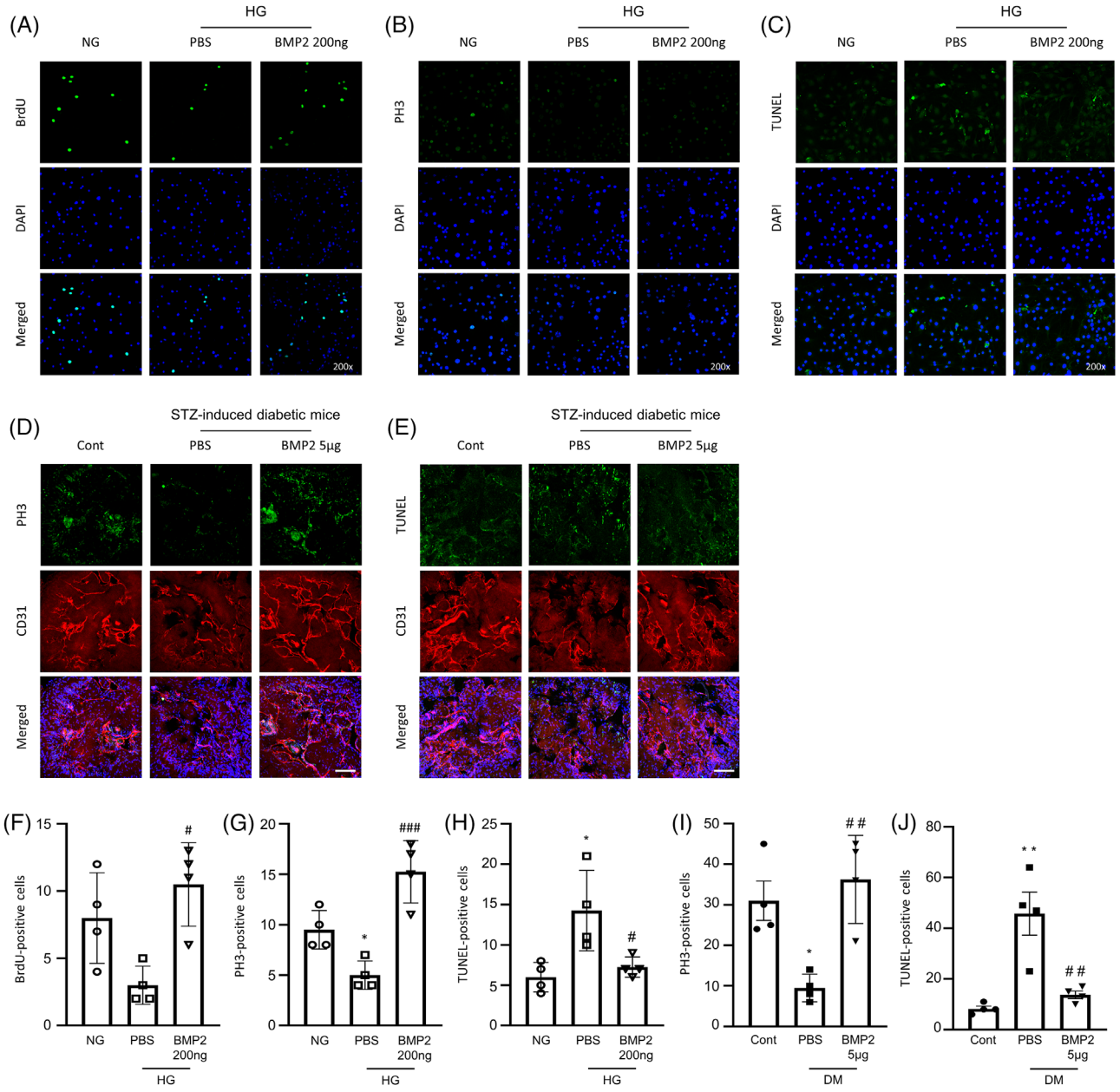

FIGURE 4 Bone morphogenetic protein 2 (BMP2) protein improved endothelial cell proliferation and abridged cavernous endothelial cell apoptosis under diabetic conditions. (A–C) Mouse cavernous endothelial cells (MCECs) that were exposed to normal glucose (NG), high glucose (HG) treated with phosphate-buffered saline (PBS), or HG treated with BMP2 protein (200 ng/mL) and stained with antibodies against BrdU (green), PH3 (green), and TUNEL (green). 4,6-Diamidino-2-phenylindole (DAPI) (blue) was used to identify the nuclei. Original magnification 200×. (D and E) Cavernous tissue from age-matched controls and streptozotocin (STZ)-induced diabetic mice 2 weeks post-treatment with PBS or BMP2 protein (5 μg/20 μL) stained with antibodies against PH3 (green) and TUNEL (green). Nuclei were labeled by costaining with DAPI (blue). Scale bar = 100 μm. (F–J) Numbers of BrdU-positive, PH3-positive, and endothelial cells that were TUNEL-positive for each high-power field. Bars denote the average and standard errors of each of the four separate experiments ($N = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the NG group or control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus the HG + PBS group or the PBS-treated diabetes mellitus (DM) group.

whether BMP2 treatment results in long-term maintenance of erectile function, further research is required. Second, although the Food and Drug Administration has already approved BMP2 as an osteoinductive growth factor for replacements of bone grafting, it has been linked to adverse effects, including ectopic bone production, inappropriate adipogenesis, as well as osteoclast-mediated bone resorption.[29] Therefore, although BMP2 protein produced promising results in the current study, more detailed studies are needed before clinical application. Nonetheless, we believe our findings provide valuable clues for the treatment of EDDM.

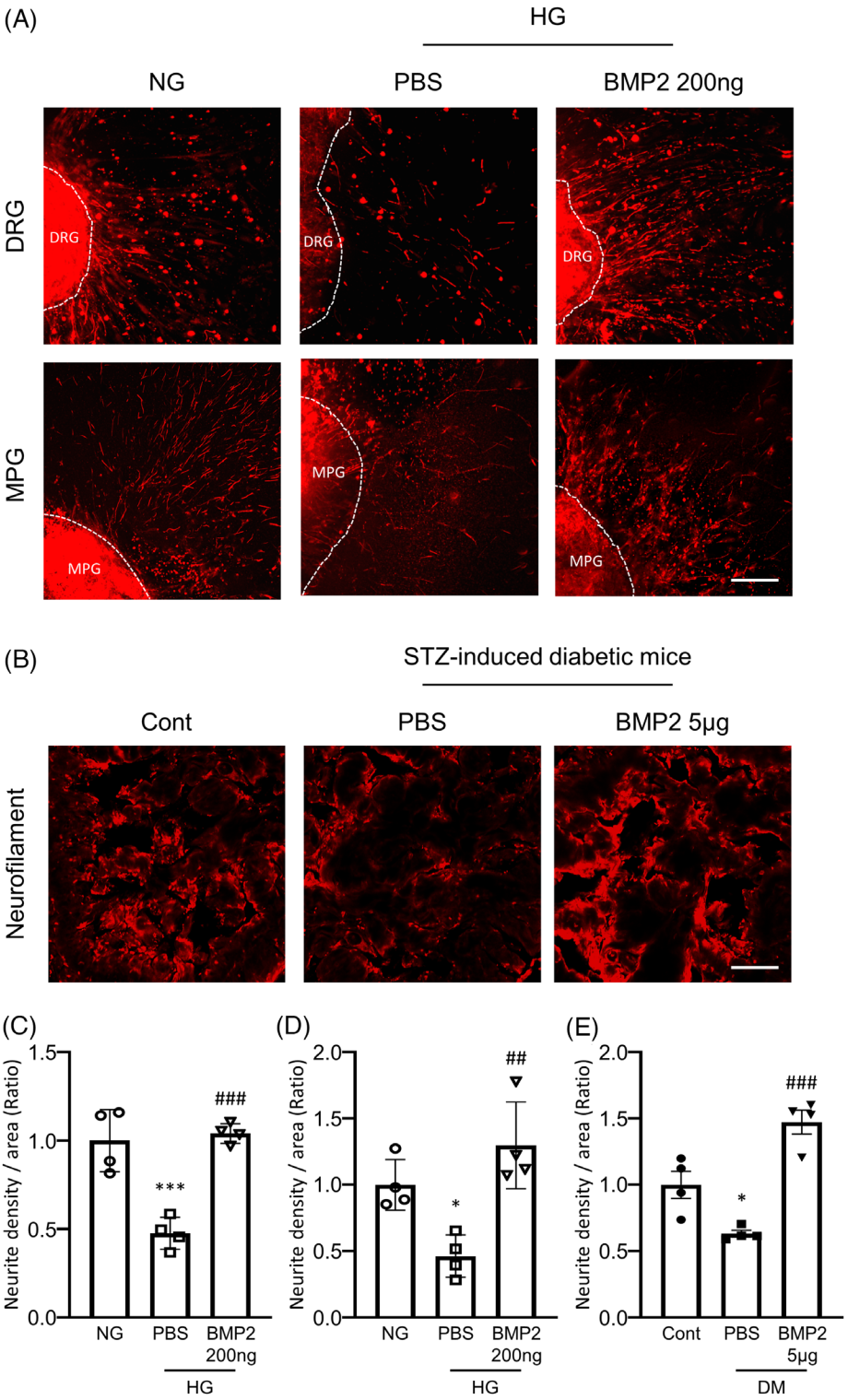

FIGURE 5 Under diabetic circumstances, the bone morphogenetic protein 2 (BMP2) protein was shown to stimulate neuronal regeneration. (A) Immunofluorescent staining of dorsal root ganglion (DRG) and major pelvic ganglion (MPG) tissues using an antibody against neurofilament in tissues that were either subjected to conditions of normal glucose (NG) or high glucose (HG) with phosphate-buffered saline (PBS) or BMP2 protein (200 ng/mL) for 48 h. Scale bar = 100 μm. (B) Immunofluorescent staining of corpus cavernosum with an antibody against neurofilament in age-matched control and streptozotocin (STZ)-induced diabetic mice at 2 weeks after repeated intracavernous injections of PBS (20 μL) or BMP2 protein (5 μg/20 μL). Scale bar = 100 μm. (C and D) Mean neurite lengths were determined using an image analyzer ($N = 4$). Relative ratios were determined with respect to the NG group. $^{*}p < 0.05$, $^{***}p < 0.001$ versus the NG group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus the HG + PBS group. (E) ImageJ was used to evaluate the contents of neurofilament. Bars represent means (±SEs) of $N = 4$ animals per group. $^{*}p < 0.05$ versus control group; $^{\#\#\#}p < 0.001$ versus the PBS-treated diabetes mellitus (DM) group.

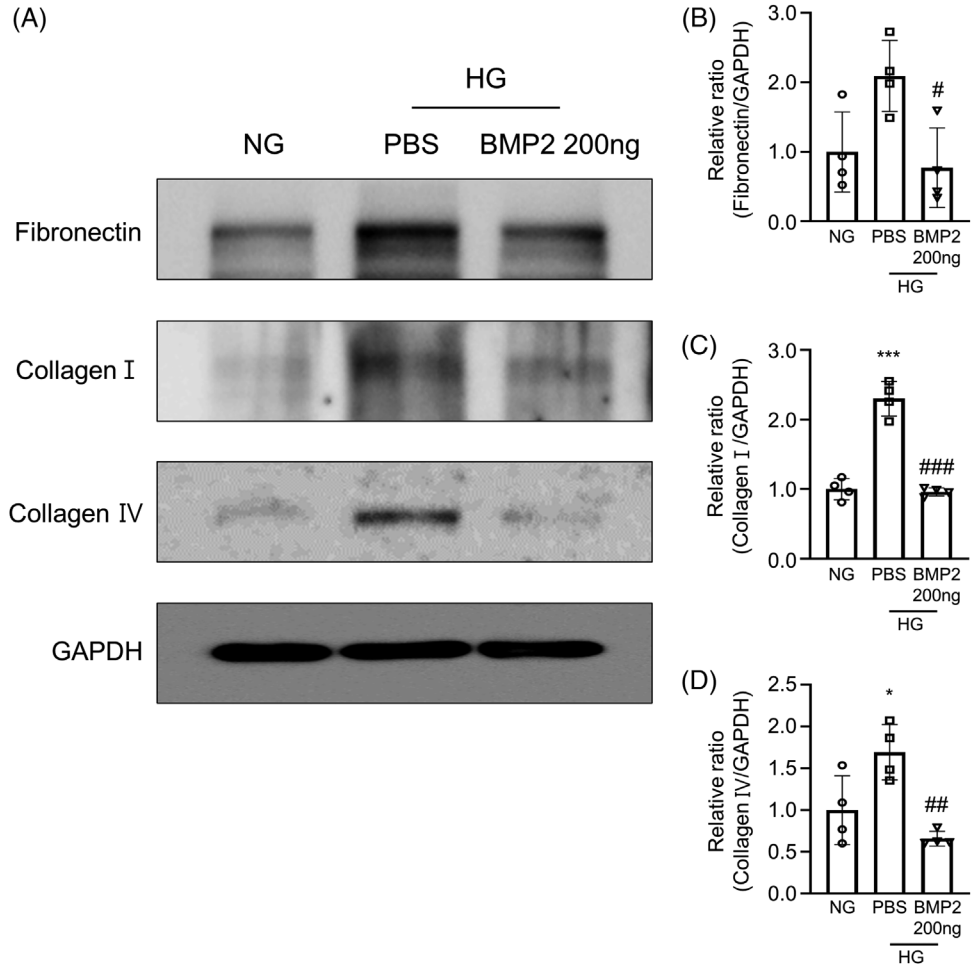

FIGURE 6 Bone morphogenetic protein 2 (BMP2) reduced the expressions of fibrogenic proteins in high glucose (HG)-treated mouse cavernous endothelial cells (MCECs). (A) Representative western blot expression of fibronectin, collagen I, and collagen IV in MCECs subjected to either normal glucose (NG) or HG conditions over a period of 48 h in the presence of phosphate-buffered saline (PBS) or BMP protein (200 ng/mL). (B–D) Values of the band intensities after normalization ($N = 4$). $^{*}p < 0.05$, $^{***}p < 0.001$ versus the NG group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus the HG + PBS group. Relative ratios were determined with respect to the NG group.

AUTHOR CONTRIBUTIONS

Conceptualization and research design: Mi-Hye Kwon, Beom Yong Rho, Doo Yong Chung, and Ji-Kan Ryu. *Experiments*: Mi-Hye Kwon, Beom Yong Rho, Min-Ji Choi, and Anita Limanjaya. *Data acquisition*: Mi-Hye Kwon and Beom Yong Rho. *Deciphering information and data analysis*: Jiyeon Ock and Guo Nan Yin. *Statistical examination*: Guo Nan Yin and Jiyeon Ock. *Drafting of the manuscript*: Mi-Hye Kwon, Beom Yong Rho, Doo Yong Chung, and Ji-Kan Ryu. *Manuscript critical appraisal*: Jun-Kyu Suh. *Grants receiver*: Doo Yong Chung and Ji-Kan Ryu. All authors gave their approval before the work was published.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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ANDROLOGY WILEY

ORIGINAL ARTICLE

Lactate as the sole energy substrate induces spontaneous acrosome reaction in viable stallion spermatozoa

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Abstract

Background: Equine spermatozoa appear to differ from spermatozoa of other species in using oxidative phosphorylation preferentially over glycolysis. However, there is little information regarding effects of different energy sources on measured parameters in equine spermatozoa.

Objective: To determine the effect of three individual energy substrates, glucose, pyruvate, and lactate, on motion characteristics, membrane integrity, and acrosomal status of stallion spermatozoa.

Materials and methods: Freshly ejaculated stallion spermatozoa were incubated with combinations of glucose (5 mM), pyruvate (10 mM), and lactate (10 mM) for 0.5 to 4 h. Response to calcium ionophore A23187 (5 μM) was used to evaluate capacitation status. Motility was evaluated using computer-assisted sperm analysis, and plasma membrane and acrosomal integrity were evaluated by flow cytometry.

Results: Incubation with lactate alone for 2 h increased acrosomal sensitivity to A23187. Notably, incubation with lactate alone for 4 h induced a significant spontaneous increase in acrosome-reacted, membrane-intact (viable) spermatozoa, to approximately 50% of the live population, whereas no increase was seen with incubation in glucose or pyruvate alone. This acrosomal effect was observed in spermatozoa incubated at physiological pH as well as under alkaline conditions (medium pH approximately 8.5). Sperm motility declined concomitantly with the increase in acrosome-reacted spermatozoa. Sperm motility was significantly higher in pyruvate-only medium than in glucose or lactate. The addition of pyruvate to lactate-containing medium increased sperm motility but reduced the proportion of live acrosome-reacted spermatozoa in a dose-dependent fashion.

Discussion: This is the first study to demonstrate that incubation with a specific energy substrate, lactate, is associated with spontaneous acrosome reaction in spermatozoa. The proportion of live, acrosome-reacted spermatozoa obtained is among the highest reported for equine spermatozoa.

Conclusion: These findings highlight the delicate control of key sperm functions, and may serve as a basis to increase our understanding of stallion sperm physiology.

KEYWORDS
acrosome reaction, capacitation, equine, glucose, lactate, spermatozoa

1 | INTRODUCTION

Sperm capacitation is associated with biochemical and physical changes both on the sperm surface and intracellularly, which prepare the spermatozoa for acrosomal exocytosis and sperm–oocyte interaction during fertilization.[1–3] These changes typically include plasma membrane reorganization and increased membrane permeability associated with cholesterol loss, increased intracellular concentrations of calcium and bicarbonate, and activation of secondary messenger systems (as reviewed by[4–6]). Many of the processes associated with capacitation require ATP, and thus rely on effective sperm metabolic function (reviewed by[7]).

Historically, the efficiency of conventional in vitro fertilization (IVF) in the horse has been poor, and results have generally not been repeatable,[8–11] reviewed by.[12] Recently, Felix et al. (2022)[13] reported that high rates of equine IVF, resulting in embryo development and birth of live foals, could be achieved after prolonged (22 h) incubation of stallion spermatozoa in a modified Tyrode's albumin lactate pyruvate (TALP) medium in the presence of penicillamine, hypotaurine, and epinephrine. It is possible that given a more appropriate or physiological environment, the requirements for capacitation in equine spermatozoa may be less extensive.

Stallion spermatozoa appear to diverge from spermatozoa of most other species studied in terms of metabolic activity, with a preference for oxidative phosphorylation (OXPHOS) over glycolysis for energy production.[14–17] Inhibition of mitochondrial activity profoundly affects motility and membrane integrity of equine spermatozoa, in contrast to its limited effect on human spermatozoa, suggesting that motility and plasma membrane integrity in equine spermatozoa rely on ATP produced by OXPHOS.[14–16] Within potential OXPHOS substrates, Darr and coworkers[17] found in separate experiments that after 1-h incubation, stallion spermatozoa showed either similar or higher motility when incubated in a medium containing only lactate as compared to only pyruvate or glucose. Notably in this context, a recent study of the carbohydrate content of equine oviductal fluid reported high levels of lactate in comparison with those for glucose and pyruvate (>50 mM vs. 0.6 mM and approximately 1 mM, respectively[18]), whereas in the mouse, oviductal fluid contains approximately equal concentrations of glucose and lactate (approximately 5 mM each[19]). Typically, work on equine spermatozoa utilizes media that were originally formulated based on the makeup of mouse oviductal fluid.

It is possible, given the metabolic differences found in equine spermatozoa, that the composition of the medium used for incubation, that is, the energy substrates available to spermatozoa and thus the metabolic pathways supported, may influence the ability of these spermatozoa to undergo capacitation. Little is known about the effects that different energy substrates might have on capacitation-related events in equine spermatozoa in vitro. One measure of capacitation status is the sensitivity of spermatozoa to the actions of inducers of the acrosome reaction. In human spermatozoa, acrosomal responsiveness to low concentrations of the calcium ionophore A23187 is dependent upon capacitation[20] and associated changes in redox status and protein tyrosine phosphorylation,[21] and acrosomal response to A23187 has been used as a marker for capacitation in other species.[22,23]

González-Fernández et al. (2012)[24] reported that protein tyrosine phosphorylation of equine spermatozoa was induced by an alkaline-pH medium, and Loux et al. (2013)[25] found that increasing medium pH was associated with the capacitation-related changes of increased sperm intracellular pH and intracellular calcium, the latter apparently through the action of the voltage-gated CATSPER calcium channel. Increasing medium pH was also associated with mildly increased indicators of hyperactivated sperm motility.[26] The pH of the equine oviduct has not been clearly defined; however, in many other species, the oviduct maintains a pH of around 8,[27–29] thus alkaline external pH may be a physiological contributor to sperm capacitation.

The objective of the present study was to determine the effect of the energy substrates glucose, pyruvate, and lactate on motility, viability, and acrosome status of stallion spermatozoa incubated in vitro, and the effect of environmental (medium) pH on these parameters. To further explore actions of the different energy substrates on capacitation-related responses, we determined whether these substrates had differential effects on the response of equine spermatozoa to the calcium ionophore A23187.

2 | MATERIALS AND METHODS

2.1 | Chemicals, reagents, and media

Triton X-100, bovine serum albumin (BSA; A9647 and A3803), calcium ionophore A23187 (C7522), dimethyl sulfoxide (DMSO; D2650), and fluorescein-isothiocyanate labeled *Pisum sativum* agglutinin (FITC-PSA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Fixable Live/Dead Red Stain was purchased from Thermo Fisher Scientific (Eugene, OR, USA). The base medium used was a modified Whitten's (MW) medium,[30] consisting of 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.9 mM $CaCl_2$, and 50 μL/mL gentamicin sulfate. This

medium was selected as it has been shown previously to support some capacitation-related changes in equine spermatozoa.[24,31–33] For initial washing of the ejaculate after collection, a HEPES-containing MW medium (HEPES-MW) was prepared by adding 22 mM HEPES sodium salt to the base medium. This HEPES-buffered medium had no energy substrates. The media used for subsequent incubation contained sodium bicarbonate (25 mM) instead of HEPES, and contained BSA (7 mg/mL). These media had only one energy substrate added: 5 mM D-glucose (MW-Glu), 10 mM lactate (MW-Lac), or 10 mM pyruvate (MW-Pyr). The concentrations were set to allow the same amount of OXPHOS substrate (after glycolysis, in the case of glucose) in each preparation. All media were adjusted with NaCl to an osmolality of 280–290 mOsm/kg. After the media were prepared, the pH of each medium was adjusted to 7.25 using 1 M NaOH. The media were maintained at 38.5°C in air (HEPES-buffered medium) or 5% CO_2 (bicarbonate-buffered media) until the beginning of the experiment (approximately 1–2 h).

For experiments incorporating calcium ionophore A23187, a stock solution was prepared by diluting 1 mg A23187 with DMSO to a concentration of 10 mg/mL (19.1 mM). Aliquots of 5 μL of the stock solution were kept frozen at −80°C until use. On the day of use, one aliquot of the stock solution was thawed and diluted with 377 μL distilled water to make a secondary stock solution of 250 μM A23187 in 1.3% DMSO. This secondary stock solution was added, immediately after it was prepared, to the sperm suspensions to result in a final concentration of 5 μM A23187. The vehicle used as a control in the experiments was 5 μL DMSO diluted with 377 μL distilled water. The final concentration of DMSO in the sperm suspensions in both A23187 and vehicle treatments was 0.026%.

2.1.1 | Semen collection and initial processing

Three mature (16−23 years) American Quarter Qorse or Thoroughbred stallions were used in this study. All animal procedures were performed according to the *United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* and were approved by the Institutional Animal Care and Use Committee at Texas A&M University (AUPs 2015-0026 and 2018-0032). Semen was collected with the stallion mounted on a breeding phantom, using a Missouri-type artificial vagina (Nasco, Ft. Atkinson, WI, USA). An in-line nylon-micromesh filter (Animal Reproduction Systems, Chino, CA, USA) was used to remove the gel fraction from the ejaculate. The gel-free ejaculate volume was determined by weight (1 g = 1 mL). The semen was then placed in an incubator during the evaluation of an aliquot for concentration, viability, and motility. Sperm concentration and the percentage of spermatozoa having an intact plasma membrane (viability) were assessed using a NucleoCounter SP-100 (Chemometec, Allerød, Denmark), as previously validated.[34,35] Sperm motion characteristics were determined by computer-assisted sperm motion analysis (CASMA), as described below. Only ejaculates with both total sperm motility and sperm viability greater than 70% were included in the experiments.

2.1.2 | Sperm motion characteristic analysis

Sperm motion characteristics were analyzed using the IVOS I (Hamilton-Thorne, South Hamilton, MA, USA), as previously described.[26] A minimum of 10 fields and 500 spermatozoa were measured for each sample. Pre-set values for the IVOS system consisted of the following: frames acquired: 45/s; frame rate: 60 Hz; minimum contrast: 70; minimum cell size: 4 pixels; minimum static contrast: 30; straightness (STR) threshold for progressive motility: 50%; average path velocity (VAP) threshold for progressive motility: 30%; VAP threshold for static cells: 15 μm/s; cell intensity: 106 pixels; static head size: 0.60–2.00 μm; static head intensity: 0.20–2.01; static elongation: 40–85; illumination intensity: 2200. Experimental endpoints included: percentage of total motile spermatozoa (TMOT), the percentage of progressively motile spermatozoa (PMOT), mean curvilinear velocity (VCL; μm/s), percentage of straightness (STR), and amplitude of lateral head movement (ALH; μm).

2.1.3 | Sperm washing and incubation

Gel-free semen was diluted at a 1:10 ratio (v/v) with HEPES-MW medium and centrifuged at 600 × *g* for 5 min. The pellet was resuspended with 10 mL HEPES-MW and the centrifugation was repeated. Subsequently, the sperm pellet was diluted with bicarbonate-containing MW medium supplemented with energy substrates as outlined under *Experimental design* for the different experiments. The sperm concentration was adjusted to 30×10^6 spermatozoa/mL in each of the treatments. Multiple aliquots of 500 μL of the sperm suspensions were placed in prewarmed 5-mL snap-cap tubes (Falcon BD) and incubated at 38.2°C in a humidified atmosphere of air or 5% CO_2 in air, as specified under *Experimental design* for each experiment. When the pH of the sperm suspension was obtained, this was achieved by completely capping the tube within the incubator, to limit the entry of air, and taking the pH measurement within 15–20 s of capping the tube. The pH measurements were taken using a pH meter coupled to a gel-filled pencil-thin electrode (Fisherbrand Accumet AB150, Fisher Scientific, Hampton, NH, USA), both placed beside the incubator. The probe diameter matched the diameter of the tube, minimizing contact of the medium with air while obtaining the pH.

2.1.4 | Treatment of spermatozoa with calcium ionophore A23187

In some experiments, sperm preparations were treated with A23187 or vehicle. To do this, the designated aliquot was removed from the incubator, and the sperm suspension transferred into a 1.5-mL microcentrifuge tube. Either A23187 secondary stock solution (to a final concentration of 5 μM; CaI) or vehicle (V) were added, and the tube incubated in air or 5% CO_2, as assigned to that treatment, for 10 min. Following this incubation, the tubes were centrifuged at 400 × *g* for 5 min and the pellet resuspended with the corresponding energy-source medium, which had been previously equilibrated for 2 h in the

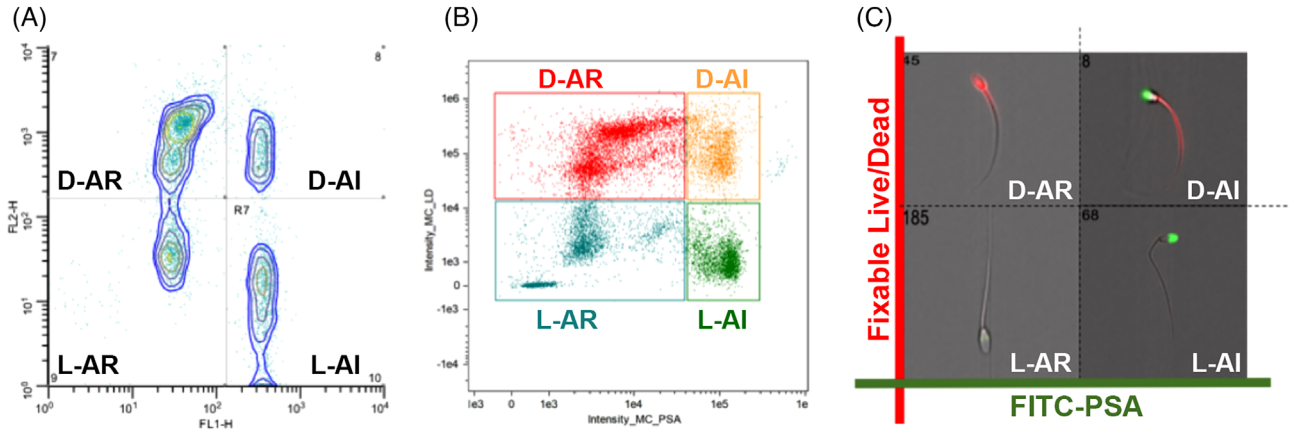

FIGURE 1 Representative scattergrams of stallion spermatozoa stained for plasma membrane and acrosomal status. Spermatozoa were incubated in MW-Lac for 4 h, stained with Fixable Live/Dead Red Stain for membrane integrity and FITC-PSA for presence of the acrosome, then analyzed using either the FACScan instrument (A), or the imaging flow cytometer (B). (C) Images of stallion spermatozoa captured by the imaging flow cytometer in the different quadrants of the scattergram. D-AR, dead (membrane-damaged) acrosome-reacted; D-AI, dead acrosome-intact; L-AR, live (membrane-intact) acrosome-reacted; L-AI, live acrosome-intact.

corresponding atmosphere (i.e., air or 5% CO_2). The sperm suspensions were then incubated for 30 min to allow response to the A23187.

2.1.5 | Sperm plasma membrane/acrosomal status analysis

Sperm plasma membrane and acrosomal integrity were analyzed via flow cytometry after LD/PSA staining, as reported by Ortíz et al.[36] Briefly, an aliquot containing 1×10^6 spermatozoa was diluted with Dulbecco's phosphate buffered saline (DPBS) containing calcium and magnesium, to a volume of 1 mL. Then, the sperm suspension was stained with 1 μL Fixable Live/Dead Red Stain (Thermo Fisher; fluorophore excitation: 595 nm; emission: 615 nm), and incubated for 20 min in the dark at room temperature. After this, paraformaldehyde was added to a final proportion of 2% (v/v), and the suspension was held at 4°C for 45 min. Subsequently, the spermatozoa were washed, permeabilized with Triton-X 100 (1% v/v), washed again, and diluted with Accumax cell dissociation solution (Innovative Cell Technologies). FITC-PSA (Sigma-Aldrich) was then added to a concentration of 0.0375 mg/mL. The sperm suspension was incubated for 10 min, diluted further with Accumax, and processed immediately by flow cytometry (FACScan, Beckton Dickinson, Mountain View, CA, USA).

The flow cytometer was equipped with a 488-nm argon laser. The voltage settings on the flow cytometer were: FSC 553, SSC 240, FL1 741, FL2 821, FL3 150. The compensation was set FL2 as 98% of FL1. A minimum of 5000 events were evaluated per sample, at a flow rate of 200–400 events/s. Data were acquired using a log-scale and analyzed by WinList software (Verity Software House, Topsham, ME, USA). Data included as experimental endpoints were: percentage of membrane-intact (live) spermatozoa (quadrants [L-AR + L-AI] live-acrosome reacted and live-acrosome intact, respectively), and the percentage of live spermatozoa that was acrosome reacted (live-acrosome reacted/live [L-AR/L]).

To validate the staining protocol, a separate trial was performed to visualize spermatozoa stained via this technique using an imaging flow cytometer (Amnis ImageStream MK II, Luminex Corp., Seattle, WA, USA), equipped with a 375, 405, 488, 561, 592, 642, and 786 nm argon lasers and a brightfield microscope with 20×, 40×, and 60× objectives. The voltage settings in the Amnis ImageStream were set as 488-nm laser power at 200 mW, 785-nm laser power at 2 mW, brightfield magnification at 40×. Representative images from the imaging flow cytometer and scattergrams from both flow cytometers are shown in Figure 1.

2.2 | Experimental design

2.2.1 | Experiment 1: Effect of glucose, pyruvate, and lactate individually on stallion sperm motion characteristics and A23187-induced acrosome reaction

Spermatozoa from three ejaculates from each of three stallions ($n = 9$ ejaculates) were used. The design of the experiment is diagrammed in Figure 2. After washing in HEPES-MW, the sperm pellet was suspended in MW-Glu, MW-Lac, or MW-Pyr and incubated in air for 0.5, 2, or 4 h. At each of these time points, an aliquot was treated with either 5 μM A23187 (CaI) or vehicle (V) and incubated for 10 min. After washing, the spermatozoa were suspended in their corresponding medium and incubated for an additional 30 min to allow response to the A23187. Aliquots were then taken for analysis of sperm motion characteristics by CASMA, and plasma membrane/acrosomal status by LD/PSA staining and flow cytometry.

To clarify interpretation of the different phases of the design, we designated the initial incubation period "incubation" and the additional period of incubation after CaI or V treatment the "response interval."

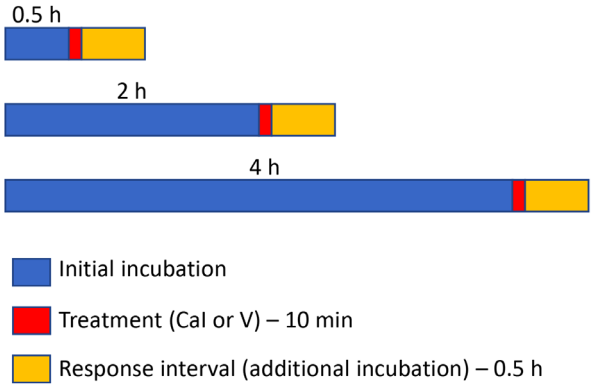

FIGURE 2 Diagram of experimental design for Experiment 1. Sperm suspensions were incubated in different media for 0.5, 2, or 4 h before treatment with 5 μM A23187 (CaI) or vehicle (V) for 10 min, followed by washing and additional incubation in the designated media for 0.5 h to allow response to treatment.

2.2.2 | Experiment 2: Effect of incubation atmosphere on sperm motility and spontaneous acrosome reaction in spermatozoa incubated in MW-Lac for 4 h

The results of Experiment 1 (see *Results*) indicated, unexpectedly, that incubation for 4 h in MW-Lac was associated with a notable and significant increase in spontaneous acrosome reaction in membrane-intact equine spermatozoa, which was not related to A23187 treatment. In Experiment 1, the media, which contained bicarbonate, were incubated in air to induce an increase in medium pH over time, as this has been previously reported to affect capacitation-related changes in equine spermatozoa.[24] Experiment 2 was conducted to determine whether the observed increase in spontaneous acrosome reaction at 4 h in MW-Lac was dependent on atmosphere and thus pH-dependent.

For this experiment, spermatozoa from two ejaculates from each of three stallions (n = 6 ejaculates) were used. After washing, the sperm pellets were suspended in MW-Lac and incubated in either air or 5% CO_2. To determine the effect of different durations of exposure to an air atmosphere on acrosomal exocytosis at 4 h, some preparations in the air atmosphere were removed at 1, 2, or 3 h, the medium pH measured, and the preparation placed in 5% CO_2 for the remaining period. The total incubation time in all treatments was 4 h; no A23187 or V treatment or response-interval incubation was performed. Immediately after completing the 4-h incubation period, the medium pH was assessed, and aliquots of the sperm suspension were analyzed for sperm motion characteristics by CASMA and plasma membrane/acrosome status by LD/PSA staining and flow cytometry.

2.2.3 | Experiment 3: Effect of incubation in complete medium (with all three energy substrates) versus medium with lactate alone on sperm motility and spontaneous acrosome reaction

The results of Experiment 2 indicated that the increase in spontaneous acrosome reaction in stallion spermatozoa incubated in lactate-only medium (MW-Lac) for 4 h was not dependent on incubation atmosphere or the associated medium pH (see *Results*). Because in Experiment 1, we evaluated only MW media with various sole energy sources, Experiment 3 was designed to determine if MW medium as conventionally formulated with 5.5 mM glucose, 1.0 mM sodium pyruvate, and 2.4 mM sodium lactate (MW+++)[24,31] performed similarly to MW-Lac, or perhaps did so only in one atmosphere, or only when treated with CaI.

Two ejaculates from each of three stallions (n = 6) were used. After washing, the sperm pellets were suspended in MW-Lac or MW+++ and incubated in either air or 5% CO_2 for 2 or 4 h. At each of these time points, an aliquot was treated with either A23187 or vehicle (V) and incubated under the corresponding atmosphere for 10 min. After washing, the spermatozoa were suspended in their corresponding media and incubated under the corresponding atmosphere for a 30-min response interval before an aliquot was taken for analysis of sperm motion characteristics by CASMA, and plasma membrane/acrosomal status by LD/PSA staining and flow cytometry.

2.2.4 | Experiment 4: Effect of BSA versus PVA in incubation medium on sperm motility and spontaneous acrosome reaction in spermatozoa incubated in MW-Lac or MW-Pyr

In Experiments 1–3, we utilized BSA in the incubation medium. In other species, BSA serves as an important component of capacitation, as it is thought to act as a cholesterol acceptor during capacitation-related membrane reorganization.[37,38] Thus, to determine the role of BSA in the acrosome reaction seen in MW-Lac medium, in Experiment 4 the BSA (7 mg/mL) was replaced with polyvinyl-alcohol (PVA; 0.02% v/v[25]) or a combination was used, and the effect on sperm motion characteristics and rate of A23187-induced and spontaneous acrosome reactions was assessed.

Spermatozoa from one ejaculate from each of the three stallions (n = 3 ejaculates) was used. After washing, the sperm pellet was suspended in MW-Lac containing either the standard BSA, no BSA but added PVA, or with both BSA and PVA, and incubated in 5% CO_2 for 2 or 4 h. Aliquots of the sperm suspension were then analyzed for sperm motion characteristics by CASMA and plasma membrane/acrosome status by LD/PSA and flow cytometry.

2.2.5 | Experiment 5: Effect of addition of pyruvate to MW-Lac on sperm motility and spontaneous acrosome reaction rate

The previous experiments showed consistently that live equine spermatozoa incubated in medium containing only lactate (MW-Lac) underwent spontaneous acrosome reaction after 4-h incubation, and that this effect was not dependent on incubation atmosphere, pH of the medium, or presence of BSA. However, in every case, the occurrence of a significant increase in the proportion of acrosome-reacted spermatozoa was associated with a precipitous decline in both total and

progressive motility, which would decrease the chances that this treatment might be effective to prepare equine spermatozoa for achieving fertilization in vitro. As Experiments 1 and 4 showed that motility of stallion spermatozoa was higher after incubation in MW-Pyr than in MW-Lac, Experiment 5 was conducted to determine whether the addition of pyruvate into MW-Lac medium would support sperm motility while maintaining the MW-Lac-associated increase in proportion of live acrosome-reacted spermatozoa. Because addition of PVA was associated with a decrease in sperm agglutination in Experiment 4, for this experiment, the media contained 7 mg/mL BSA and 0.02% PVA.

Three ejaculates from each of the three stallions ($n = 9$) were used. After washing, the sperm pellet was suspended in MW-Lac, or in MW-Lac supplemented with 0.5, 1, 5, or 10 mM pyruvate, or in MW-Lac supplemented with 0.5, 1, 5, or 10 mM additional lactate, as a control for the change in total energy substrate and osmolarity of the media in the pyruvate-containing treatments. The sperm suspensions were incubated in 5% CO_2 for 2 or 4 h; no A23187 or V treatment was performed. Aliquots of the sperm suspension were then analyzed for sperm motion characteristics by CASMA and plasma membrane/acrosome status by LD/PSA staining and flow cytometry.

2.3 | Statistical analysis

Statistical analysis was performed using SAS version 9.4 (SAS Institute, Inc., Corp., Cary, NC, USA). In all experiments, the Shapiro–Wilk test was conducted to test data distribution for normality. As most of the data were not normally distributed, a rank-transformation procedure (PROC RANK) was performed before analysis using the general linear model (PROC GLM) procedure. All comparisons were performed between treatments, within each time period tested. Tabular data are presented as non-transformed values for ease of interpretation. The LS means Tukey test was used to separate the main-effects means when treatment *F*-ratios were significant ($p < 0.05$).

3 | RESULTS

3.1 | Experiment 1: Effect of glucose, pyruvate, and lactate individually on stallion sperm motion characteristics and A23187-induced acrosome reaction

There were no significant differences in medium pH between CaI and V treatments. There were no differences in pH values between the different energy substrate media at 0.5 h (8.29 ± 0.03 to 8.32 ± 0.01; $p > 0.05$) or 2 h (8.30 ± 0.02 to 8.33 ± 0.02; $p > 0.05$). At 4 h, the pH of MW-Lac (8.48 ± 0.03) was higher than that for MW-Glu (8.40 ± 0.03; $p > 0.05$) or MW-Pyr (8.45 ± 0.01; $p < 0.05$).

The main effects of energy substrate on TMOT, PMOT, and plasma membrane/acrosome status are presented in Figure 3. There were no significant differences between CaI and V treatments within energy substrate for any experimental endpoint at any time point analyzed ($p > 0.05$), and no energy substrate-by-treatment interactions ($p > 0.05$). PMOT was lower in MW-Glu than in MW-Lac or MW-Pyr at all times. The mean percentage of live spermatozoa was highest at all time points for MW-Glu ($p < 0.05$). No reduction in the mean percentage of live spermatozoa was observed within treatments over time.

Notably, at 4 h of incubation, there was a dramatic and significant increase in the percentage of live spermatozoa that were acrosome-reacted (L-AR/L) in MW-Lac, as compared to MW-Pyr or MW-Glu ($p < 0.05$). At 4 h, over 50% of live spermatozoa in MW-Lac were acrosome-reacted. This increase was independent of CaI treatment.

Values for ALH and VCL are presented in Figure S1. VCL was lowest in MW-Glu at 0.5 and 2 h. No consistent pattern was seen for ALH.

3.2 | Experiment 2: Effect of incubation atmosphere on sperm motility and spontaneous acrosome reaction in spermatozoa incubated in MW-Lac for 4 h

The effect of the incubation atmosphere (air vs. 5% CO_2) on MW-Lac medium pH and sperm quality parameters is presented in Figure 4. Media incubated in air for 4 h had a higher pH (8.54 ± 0.03) than did media incubated in 5% CO_2 for 4 h (7.37 ± 0.02; $p < 0.05$). In media incubated in air for a period, then changed to 5% CO_2, the medium pH increased with longer air exposure time. Mean TMOT was similar among all incubator conditions ($p > 0.05$), while PMOT varied inconsistently among conditions. The mean percentage of live spermatozoa was not affected by incubator conditions ($p > 0.05$). The mean percentage of live spermatozoa that were acrosome-reacted (L-AR/L) at 4-h incubation increased similarly in air or 5% CO_2 atmosphere, and tended to be higher in groups incubated for 4 h under the same conditions (4 h in 5% CO_2 or 4 h in air) than in groups that were switched between conditions part-way through the 4-h period.

Mean VCL and ALH were not affected by incubator conditions ($p > 0.05$; Figure S2).

3.3 | Experiment 3: Effect of incubation in complete medium (with all three energy substrates) versus medium with lactate alone on sperm motility and spontaneous acrosome reaction

The results of this experiment are represented in Figure 5. No differences in any of the variables measured were observed between air and CO_2 conditions. At both 2 and 4 h, higher values for TMOT and PMOT were observed in spermatozoa incubated in MW+++ than in MW-Lac ($p < 0.05$). The mean percentage of live spermatozoa was similar between MW+++ and MW-Lac at both time periods ($p > 0.05$). Spermatozoa incubated in MW-Lac for 2 h and then treated with CaI showed a significant increase in L-AR/L, compared to spermatozoa in the same medium treated with vehicle, or

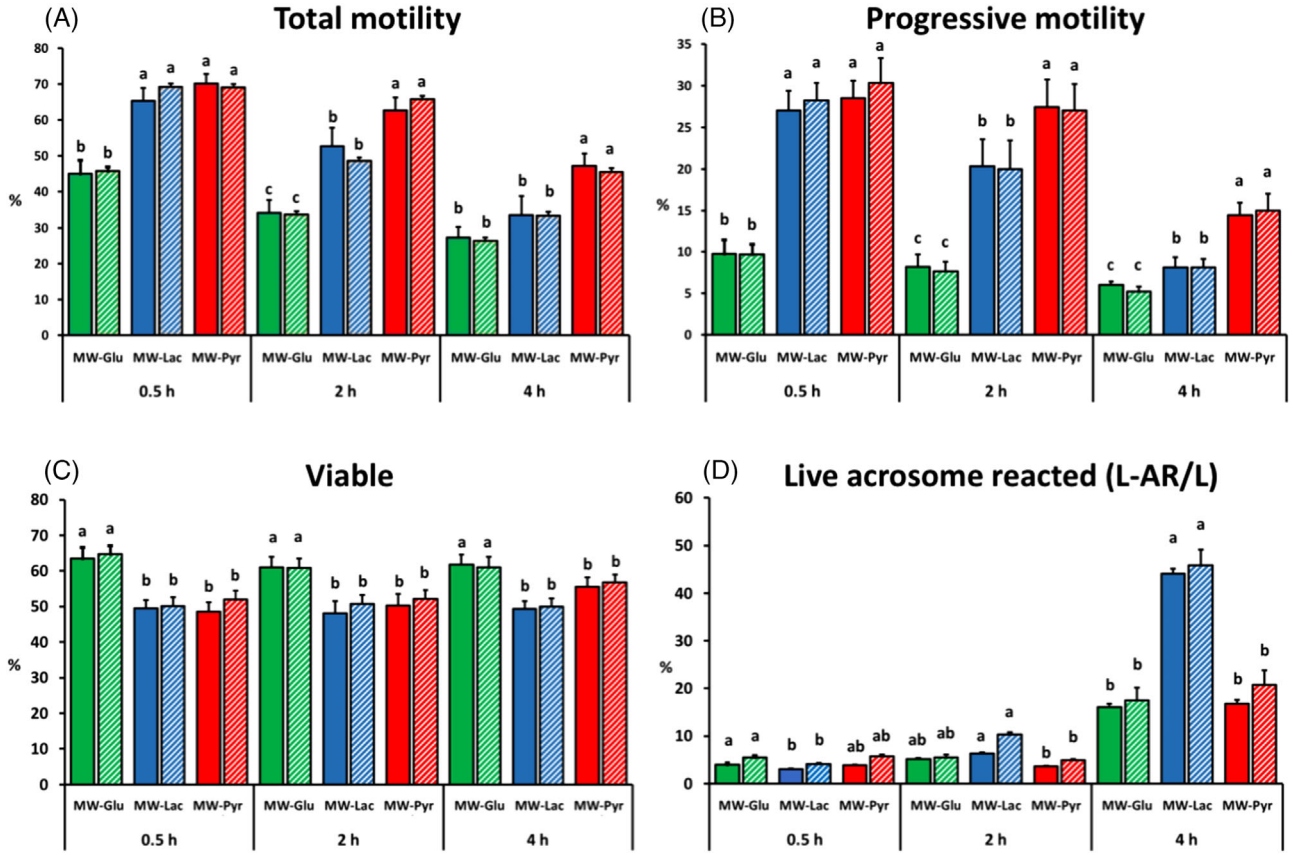

FIGURE 3 The effect of three energy substrates (5 mM glucose [Glu], 10 mM lactate [Lac], or 10 mM pyruvate [Pyr]), added to modified Whitten's base medium as the sole energy source, on sperm quality parameters over time. Spermatozoa were incubated in air, to allow an increase in medium pH, for 0.5, 2, or 4 h, then treated with vehicle (V; DMSO [solid bars]) or 5 μM calcium ionophore A23187 (CaI [patterned bars]), followed by a 30-min response interval incubation. Error bars represent SEM ($n = 9$ ejaculates). $^{a-c}$Within time period (0.5, 2, or 4 h), different superscripts indicate differences among energy sources ($p < 0.05$).

to spermatozoa incubated in MW+++ with or without CaI treatment. Independent of CaI treatment, at 4 h the L-AR/L was notably higher in spermatozoa incubated in MW-Lac than in MW+++ ($p < 0.05$).

Mean VCL values were higher in MW+++ than in MW-Lac at both 2 and 4 h; ALH values were higher in MW+++ than in MW-Lac at 4 h ($p < 0.05$; Figure S3).

3.4 | Experiment 4: Effect of BSA versus PVA in incubation medium on sperm motility and spontaneous acrosome reaction in spermatozoa incubated in MW-Lac or MW-Pyr

At 4 h, spermatozoa suspended with PVA alone had higher TMOT in both MW-Lac and MW-Pyr ($p < 0.05$; Figure 6). Spermatozoa suspended in PVA+BSA had a higher percentage live spermatozoa at 2 h than did spermatozoa suspended with PVA alone, and at 4 h, a higher percentage live spermatozoa than either BSA alone or PVA alone ($p < 0.05$). There was no effect of macromolecule on L-AR/L ($p > 0.05$).

Spermatozoa suspended with PVA alone had a higher VCL (Figure S4) at both 2 and 4 h than did spermatozoa suspended with BSA alone ($p < 0.05$).

3.5 | Experiment 5: Effect of addition of pyruvate to MW-Lac on sperm motility and spontaneous acrosome reaction rate

The effects of adding increasing concentrations of pyruvate or lactate to MW-Lac on the sperm quality parameters analyzed are represented in Figure 7. In general, addition of increasing pyruvate to MW-Lac resulted in increasing mean TMOT and PMOT, with significant differences seen at 2-h incubation in media with 5 mM added pyruvate ($p < 0.05$) and at 4-h incubation in media with 1, 5, or 10 mM added pyruvate. No effect of addition of pyruvate or lactate was observed on the percentage of live spermatozoa at any time period ($p > 0.05$). Notably, the L-AR/L was markedly reduced by pyruvate in a dose-dependent fashion, but was not affected by added lactate. The decrease in L-AR/L was significant at 2 h in media with 5 or 10 mM

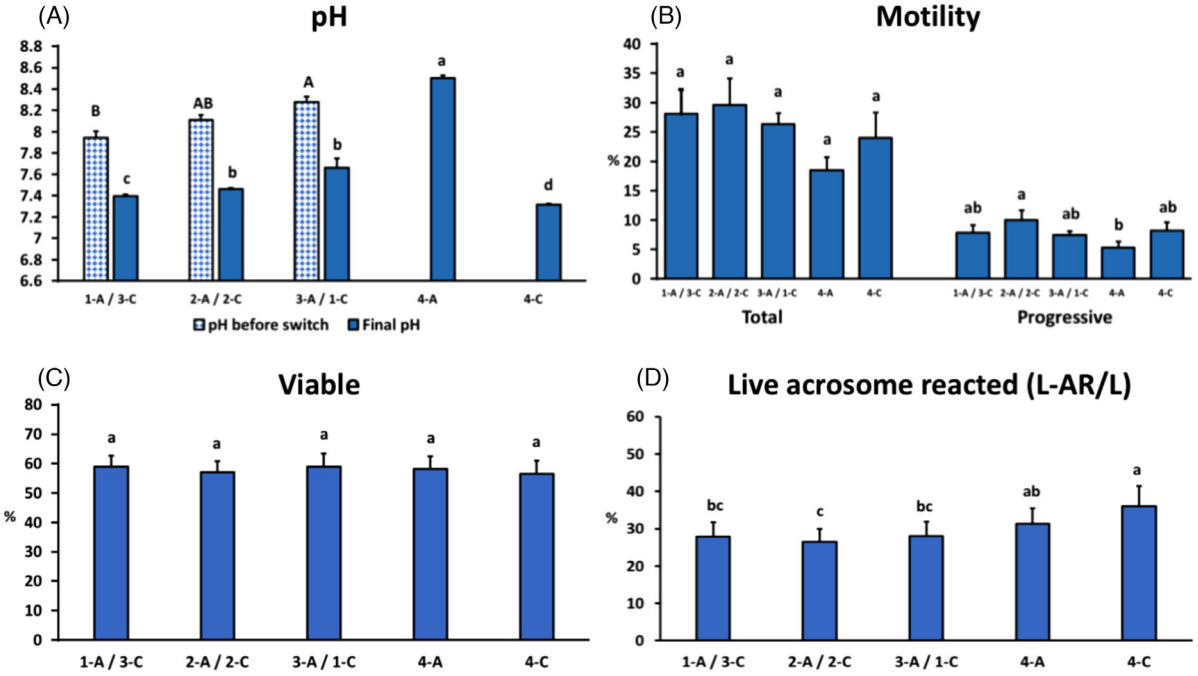

FIGURE 4 The effect of three different atmospheric conditions, air, CO_2, and different exposures to air versus CO_2, on medium pH and sperm quality parameters over time in samples incubated in MW-Lac. Error bars represent SEM ($n = 6$ ejaculates). ^{A,B}Different superscripts indicate differences in the medium pH among atmospheric conditions at the time of atmosphere change ($p < 0.05$). $^{a-c}$Different superscripts indicate differences in the final pH and sperm quality parameters among atmospheric conditions ($p < 0.05$). 1-A/3-C = sperm suspension incubated in air for 1 h then in 5% CO_2 for additional 3 h, and so forth.

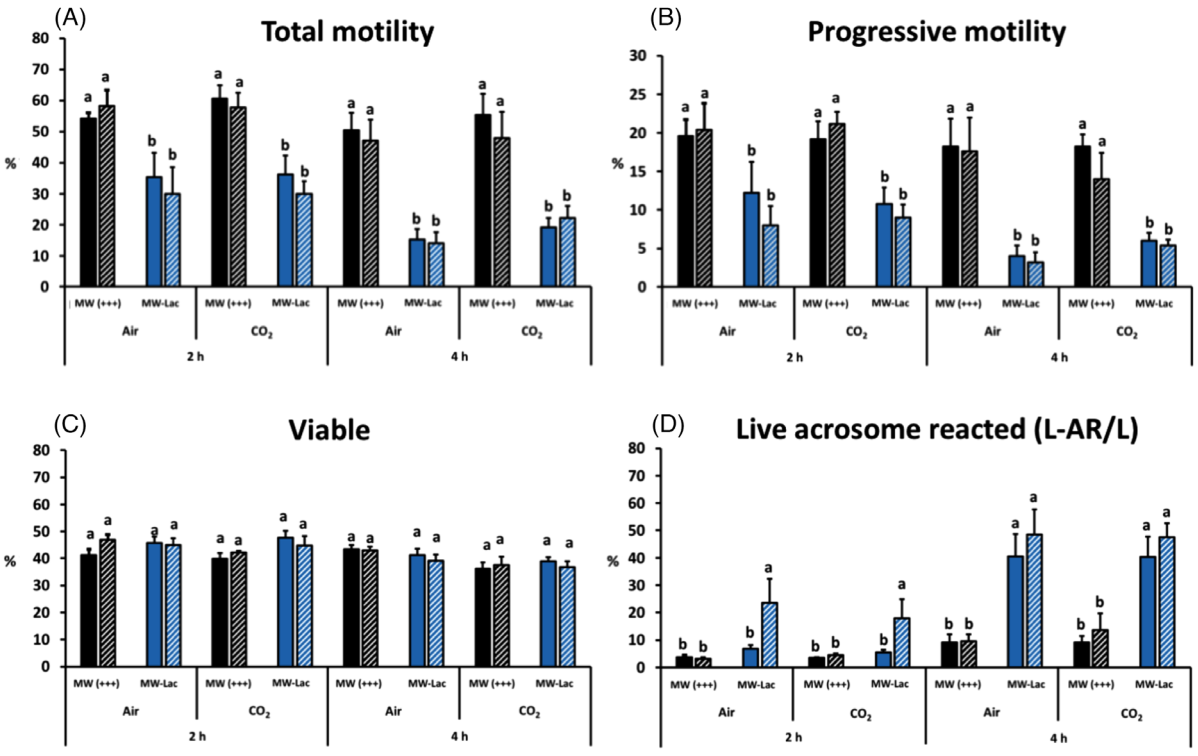

FIGURE 5 The effect of energy source (modified Whitten's base medium with glucose, pyruvate, and lactate [MW+++], or with only lactate [MW-Lac]) and two incubation atmospheres (air or 5% CO_2) on sperm quality parameters over time. Spermatozoa were incubated for 2 or 4 h, then treated with vehicle (V; DMSO [solid bars]) or 5 μM calcium ionophore A23187 (CaI [patterned bars]) for 10 min, followed by a 30-min response interval incubation. Error bars represent SEM ($n = 6$ ejaculates). ^{a,b}Within time period, different superscripts indicate differences among incubation conditions and incubation media ($p < 0.05$).

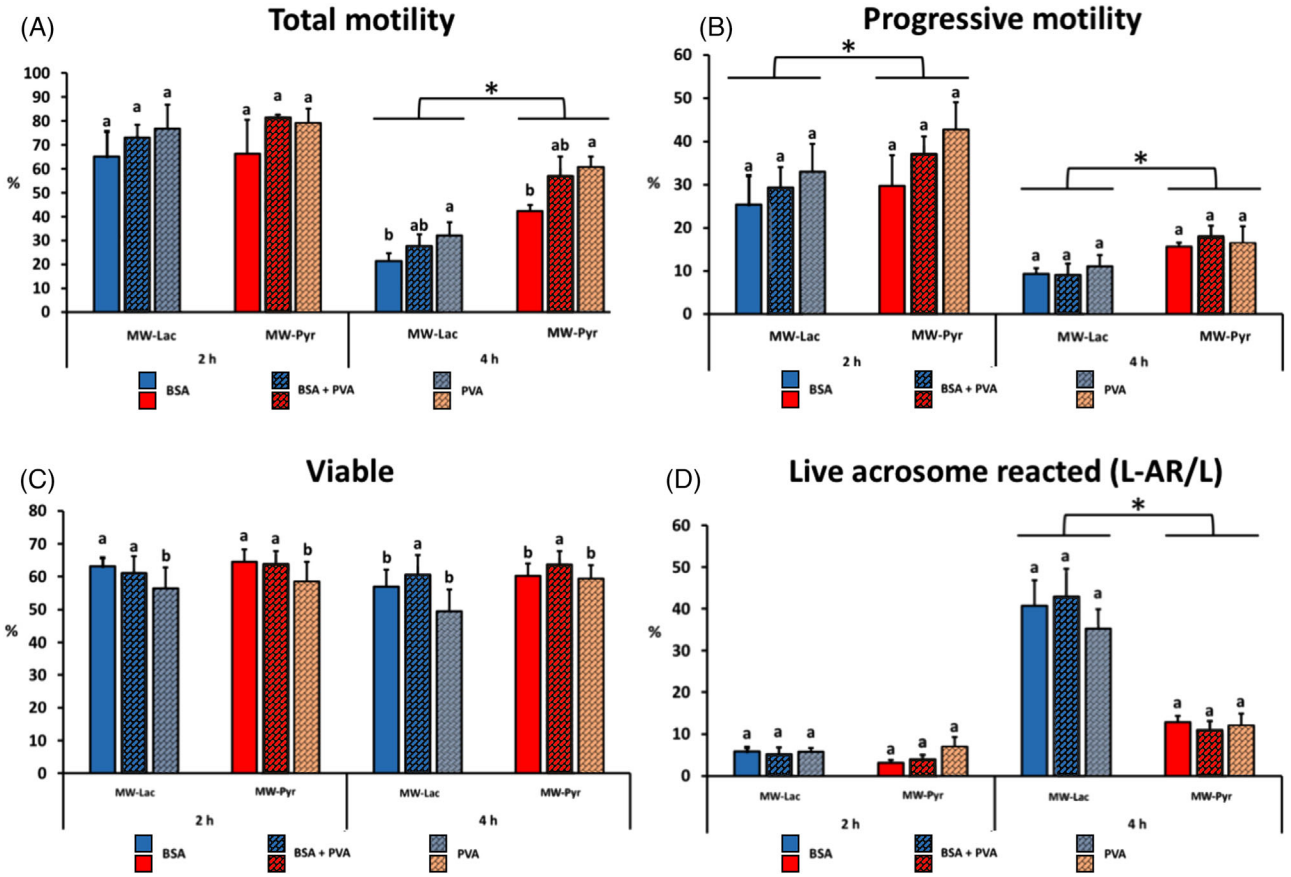

FIGURE 6 The effect of energy substrates (10 mM lactate [MW-Lac] or 10 mM pyruvate [MW-Pyr]), and three macromolecules (7 mg/mL BSA; 0.02% PVA; BSA + PVA) on sperm quality parameters after incubation for 2 or 4 h in a 5% CO_2 atmosphere. Error bars represent SEM ($n = 3$ ejaculates). [a,b]Within time period (2 or 4 h), different superscripts indicate differences among macromolecules in sperm quality parameters ($p < 0.05$). *Indicate differences between medium energy substrate within BSA/PVA treatment on sperm quality parameters at either 2 or 4 h ($p < 0.05$).

added pyruvate ($p < 0.05$) and at 4 h in media with 1, 5, or 10 mM added pyruvate ($p < 0.05$).

Addition of pyruvate increased VCL and ALH values at 4 h (Figure S5; $p < 0.05$).

4 | DISCUSSION

To the best of our knowledge, this is the first critical evaluation of the effect of different energy sources on motility parameters, plasma membrane integrity, and acrosome status of equine spermatozoa during in vitro incubation. We found that presence of the oxidative phosphorylation substrates pyruvate or lactate, when added solely in the medium, resulted in higher sperm motility characteristics than did addition of only glucose, and that pyruvate stimulated higher motility than did lactate. Incubation in lactate-only medium for 2 h sensitized the spermatozoa to undergo acrosomal exocytosis in response to low-dose A23187. However, the most notable finding of these studies was the markedly high rate of spontaneous acrosome reaction in stallion spermatozoa incubated in medium containing only lactate, with approximately 50% of live sperm acrosome-reacted at 4 h. This effect was not dependent on medium pH and was suppressed in a dose-dependent fashion by addition of pyruvate to the medium. The proportion of live acrosome-reacted spermatozoa achieved in lactate-only medium is among the highest reported for stallion spermatozoa.

Our finding that motility is higher when OXPHOS substrates are available agrees with the findings of Darr et al. (2016)[17] after incubation of stallion spermatozoa for 1 h at 38.5°C in 5% CO_2. These authors reported that spermatozoa preferentially metabolized energy substrates that are used immediately by the oxidative phosphorylation pathway (pyruvate or lactate) over glucose. This conclusion was based on higher values of mitochondrial oxygen consumption in spermatozoa incubated with 5.5 mM pyruvate or lactate versus standard (5.5 mM) or high (67 mM) concentrations of glucose. These findings agree with those of Gibb et al.[14] and Plaza-Dávila et al.,[15,16] who reported that inhibition of mitochondrial function had a more dramatic effect on stallion sperm function than did inhibition of glycolysis.

In Experiment 1, we found similar values for sperm motion characteristics after incubation of stallion spermatozoa for 0.5 h in media that contained only lactate or only pyruvate, and these values were higher than those for glucose. Interestingly, after 2 or 4 h of incubation, spermatozoa in medium supplemented with only pyruvate had

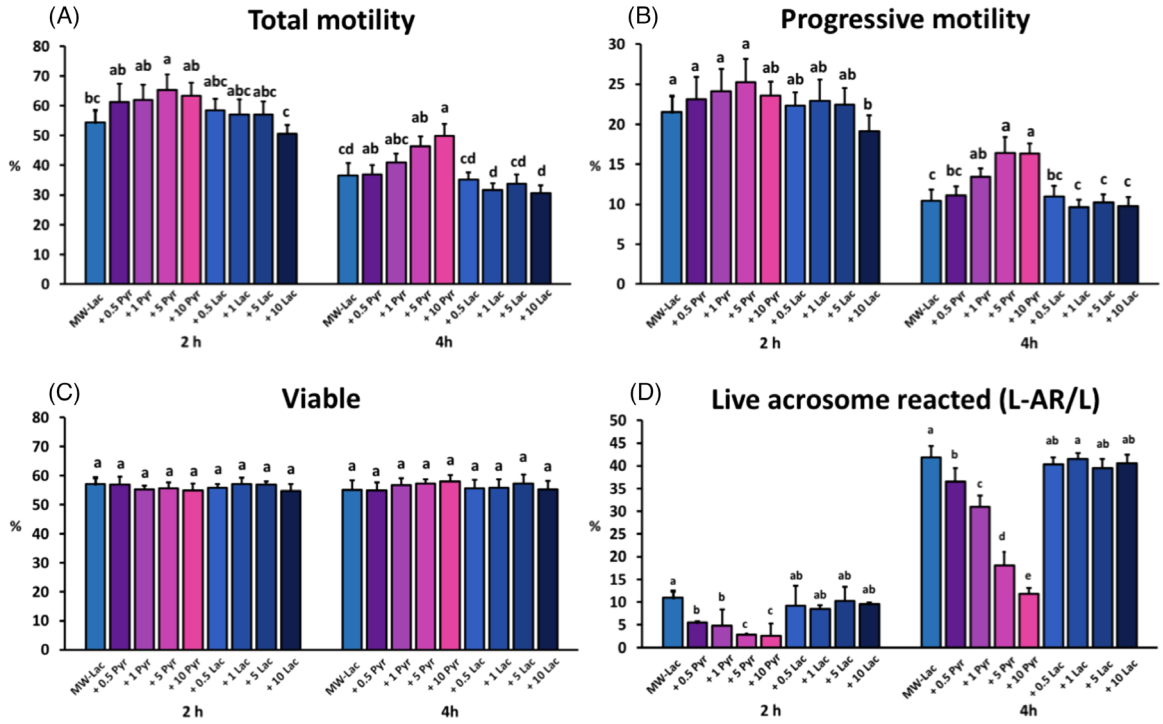

FIGURE 7 The effect of two energy substrates (pyruvate [Pyr] or lactate [Lac]) added at different concentrations (0.5, 1, 5, or 10 mM) to MW-Lac medium (already containing 10 mM sodium lactate) on sperm quality parameters over time. Error bars represent SEM ($n = 9$ ejaculates). $^{a-e}$Within time period (2 or 4 h), different superscripts indicate differences among concentrations of energy source on sperm quality parameters ($p < 0.05$). MW-Lac = 10 mM lactate. +0.5 Pyr, +1 Pyr, and so forth = MW-Lac with added 0.5 mM sodium pyruvate, 1 mM sodium pyruvate, and so forth; +0.5 Lac, +1 Lac, and so forth = MW-Lac with additional added 0.5 mM sodium lactate, 1 mM sodium lactate, and so forth.

significantly higher motility values than did those in lactate or glucose only. Similar findings were also observed in Experiments 3–5. These differences in effects of lactate and pyruvate were unanticipated. Darr et al. (2016)[17] found no significant differences in basal or maximal oxygen consumption or ROS production (markers of mitochondrial function and thus presumably ATP generation) between stallion spermatozoa in media supplemented with lactate or pyruvate, and reported equivalent or higher motility in lactate than in pyruvate. However, these authors evaluated spermatozoa only at 1h incubation. Thus, it is possible in the study of Darr et al. that after only 1 h, stored intracellular substrate was still available to the spermatozoa and the effects of the supplied substrate were muted.

Reduced motility in spermatozoa incubated in lactate alone, versus pyruvate alone, for 2 or 4 h suggests impairment of mitochondrial ATP production, as motility is a sensitive marker of OXPHOS function in stallion spermatozoa.[15,16] Lactate and pyruvate are rapidly interconverted by the highly active cytoplasmic enzyme lactate dehydrogenase, which greatly favors formation of lactate; lactate to pyruvate ratios in different somatic cell types range from 7:1 to 23:1 (reviewed by[39]). Lactate dehydrogenase C4 (LDH-C4), a homotetramer of the LDH-C polypeptide, is the major LDH found in spermatozoa; this enzyme has the highest affinity for pyruvate of the LDH isoforms, with up to 90-fold higher affinity for pyruvate than lactate,[40] resulting in a lactate: pyruvate ratio in spermatozoa of about 50:1.[41] This implies that supplementation of spermatozoa with pyruvate alone will result in immediate conversion of the majority of pyruvate to lactate. As this is associated with oxidation of NADH to NAD^+, the lactate:pyruvate ratio directly affects NADH:NAD^+ homeostasis and cellular redox status. Conversion of pyruvate to lactate also uses an H^+ ion, but changes in intracellular pH are typically neutralized and not considered meaningful (reviewed by[42]).

Incubation of cells in the absence of pyruvate removes pyruvate both as a substrate for OXPHOS and as a source (via conversion to lactate) of NAD^+. It is generally held that pyruvate, but not lactate, can enter the mitochondria. Thus, absence of pyruvate necessitates conversion of lactate to pyruvate to provide a substrate for OXPHOS. This conversion is poorly supported by LDH-C_4, and is associated with reduction of NAD^+ to NADH; thus, OXPHOS function may be impaired. In our system, then, the operative factor in the results seen on spermatozoa motility in lactate-only incubation may not be an effect of lactate, but rather of absence of pyruvate.

Our novel finding that incubation of stallion spermatozoa in lactate alone is associated with apparent acrosomal exocytosis, which is depressed in a concentration-dependent manner by addition of pyruvate, suggests that the observed acrosomal changes are related to cytoplasmic lactate:pyruvate ratio. The underlying mechanism is unclear and may possibly be species-specific, as incubation in lactate alone, or supplementation of media to produce high lactate:pyruvate ratios, inhibits the acrosome reaction in guinea pigs and hamsters.[43,44] The effect of lactate on the equine sperm acrosome could involve

influence of lactate:pyruvate ratio on mitochondrial function (ATP generation) as discussed above for motility; however, the effects on motility are the opposite of that on the acrosome, and both motility and the acrosome reaction are ATP-requiring processes.

The dose-dependent "quenching" of acrosomal exocytosis by pyruvate was much more vivid than were the positive effects of added pyruvate on motility (Figure 6). While little is known about sperm capacitation in the horse, in other species, sperm capacitation is considered to be an oxidative process (reviewed by[45]). Lactate is a reducing agent, thus the mechanism involved in inducing the acrosome reaction in lactate-only medium would not appear to be via "capacitation," as it is currently understood. Notably, LDH-C_4 is expressed in the acrosomal matrix of spermatozoa from several species, as well as in the mitochondrial matrix,[46–48] and inhibition of LDH function suppresses the acrosome reaction in mouse and bull spermatozoa.[49–51] Although proteomic evidence indicates that this enzyme is expressed in stallion spermatozoa,[52] it has not yet been characterized. Function of LDH within the acrosome under physiological conditions (presence of pyruvate, lactate, and glucose) would presumably be in the direction of pyruvate conversion to lactate. In the lactate-only treatments in our study, absence of pyruvate would lead to LDH-induced conversion of lactate to pyruvate within the acrosome, according to the enzyme equilibrium, which would be associated with production of H^+ within the acrosome. The physiological acrosome reaction is triggered by acrosomal alkalinization (reviewed by[53]), so again this effect of lactate-only medium is counterintuitive. Lastly, lactate is known to be a signaling molecule (reviewed by[42]) and NADH is a cofactor in numerous enzymes, and thus the effects of lactate-only medium may be through direct action, or indirectly via generation of NADH during conversion to pyruvate, on components of the acrosomal exocytosis pathway.

It is possible that the acrosomal loss seen in lactate-only or high lactate:pyruvate medium in our study does not reflect a physiological acrosome reaction. However, the equine oviduct appears to be rich in lactate (54—69 mM), while glucose and pyruvate are 1 mM or lower.[18] Thus, our findings are compatible with the hypothesis that high lactate:pyruvate ratio may aid in the physiological induction of the acrosome reaction of stallion spermatozoa. The mechanism by which incubation with lactate alone (i.e., absence of pyruvate) sensitizes stallion spermatozoa to the effects of A23187, and results in eventual spontaneous acrosomal loss in viable spermatozoa, requires further study. It would be meaningful to confirm whether these findings differ from those seen in spermatozoa of other species.

An interesting phenomenon observed in our study is that the treatments that supported the highest rates of spontaneous acrosome reaction in live spermatozoa were also the treatments associated with the lowest motility values. This phenomenon was presented by Ortíz et al.[36] in their work with A23187—in every condition in which an increase in viable acrosome-reacted spermatozoa occurred, an essentially concomitant decrease in motility was seen. Ortíz et al. hypothesized that A23187-induced calcium overload led to induction of the acrosome reaction, but also to mitochondrial malfunction and thus loss of motility. Another possibility for the reciprocal relationship between motility and acrosomal exocytosis is that because the energy requirements for sperm capacitation-related processes are high,[7,54] the triggering of the acrosome reaction results in lack of availability of ATP for the maintenance of sperm motility. In our study, while we did see a decrease in motility concomitant with increasing viable acrosome-reacted spermatozoa, the decrease in motility (e.g., 50% live sperm acrosome-reacted; 30% total motility) was not as profound as that seen after induction of the acrosome reaction by A23187 in the study of Ortíz et al. (e.g., 20% live acrosome-reacted spermatozoa, <5% total motility[36]). Thus, lactate-only treatment may offer an alternative or complementary approach to the recently reported successful treatment (22-h pre-incubation in the presence of penicillamine, hypotaurine, and epinephrine) for preparation of equine spermatozoa for IVF,[13] as lactate treatment may be compatible with a population of motile, acrosome-reacted spermatozoa.

In these studies, we found that presence of PVA in the medium, with or without BSA, resulted in similar rates of spontaneous acrosome reaction as did medium with only BSA. BSA is included in capacitation media in other species as a cholesterol acceptor, promoting loss of cholesterol from the sperm plasma membrane, which destabilizes the membrane and facilitates capacitation and the occurrence of the acrosome reaction.[55–57] Our results suggest that BSA is not required to support the lactate-associated spontaneous acrosome reaction. This could be beneficial in the quest of a more chemically defined media for in vitro capacitation of stallion spermatozoa. Of interest, in the current study, we noted that sperm viability as recorded on flow cytometry was lower than sperm motility as recorded by CASA in some treatments. In a previous investigation of this issue, we found that viable spermatozoa tend to aggregate in capacitating media (media with calcium, bicarbonate, and albumin), and these sperm aggregates are disregarded by the flow cytometer. This increases the relative proportion of dead spermatozoa in the analyzed (individual spermatozoa) population.[36] In that report, we found that use of the Live/Dead Fixable Stain with Accumax treatment, as performed in the current studies, reduces this artifact greatly compared to that obtained with standard PI staining for viability, but does not eliminate it completely.

5 | CONCLUSION

In conclusion, the findings of the present study indicate that presence of lactate alone sensitizes stallion spermatozoa to the effects of calcium ionophore A23187 and, with prolonged incubation, supports spontaneous acrosome reaction in live spermatozoa. This effect was not pH-dependent and was significantly reduced in a dose-dependent fashion when pyruvate was added to lactate-containing medium. The pronounced effects of the different energy substrates raise questions about the mechanisms for capacitation and the acrosome reaction in horses, and suggest that in vitro capacitation media for equine spermatozoa must be carefully formulated, taking into consideration possible substrate effects on energy metabolism and directly on the process of acrosomal exocytosis. The results of this work could be used to refine the methods used for in vitro fertilization in horses.

AUTHOR CONTRIBUTIONS

Luisa Ramírez-Agámez contributed to experimental conception and design, data acquisition and interpretation, literature search, and manuscript preparation. Camilo Hernández-Avilés contributed to data acquisition, statistical analysis, and manuscript preparation. Isabel Ortíz contributed to data acquisition. Charles C. Love contributed to financial support and data acquisition and interpretation. Dickson D. Varner contributed to financial support and data discussion. Katrin Hinrichs contributed to financial support, experimental conception and design, data interpretation, literature search, and manuscript preparation.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

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DATA AVAILABILITY STATEMENT

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

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