# MALE IDIOPATHIC INFERTILITY: NOVEL POSSIBLE TARGETS, VOLUME I

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# MALE IDIOPATHIC INFERTILITY: NOVEL POSSIBLE TARGETS, VOLUME I

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EDITORIAL
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# Editorial: Male Idiopathic Infertility: Novel Possible Targets, Volume I

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**Editorial on the Research Topic**

**Male Idiopathic Infertility: Novel Possible Targets, Volume I**

Male reproductive health represents an issue of increasing importance, especially considering that the total sperm count has halved is the last forty years (1). In Western countries, infertility affects about 15% of couples of childbearing ages (2). About half of these cases involve the male partner alone or is associated with a female factor of infertility (3). However, despite a thorough diagnostic work-up, the cause of infertility remains elusive in up to ~70% of cases (4). Therefore, identifying novel possible targets of male idiopathic infertility is of paramount relevance to better understand its pathophysiological mechanisms and etiology. This, in turn, will possibly help in identifying targeted treatments in the near future.

Several recent lines of evidence suggest a role for the sperm transcriptome in human fertility. The review article by Pantos et al. timely describes the importance of the miRNA-34/449 for male infertility. The deregulation of this miRNA could lead to sperm aggregation and agglutination, abnormalities of sperm ciliogenesis in the efferent ducts, and defective reabsorption of the fluid present in the seminiferous tubules with a consequent increase in intratubular hydrostatic pressure. These mechanisms are compatible with the onset of obstructive infertility. Furthermore, it has been suggested that this miRNA is involved in the first stage of meiotic division. Hence, its downregulation could also lead to spermatogenic failure. All this results in a wide range of phenotypes ranging from oligozoospermia to azoospermia. However, further studies are still needed to confirm the role of miRNA-34/449 in male infertility.

The study by Hong et al. showed that piRNA levels in seminal plasma are lower in asthenozoospermic patients than in normozoospermic controls. The topic of the study is of great interest as previous evidence, mainly in animals, has shown a cross-talk between piRNAs in epididymal exosomes and spermatozoa. Particularly, piRNAs are distributed to spermatozoa in the caput of the epididymis, and their content in the exosomes of the cauda is significantly lower than that of the caput (5).

Human papillomavirus (HPV) is another under-diagnosed cause of male infertility, able to cause asthenozoospermia and negatively influence embryo growth. A deep and comprehensive review of the animal and human evidence is provided by Muscianisi et al. According to their data, patients with apparently idiopathic male infertility should also be evaluated for HPV infection. This may help to decrease the rate of idiopathic infertile male patients.

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Obesity and metabolic impairment may represent another potential cause of apparently idiopathic male infertility. The meta-analysis by Zhao and Pang including 1731 patients have indeed shown that patients with metabolic syndrome (MetS) have statistically significantly lower sperm concentration, total sperm count, progressive motility, and sperm vitality, and increased percentage of spermatozoa with DNA fragmentation (SDF) and of the percentage of spermatozoa with low mitochondrial membrane potential. Insulin has been shown to influence Sertoli cell (SC) function and proliferation (6), and the action of endogenous follicle-stimulating hormone (FSH) is impaired in patients with MetS (7). These data may explain, at least partially, the findings by Zhao and Pang (Arato et al.).

Two articles of this special issue focused on porcine SCs. The first shows how diverse FSH molecules can impact SC function differently in terms of comparative proteomic analysis. Although no definitive conclusion can be drawn in humans, this finding may point toward a personalized therapeutic approach for the treatment of male infertility (Arato et al.). The second one focuses on the immunological properties of SCs. Accordingly, *in vitro* exposure to bacteria and lipopolysaccharides (LPS) resulted in a cell switch from the typical SC to an antigen-presenting cell (8).

Published studies support the concept that already in late adolescence (about 18 years), a proportion of boys ranging from 1:4 to 1:7 has testicular hypotrophy (8, 9). Hence, it would be essential to understand the pre-adolescent stimuli that are implicated in SC proliferation and, hence, contributing to reach an adequate testicular volume (TV). One of these stimuli is insulin-like growth factor 1 that has shown proliferative effects in porcine SCs *in vitro* (10). A retrospective analysis in patients with growth hormone deficiency (GHD) showed that, when treated with growth hormone (GH), patients had an increase of the TV over time as well as the age of puberty onset. Those who received treatment late had testicular hypotrophy and puberty delay (Cannarella et al.). These findings are in line with the effect of GH/IGF1 on testicular growth. Hypothetically, borderline-low GH-IGF1 serum levels in childhood and adolescence may be responsible for borderline-low testicular volumes in adulthood, although further evidence is needed to confirm this.

Next-generation sequencing (NGS) analyses represent a novel approach that may help to clarify the genetic etiology of oligozoospermia or azoospermia. Genetic panels for spermatogenetic failure have been developed to address this issue. As shown by the study of Precone et al., this panel could help to increase the diagnostic rate of male infertility.

Interestingly, oxidative stress (OS) is known as a cause of male infertility. Increased OS is indeed able to cause DNA double-strand breaks, in turn damaging sperm function. The review article by Aitken and Baker supports the concept that OS may damage susceptible regions of sperm DNA that encode for genes involved in male infertility, cancer, imprinted diseases, and behavioral disorders.

Abnormal sperm methylation has been observed in patients with infertility. On this account, the article by Franzago et al. evaluated the effect of a vegan diet on sperm epigenetics. Particularly, they found that a vegan diet can change the methylation rate of the *FTO* gene at the sperm level, which may impact transgenerational inheritance.

Lastly, the study by Li et al. evaluated seminal plasma proteomic profiles to understand any difference in patients with Kallmann syndrome compared to healthy men.

Taken together, the studies published in this Research Topic provide novel epigenetic, proteomic, transcriptomic targets of idiopathic male infertility, suggesting a unifying hypothesis and highlighting novel aspects of SC function. These data may help to reach a better comprehension of the causes of infertility. However, further efforts from the scientific community are still expected to understand the causes of idiopathic male infertility better.

## AUTHOR CONTRIBUTIONS

RC and AEC conceived the study. RC wrote the original draft. AEC and DJ revised the draft and made revisions. RAC approved the manuscript. All authors contributed to the article and approved the submitted version.

## REFERENCES


1. Levine H, Jørgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, et al. Temporal Trends in Sperm Count: A Systematic Review and Meta-Regression Analysis. *Hum Reprod Update* (2017) 23(6):646–59. doi: 10.1093/humupd/dmx022
2. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A Unique View on Male Infertility Around the Globe. *Reprod Biol Endocrinol* (2015) 13:37. doi: 10.1186/s12958-015-0032-1
3. Valenti D, La Vignera S, Condorelli RA, Rago R, Barone N, Vicari E, et al. Follicle-Stimulating Hormone Treatment in Normogonadotropic Infertile Men. *Nat Rev Urol* (2013) 10:55–62. doi: 10.1038/nrurol.2012.234
4. Punab M, Poolamets O, Paju P, Vihljajev V, Pomm K, Ladva R, et al. Causes of Male Infertility: A 9-Year Prospective Monocentre Study on 1737 Patients With Reduced Total Sperm Counts. *Hum Reprod* (2017) 32(1):18–31. doi: 10.1093/humrep/dew284
5. Cannarella R, Barbagallo F, Crafa A, La Vignera S, Condorelli RA, Calogero AE. Seminal Plasma Transcriptome and Proteome: Towards a Molecular Approach in the Diagnosis of Idiopathic Male Infertility. *Int J Mol Sci* (2020) 21(19):7308. doi: 10.3390/ijms21197308
6. Cannarella R, Arato I, Condorelli RA, Mongioì LM, Lilli C, Bellucci C, et al. Effects of Insulin on Porcine Neonatal Sertoli Cell Responsiveness to FSH In Vitro. *J Clin Med* (2019) 8(6):809. doi: 10.3390/jcm8060809
7. La Vignera S, Condorelli RA, Duca Y, Cannarella R, Giacone F, Calogero AE. FSH Treatment for Normogonadotropic Male Infertility: A Synergistic Role for Metformin? *Eur Rev Med Pharmacol Sci* (2019) 23(13):5994–8. doi: 10.26355/eurrev_201907_18346
8. Gianfrilli D, Ferlin A, Isidori AM, Garolla A, Maggi M, Pivonello R, et al. Risk Behaviours and Alcohol in Adolescence are Negatively Associated With Testicular Volume: Results From the Amico-Andrologo Survey. *Andrology* (2019) 7(6):769–77. doi: 10.1111/andr.12659
9. Perri A, Lofaro D, Izzo G, Aquino B, Bitonti M, Ciambrone G, et al. The Risky Health Behaviours of Male Adolescents in the Southern Italian Region:

Implications for Sexual and Reproductive Disease. *J Clin Med* (2019) 8 (9):1414. doi: 10.3390/jcm8091414

10. Cannarella R, Mancuso F, Condorelli RA, Arato I, Mongioì LM, Giacone F, et al. Effects of GH and IGF1 on Basal and FSH-Modulated Porcine Sertoli Cells in-Vitro. *J Clin Med* (2019) 8(6):811. doi: 10.3390/jcm8060811

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# "*In vitro*" Effect of Different Follicle—Stimulating Hormone Preparations on Sertoli Cells: Toward a Personalized Treatment for Male Infertility

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Follicle-stimulating hormone (FSH), a major regulator of spermatogenesis, has a crucial function in the development and function of the testis and it is extensively given as a fertility treatment to stimulate spermatogenesis. We analyzed the effects of different FSH preparations (α-follitropin, β-follitropin, and urofollitropin) in combination with testosterone on porcine pre-pubertal Sertoli cells. To study the effect of the different FSH treatments in the Sertoli cell function we performed Real Time PCR analysis of AMH, inhibin B, and FSH-r, an ELISA assay for AMH and inhibin B, and a high-throughput comparative proteomic analysis. We verified that all three preparations induced a reduction of AMH in terms of mRNA and secreted proteins, and an increase of inhibin B in terms of mRNA in all the FSH formulations, while solely α-follitropin produced an increase of secreted inhibin B in the culture medium. Comparative proteomic analysis of the three FSH preparations identified 46 proteins, 11 up-regulated and 2 down-regulated. Surprisingly, the combination of testosterone with β-follitropin specifically induced an up-regulation of eight specific secreted proteins. Our study, showing that the three different FSH preparations induce different effects, could offer the opportunity to shed light inside new applications to a personalized reproductive medicine.

**Keywords: Sertoli cells, alpha follitropin, beta follitropin, urofollitropin, proteomic analysis**

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## INTRODUCTION

Follicle-stimulating hormone (FSH), a glycoprotein hormone secreted by the anterior pituitary gland, plays a key function in the treatment of human infertility. In infertile women it is widely prescribed to stimulate follicular development, meanwhile, in males, it is used alone or in association with Human chorionic gonadotropin (hCG) to trigger off and maintain spermatogenesis both in hypogonadotropic hypogonadism (1), and in oligozoospermic subjects with normogonadotropic hypogonadism (2).

FSH comprises two subunits, α and β, which are both glycosylated and contain four N-linked carbohydrates. The different content in sialic acid at the C-terminal determines a family of glycoforms that explain the structural and functional heterogeneity of the different FSH formulations (3).

The preparations of FSH available in the market are derived by either recombinant DNA technology (rFSH such as α- and β-follitropin) or post-menopausal urines (urofollitropin). α- and β-follitropins are synthesized by the same recombinant technology, producing identical dimeric α-FSH and β-FSH subunits, but with differences in the further glycosylation and in the procedures of purification. In contrast, urofollitropin consists of FSH with a minimal LH activity, and it has low specific activity (∼100–150 IU FSH/mg protein). The low specific activity of this preparation could be explained by the fact that more than 95% of the protein content correspond to non-specific co-purified urinary proteins (1).

Regarding the efficacy of the different FSH preparations in the female, many contradictory results have been published in the last 2 decades. The meta-analyses regarding the clinical efficacy of different FSH preparations demonstrated no significant differences in clinical or ongoing pregnancy and in the live-birth rate, in the miscarriage rate, or for the incidence of multiple pregnancy rate or ovarian hyperstimulation syndrome (OHSS) between rFSH and urofollitropin (4–8).

Up to now, in the male, no data exist regarding the efficacy of the treatment in relation to the FSH-therapy used. However, a meta-analysis reported a significant positive effect of the treatment with FSH both on sperm parameters and on pregnancy rate in oligozoospermic patients with normal FSH levels (9). Unfortunately, the studies included in the meta-analyses have an extremely heterogeneity in the selection criteria of the patients, in primary and secondary end-points, in the doses of FSH treatment and in time of treatment (10).

Sertoli cell (SC) is one of the principal actors in spermatogenesis as it provides nourishment, and structural and functional support to germ cells. Moreover, it protects germ cells by the blood-testis barrier (BTB) and by the production of immunomodulatory factors (11). In testis, FSH controls the function of SC through FSH receptors (FSH-r), which are only present in SC. In particular, FSH plays a pivotal role in the early stages of spermatogenesis, while testosterone has a major role in spermiogenesis (12).

In the prepubertal testis, SC is the most representative cell population. However, during this stage, there is a low activity of the hypothalamic–pituitary–gonadal axis reflected by the high levels of Anti-Müllerian Hormone (AMH) and inhibin B in serum (13). In contrast, during puberty, testosterone induces SC maturation and inhibits AMH production (13).

The aim of this research was to assess the effects of the different FSH formulation in an "*in vitro*" model of porcine pre-pubertal SC, in order to evaluate the SC responsiveness to pharmacological treatment of different FSH preparations, never assessed until now.

## MATERIALS AND METHODS

### SC Culture, Characterization, and Stimulation

Pure porcine pre-pubertal SC were isolated and characterized according to previously reported methods (14–16).

Purified SC cultures, as previously stated (14–16), were treated for 48 h as follows, and stimulation was performed according to the previously described protocol (17):

- Stimulated with testosterone (0.2 μg/ml; SIT, Pavia, Italy) (control group);
- Stimulated with α-follitropin (α-FSH) 100 nM and testosterone (0.2 μg/ml);
- Stimulated with β-follitropin (β-FSH) 100 nM and testosterone (0.2 μg/ml);
- Stimulated with Urofollitropin (u-FSH) 100 nM and testosterone (0.2 μg/ml).

We used testosterone in addition to any of the FSH formulation to mimic a physiological condition in testis, considering that both FSH and testosterone are essential for the adequate spermatogenesis (12).

### Quantitative, Real-Time PCR

Analyses for AMH, inhibin B, and FSH receptor (FSH-r) were performed as previously described (17) employing the primers listed in **Table 1**. Total RNA was extracted from SC monolayers obtained in the experimental groups using Trizol reagent (Sigma-Aldrich, Milan, Italy), and quantified by reading the optical density at 260 nm. In detail, 2.5 μg of total RNA was subjected to reverse transcription (RT, Thermo Scientific, Waltham, MA, USA) to a final volume of 20 μl. We performed the qPCR with the use of 25 ng of the cDNA obtained by RT and a SYBR Green

**TABLE 1** | Primer sequences for PCR analyses.

| Gene | Forward sequences (5′-3′) | Reverse sequences (5′-3′) |
|---|---|---|
| AMH | GCGAACTTAGCGTGGACCTG | CTTGGCAGTTGTTGGCTTGATATG |
| Inhibin B | CCGTGTGGAAGGATGAGG | TGGCTGGAGTGACTGGAT |
| FSH-r | TGAGTATAGCAGCCACAGATGACC | TTTCACAGTCGCCCTCTTTCCC |
| β-actin | ATGGTGGGTATGGGTCAGAA | CTTCTCCATGTCGTCCCAGT |

Master Mix (Stratagene, Amsterdam, The Netherlands). This procedure was performed in an Mx3000P cycler (Stratagene), using FAM for detection and ROX as the reference dye. We normalized the mRNA level of each sample against β-actin mRNA and expressed it as fold changes vs. the levels in the control group.

## Culture Media Isolation

Aliquots of the culture media (CM) of SC were collected after 48 h of stimulation, centrifuged at 1,500 g for 10 min, and the supernatant was saved at −20°C for proteomic analysis and for an ELISA assay for Inhibin B and AMH secretion performed as previously described (18).

## Proteomic Analysis of the SC Secretome

The SC CM for each sample was thawed and centrifuged at 3,000 g for 20 min at 4°C. The resulting supernatants were filtered (0.45 μm pore size) to remove cell debris and other impurities if any. Afterward, to perform protein solubilization, sodium dodecyl sulfate (SDS) and phenylmethylsulfonyl fluoride (PMSF) were added to a final concentration of 2% (w/v) and 1 mM, respectively. After 30 min, all samples were centrifuged at 4°C at 16,000 g for 10 min, and the supernatants (soluble proteins) were kept, and proteins were precipitated overnight in 80% cold acetone (v/v) at −20°C. After centrifugation at 17,530 g for 15 min at 4°C, the protein precipitates were collected and resuspended in 1% SDS, 1 mM PMSF in phosphate-buffered saline (PBS) to perform protein quantification using the BCA protein Assay Kit (Pierce™ BCA protein Assay Kit, Thermo Fisher Scientific, Rockford, IL), according to manufacturer's recommendations.

The TMT labeling was performed as described previously (19, 20) and according to the manufacturer's instructions. Briefly, 50 μg of proteins from each sample were transferred to a new tube and adjusted to a final volume of 50 μl with 100 mM triethylammonium bicarbonate (TEAB) to obtain a 1 μg/μl concentration. Proteins were reduced in 9.5 mM tris (2-carboxyethyl) phosphine (TCEP), alkylated with 17 mM iodoacetamide (IAA), and precipitated by adding six volumes of 100% cold acetone. Then, samples were centrifuged at 17,500 g, and the acetone-precipitated protein pellets (containing 50 μg of proteins) were resuspended in 50 μl of 100 mM TEAB. Trypsin was added at a 1:22 protease-to-protein ratio and incubated overnight at 37°C. Prior to peptide labeling, an aliquot from each sample was taken and combined at equal amounts to form the internal control. After, 35 μg of peptides from each sample (including the internal control) were labeled with TMT isobaric tags (TMT 10-plex Mass Tag Labeling; Thermo Fisher Scientific, Rockford, IL). The technical reproducibility and analytical reliability of the approach were assessed by performing duplicate analyses on the internal control. Then, 15 μl of the TMT label reagents, previously resuspended in acetonitrile anhydrous (ACN), were added to the corresponding sample, followed by 1 h incubation at RT. Afterward, the reaction was stopped by adding 5% hydroxylamine. The TMT-labeled samples were combined at equal amounts constituting one multiplex pool, which was dried in a vacuum centrifuge and resuspended in 50 μl of 0.5% trifluoroacetic acid in 5% ACN. After, labeled peptides were cleaned up via reversed-phase C18 spin columns (Pierce C18 Spin Columns, Thermo Fisher Scientific, Rockford, IL), according to manufacturer's instructions. Then, the peptides were reconstituted in 0.1% formic acid (FA) to be processed by LC-MS/MS.

Our MS data was collected using a nano-LC Ultra 2D Eksigent (AB Sciex, Brugg, Switzerland) attached to an LTQ-Orbitrap Velos (Thermo scientific, San Jose, CA). Peptides were injected onto a C18 trap column (L 2 cm, 100 μm ID, 5 μm, 120 Å; NanoSeparations, Nieuwkoop, the Netherlands) and chromatographic analyses were performed using an analytical column (L 15 cm, 75 μm ID, 3 μm, 100 Å; Thermo scientific, San Jose, CA). The buffers used for the analysis were buffer A (97% $H_2O$-3% ACN, 0.1% FA) and buffer B (3% $H_2O$-97% ACN, 0.1% FA). A peptide mixture was loaded onto the analytical column with the following gradient: time 0–5 min, 0% of B; 5–180 min, 0–32.5% of B; 180–185 min, 32.5–100% of B at a flow rate of 400 ml/min; and 185–200 min, 100% of B at a flow rate of 400 nl/min to avoid carry-over. MS/MS analyses were performed using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA) with a nanoelectrospray ion source. The LTQ-Orbitrap Velos settings included one 30,000 resolution at 400 m/z MS1 scan for precursor ions followed by MS2 scans of the 15 most intense precursor ions, at 30,000 resolution at 400 m/z, in positive ion mode. The lock mass option was enabled, and mass calibration was performed on polysiloxane (m/z 445.12003). MS/MS data acquisition was completed using Xcalibur 2.1 (Thermo Fisher Scientific, Waltham, MA). MS2 experiments were performed using higher-energy collision dissociation (HCD) with a normalized collision energy of 42%.

LC-MS/MS data was analyzed using Proteome Discoverer 1.4.1.14 (Thermo Fisher Scientific, Waltham, MA, USA) based on SEQUEST HT cluster as search engine (University of Washington, licensed to Thermo Electron Corp., San Jose, CA) against UniProtKB/Swiss-Prot database with Sus scrofa (released September 2018; 3,339 sequences). Searches were run applying the following parameters: two maximum missed cleavage sites for trypsin, TMT-labeled lysine (+229.163 Da) and methionine oxidation (+15.995 Da) as dynamic modifications, cysteine carbamidomethylation (+57.021 Da) as a static modification, 20 ppm precursor mass tolerance, 0.6 Da fragment mass tolerance. Percolator was used for protein identification, applying the following identification criteria: at least one unique peptide per protein and a FDR of 1%. The reporter ion intensities were corrected according to the isotopic purities provided by the manufacturer.

For protein quantification purposes, only unique peptides were used, and protein ratios (i.e., TMT-127/TMT-126) were normalized to protein median. The cut-off for up-regulated proteins was $\geq$1.500, and for down-regulated proteins was $\leq$ 0.667 as previously reported (21).

## Statistical Analysis

Values reported in the figures are the mean ± S.D. of three independent experiments, each one performed in triplicate. Statistical analysis was performed by the paired Student's *t*-test

using SigmaStat 4.0 software (Systat Software Inc., CA, USA). All tests were performed in triplicate, and statistically significance was assigned for $p < 0.05$.

# RESULTS

## Purification and Characterization of SC

The isolated SC culture was 95% pure as indicated by immunostaining for AMH (**Figure 1a**) with an extremely low percentage of non-SC cells (<5%) characterized by immunostaining for insulin-like 3-positive (Leydig) cells (INSL-3) (**Figure 1b**), alpha-smooth muscle actin positive (peritubular myoid) cells (ASMA) (**Figure 1c**) and protein gene product 9.5-positive (gonocytes and spermatogonial) cells (PGP9.5) (**Figure 1d**).

## Inhibin B, AMH, and FSH-r Gene Expression in SC

AMH gene expression in SC was significantly down-regulated by treatment with α-, β-follitropin, and urofollitropin in combination with testosterone treatment compared with testosterone alone (**Figure 2A**, $p < 0.001$).

In contrast, inhibin B expression was significantly increased after treatment with α-, β-follitropin, and urofollitropin, each other combined with testosterone treatment compared with testosterone alone (**Figure 2A**, $p < 0.001$).

Moreover, we found a statistically significant reduction of FSH-r upon all three FSH preparations plus testosterone compared with testosterone alone (**Figure 2A**, $p < 0.001$).

## Inhibin B and AMH Secretion Assay

The secretion of AMH was significantly down-regulated by α-, β-follitropin, and urofollitropin plus testosterone treatments compared with testosterone alone, consistent with the results of gene expression showed above (**Figure 2B**, $p < 0.001$).

Meanwhile, inhibin B was significantly increased in culture medium only after exposure to α-follitropin plus testosterone (**Figure 2B**, $p < 0.001$), while no changes were observed after β-follitropin plus testosterone stimulation. Interestingly, we observed a significant reduction of inhibin B after the stimulation with urofollitropin plus testosterone compared with testosterone alone (**Figure 2B**, $p < 0.05$).

## Secretomic Protein Profiling

In order to evaluate the differences induced by the different FSH preparations on SC secretomic profiles, we performed a comparative proteomic analysis of the SC culture media in the groups treated with testosterone associated with α-, β-follitropin, and urofollitropin, and we compared it with the group of the testosterone treatment alone.

The proteomic analysis resulted in the identification of 46 TMT-labeled proteins in all the SC culture media proteomes (**Table 2**). Of those, 13 proteins were detected in a significantly altered abundance (**Table 3**). Specifically, 11 proteins were observed as up-regulated (**Table 3**; cut-off $\geq 1.500$) and 2 proteins as down-regulated (**Table 3**; cut-off $\leq 0.667$) by the different FSH preparations. All the different FSH preparations showed a down-regulation of the secreted SPARC protein

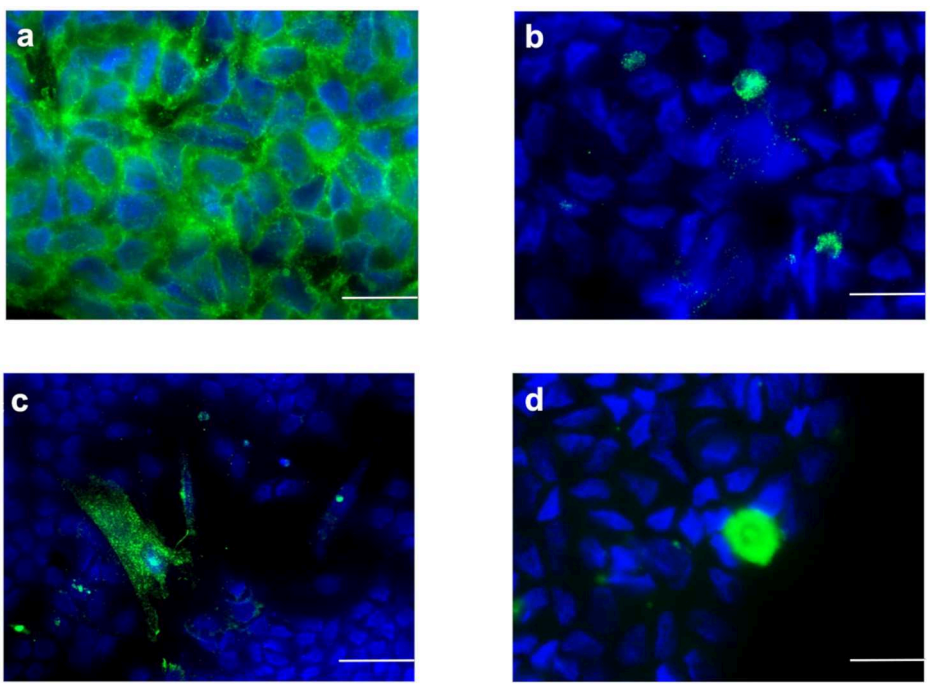


**FIGURE 1 |** Sertoli cells characterization by immunofluorescence staining (green color). Sertoli cell monolayers were characterized by the expression of **(a)** AMH, **(b)** INSL3, **(c)** ASMA, and **(d)** (PGP9.5). Nuclei are labeled with DAPI in blue color. Bars = 20 μm.

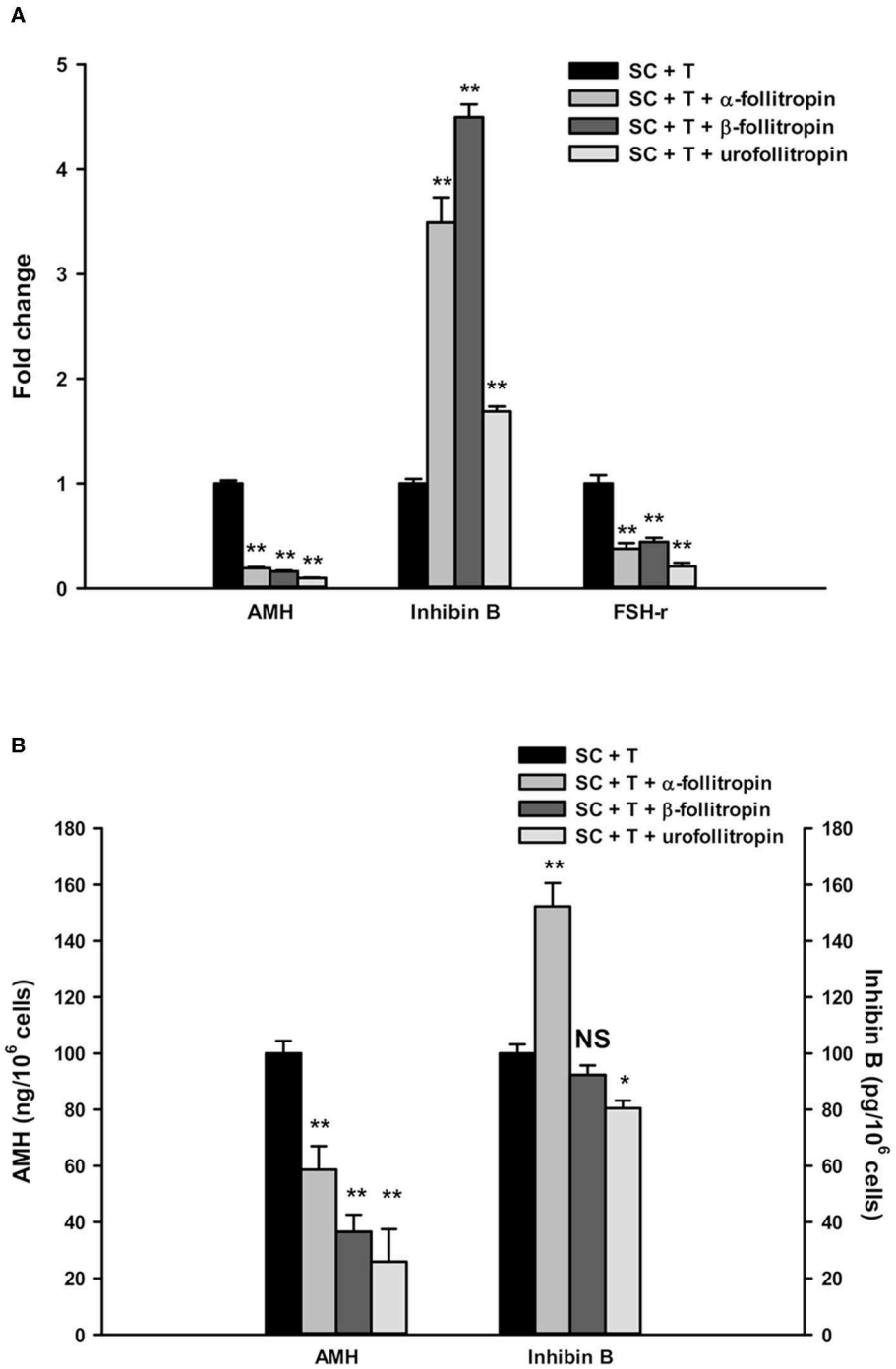


**FIGURE 2 |** Real-time PCR analysis and ELISA assays. **(A)** Gene expression in SC of AMH, inhibin B, and FSH-r. **(B)** AMH and inhibin B secretion in SC culture medium. Data represent the mean ± S.D. (*$p < 0.05$, **$p < 0.001$) of three independent experiments performed in triplicate.

and an up-regulation of 3 proteins (PLAT, INHBA, and TPI1, **Table 3**).

Interestingly, an up-regulation of 8 additional secreted proteins (ACTB, UBC, PRDX2, PPIA, SPP1, HBB, MIF, and S100A6) and a down-regulation of Histone H4 were specifically observed after the use of β-FSH with T, as reported in **Table 3**.

## DISCUSSION

In the present work, we have focused on the "*in vitro*" effect of different FSH preparations on pre-pubertal porcine SC culture, evaluating the modulation of specific markers determined through different approaches. All the FSH preparations assessed

**TABLE 2 |** List of the 46 TMT-labeled proteins identified in the Sertoli cell culture.

| Accession | Gene name | Description | Coverage | # Proteins | # Peptides | # Unique Peptides | MW (kDa) |
|---|---|---|---|---|---|---|---|
| P08835 | ALB | Serum albumin | 14.00 | 1 | 12 | 12 | 69.6 |
| Q29549 | CLU | Clusterin | 40.36 | 1 | 14 | 14 | 51.7 |
| P01025 | C3 | Complement C3 | 16.68 | 1 | 21 | 21 | 186.7 |
| P04087 | INHA | Inhibin alpha chain | 31.04 | 1 | 8 | 8 | 39.2 |
| Q6QAQ1 | ACTB | Actin, cytoplasmic 1 | 22.40 | 1 | 6 | 2 | 41.7 |
| P20305 | GSN | Gelsolin (Fragment) | 11.79 | 1 | 7 | 7 | 84.7 |
| Q9GKQ6 | BGN | Biglycan (Fragments) | 31.99 | 1 | 7 | 7 | 30.4 |
| P00761 | N/A | Trypsin | 9.96 | 1 | 2 | 2 | 24.4 |
| P18648 | APOA1 | Apolipoprotein A-I | 32.83 | 1 | 7 | 7 | 30.3 |
| P02543 | VIM | Vimentin | 8.37 | 1 | 4 | 4 | 53.6 |
| P04404 | CHGA | Chromogranin-A (Fragment) | 16.59 | 1 | 5 | 5 | 49.3 |
| P20112 | SPARC | SPARC | 24.67 | 1 | 6 | 6 | 34.2 |
| P68137 | ACTA1 | Actin, alpha skeletal muscle | 15.65 | 1 | 5 | 1 | 42.0 |
| P0CG68 | UBC | Polyubiquitin-C | 32.83 | 2 | 2 | 2 | 60.0 |
| Q29095 | PTGDS | Prostaglandin-H2 D-isomerase | 16.40 | 1 | 3 | 3 | 20.6 |
| Q8SQ23 | PLAT | Tissue-type plasminogen activator | 8.54 | 1 | 3 | 3 | 63.6 |
| Q1KYT0 | ENO3 | Beta-enolase | 8.06 | 1 | 2 | 2 | 47.1 |
| P80031 | GSTP1 | Glutathione S-transferase P | 7.73 | 1 | 1 | 1 | 23.5 |
| P35624 | TIMP1 | Metalloproteinase inhibitor 1 | 9.18 | 1 | 1 | 1 | 23.1 |
| P62802 | N/A | Histone H4 | 11.65 | 1 | 1 | 1 | 11.4 |
| A5A8V7 | HSPA1L | Heat shock 70 kDa protein 1-like | 6.08 | 1 | 2 | 2 | 70.3 |
| P10668 | CFL1 | Cofilin-1 | 18.67 | 2 | 2 | 2 | 18.5 |
| Q9XSD9 | DCN | Decorin | 5.28 | 1 | 2 | 2 | 39.9 |
| Q49I35 | LGALS1 | Galectin-1 | 5.93 | 1 | 1 | 1 | 14.7 |
| P52552 | PRDX2 | Peroxiredoxin-2 (Fragment) | 7.09 | 1 | 1 | 1 | 14.2 |
| O97763 | NPC2 | NPC intracellular cholesterol transporter 2 | 16.78 | 1 | 2 | 2 | 16.3 |
| P79295 | AMH | Muellerian-inhibiting factor | 7.65 | 1 | 3 | 3 | 61.5 |
| P62936 | PPIA | Peptidyl-prolyl cis-trans isomerase A | 5.49 | 1 | 1 | 1 | 17.9 |
| Q29243 | DAG1 | Dystroglycan | 1.82 | 1 | 1 | 1 | 95.4 |
| P14287 | SPP1 | Osteopontin | 5.28 | 1 | 1 | 1 | 33.6 |
| P00690 | AMY2 | Pancreatic alpha-amylase | 2.74 | 1 | 1 | 1 | 57.0 |
| P43368 | CAPN3 | Calpain-3 | 1.83 | 1 | 1 | 1 | 94.5 |
| P00172 | CYB5A | Cytochrome b5 | 6.72 | 1 | 1 | 1 | 15.3 |
| P29412 | EEF1B | Elongation factor 1-beta | 6.70 | 1 | 1 | 1 | 24.6 |
| P02067 | HBB | Hemoglobin subunit beta | 6.12 | 1 | 1 | 1 | 16.2 |
| O02705 | HSP90AA1 | Heat shock protein HSP 90-alpha | 1.91 | 1 | 1 | 1 | 84.7 |
| P03970 | INHBA | Inhibin beta A chain | 7.08 | 1 | 2 | 2 | 47.4 |
| P01315 | INS | Insulin | 19.44 | 1 | 1 | 1 | 11.7 |
| P80928 | MIF | Macrophage migration inhibitory factor | 7.83 | 1 | 1 | 1 | 12.4 |
| Q2EN75 | S100A6 | Protein S100-A6 | 8.89 | 1 | 1 | 1 | 10.1 |
| P03974 | VCP | Transitional endoplasmic reticulum ATPase | 2.11 | 1 | 1 | 1 | 89.2 |
| Q29371 | TPI1 | Triosephosphate isomerase | 6.05 | 1 | 1 | 1 | 26.7 |
| P42639 | TPM1 | Tropomyosin alpha-1 chain | 4.58 | 1 | 1 | 1 | 32.7 |
| P09571 | TF | Serotransferrin | 2.01 | 1 | 1 | 1 | 76.9 |
| P50390 | TTR | Transthyretin | 4.67 | 1 | 1 | 1 | 16.1 |
| P04185 | PLAU | Urokinase-type plasminogen activator | 3.85 | 1 | 1 | 1 | 49.1 |

*N/A, Not applicable.*

in the current study, α-, β-follitropin, and urofollitropin, induced a significant and similar response in terms of down-regulation of both AMH gene expression and AMH secretion, up-regulation of inhibin B gene expression and down-regulation of the expression of FSH-r gene expression. AMH is a glycoprotein dimeric hormone, a member of the transforming growth factor β (TGF-β)

**TABLE 3 |** List of the up- and down-regulated proteins in Sertoli cell medium after stimulation with the different FSH preparations plus testosterone, compared with testosterone treatment alone.

| Accession | Gene name | Description | αFSH+T/T | βFSH+T/T | uFSH+T/T |
|---|---|---|---|---|---|
| P03970 | INHBA | Inhibin beta A chain | **1.804** | **1.835** | **3.084** |
| Q8SQ23 | PLAT | Tissue-type plasminogen activator | **1.881** | **2.533** | **1.857** |
| Q29371 | TPI1 | Triosephosphate isomerase | **1.786** | **2.132** | **1.562** |
| Q6QAQ1 | ACTB | Actin, cytoplasmic 1 | 1.377 | **1.618** | 1.147 |
| P0CG68 | UBC | Polyubiquitin-C | 1.185 | **1.500** | 1.357 |
| P52552 | PRDX2 | Peroxiredoxin-2 (Fragment) | 1.459 | **1.634** | 1.077 |
| P62936 | PPIA | Peptidyl-prolyl cis-trans isomerase A | 1.358 | **1.980** | 1.076 |
| P14287 | SPP1 | Osteopontin | 1.016 | **2.134** | 1.191 |
| P02067 | HBB | Hemoglobin subunit beta | 1.270 | **1.814** | 1.336 |
| P80928 | MIF | Macrophage migration inhibitory factor | 1.412 | **1.513** | 1.233 |
| Q2EN75 | S100A6 | Protein S100-A6 | 1.302 | **1.564** | 1.100 |
| P20112 | SPARC | SPARC | **0.589** | **0.474** | **0.485** |
| P62802 | N/A | Histone H4 | 1.275 | **0.630** | 1.049 |

*N/A, Not applicable.*
*The values in bold indicate the cut-off ≥1.500 for up-regulated proteins, and ≤ 0.667 for down-regulated proteins.*

family, that plays a pivotal function in fetal sex differentiation, being involved in the regression of the Müllerian ducts (22). In the male, SC secretes high amounts of AMH from fetal life until the onset of puberty. AMH is exclusively secreted by SC and, for this reason, it is widely considered an important marker of the testicular function during the pre-pubertal life (22). We observed that testosterone alone and in combination with the three FSH preparations induced a significant down-regulation in AMH mRNA expression and secretion. As expected, we also demonstrated a statistically significant reduction in FSH-r expression independently of the FSH preparation. These data are in accordance with literature reporting how the interaction of the hormone with its receptor leads to the down-regulation of FSH-r mRNA expression by a cAMP-dependent post-transcriptional mechanism (23).

Another specific and important marker of SC functionality is inhibin B, which provides for a negative feedback on FSH secretion. In particular, serum inhibin B concentration is high during early postnatal life, and then gradually is reduced to a detectable plateau-level until its increase at the beginning of puberty (12). The assay of inhibin B is used in clinical practice to evaluate the presence and function of SC during childhood. Additionally, in adult life, the inhibin B levels depend on the presence of germ cells thus reflecting the efficiency of spermatogenesis (24). Our results demonstrated that all three FSH preparations plus testosterone significantly induced an up-regulation in the levels of inhibin B mRNA, confirming the role of FSH in inducing the transcription of inhibin B gene.

Our SC secretomic analysis uncovered a similar response after stimulation of SC with the different FSH preparations. On the one hand, we demonstrated the reduction levels of secreted SPARC protein after stimulation of SC with the different FSH preparations. SPARC, also known as osteonectin or BM-40, is a multifunctional protein that can modulate cell shape, proliferation, differentiation, and migration (25). SPARC has been found to interact with structural matrix proteins and may act to mediate their interactions with cells (24–28). In addition, SPARC can regulate the activity of several signaling molecules, either by direct interaction or by interfering with their signaling pathways (29–31). In our scenario, it is known that SPARC is produced by Leydig and Sertoli cells (32, 33), and that it is internalized in Sertoli, Leydig, and germ cells (34), playing a paracrine regulatory role during fetal testis development. Furthermore, the expression of SPARC in SC bearing late-stage elongate spermatids might suggest a role in the spermiation of elongated spermatids (33). Future studies will comprehensively define the function of SPARC in Sertoli-germ cell interaction and spermiogenesis. Here we provide for the first time information about the down-regulation of SPARC secretion by FSH.

On the other hand, the proteomic analysis also showed that tissue-type plasminogen activator (PLAT), triosephosphate isomerase (TPI1), and inhibin beta A chain (INHBA) proteins were up-regulated by the stimulation with α-, β-follitropin, and urofollitropin in presence of normal androgen milieu. Specifically, the inhibin beta A chain was observed as upregulated in proteomics by all the FSH preparations, and the higher increase was observed for urofollitropin. Despite this evidence, only α-follitropin stimulation induced a significative increase in the inhibin B levels in the medium, as documented by ELISA. INHBA is a subunit of both activin B and inhibin B (24). In the testis, it has been postulated that activin B acts as an autocrine and paracrine regulator of spermatogenesis (35, 36), modulating the proliferation in the testis of germ cells and SC (37). We might speculate that β-follitropin and, especially, urofollitropin, induce the activation of the INHB gene and the production of INHB, but do not increase the levels of INHB in the medium since they might increase the levels of activin B instead of inhibin B. Further studies are so needed to understand how the different FSH preparations modify the inhibin B and activin B balance.

The remaining two up-regulated proteins have been previously associated with SC. For example, previous studies demonstrated low levels of PLAT activity in cultured SC under basal conditions, whereas FSH stimulation induces PLAT (38, 39). Our results through a quantitative *in vitro* secretomic approach support the increased secretion of PLAT after FSH treatment independently of the FSH preparation. Interestingly, the plasminogen activator system acts in the process of spermiation (40), the detachment of residual bodies from the mature spermatids (41), and the residual body phagocytosis by SC (42). Triosephosphate isomerase has moreover been previously reported to be expressed in SC (43). An increased TPI1 expression in SC may influence the early activities of spermatogenesis, such as mitosis or initiation of meiosis, by spermatogonia or pre-leptotene spermatocytes, respectively (43).

Surprisingly, the combination of β-follitropin with testosterone revealed specific effects in the SC function besides the aforementioned similar effect of all tested FSH treatments. Specifically, the levels of eight additional proteins were up-regulated, which were: Actin (ACTB), Polyubiquitin-C (UBC), Peroxiredoxin-2 (PRDX2), Peptidyl-prolyl cis-trans isomerase A (PPIA), Osteopontin (SPP1), Hemoglobin subunit beta (HBB), Macrophage migration inhibitory factor (MIF), and Protein S100-A6 (S100A6); and just one protein, Histone H4, was down-regulated.

It is important to underline that some of these proteins might have a pivotal role both in the germ cell migration and in the cell-to-cell contact at the blood-testis barrier (BTB). For example, actin filament bundles have been described in specific Sertoli cell regions that are adjoining to tight junctions and to the sites of adhesion to spermatogenic cells (44). During spermatogenesis, these actin bundles undergo organizational changes, which might play a role in changing the interrelationship between SC and germ cells by facilitating the movement of spermatogenic cells (44). Similarly, SPP1, synthesized by SC and germ cells, is involved in cell adhesion and migration (45), and MIF, produced by SC under basal conditions, induces the migration of spermatogonial cells (46). Also, we found up-regulation of PPIA, a protein highly expressed in SC that has been recognized as a crucial factor in BTB integrity and maintenance (47), as well as S100A6, a protein that promotes cell migration and influences cell junction of SC (48) that could be implied in the spermatogenesis and modulation of BTB.

Interestingly, β-follitropin also increased PRDX2, an antioxidant protein preferentially expressed in SC that might play a role in removing or regulating the intracellular levels of peroxides produced during metabolism (49, 50). Additional up-regulated proteins were HBB, a metalloprotein that acts as a scavenger balancing the level of carbon monoxide (CO) in testis (51), and UBC, which is required for normal spermatogenesis development (52).

In conclusion, α-, β-follitropin, and urofollitropin induced a similar response as expressed by the down-regulation of AMH gene expression and AMH secretion, and an up-regulation and down-regulation of inhibin B and FSH-r gene expression, respectively, thus exerting an interesting effect in inducing maturation of SC from a pre-pubertal to an adult phenotype. This data confirms that the FSH in presence of an androgenic milieu regulates the proliferation and functional maturation of Sertoli cell type (53). Moreover, all three FSH preparations induced a down-regulation of a spermiation related protein, the SPARC, supporting the role of FSH in the regulation of spermatogenesis. Nevertheless, there are some specific effects of each FSH preparation, which need consideration before their prescription to infertile males.

For instance, only α-follitropin, in association with testosterone, induced the secretion in the media of inhibin B. Since inhibin B secreted by Sertoli cells could serve as negative feedback control on the hypothalamic-pituitary system to decrease FSH release (54), we might suppose that α-follitropin could have an inhibitory effect on the hypothalamic/pituitary axis *in vivo*. If these preliminary data obtained in our *in vitro* model would be confirmed by further *in vitro* and *in vivo* studies, we might conclude that male secondary hypogonadism would represent the best indication for this treatment since it induces a good response in terms of Sertoli activation, and the increase in Inhibin B secretion does not have any clinical relevance. In contrast, the increase of Inhibin B secretion as a response to α-follitropin treatment might represent a problem for normogonadotropic infertile patients since it might inhibit physiological pituitary FSH secretion.

In the case of urofollitropin, it induces a similar secretomic profile but also an increase in the release of INHBA without increasing the levels of secreted inhibin B, suggestive of an increasing release of activin B. This panel of action might be useful for treating oligozoospermic patients with normal FSH secretion. Therefore, we might speculate that—if these preliminary data would be confirmed—urofollitropin might represent the best treatment option for patients with normogonadotropic hypogonadism, since it seems to induce an increase in the secreted proteins, including the A chain of inhibin B and, consequentially, activin B, without increasing the levels of inhibin B, which might interfere with pituitary FSH secretion.

Finally, β-FSH stimulation exhibits additional effect up-regulating specific proteins mainly related to spermatogenic cell migration and BTB maintenance. In this context, the stimulation of SC with β-follitropin exerts his effect in the modulation of additional proteins implicated in the last stage of spermiation and in the related antioxidant activity. Further studies are needed to confirm that β-follitropin treatment is the best treatment option for patients with a spermatogenic arrest at the spermatid stage, as suggested by these preliminary *in vitro* data.

This molecular and proteomic approach demonstrated how some molecular effects seem to have a specific signature depending on each FSH preparation. The different molecular responses could help to choose which of the different FSH preparations could be used for infertility treatment according to the different present physio-pathological conditions. We can argue that α-follitropin can find a specific clinical use in hypospermatogenesis due to hypogonadotropic stimulus or in inducing spermatogenesis in puberty; β-follitropin could be specifically indicated to improve spermiation or in case of spermatidic arrest; and, finally, urofollitropin could be useful

in idiopathic infertility in normogonadotropic patients. In conclusion, we performed for the first time a comparative study on the effects of different preparations of FSH on *in vitro* porcine pre-pubertal SC model. In the landscape of a personalized medicine, this study opens a window on the different use of the FSH formulations in relation to various clinical therapeutic targets.

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

## ETHICS STATEMENT

The animal study was reviewed and approved by Italian Approved Animal Welfare Assurance (A-3143-01).

## AUTHOR CONTRIBUTIONS

All authors had critically revised and approved the final version of the manuscript. IA, GG, and FB designed and drafted the manuscript. The experimental procedures and data analysis were performed by GG, FB, CB, CL, MJ, MA, FV, and FManci. AP, RC, and RO gave experimental guidance. GL, FMancu, and DM revised the manuscript.

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## REFERENCES

1. Recombinant Human FSH Study Group. Clinical assessment of recombinant human follicle-stimulating hormone in stimulating ovarian follicular development before *in vitro* fertilization. *Fertil Steril.* (1995) 63:77–86. doi: 10.1016/S0015-0282(16)57300-2
2. Foresta C, Selice R, Ferlin A, Garolla A. Recombinant FSH in the treatment of oligozoospermia. *Expert Opin Biol Ther.* (2009) 9:659–66. doi: 10.1517/14712590902932673
3. Ulloa-Aguirre A, Timossi C. Structure-function relationship of follicle-stimulating hormone and its receptor. *Hum Reprod Update.* (1998) 4:260–83. doi: 10.1093/humupd/4.3.260
4. Al-Inany H, Aboulghar MA, Mansour RT, Serour GI. Ovulation induction in the new millennium: recombinant follicle-stimulating hormone versus human menopausal gonadotropin. *Gynecol Endocrinol.* (2005) 20:161–9. doi: 10.1080/09513590400027232
5. European and Israeli Study Group on Highly Purified Menotropin versus Recombinant Follicle-Stimulating Hormone (EISG). Efficacy and safety of highly purified menotropin versus recombinant follicle-stimulating hormone in in vitro fertilization/intracytoplasmic. *Fertil Steril.* (2002) 78:520–8. doi: 10.1016/S0015-0282(02)03250-8
6. Al-Inany H, Aboulghar M, Mansour R, Serour G. Meta-analysis of recombinant versus urinary-derived FSH: an update. *Hum Reprod.* (2003) 18:305–13. doi: 10.1093/humrep/deg088
7. Coomarasamy A, Afnan M, Cheema D, van der Veen F, Bossuyt PM, van Wely M. Urinary hMG versus recombinant FSH for controlled ovarian hyperstimulation following an agonist long down-regulation protocol in IVF or ICSI treatment: a systematic review and meta-analysis. *Hum Reprod.* (2008) 23:310–15. doi: 10.1093/humrep/dem305
8. van Wely M, Kwan I, Burt AL, Thomas J, Vail A, Van der Veen F, et al. Recombinant versus urinary gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. *Cochrane Database Syst Rev.* (2011) 16:CD005354. doi: 10.1002/14651858.CD005354.pub2
9. Attia AM, Abou-Setta AM, Al-Inany HG. Gonadotrophins for idiopathic male factor subfertility. *Cochrane Database Syst Rev.* (2013) 23:CD005071. doi: 10.1002/14651858.CD005071.pub4
10. Barbonetti A, Calogero AE, Balercia G, Garolla A, Krausz C, La Vignera S, et al. The use of follicle stimulating hormone (FSH) for the treatment of the infertile man: position statement from the Italian Society of Andrology and Sexual Medicine (SIAMS). *J Endocrinol Invest.* (2018) 41:1107–22. doi: 10.1007/s40618-018-0843-y
11. Kaur G, Thompson LA, Dufour JM. SC-immunological sentinels of spermatogenesis. *Semin Cell Dev Biol.* (2014) 30:36–44. doi: 10.1016/j.semcdb.2014.02.011
12. Dimitriadis F, Tsiampali C, Chaliasos N, Tsounapi P, Takenaka A, Sofikitis N. The Sertoli cell as the orchestra conductor of spermatogenesis: spermatogenic cells dance to the tune of testosterone. *Hormones (Athens).* (2015) 14:479–503. doi: 10.14310/horm.2002.1633
13. Edelsztein NY Grinspon RP, Schteingart HF, Rey RA. Anti-Müllerian hormone as a marker of steroid and gonadotropin action in the testis of children and adolescents with disorders of the gonadal axis. *Int J Pediatr Endocrinol.* (2016) 2016:20. doi: 10.1186/s13633-016-0038-2
14. Golat BT, Cameron DF. Sertoli cells enhance formation of capillary-like structures *in vitro*. *Cell Transplant.* (2008) 17:1135–44. doi: 10.3727/096368908787236512
15. Luca G, Fallarino F, Calvitti M, Mancuso F, Nastruzzi C, Arato I, et al. Xenograft of microencapsulated sertoli cells reverses T1DM in NOD mice by inducing neogenesis of beta-cells. *Transplantation.* (2010) 90:1352–57. doi: 10.1097/TP.0b013e3181ffb9d2
16. Luca G, Bellezza I, Arato I, Di Pardo A, Mancuso F, Calvitti M, et al. Terapeutic potential of microencapsulated sertoli cells in huntington disease. *CNS Neurosci Ther.* (2016) 22:686–90. doi: 10.1111/cns.12569
17. Arato I, Luca G, Mancuso F, Bellucci C, Lilli C, Calvitti M, et al. An *in vitro* prototype of a porcine biomimetic testis-like cell culture system: a novel tool for the study of reassembled Sertoli and Leydig cells. *Asian J Androl.* (2018) 20:160–5. doi: 10.4103/aja.ja_47_17
18. Mancuso F, Arato I, Lilli C, Bellucci C, Bodo M, Calvitti M, et al. Acute effects of lead on porcine neonatal Sertoli cells *in vitro*. *Toxicol In Vitro.* (2018) 48:45–52. doi: 10.1016/j.tiv.2017.12.013
19. Codina C, Estanyol JM, Fidalgo MJ, Ballescà JL, Oliva R. Advances in sperm proteomics: best-practise methodology and clinical potential. *Expert Rev Proteomics.* (2015) 12:255–77. doi: 10.1586/14789450.2015.1040769

20. Barrachina F, Jodar M, Delgado-Dueñas D, Soler-Ventura A, Estanyol JM, Mallofré C, et al. Stable-protein pair analysis as a novel strategy to identify proteomic signatures: application to seminal plasma from infertile patients. *Mol Cell Proteomics.* (2019) 18:S77–S90. doi: 10.1074/mcp.RA118.001248
21. Grande G, Vincenzoni F, Mancini F, Baroni S, Luca G, Calafiore R, et al. Semen Proteomics reveals the impact of enterococcus faecalis on male fertility. *Protein Pept Lett.* (2018) 25:472–77. doi: 10.2174/0929866525666180412161818
22. Josso N, Rey RA, Picard JY. Anti-müllerian hormone: a valuable addition to the toolbox of the pediatric endocrinologist. *Int J Endocrinol.* (2013) 2013:674105. doi: 10.1155/2013/674105
23. Themmen AP, Blok LJ, Post M, Baarends WM, Hoogerbrugge JW, Parmentier M, et al. Follitropin receptor down-regulation involves a cAMP-dependent post-transcriptional decrease of receptor mRNA expression. *Mol Cell Endocrinol.* (1991) 78:R7–R13. doi: 10.1016/0303-7207(91)90130-K
24. Meachem SJ, Nieschlag E, Simoni M. Inhibin B in male reproduction: pathophysiology and clinical relevance. *Eur J Endocrinol.* (2001) 145:561–71. doi: 10.1530/eje.0.1450561
25. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix. *Matrix Biol.* (2000) 19:569–80. doi: 10.1016/S0945-053X(00)00105-0
26. Sage H, Vernon RB, Funk SE, Everitt EA, Angello J. SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading *in vitro* and exhibits $Ca^{+}2$-dependent binding to the extracellular matrix. *J Cell Biol.* (1989) 109:341–56. doi: 10.1083/jcb.109.1.341
27. Mayer U, Aumailley M, Mann K, Timpl R, Engel J. Calcium-dependent binding of basement membrane protein BM-40 (osteonectin, SPARC) to basement membrane collagen type IV. *Eur J Biochem.* (1991) 198:141–50. doi: 10.1111/j.1432-1033.1991.tb15996.x
28. Lane TF, Sage EH. The biology of SPARC, a protein that modulates cell-matrix interactions. *FASEB J.* (1994) 8:163–73. doi: 10.1096/fasebj.8.2.8119487
29. Raines EW, Lane TF, Iruela-Arispe ML, Ross R, Sage EH. The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors. *Proc Natl Acad Sci U S A.* (1992) 89:1281–5. doi: 10.1073/pnas.89.4.1281
30. Kupprion C, Motamed K, Sage EH. SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. *J Biol Chem.* (1998) 273:29635–40. doi: 10.1074/jbc.273.45.29635
31. Motamed K, Blake DJ, Angello JC, Allen BL, Rapraeger AC, Hauschka SD, et al. Fibroblast growth factor receptor-1 mediates the inhibition of endothelial cell proliferation and the promotion of skeletal myoblast differentiation by SPARC: a role for protein kinase A. *J Cell Biochem.* (2003) 90:408–23. doi: 10.1002/jcb.10645
32. Howe CC, Overton GC, Sawicki J, Solter D, Stein P, Strickland S. Expression of SPARC/osteonectin transcript in murine embryos and gonads. *Differentiation.* (1988) 37:20–5. doi: 10.1111/j.1432-0436.1988.tb00792.x
33. Vernon RB, Sage H. The calcium-binding protein SPARC is secreted by Leydig and Sertoli cells of the adult mouse testis. *Biol Reprod.* (1989) 40:1329–40. doi: 10.1095/biolreprod40.6.1329
34. Wilson MJ, Bowles J, Koopman P. The matricellular protein SPARC is internalized in Sertoli, Leydig, and germ cells during testis differentiation. *Mol Reprod Dev.* (2006) 73:531–9. doi: 10.1002/mrd.20394
35. Mather JP, Woodruff TK, Krummen LA. Paracrine regulation of reproductive function by inhibin and activin. *Proc Soc Exp Biol Med.* (1992) 201:1–15. doi: 10.3181/00379727-201-43473
36. Bläuer M, Husgafvel S, Syvälä H, Tuohimaa P, Ylikomi T. Identification of nuclear localization signal in activin/inhibin betaA subunit; intranuclear βA in rat spermatogenic cells. *Biol Reprod.* (1999) 60:588–93. doi: 10.1095/biolreprod60.3.588
37. Ying SY, Zhang Z, Furst B, Batres Y, Huang G, Li G. Activin and activin receptors in cell growth. *Proc Soc Exp Biol Med.* (1997) 214:114–22. doi: 10.3181/00379727-214-44077
38. Liu YX, Du Q, Liu K, Fu GQ. Hormonal regulation of plasminogen activator in rat and mouse seminiferous epithelium. *Biol Signals.* (1995) 4:232–40. doi: 10.1159/000109447
39. Liu YX, Liu K, Zhou HM, Du Q, Hu ZY, Zou RJ. Hormonal regulation of tissue-type plasminogen activator and plasminogen activator inhibitor type-1 in cultured monkey Sertoli cells. *Hum Reprod.* (1995) 10:719–27. doi: 10.1093/oxfordjournals.humrep.a136022
40. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann N Y Acad Sci.* (1952) 55:548–73. doi: 10.1111/j.1749-6632.1952.tb26576.x
41. Morales C, Clermont Y, Nadler NJ. Cyclic endocytic activity and kinetics of lysosomes in Sertoli cells of the rat: a morphometric analysis. *Biol Reprod.* (1986) 34:207–18. doi: 10.1095/biolreprod34.1.207
42. Sigillo F, Pernod G, Kolodie L, Benahmed M, Le Magueresse-Battistoni B. Residual bodies stimulate rat Sertoli cell plasminogen activator activity. *Biochem Biophys Res Commun.* (1998) 250:59–62. doi: 10.1006/bbrc.1998.9264
43. Russell DL, Kim KH. Expression of triosephosphate isomerase transcripts in rat testis: evidence for retinol regulation and a novel germ cell transcript. *Biol Reprod.* (1996) 55:11–8. doi: 10.1095/biolreprod55.1.11
44. Vogl AW, Soucy LJ. Arrangement and possible function of actin filament bundles in ectoplasmic specializations of ground squirrel Sertoli cells. *J Cell Biol.* (1985) 100:814–25. doi: 10.1083/jcb.100.3.814
45. Moura AA. Seminal plasma proteins and fertility indexes in the bull: the case for osteopontin. *Anim Reprod.* (2005) 2:3–10.
46. Huleihel M, Abofoul-Azab M, Abarbanel Y, Einav I, Levitas E, Lunenfeld E. Production of macrophage inhibitory factor (MIF) by primary Sertoli cells; its possible involvement in migration of spermatogonial. *Cells J Cell Physiol.* (2017) 232:2869–77. doi: 10.1002/jcp.25718
47. Islam R, Yoon H, Kim BS, Bae HS, Shin HR, Kim WJ, et al. Blood-testis barrier integrity depends on Pin1 expression in Sertoli cells. *Sci Rep.* (2017) 7:6977. doi: 10.1038/s41598-017-07229-1
48. Ge LC, Chen ZJ, Liu H, Zhang KS, Sue Q, Ma XY, et al. Signaling related with biphasic effects of bisphenol A (BPA) on Sertoli cell proliferation: a comparative proteomic analysis. *Biochim Biophys Acta.* (2014) 1840:2663–73. doi: 10.1016/j.bbagen.2014.05.018
49. Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG. Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *J Biol Chem.* (1998) 273:6297–302. doi: 10.1074/jbc.273.11.6297
50. Lee K, Park JS, Kim YJ, Lee YS, Hwang TS, Kim DJ, et al. Differential expression of Prx I and II in mouse testis and their up-regulation by radiation. *Biochem Biophys Res Commun.* (2002) 296:337–42. doi: 10.1016/S0006-291X(02)00801-X
51. Middendorff R, Kumm M, Davidoff MS, Holstein AF, Müller D. Generation of cyclic guanosine monophosphate by heme oxygenases in the human testis-A regulatory role for carbon monoxide in sertoli cells? *Biol Reprod.* (2000) 63:651–7. doi: 10.1095/biolreprod63.2.651
52. Kwon J, Mochida K, Wang YL, Sekiguchi S, Sankai T, Aoki S, et al. Ubiquitin C-terminal hydrolase L-1 is essential for the early apoptotic wave of germinal cells and for sperm quality control during spermatogenesis. *Biol Reprod.* (2005) 73:29–35. doi: 10.1095/biolreprod.104.037077
53. Griswold MD, Solari A, Tung PS, Fritz IB. Stimulation by follicle-stimulating hormone of DNA synthesis and of mitosis in cultured Sertoli cells prepared from testes of immature rats. *Mol Cell Endocrinol.* (1997) 7:151–65. doi: 10.1016/0303-7207(77)90064-8
54. Heindel JJ, Treinen KA. Physiology of the male reproductive system: endocrine, paracrine and autocrine regulation. *Toxicol Pathol.* (1989) 17:411–45. doi: 10.1177/019262338901700219


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# Effects of Metabolic Syndrome on Semen Quality and Circulating Sex Hormones: A Systematic Review and Meta-Analysis

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Previous studies were controversial in the effects of metabolic syndrome (MetS) on semen quality and circulating sex hormones, and thus we conducted a systematic review and meta-analysis to clarify the association. A systematic search was conducted in public databases to identify all relevant studies, and study-specific standardized mean differences (SMD) and 95% confidence intervals (CI) were pooled using a random-effects model. Finally, 11 studies were identified with a total of 1,731 MetS cases and 11,740 controls. Compared with the controls, MetS cases had a statistically significant decrease of sperm total count (SMD: −0.96, 95% CI: −1.58 to −0.31), sperm concentration (SMD: −1.13, 95% CI: −1.85 to −0.41), sperm normal morphology (SMD: −0.61, 95% CI: −1.01 to −0.21), sperm progressive motility (SMD: −0.58, 95% CI: −1.00 to −0.17), sperm vitality (SMD: −0.83, 95% CI: −1.11 to −0.54), circulating follicle-stimulating hormone (SMD: −0.87, 95% CI: −1.53 to −0.21), testosterone (SMD: −5.61, 95% CI: −10.90 to −0.31), and inhibin B (SMD: −2.42, 95% CI: −4.52 to −0.32), and a statistically significant increase of sperm DNA fragmentation (SMD: 0.76, 95% CI: 0.45 to 1.06) and mitochondrial membrane potential (SMD: 0.89, 95% CI: 0.49 to 1.28). No significant difference was found in semen volume, sperm total motility, circulating luteinizing hormone (LH), estradiol, prolactin and anti-Müllerian hormone (AMH) ($P > 0.05$). In conclusion, this meta-analysis demonstrated the effects of MetS on almost all the semen parameters and part of the circulating sex hormones, and MetS tended to be a risk factor for male infertility. Further larger-scale prospective designed studies were needed to confirm our findings.

**Keywords: metabolic syndrome, semen quality, sex hormones, meta-analysis, male infertility**

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## INTRODUCTION

Metabolic syndrome (MetS) is a complex of clinical conditions characterized by abdominal obesity, dyslipidemia, hypertension and insulin resistance (1, 2). Despite a link between MetS and poor health status, its impact on male infertility is still under discussion (3). First, as an important feature of MetS, obesity was found to reduce semen quality by altering the sex hormone levels and semen microenvironment, and inducing oxidative stress damage in sperms and interstitial glands (4, 5). However, the meta-analysis by MacDonald et al. (6) found no significant

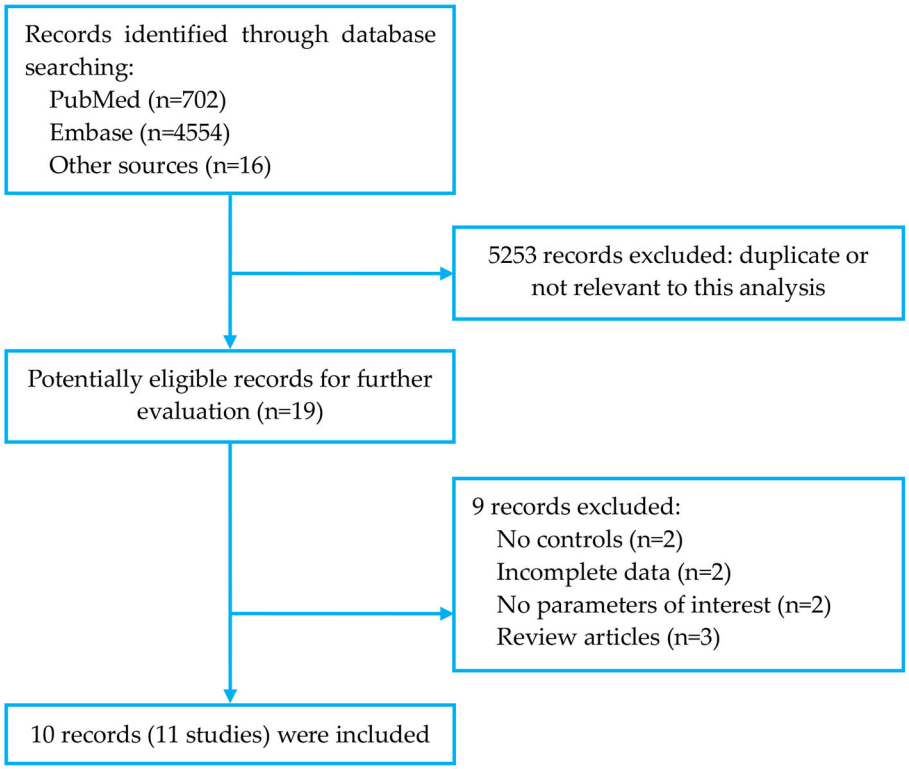


**FIGURE 1 |** Flowchart of literature search.

association between body mass index (BMI) and semen parameters, while it was associated with the prevalence of azoospermia or oligozoospermia in the meta-analysis of Sermondade et al. (7). Second, dyslipidemia, hypertension, and diabetes mellitus could also affect male reproductivity by decreasing testosterone secretion and causing testicular damage and erectile dysfunction (8–10).

As a collection of these features, MetS was thought to be involved in the pathogenesis of male infertility. The meta-analysis by Brand et al. (11) indicated a lower level of testosterone in men with MetS, but it failed to evaluate the effects of MetS on other circulating sex hormones and semen quality. The review by Corona et al. (12) analyzed the relationship between obesity, its metabolic complications and male hypogonadism (HG), and their contribution to the pathogenesis of erectile dysfunction. The meta-analysis by Rastrelli et al. (13) evaluated the association between MetS and HG, the association between HG and specific MetS components (central obesity, glucose tolerance, dyslipidemia, and hypertension), the association between MetS and sexual symptoms, the effects of MetS treatment on HG, and the effects of HG treatment on MetS. However, no meta-analyses have systematically and quantitatively evaluated the effects of MetS on both semen quality and several sex hormones in men, although there existed obvious controversies in original studies. Thus, this meta-analysis aimed to clarify the role of MetS in male infertility by assessing its impact on both semen and hormonal parameters.

# MATERIALS AND METHODS

## Search Strategy

The databases of PubMed and Embase were searched for relevant studies published up to March 1st, 2020, using the key words: ("metabolic syndrome" OR "syndrome X" OR "insulin resistance syndrome" OR "metabolic X syndrome" OR "dysmetabolic syndrome" OR "reaven syndrome" OR "metabolic cardiovascular syndrome") AND ("sperm" OR "semen" OR "spermatozoa" OR "sperm count" OR "sperm concentration" OR "semen quality" OR "semen parameters" OR "sperm quantity" OR "total sperm count" OR "azoospermia" OR "oligozoospermia"). We also reviewed the references of related studies and reviews for undetected studies. This study was approved by the ethics committee of Linyi People's Hospital (No. 2020006).

## Study Selection and Exclusion

Two authors (LZ and AP) reviewed the studies independently. The inclusion criteria were as follows: (i) focused on MetS cases and controls; (ii) any measurement of semen volume, total sperm count, sperm concentration, sperm normal morphology, sperm total motility, sperm progressive motility, sperm vitality, sperm DNA fragmentation or mitochondrial membrane potential (MMP); (iii) measurement levels were presented as mean or median with standard error (SD), 95% confidence interval (95% CI), range or inter-quartile range (IQR). The exclusion criteria were as follows: abstracts without full texts, reviews,

**TABLE 1 |** Characteristics of included studies.

| References | Area | Study population | MetS diagnosis criteria | Parameters | MetS | | Controls | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Num | Levels | Num | Levels |
| Saikia et al. (17) | Gauhati, India | MetS and age-matched controls (20∼40y) | International Diabetes Federation (IDF) criteria in 2005 | FSH (IU/L), median (range) | 50 | 0.97 (0.76∼1.1) | 30 | 3.8 (3∼4.2) |
| | | | | T (ng/mL), median (range) | | 2.32 (1.5∼4.5) | | 4.04 (1.98∼5.98) |
| | | | | InhB (pg/mL), median (range) | | 22.25 (14.42∼36) | | 124.43 (88.84∼198.94) |
| | | | | Total sperm count (million/mL), median (range) | | 14 (10∼22) | | 70 (50∼78) |
| | | | | Total sperm volume (mL), median (range) | | 3.15 (2.4∼4.2) | | 3.45 (2.5∼4.2) |
| | | | | Total sperm motility (%), median (range) | | 69.5 (58∼82) | | 79.5 (70∼86) |
| | | | | Progressive motility (%), median (range) | | 36 (32∼45) | | 54 (50∼59) |
| | | | | Normal morphology (%), median (range) | | 82 (64∼90) | | 80 (70∼85) |
| Chen et al. (18) | Taipei, China | Participants of reproductive age (mean age 32∼34y) | Harmonized criteria in 2009 | Sperm concentration, mean (SD) | 885 | 51.4 (40.21) | 7,510 | 53.16 (40.89) |
| | | | | Sperm total motility (%), mean (SD) | | 63.3 (18.11) | | 64.49 (16.17) |
| | | | | Sperm progressive motility (%), mean (SD) | | 46.08 (18.05) | | 45.88 (16.68) |
| | | | | Sperm normal morphology (%), mean (SD) | | 65.55 (17.59) | | 67.33 (16.41) |
| Ehala-Aleksejev and Punab (FM) (19) | Tartu, Estonia | Partners of pregnant women (FM) (mean age 32y) | National Cholesterol Education in Program (NCEP) criteria in 2004 | Semen volume (mL), mean (95% CI) | 29 | 4.2 (3.5∼5) | 209 | 3.7 (3.5∼4) |
| | | | | Sperm concentration ($10^6$/mL), mean (95% CI) | | 55.9 (41.4∼75.4) | | 77.7 (69.9∼86.3) |
| | | | | Total sperm count ($10^6$), mean (95% CI) | | 234.9 (175.2∼314.5) | | 289.5 (257.8∼325.1) |
| | | | | Motile spermatozoa (%), mean (95% CI) | | 51.8 (48.3∼55.4) | | 51.5 (49.9∼53.2) |
| | | | | Normal morphology (%), mean (95% CI) | | 9.2 (7.3∼11.2) | | 11.2 (10.5∼12) |
| | | | | FSH (IU/L), mean (95% CI) | | 4 (3.3∼5.1) | | 4.2 (3.8∼4.6) |
| | | | | LH (IU/L), mean (95% CI) | | 4.2 (3.6∼4.9) | | 3.7 (3.4∼3.9) |
| | | | | Testosterone (nmol/L), mean (95% CI) | | 13.5 (11.4∼15.7) | | 17.3 (16.5∼18.1) |
| | | | | Estradiol (pmol/L), mean (95% CI) | | 120.8 (101∼140.4) | | 124 (117.2∼130.9) |
| Ehala-Aleksejev and Punab (MPIC) (19) | Tartu, Estonia | Male partners of infertile couples (MPIC) (mean age 33y) | National Cholesterol Education in Program (NCEP) criteria in 2004 | Semen volume (mL), mean (95% CI) | 471 | 3.6 (3.5∼3.8) | 2,171 | 3.9 (3.8∼4) |
| | | | | Sperm concentration ($10^6$/mL), mean (95% CI) | | 40 (36.5∼45.9) | | 37.8 (35.8∼40) |
| | | | | Total sperm count ($10^6$), mean (95% CI) | | 142.9 (126.1∼162.1) | | 144.3 (136.3∼152.6) |
| | | | | Motile spermatozoa (%), mean (95% CI) | | 40.9 (39.4∼42.4) | | 41.5 (40.8∼42.1) |
| | | | | Normal morphology (%), mean (95% CI) | | 6.9 (6.4∼7.4) | | 7 (6.7∼7.2) |
| | | | | FSH (IU/L), mean (95% CI) | | 4.3 (4∼4.6) | | 4.3 (4.2∼4.5) |

*(Continued)*

**TABLE 1 |** Continued

| References | Area | Study population | MetS diagnosis criteria | Parameters | MetS | | Controls | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Num | Levels | Num | Levels |
| | | | | LH (IU/L), mean (95% CI) | | 3.3 (3.1∼3.5) | | 3.6 (3.5∼3.7) |
| | | | | Testosterone (nmol/L), mean (95% CI) | | 13.2 (12.5∼13.8) | | 17.4 (17.2∼17.7) |
| | | | | Estradiol (pmol/L), mean (95% CI) | | 139.6 (133.5∼145.7) | | 130.6 (127.7∼133.5) |
| Ventimiglia et al. (20) | Milan, Italy | Secondary infertile men (22∼68y) | National Cholesterol Education in Program (NCEP) criteria in 2004 | FSH (mIU/mL), mean (range) | 20 | 9.49 (0.3∼20.4) | 147 | 6.74 (0.1∼93.97) |
| | | | | LH (mIU/mL), mean (range) | | 4.88 (0.1∼10) | | 4.47 (0.6∼32.8) |
| | | | | InhB (pg/mL), mean (range) | | 75.3 (6∼129.2) | | 114.6 (0.5∼245.7) |
| | | | | AMH (ng/mL), mean (range) | | 2.52 (1.3∼4.4) | | 7.04 (0.6∼19.3) |
| | | | | tT (ng/mL), mean (range) | | 3.44 (2∼6.26) | | 4.92 (1.75∼9.73) |
| | | | | E2 (pg/mL), mean (range) | | 35.89 (12∼69) | | 34.91 (11∼104) |
| | | | | PRL (ng/mL), mean (range) | | 15.58 (1.22∼319) | | 14.29 (1.08∼751) |
| | | | | TSH (μUI/mL), mean (range) | | 1.98 (0.65∼5.06) | | 1.83 (0.01∼15.58) |
| | | | | Semen volume (mL), mean (range) | | 1.31 (0.1∼5) | | 2.58 (0.1∼10) |
| | | | | Sperm concentration ($10^6$/mL), mean (range) | | 20.08 (0∼52.2) | | 34.53 (0∼167) |
| | | | | Progressive motility (%), mean (range) | | 18.78 (0∼50) | | 25.28 (0∼78) |
| | | | | Normal morphology (%), mean (range) | | 1.44 (0∼6) | | 8.01 (0∼70) |
| Ventimiglia et al. (21) | Milan, Italy | Primary infertile men (mean age 36y) | National Cholesterol Education in Program (NCEP) criteria in 2004 | FSH (mIU/mL), median (IQR) | 128 | 5.2 (3.3∼17) | 1,209 | 5.7 (3.1∼12.7) |
| | | | | LH (mIU/mL), median (IQR) | | 4 (2.8∼6.6) | | 4.1 (2.7∼6.1) |
| | | | | InhB (pg/mL), median (IQR) | | 40 (27.3∼114.7) | | 85.8 (24.3∼142.9) |
| | | | | AMH (ng/mL), median (IQR) | | 4.1 (1.6∼5.4) | | 4.7 (2.4∼9.6) |
| | | | | tT (ng/mL), median (IQR) | | 3.8 (2.7∼5.3) | | 4.7 (3.6∼6) |
| | | | | E2 (pg/mL), median (IQR) | | 32 (24∼41) | | 32 (25∼42) |
| | | | | PRL (ng/mL), median (IQR) | | 8.5 (3.4∼18.2) | | 8 (3∼18) |
| | | | | TSH (μUI/mL), median (IQR) | | 1.7 (1.2∼2.6) | | 1.6 (1.1∼2.2) |
| | | | | Semen volume (mL), median (IQR) | | 2 (0.1∼3) | | 2 (0.1∼2.5) |
| | | | | Sperm concentration ($10^6$/mL), median (IQR) | | 13.8 (2.2∼40.8) | | 14.2 (3.8∼44.1) |
| | | | | Progressive motility (%), median (IQR) | | 25 (11∼44) | | 25 (10∼40) |
| | | | | Normal morphology (%), median (IQR) | | 5 (0∼16) | | 4 (0∼12) |
| | | | | Total sperm count, median (IQR) | | 25.3 (5.7∼72.8) | | 28.7 (6.3∼75.4) |
| Pilatz et al. (22) | Giessen, Germany | MetS and controls (30∼62y) | National Cholesterol Education Program (NCEP) criteria in 2001 and International Diabetes Federation (IDF) criteria in 2009 | Volume (mL), median (range) | 27 | 2.7 (0.2∼8.5) | 27 | 3 (1∼7.8) |

*(Continued)*

**TABLE 1 |** Continued

| References | Area | Study population | MetS diagnosis criteria | Parameters | MetS | | Controls | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Num | Levels | Num | Levels |
| | | | | Sperm concentration ($10^6$/mL), median (range) | | 52 (0.01∼379) | | 58 (5.8∼404) |
| | | | | Progressive motility (%), median (range) | | 48 (16∼64) | | 43 (0∼72) |
| | | | | Sperm morphology (normal forms, %), median (range) | | 4 (0∼14) | | 5 (0∼18) |
| Leisegang et al. (23) | Bellville and Stellenbosch, South Africa | MetS and controls (25∼65y) | International Diabetes Federation (IDF) criteria in 2009 | Ejaculation volume (mL), median (IQR) | 32 | 2 (1.2∼2.5) | 42 | 2.55 (1.95∼3.5) |
| | | | | Sperm concentration (million/mL), median (IQR) | | 26.7 (15.8) | | 43.7 (24.6) |
| | | | | Total sperm count (million), median (IQR) | | 48.1 (25.5∼65.8) | | 103.6 (65.6∼139.5) |
| | | | | Sperm vitality (% sperm alive), median (IQR) | | 50 (23.2) | | 67.3 (15.4) |
| | | | | Progressive motility (% motile), median (IQR) | | 20 (17.1) | | 29.4 (17.2) |
| | | | | Total motility (% motile), median (IQR) | | 42.9 (19.9) | | 57.5 (20.8) |
| | | | | MMP (% abnormal), median (IQR) | | 63.1 (22.2) | | 42.1 (25.8) |
| | | | | DNA fragmentation (% abnormal), median (IQR) | | 26.9 (19.7) | | 13.9 (9.8) |
| Elsamanoudy et al. (24) | Mansoura, Egypt | Fertile MetS and controls (mean age 39∼40y) | International Diabetes Federation (IDF) criteria in 2009 | Volume (mL), mean (SD) | 38 | 2.37 (0.67) | 45 | 2.18 (0.54) |
| | | | | Sperm concentration ($10^6$/mL), mean (SD) | | 37.78 (9.91) | | 39.45 (14.2) |
| | | | | Progressive motility (%), mean (SD) | | 43.68 (11.24) | | 49.67 (14.66) |
| | | | | Vitality (%), mean (SD) | | 54.73 (16.14) | | 68.7 (22.04) |
| | | | | Normal morphology (%), mean (SD) | | 22.44 (5.02) | | 23.53 (6.78) |
| | | | | DNA fragmentation (%), mean (SD) | | 26.95 (9.43) | | 20.78 (7.15) |
| Leisegang et al. (25) | Western Cape, South Africa | MetS and controls (24∼67y) | International Diabetes Federation (IDF) criteria in 2009 | Ejaculation volume (mL), mean (SD) | 24 | 2.3 (1.6) | 26 | 2.7 (1) |
| | | | | Sperm concentration ($10^6$/mL), mean (SD) | | 24.6 (14.6) | | 43.2 (25.4) |
| | | | | Total sperm count ($10^6$), mean (SD) | | 59.3 (57.1) | | 122 (108.2) |
| | | | | Progressive motility (%), mean (SD) | | 21.7 (18.3) | | 31 (17.6) |
| | | | | Total motility (%), mean (SD) | | 41.8 (20.6) | | 54.8 (20.2) |
| | | | | Vitality (%), mean (SD) | | 47.2 (25) | | 67 (16) |
| | | | | Disturbed MMP (%), mean (SD) | | 62.4 (22.3) | | 40.3 (24.5) |
| | | | | TUNEL-pos (%), mean (SD) | | 29.8 (20.4) | | 17.8 (12.1) |
| Lotti et al. (26) | Florence, Italy | Male members of infertile couples | International Diabetes Federation (IDF) criteria in 2009 | FSH (IU/L), median (IQR) | 27 | 5.6 (3.3∼9.2) | 324 | 4.8 (3∼7.7) |

*(Continued)*

**TABLE 1** | Continued

| References | Area | Study population | MetS diagnosis criteria | Parameters | MetS | | Controls | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Num | Levels | Num | Levels |
| | | | | LH (IU/L), median (IQR) | | 3.9 (2.8∼4.5) | | 3.7 (2.6∼5.2) |
| | | | | PRL (pmol/L), median (IQR) | | 282 (234∼489) | | 294 (226∼435) |
| | | | | TSH (mIU/L), median (IQR) | | 1.84 (1.13∼2.26) | | 1.51 (1.08∼2.09) |
| | | | | Total testosterone (nmol/L), mean (SD) | | 13.8 (6.5) | | 16.7 (6.2) |
| | | | | Semen volume (mL), median (IQR) | | 2.8 (1.3∼3.8) | | 3 (2∼4.2) |
| | | | | Sperm concentration ($10^6$/mL), median (IQR) | | 16.1 (3.9∼49.5) | | 13 (1.6∼46) |
| | | | | Sperm progressive motility (%), mean (SD) | | 39.3 (16.9) | | 36.2 (20.7) |
| | | | | Sperm morphology (% normal), median (IQR) | | 4 (2∼6.3) | | 5 (2∼10) |

*MetS, metabolic syndrome; FSH, follicle-stimulating hormone; T, testosterone; tT, total testosterone; InhB, inhibin B; LH, luteinizing hormone; AMH, anti-Müllerian hormone; TSH, thyroid-stimulating hormone; E2, estradiol; PRL, prolactin; MMP, mitochondrial membrane potential; IQR, inter-quartile range; SD, standard error; Num, number.*

case reports, animal studies, and studies in languages other than English.

## Data Extraction and Quality Assessment

Two authors (LZ and AP) extracted the data by a standardized collection form. All differences were resolved by discussion. In each study, the following information was extracted: first author, publication year, study area, study population, diagnosis criteria of MetS, semen or sex hormone parameters, sample size per group, and the testing values of semen parameters and circulating sex hormones. For studies from the same area, we also reviewed the medical center and study time to exclude duplicate publications. The Newcastle-Ottawa Scale (NOS) was used to assess the methodological quality of included studies (http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp).

## Statistical Analysis

If the measurement levels were presented as mean or median with range or IQR, they were converted to mean ± SD according to the methods by Wan et al. (14). Study-specific standardized mean differences (SMD) and the corresponding 95% CI were pooled by the Inverse Variance method to evaluate the effects of MetS on selected parameters. A random-effects model was used as the pooling method, which considered both within-study and between-study variation. The heterogeneity among studies was estimated by $Q$ test and $I^2$ statistic, and $I^2 >$ 50% represented substantial heterogeneity (15). Egger's test was used to detect publication bias (16). Sensitivity analysis was conducted to estimate the stability of the meta-analysis by omitting one study at a time during repeated analyses. Subgroup analysis was conducted on the study cohorts [including fertile cohort, infertile cohort, and the general cohort (not specified)], ethnicity and study area (from developed or developing area) to evaluate the effects of potential confounding factors on the primary results. Statistical analyses were performed using Review Manager 5.3 (RevMan, The Nordic Cochrane Center, The Cochrane Collaboration), and Egger's test was realized with software STATA version 12.0 (StataCorp LP, College Station). $P < 0.05$ was considered statistically significant.

# RESULTS

## Characteristics of Included Studies

The search strategy identified 5,272 records: 702 from PubMed, 4,554 from Embase, and 16 from other sources (**Figure 1**). After eliminating duplicated and irrelevant records, 10 recodes (11 studies) were included in the meta-analysis, with a total of 1,731 MetS cases and 11,740 controls (**Table 1**) (17–26). The research by Ehala-Aleksejev and Punab (19) was based on two cohorts (fertile men and male partners of infertile couples), and thus it was divided into two individual studies. All studies were cross-sectional designed. Four studies focused on infertile cohorts, two on fertile cohorts and five on the general cohort (not specified). Six studies were from Europe, three from Africa and two from Asia. In quality assessment (NOS score 0∼9), the included studies had an average score of 6.73 (**Table S1**).

## MetS and Semen Quality

Compared with the controls, MetS cases had a statistically significant decrease of sperm total count (SMD: −0.96, 95% CI: −1.58 to −0.31; $I^2 = 97\%$, $n = 5$), sperm concentration (SMD: −1.13, 95% CI: −1.85 to −0.41; $I^2 = 99\%$, $n = 11$), sperm normal morphology (SMD: −0.61, 95% CI: −1.01 to −0.21; $I^2 = 97\%$, $n = 9$), sperm progressive motility (SMD: −0.58, 95% CI: −1.00 to −0.17; $I^2 = 94\%$, $n = 9$), and sperm vitality (SMD: −0.83, 95% CI: −1.11 to −0.54; $I^2 = 0\%$, $n = 3$) (**Figures 2–4**). There was found a weak decrease of semen volume (SMD: −0.46, 95% CI: −2.30 to 1.37; $I^2 = 100\%$, $n = 10$) and sperm total motility in MetS cases (SMD: −0.68, 95% CI: −1.39 to 0.02; $I^2 = 99\%$, $n =$

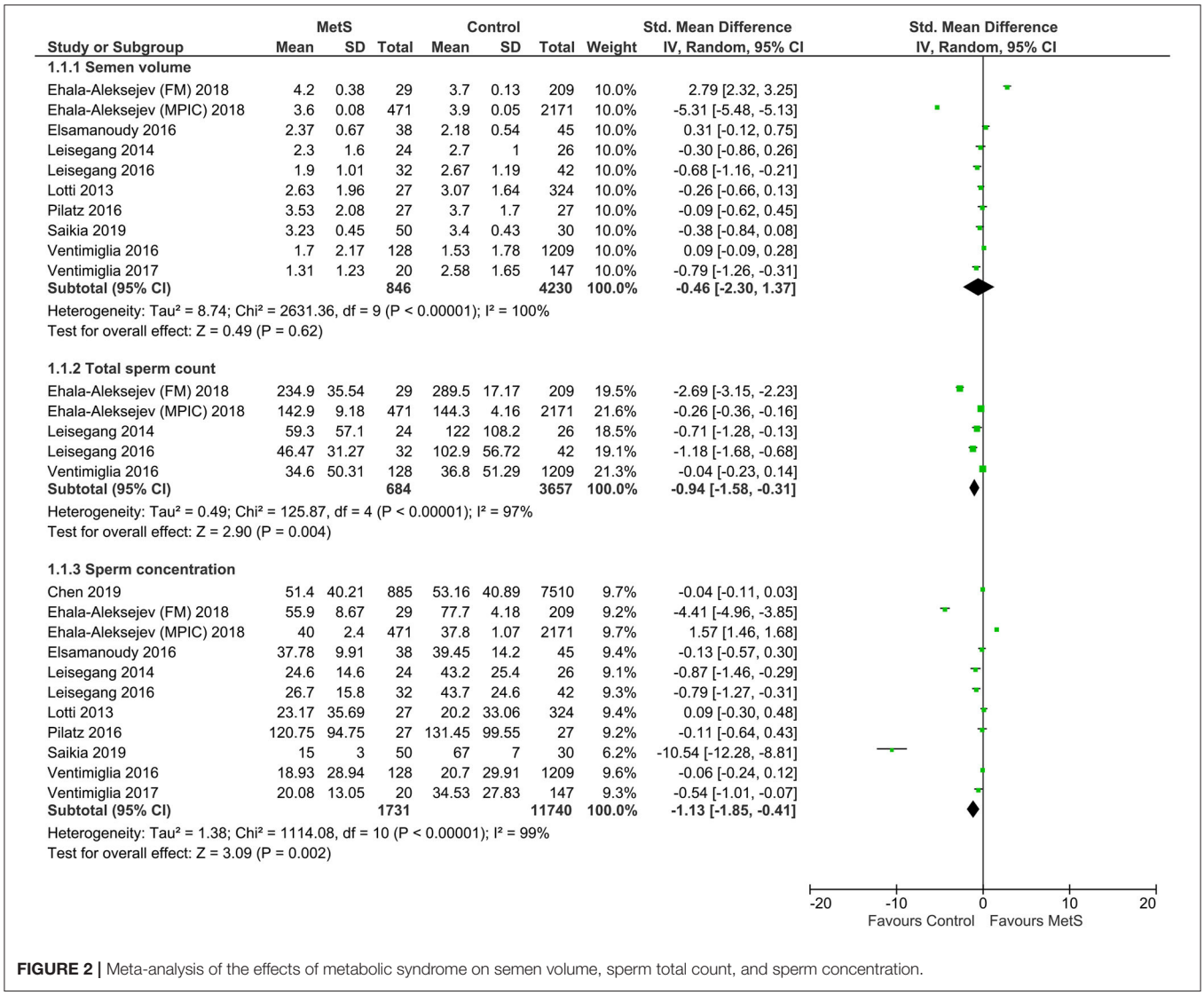


**FIGURE 2 |** Meta-analysis of the effects of metabolic syndrome on semen volume, sperm total count, and sperm concentration.

6). Furthermore, MetS cases had a statistically significant increase of sperm DNA fragmentation (SMD: 0.76, 95% CI: 0.45 to 1.06; $I^2 = 0\%$, $n = 3$) and MMP (SMD: 0.89, 95% CI: 0.49 to 1.28; $I^2 = 0\%$, $n = 2$).

## MetS and Circulating Sex Hormones

Compared with the controls, MetS cases had a statistically significant decrease of follicle-stimulating hormone (FSH) (SMD: −0.87, 95% CI: −1.53 to −0.21; $I^2 = 97\%$, $n = 6$), testosterone (SMD: −5.61, 95% CI: −10.90 to −0.31; $I^2 = 100\%$, $n = 6$), and inhibin B (SMD: −2.42, 95% CI: −4.52 to −0.32; $I^2 = 0\%$, $n = 3$) (**Figures 5**, **6**). There was found a weak decrease of luteinizing hormone (LH) (SMD: −0.36, 95% CI: −3.24 to 2.52; $I^2 = 100\%$, $n = 5$) and anti-Müllerian hormone (AMH) (SMD: −0.92, 95% CI: −2.06 to 0.22; $I^2 = 95\%$, $n = 2$), and a weak increase of estradiol (SMD: 1.04, 95% CI: −2.05 to 4.12; $I^2 = 100\%$, $n = 4$) in MetS cases.

## Sensitivity Analyses

Sensitivity analysis showed that the results were robust in semen parameters. In the meta-analysis of circulating FSH, the result showed no statistical difference when omitting the study by Saikia et al. (17) (**Table S2**). In the meta-analysis of testosterone, the result also showed no statistical difference when omitting the study by Ehala-Aleksejev and Punab (19) (FM), Lotti et al. (26), or Ventimiglia et al. (21).

## Subgroup Analyses

Subgroup analyses were conducted on the study cohort, ethnicity and study area. Compared with the infertile cohort, MetS cases in the fertile cohort had a significantly higher incidence of a decrease in sperm total count and sperm progressive motility, FSH, testosterone and estradiol, and an increase in LH (**Table 2**). Comparably, MetS cases in the general cohort also had a significantly higher incidence of the decrease in semen volume, sperm total count, sperm concentration, sperm normal

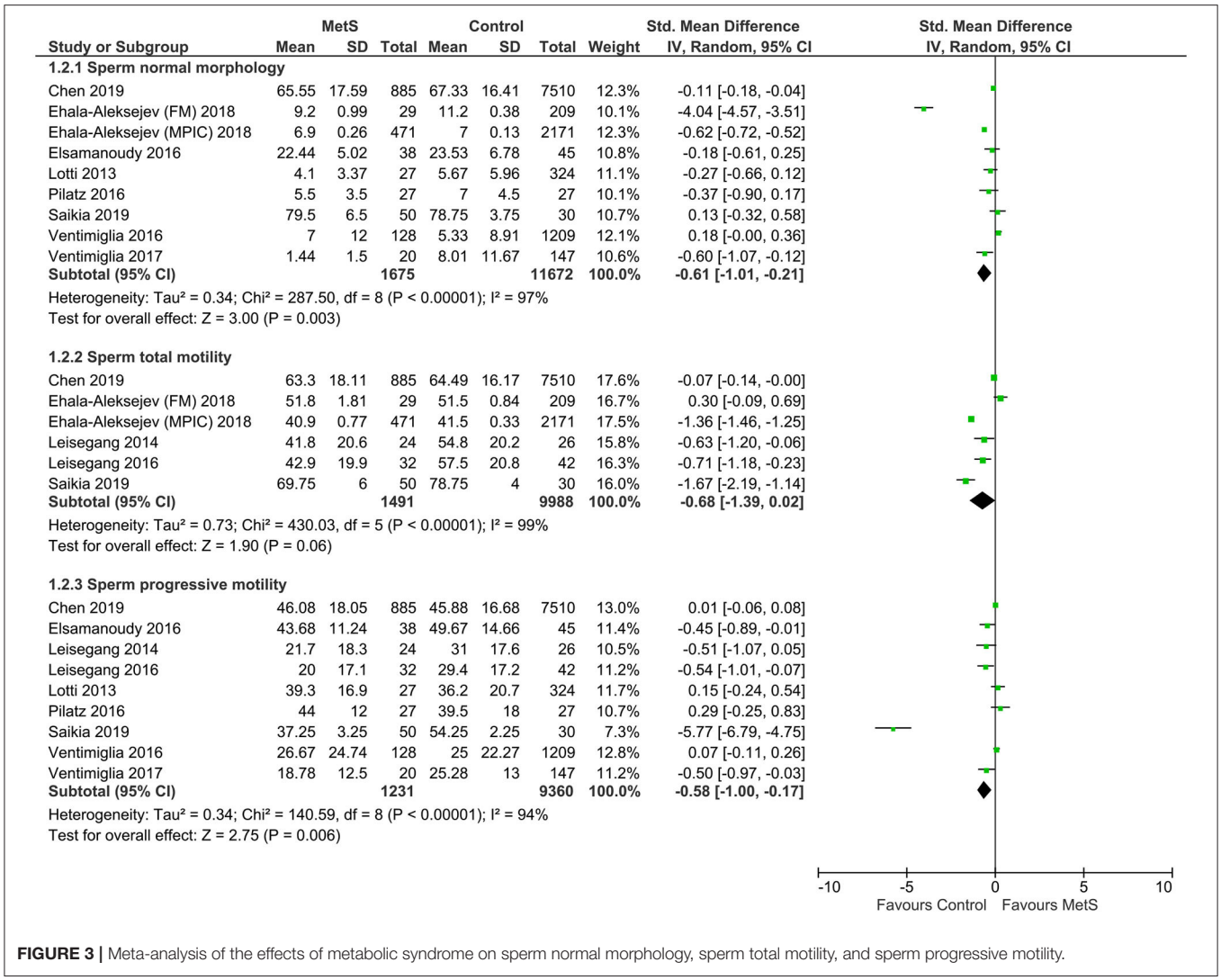


**FIGURE 3 |** Meta-analysis of the effects of metabolic syndrome on sperm normal morphology, sperm total motility, and sperm progressive motility.

morphology, sperm progressive motility, FSH, testosterone, and inhibin B. Generally, MetS had the most impact on semen quality and circulating sex hormones of the general cohort, moderate impact on fertile cohort, and the least impact on the infertile cohort.

Compared with the Caucasian cohort or the cohort from developed area, MetS cases from the non-Caucasian cohort or the cohort from developing area had a significantly higher incidence of the decrease in sperm concentration, sperm total motility, sperm progressive motility, FSH, and inhibin B (**Table 3**). MetS tended to have more impact on the individuals from the non-Caucasian cohort or the cohort from developing area.

## Publication Bias

Egger's test was conducted on the indicators with more than four included studies. Finally, we detected no significant publication bias in semen volume ($P = 0.122$), sperm total count ($P = 0.200$), sperm concentration ($P = 0.185$), sperm normal morphology ($P = 0.400$), sperm total motility ($P = 0.659$), sperm progressive motility ($P = 0.120$), circulating FSH ($P = 0.199$), testosterone ($P = 0.215$), or LH ($P = 0.293$) (**Figures S1**–**S3**).

# DISCUSSION

According to the World Health Organization (WHO), infertility had an incidence of 8∼12% in childbearing couples worldwide, among which male infertility accounted for 40∼50% (27). Along with the modernized lifestyles of recent decades, metabolic disorders were increasingly prevalent, while semen quality was gradually decreasing (28). Thus, as a collection of metabolic disorders characterized by abdominal obesity, dyslipidemia, hypertension and insulin resistance, MetS was thought to be involved in the pathogenesis of male infertility (28).

The mechanism has been not clarified, and currently many researchers indicate a central role of insulin resistance in the pathogenesis. Abnormal blood glucose could cause the impairment of multiple organs, including erectile and ejaculation disorders. In the meta-analysis by Pergialiotis et al. (29) the

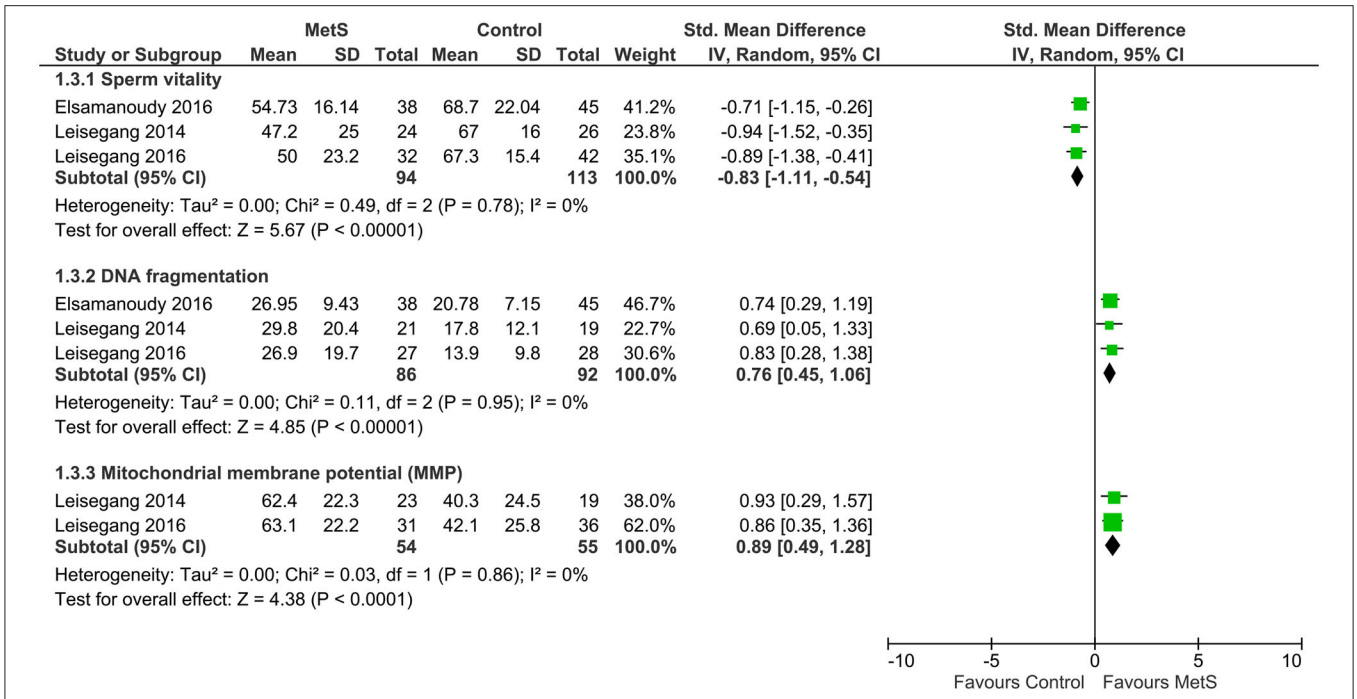


**FIGURE 4 |** Meta-analysis of the effects of metabolic syndrome on sperm vitality, DNA fragmentation, and mitochondrial membrane potential (MMP).

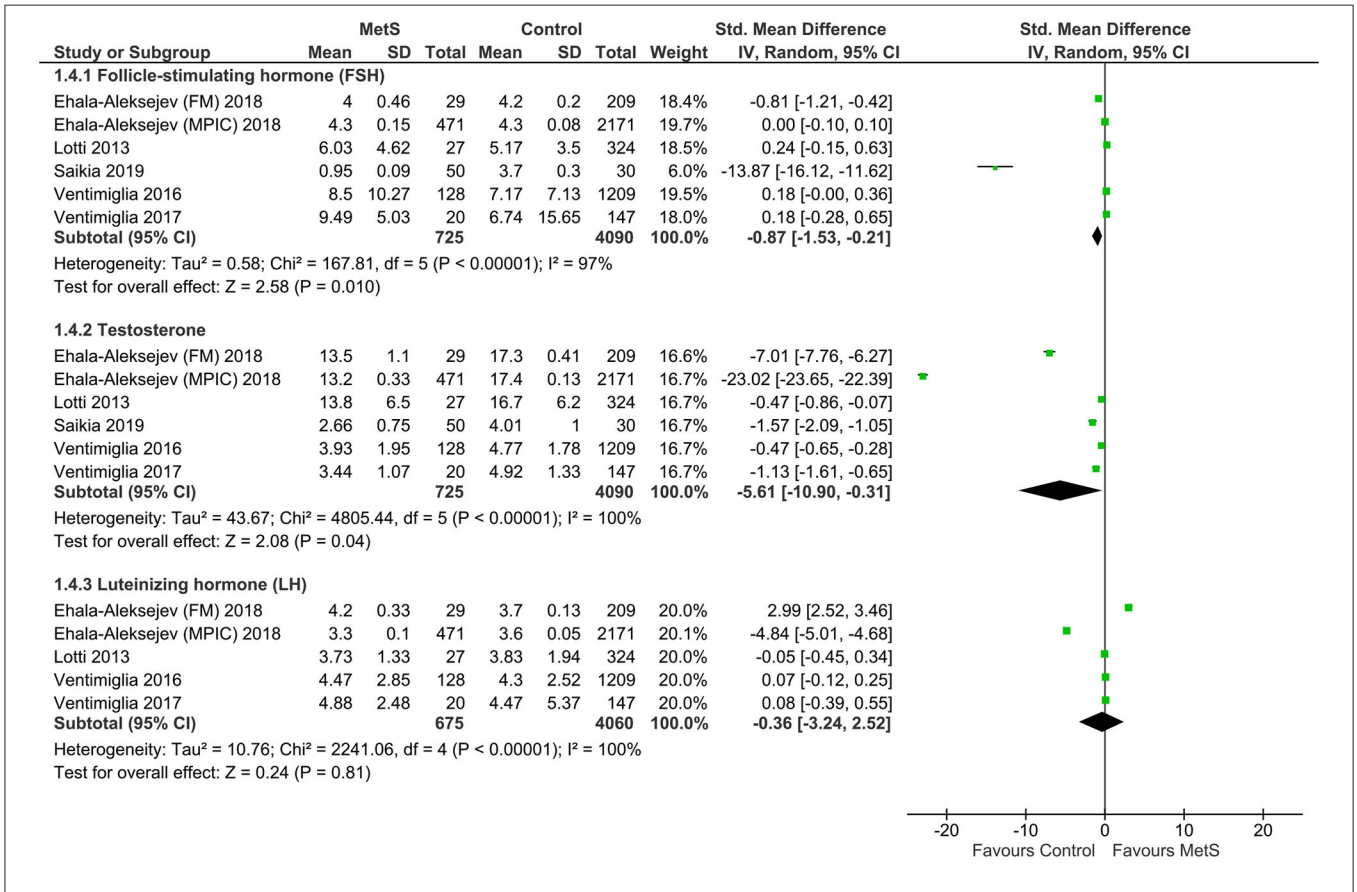


**FIGURE 5 |** Meta-analysis of the effects of metabolic syndrome on circulating follicle-stimulating hormone (FSH), testosterone, and luteinizing hormone (LH).

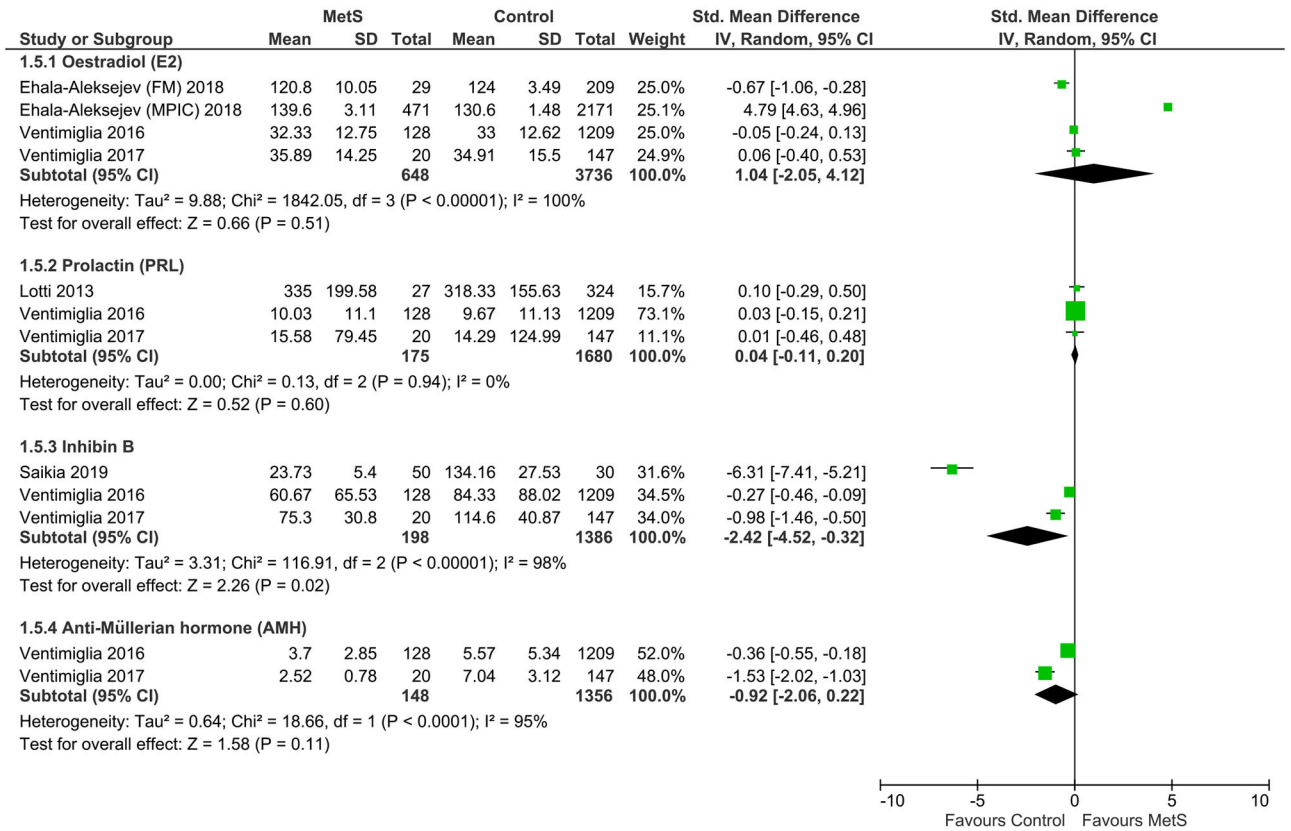


**FIGURE 6 |** Meta-analysis of the effects of metabolic syndrome on estradiol, inhibin B, and anti-Müllerian hormone (AMH).

**TABLE 2 |** Subgroup analysis of the effects of metabolic syndrome on semen quality and circulating sex hormones according to the study cohort.

| Variables | Fertile cohort | | Infertile cohort | | Not specified | |
|---|---|---|---|---|---|---|
| | SMD (95% CI) | No. | SMD (95% CI) | No. | SMD (95% CI) | No. |
| Semen volume | 1.55 (−0.88 to 3.97) | 2 | −1.57 (−4.81 to 1.68) | 4 | −0.38 (−0.63 to −0.13) | 4 |
| Sperm total count | −2.69 (−3.15 to −2.23) | 1 | −0.16 (−0.37 to 0.04) | 2 | −0.96 (−1.42 to −0.50) | 2 |
| Sperm concentration | −2.27 (−6.45 to 1.92) | 2 | 0.27 (−0.83 to 1.38) | 4 | −1.99 (−3.23 to −0.75) | 5 |
| Sperm normal morphology | −2.11 (−5.89 to 1.68) | 2 | −0.32 (−0.81 to 0.17) | 4 | −0.11 (−0.17 to −0.04) | 3 |
| Sperm total motility | 0.30 (−0.09 to 0.69) | 1 | −1.36 (−1.46 to −1.25) | 1 | −0.75 (−1.47 to −0.02) | 4 |
| Sperm progressive motility | −0.45 (−0.89 to −0.01) | 1 | −0.05 (−0.37 to 0.28) | 3 | −1.18 (−2.26 to −0.11) | 5 |
| Sperm vitality | −0.71 (−1.15 to −0.26) | 1 | – | – | −0.91 (−1.28 to −0.54) | 2 |
| DNA fragmentation | 0.74 (0.29 to 1.19) | 1 | – | – | 0.77 (0.35 to 1.19) | 2 |
| Mitochondrial membrane potential (MMP) | – | – | – | – | 0.89 (0.49 to 1.28) | 2 |
| Follicle-stimulating hormone (FSH) | −0.81 (−1.21 to −0.42) | 1 | 0.08 (−0.04 to 0.21) | 4 | −13.87 (−16.12 to −11.62) | 1 |
| Testosterone | −7.01 (−7.76 to −6.27) | 1 | −6.27 (−13.71 to 1.18) | 4 | −1.57 (−2.09 to −1.05) | 1 |
| Luteinizing hormone (LH) | 2.99 (2.52 to 3.46) | 1 | −1.19 (−4.24 to 1.86) | 4 | – | – |
| estradiol | −0.67 (−1.06 to −0.28) | 1 | 1.60 (−2.07 to 5.28) | 3 | – | – |
| Prolactin | – | – | 0.04 (−0.11 to 0.20) | 3 | – | – |
| Inhibin B | – | – | −0.59 (−1.28 to 0.10) | 2 | −6.31 (−7.41 to −5.21) | 1 |
| Anti-Müllerian hormone (AMH) | – | – | −0.92 (−2.06 to 0.22) | 2 | – | – |

*SMD, standardized mean differences; CI, confidence interval; No., number of included studies.*

**TABLE 3 |** Subgroup analysis of the effects of metabolic syndrome on semen quality and circulating sex hormones according to the ethnicity and study area.

| Variables | Caucasian cohort/developed area | | Non-Caucasian cohort/developing area | |
|---|---|---|---|---|
| | SMD (95% CI) | No. | SMD (95% CI) | No. |
| Semen volume | −0.60 (−3.28 to 2.09) | 6 | −0.25 (−0.69 to 0.18) | 4 |
| Sperm total count | −0.95 (−1.79 to −0.11) | 3 | −0.96 (−1.42 to −0.50) | 2 |
| Sperm concentration | −0.56 (−1.82 to 0.70) | 6 | −1.94 (−3.10 to −0.78) | 5 |
| Sperm normal morphology | −0.93 (−1.66 to −0.19) | 6 | −0.10 (−0.17 to −0.04) | 3 |
| Sperm total motility | −0.54 (−2.16 to 1.08) | 2 | −0.75 (−1.47 to −0.02) | 4 |
| Sperm progressive motility | 0.02 (−0.25 to 0.28) | 4 | −1.32 (−2.34 to −0.30) | 5 |
| Sperm vitality | – | – | −0.83 (−1.11 to −0.54) | 3 |
| DNA fragmentation | – | – | 0.76 (0.45 to 1.06) | 3 |
| Mitochondrial membrane potential (MMP) | – | – | 0.89 (0.49 to 1.28) | 2 |
| Follicle-stimulating hormone (FSH) | −0.03 (−0.29 to 0.23) | 5 | −13.87 (−16.12 to −11.62) | 1 |
| Testosterone | −5.61 (−10.90 to −0.31) | 5 | −6.41 (−12.81 to −0.02) | 1 |
| Luteinizing hormone (LH) | −0.36 (−3.24 to 2.52) | 5 | – | – |
| estradiol | 1.04 (−2.05 to 4.12) | 4 | – | – |
| Prolactin | 0.04 (−0.11 to 0.20) | 3 | – | – |
| Inhibin B | −0.59 (−1.28 to 0.10) | 2 | −6.31 (−7.41 to −5.21) | 1 |
| Anti-Müllerian hormone (AMH) | −0.92 (−2.06 to 0.22) | 2 | – | – |

*SMD, standardized mean differences; CI, confidence interval; No., number of included studies.*

infertile male with diabetes had a decrease in seminal volume and motile cells and an increase in FSH. Moreover, diabetes patients in fertile age had a higher prevalence of male accessory gland inflammations/infections, as well as a higher failure rate of vitro fertilization (30, 31). Antidiabetic agents could not only control blood glucose levels but also improve semen quality and testosterone levels (32, 33). Besides, individuals with obesity, dyslipidemia or hypertension were also reported with a decrease in semen quality and changes in sex hormones. These might contribute to concomitant oxidative stress and inflammation, and impaired seminal antioxidant capacity (34, 35).

In this meta-analysis, we found a decrease of sperm total count, sperm concentration, sperm normal morphology, sperm progressive motility, and sperm vitality and an increase of sperm DNA fragmentation and MMP, while no significant difference was found in semen volume and sperm total motility. Generally, MetS had a negative impact on the semen quality, just like diabetes and obesity (10, 36). On the other hand, MetS cases had a decrease of FSH, testosterone and inhibin B, while no significant difference was found in LH, estradiol, prolactin, and AMH. Previous studies reported a decrease of inhibin B and an increase of FSH in infertile males (37). However, our meta-analysis indicated a similar change trend of FSH and inhibin B in MetS. This might contribute to the heterogeneity between studies, especially from the study by Saikia et al. (17). Second, LH and estradiol were usually increased in infertile males, but our meta-analysis found no obvious difference in MetS. Apart from the heterogeneity, this might also contribute to the complexity of MetS as a syndrome. For example, as one of the characteristics of MetS, obese males could have an increase of testosterone, LH and FSH after bariatric surgery (38). In general, MetS had a greater impact on semen quality than sex hormones, which might contribute to the direct impairment caused by MetS.

Sensitivity analysis indicated a relative stability for semen parameters, while FSH and testosterone turned statistically insignificant when omitting certain studies. MetS seemed to have more significant and stable effects on semen quality than sex hormones, which was consistent with our previous analysis. Second, for almost all the outcomes, the exclusion of a single specific study dramatically decreased the effect size, especially like Ehala-Aleksejev and Punab (19) (FM) in sperm concentration, sperm normal morphology and testosterone, Saikia et al. (17) in sperm progressive motility and FSH, and Lotti et al. (26) in testosterone. These studies were limited in the sample size of MetS cases ranging from 27 to 50. Small sample size could increase the risk of sampling error, and thus lead to within-study and between-study heterogeneity and the expansion of synthetic effect size. Besides, the meta-analysis of continuous data usually showed a higher heterogeneity than categorical data, just like the recent study of "Cardio-metabolic risk factors among young infertile women: a systematic review and meta-analysis" (35).

The subgroup analyses suggested more effects of MetS on the individuals from the fertile cohort, non-Caucasian cohort, or the cohort from developing area. This might contribute to less impact of MetS on the impaired reproductivity, and MetS had a stronger influence on the reproductivity of healthy individuals. Moreover, this was also consistent with the high incidence of male infertility in the Asian cohort and developing countries (27).

Although this was the first meta-analysis to evaluate the effects of MetS on both semen quality and circulating sex hormones in men, several limitations in this study should be also considered. First, not all included studies had a large sample size. Second, all included studies were cross-sectionally designed, and prospective

studies were needed to confirm our findings. Third, the protocol of our meta-analysis was not registered in the PROSPERO database. Fourth, obvious heterogeneity between studies was observed, although we conducted both sensitivity analysis and subgroup analysis to evaluate the stability of the results. We expected large-scale prospective designed studies in the future to overcome these limitations.

In conclusion, this meta-analysis demonstrated the effects of MetS on almost all the semen parameters and part of the circulating sex hormones, and MetS tended to be a risk factor for male infertility. Further larger-scale prospective designed studies were needed to confirm our findings.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

LZ and AP conceived the manuscript, performed literature search, drafted and wrote the manuscript, contributed to manuscript revision during peer review process, and contributed to manuscript revision, read, and approved the submitted version. AP critically revised the first original draft and any other version of the manuscript before and after peer review process, and provided significant content contribution and English language support.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00428/full#supplementary-material

## REFERENCES


1. Kurl S, Laaksonen DE, Jae SY, Makikallio TH, Zaccardi F, Kauhanen J, et al. Metabolic syndrome and the risk of sudden cardiac death in middle-aged men. *Int J Cardiol.* (2016) 203:792–7. doi: 10.1016/j.ijcard.2015.10.218
2. Kaneko K, Yatsuya H, Li Y, Uemura M, Chiang C, Hirakawa Y, et al. Risk and population attributable fraction of metabolic syndrome and impaired fasting glucose for incidence of type 2 diabetes mellitus among middle-aged Japanese: aichi worker's cohort study. *J Diabetes Investig.* (2020) doi: 10.1111/jdi.13230. [Epub ahead of print].
3. Greenfield DM, Snowden AJ. Cardiovascular diseases and metabolic syndrome. *Transl Res.* (2019) 415–20. doi: 10.1007/978-3-030-02278-5_55
4. Brand JS, Rovers MM, Yeap BB, Schneider HJ, Tuomainen TP, Haring R, et al. Testosterone, sex hormone-binding globulin and the metabolic syndrome in men: an individual participant data meta-analysis of observational studies. *PLoS ONE.* (2014) 9:e100409. doi: 10.1371/journal.pone.0100409
5. Benedetti S, Tagliamonte MC, Catalani S, Primiterra M, Canestrari F, De Stefani S, et al. Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. *Reprod Biomed Online.* (2012) 25:300–6 doi: 10.1016/j.rbmo.2012.05.011
6. MacDonald AA, Herbison GP, Showell M, Farquhar MC. The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. *Hum Reprod Update.* (2010) 16:293–311. doi: 10.1093/humupd/dmp047
7. Sermondade N, Faure C, Fezeu L, Shayeb AG, Bonde JP, Jensen TK, et al. BMI in relation to sperm count: an updated systematic review and collaborative meta-analysis. *Hum Reprod Update.* (2013) 19:221–31. doi: 10.1093/humupd/dms050
8. Colli LG, Belardin LB, Echem C, Akamine EH, Antoniassi MP, Andretta RR, et al. Systemic arterial hypertension leads to decreased semen quality and alterations in the testicular microcirculation in rats. *Sci Rep.* (2019) 9:11047 doi: 10.1038/s41598-019-47157-w
9. Hagiuda J, Ishikawa H, Furuuchi T, Hanawa Y, Marumo K. Relationship between dyslipidaemia and semen quality and serum sex hormone levels: an infertility study of 167 Japanese patients. *Andrologia.* (2014) 46:131–5. doi: 10.1111/and.12057
10. Lu X, Huang Y, Zhang H, Zhao J. Effect of diabetes mellitus on the quality and cytokine content of human semen. *J Reprod Immunol.* (2017) 123:1–2. doi: 10.1016/j.jri.2017.08.007
11. Brand JS, van der Tweel I, Grobbee DE, Emmelot-Vonk MH, van der Schouw TY. Testosterone, sex hormone-binding globulin and the metabolic syndrome: a systematic review and meta-analysis of observational studies. *Int J Epidemiol.* (2011) 40:189–207. doi: 10.1093/ije/dyq158
12. Corona G, Bianchini S, Sforza A, Vignozzi L, Maggi M. Hypogonadism as a possible link between metabolic diseases and erectile dysfunction in aging men. *Hormones (Athens).* (2015) 14:569–78. doi: 10.14310/horm.2002.1635
13. Rastrelli G, Filippi S, Sforza A, Maggi M, Corona G. Metabolic syndrome in male hypogonadism. *Front Horm Res.* (2018) 49:131–55. doi: 10.1159/000485999
14. Wan X, Wang W, Liu J, Tong T. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Med Res Methodol.* (2014) 14:135. doi: 10.1186/1471-2288-14-135
15. Higgins JP, Thompson SG, Deeks JJ, Altman GD. Measuring inconsistency in meta-analyses. *BMJ.* (2003) 327:557–60. doi: 10.1136/bmj.327.7414.557
16. Egger M, Davey SG, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ.* (1997) 315:629–34. doi: 10.1136/bmj.315.7109.629
17. Saikia UK, Saikia K, Sarma D, Appaiah S. Sertoli cell function in young males with metabolic syndrome. *Indian J Endocrinol Metab.* (2019) 23:251–6. doi: 10.4103/ijem.IJEM_574_18
18. Chen YY, Kao TW, Peng TC, Yang HF, Wu CJ, Chen LW. Metabolic syndrome and semen quality in adult population. *J Diabetes.* (2019) 12:294–304. doi: 10.1111/1753-0407.12995
19. Ehala-Aleksejev K, Punab M. The effect of metabolic syndrome on male reproductive health: a cross-sectional study in a group of fertile men and male partners of infertile couples. *PLoS ONE.* (2018) 13:e0194395. doi: 10.1371/journal.pone.0194395
20. Ventimiglia E, Capogrosso P, Serino A, Boeri L, Colicchia M, La Croce G, et al. Metabolic syndrome in white-European men presenting for secondary couple's infertility: an investigation of the clinical and reproductive burden. *Asian J Androl.* (2017) 19:368–73. doi: 10.4103/1008-682X.175783
21. Ventimiglia E, Capogrosso P, Colicchia M, Boeri L, Serino A, Castagna G, et al. Metabolic syndrome in white European men presenting for primary couple's infertility: investigation of the clinical and reproductive burden. *Andrology.* (2016) 4:944–51. doi: 10.1111/andr.12232
22. Pilatz A, Hudemann C, Wolf J, Halefeld I, Paradowska-Dogan A, Schuppe HC, et al. Metabolic syndrome and the seminal cytokine network in morbidly obese males. *Andrology.* (2017) 5:23–30. doi: 10.1111/andr.12296
23. Leisegang K, Bouic PJ, Henkel RR. Metabolic syndrome is associated with increased seminal inflammatory cytokines and reproductive dysfunction in a case-controlled male cohort. *Am J Reprod Immunol.* (2016) 76:155–63. doi: 10.1111/aji.12529
24. Elsamanoudy AZ, Abdalla HA, Hassanien M, Gaballah AM. Spermatozoal cell death-inducing DNA fragmentation factor-alpha-like effector A (CIDEA) gene expression and DNA fragmentation in infertile men with metabolic syndrome and normal seminogram. *Diabetol Metab Syndr.* (2016) 8:76. doi: 10.1186/s13098-016-0192-y

25. Leisegang K, Udodong A, Bouic PJ, Henkel RR. Effect of the metabolic syndrome on male reproductive function: a case-controlled pilot study. *Andrologia.* (2014) 46:167–76. doi: 10.1111/and.12060
26. Lotti F, Corona G, Degli IS, Filimberti E, Scognamiglio V, Vignozzi L, et al. Seminal, ultrasound and psychobiological parameters correlate with metabolic syndrome in male members of infertile couples. *Andrology.* (2013) 1:229–39. doi: 10.1111/j.2047-2927.2012.00031.x
27. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens AG. National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. *PLoS Med.* (2012) 9:e1001356. doi: 10.1371/journal.pmed.1001356
28. Huang C, Li B, Xu K, Liu D, Hu J, Yang Y, et al. Decline in semen quality among 30,636 young Chinese men from 2001 to 2015. *Fertil Steril.* (2017) 107:83–8.e2. doi: 10.1016/j.fertnstert.2016.09.035
29. Pergialiotis V, Prodromidou A, Frountzas M, Korou LM, Vlachos GD, Perrea D. Diabetes mellitus and functional sperm characteristics: a meta-analysis of observational studies. *J Diabetes Complicat.* (2016) 30:1167–76. doi: 10.1016/j.jdiacomp.2016.04.002
30. Condorelli RA, Calogero AE, Vicari E, Duca Y, Favilla V, Morgia G, et al. Prevalence of male accessory gland inflammations/infections in patients with Type 2 diabetes mellitus. *J Endocrinol Invest.* (2013) 36:770–4. doi: 10.3275/8950
31. Rama RG, Jaya PG, Murali KK, Madan K, Siva NT, Ravi CK. Noninsulin-dependent diabetes mellitus: effects on sperm morphological and functional characteristics, nuclear DNA integrity and outcome of assisted reproductive technique. *Andrologia.* (2012) 44(Suppl. 1):490–8. doi: 10.1111/j.1439-0272.2011.01213.x
32. Morgante G, Tosti C, Orvieto R, Musacchio MC, Piomboni P, De Leo V. Metformin improves semen characteristics of oligo-terato-asthenozoospermic men with metabolic syndrome. *Fertil Steril.* (2011) 95:2150–2. doi: 10.1016/j.fertnstert.2010.12.009
33. Bosman E, Esterhuizen AD, Rodrigues FA, Becker PJ, Hoffmann AW. Effect of metformin therapy and dietary supplements on semen parameters in hyperinsulinaemic males. *Andrologia.* (2015) 47:974–9. doi: 10.1111/and.12366
34. Pini T, Parks J, Russ J, Dzieciatkowska M, Hansen KC, Schoolcraft WB, et al. Obesity significantly alters the human sperm proteome, with potential implications for fertility. *J Assist Reprod Genet.* (2020) 37:777–87. doi: 10.1007/s10815-020-01707-8
35. Mulder CL, Lassi ZS, Grieger JA, Ali A, Jankovic-Karasoulos T, Roberts CT, et al. Cardio-metabolic risk factors among young infertile women: a systematic review and meta-analysis. *BJOG.* (2020) 127:930–9. doi: 10.1111/1471-0528.16171
36. Rufus O, James O, Michael A. Male obesity and semen quality: any association? *Int J Reprod Biomed (Yazd).* (2018) 16:2 85–90.
37. Corinne TM, Anatole PC, Jeanne YN. Comparison of serum inhibin B and follicle-stimulating hormone (FSH) level between normal and infertile men in yaounde. *Int J Reprod Med.* (2020) 2020:4765809. doi: 10.1155/2020/4765809
38. Lee Y, Dang JT, Switzer N, Yu J, Tian C, Birch DW, et al. Impact of bariatric surgery on male sex hormones and sperm quality: a systematic review and meta-analysis. *Obes Surg.* (2019) 29:334–46. doi: 10.1007/s11695-018-3557-5

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REVIEW
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# The Role of Genetics and Oxidative Stress in the Etiology of Male Infertility—A Unifying Hypothesis?

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Despite the high prevalence of male infertility, very little is known about its etiology. In recent years however, advances in gene sequencing technology have enabled us to identify a large number of rare single point mutations responsible for impeding all aspects of male reproduction from its embryonic origins, through the endocrine regulation of spermatogenesis to germ cell differentiation and sperm function. Such monogenic mutations aside, the most common genetic causes of male infertility are aneuploidies such as Klinefelter syndrome and Y-chromosome mutations which together account for around 20–25% of all cases of non-obstructive azoospermia. Oxidative stress has also emerged as a major cause of male fertility with at least 40% of patients exhibiting some evidence of redox attack, resulting in high levels of lipid peroxidation and oxidative DNA damage in the form of 8-hydroxy-2'-deoxyguanosine (8OHdG). The latter is highly mutagenic and may contribute to *de novo* mutations in our species, 75% of which are known to occur in the male germ line. An examination of 8OHdG lesions in the human sperm genome has revealed ~9,000 genomic regions vulnerable to oxidative attack in spermatozoa. While these oxidized bases are generally spread widely across the genome, a particular region on chromosome 15 appears to be a hot spot for oxidative attack. This locus maps to a genetic location which has linkages to male infertility, cancer, imprinting disorders and a variety of behavioral conditions (autism, bipolar disease, spontaneous schizophrenia) which have been linked to the age of the father at the moment of conception. We present a hypothesis whereby a number of environmental, lifestyle and clinical factors conspire to induce oxidative DNA damage in the male germ line which then triggers the formation *de novo* mutations which can have a major impact on the health of the offspring including their subsequent fertility.

**Keywords: male infertility, mutation, oxidative stress, spermatozoa, DNA damage**

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## INTRODUCTION

Spermatogenesis is an immensely complicated process involving the coordinated action of thousands of genes in order to generate one of the most complex, specialized cell types in human biology, the spermatozoon. Given this complexity, it may not be surprising that "the male factor" is held to be a major contributor to human infertility, although, the extent of this contribution is still a matter for conjecture. A recent survey of papers that have set out to determine the

causes of human infertility by assigning each case to one of four categories (male factor, female factor, both male and female factors, and unexplained) indicated that defects in the male were, on average (±SEM), thought to account for 21.1 ± 2.8% of all infertility, while the remaining causes distributed as follows: female factors (42.8 ± 3.2%), combined male and female factors (24.2 ± 4.9%) and unexplained infertility (13.2 ± 2.1%) (1). Examination of individual studies reveals a wide range of estimates for the incidence of male infertility (5–35%) that may reflect real differences between populations in terms of the quality of primary health care, occupational and environmental exposures to reproductive toxicants, age, dietary factors, obesity, climate, education, recreational exposure to drugs and genetic as well as epigenetic factors (1).

The major problem with all of these assessments is that the existence of male factor infertility was determined on the basis of a conventional semen profile. While the latter is acknowledged as a fundamental component of diagnostic andrology, with few exceptions, it is also widely understood that the criteria used to create such semen profiles (sperm count, motility and morphology) are not precisely predictive of infertility. In a prospective study of patients exhibiting unexplained infertility (normal female partner and normal conventional semen profile), these criteria were found be incapable of predicting the chances of spontaneous conception during a follow-up period lasting up to 4 years during which the patients received no further treatment (2). Similarly, in assisted conception cycles, including both *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) the conventional semen profile has been found to be of no value in predicting fertilization rates (3). However, when elements of the semen profile are combined with other data describing, for example, competence for sperm-oocyte fusion, the detailed movement characteristics of the spermatozoa and aspects of oxidative stress, then algorithms can be generated that better predict the relative fertility of males *in vitro* and *in vivo* (2, 4, 5). The exception to this rule is when the semen profile is seriously flawed, as is the case with azoospermia or severe oligozoospermia (<5 million/ml) where fertility rates are significantly reduced as might be anticipated (6). At the same time, it should be recognized normal fertility is possible in men whose sperm counts have been suppressed well into the oligospermic range (<5 million per ml) as a result of exogenous steroid administration (7).

We can conclude from such studies that the traditional approach to male infertility diagnosis, which relies on comparing each element of the semen profile with thresholds of normality established by the World Health Organization, is seriously flawed, no matter how carefully those thresholds were established (8). For the most part, male fertility is not a question of possessing more than a certain critical number of motile, morphologically normal spermatozoa in the ejaculate. It is not a binary phenomenon that allows us to classify patients as "fertile" or "infertile" groups. With the exception of patients exhibiting azoospermia or certain genetic defects such as Kartagener syndrome, most males are on a continuum of relative fertility. Where any given patient lies on this continuum cannot be reliably ascertained by conventional semenology. The diagnostic value of the conventional semen profile lies only in its ability to reflect the quality of the underlying spermatogenic process. Although there may be general low-level statistical correlations between morphology or total sperm number and fertility, the ranges for each of these criteria are so broad that their prognostic significance is very limited (9)–and they tell us nothing about etiology (**Figure 1A**). Terms such as asthenozoospermia, oligozoospermia, and teratozoospermia are convenient, descriptive terms. However, they are not diagnoses in the true sense of the word. As a consequence, we have greatly overestimated our understanding of the causes of human infertility and, in reality, have very limited insight into the overall contribution made by the "male factor." Much of male infertility is, in fact, idiopathic.

Fortunately, this bleak landscape is gradually changing as we acquire more knowledge of the mechanisms regulating sperm production and function. For example, the impact of genetics and epigenetics on male fertility has been revealed in recent years, largely as a consequence of technical advances in our ability to screen the genome for differences in DNA methylation profile and single nucleotide polymorphisms/mutations. Similarly, many studies describe oxidative stress as another significant cause of male infertility that influences the functionality of spermatozoa and the integrity of their DNA. In this review, we shall consider the progress that has been made in establishing the relative contributions of genetics and oxidative stress to the etiology of previously unexplained male infertility. We shall also tentatively present a unifying hypothesis suggesting that these two contributors to the pathophysiology of male reproduction may well be causally linked.

# GENETIC CAUSES OF MALE INFERTILITY

## Mutations Affecting Sperm Structure and Function

Mutations and epimutations in the male germ line can affect the functional competence of spermatozoa and/or their primary production. Examples of mutations that influence sperm quality include conditions that affect the morphological appearance of spermatozoa and their competence for fertilization. A classic example of such a condition is globozoospermia. Patients exhibiting globozoospermia produce spermatozoa with spherical-shaped heads, no acrosome and a disorganized midpiece characterized by excess amounts of residual cytoplasm (**Figure 1B**). Interestingly, globozoospermic spermatozoa possess normal flagella that are capable of progressive motility and, under the appropriate conditions, can exhibit hyperactivation. However, these cells are incapable of binding to the zona pellucida and achieving sperm-oocyte fusion, even after treatment with the calcium ionophore, A23187 (10). These functional defects can be circumvented through the application of ICSI, although, even with this technique, fertilization rates are low. One of the reasons for the lack of fertilization is that globozoospermic spermatozoa are deficient in phospholipase C zeta (PLCζ), the calcium oscillogen that orchestrates oocyte activation at fertilization. Artificial oocyte activation in concert

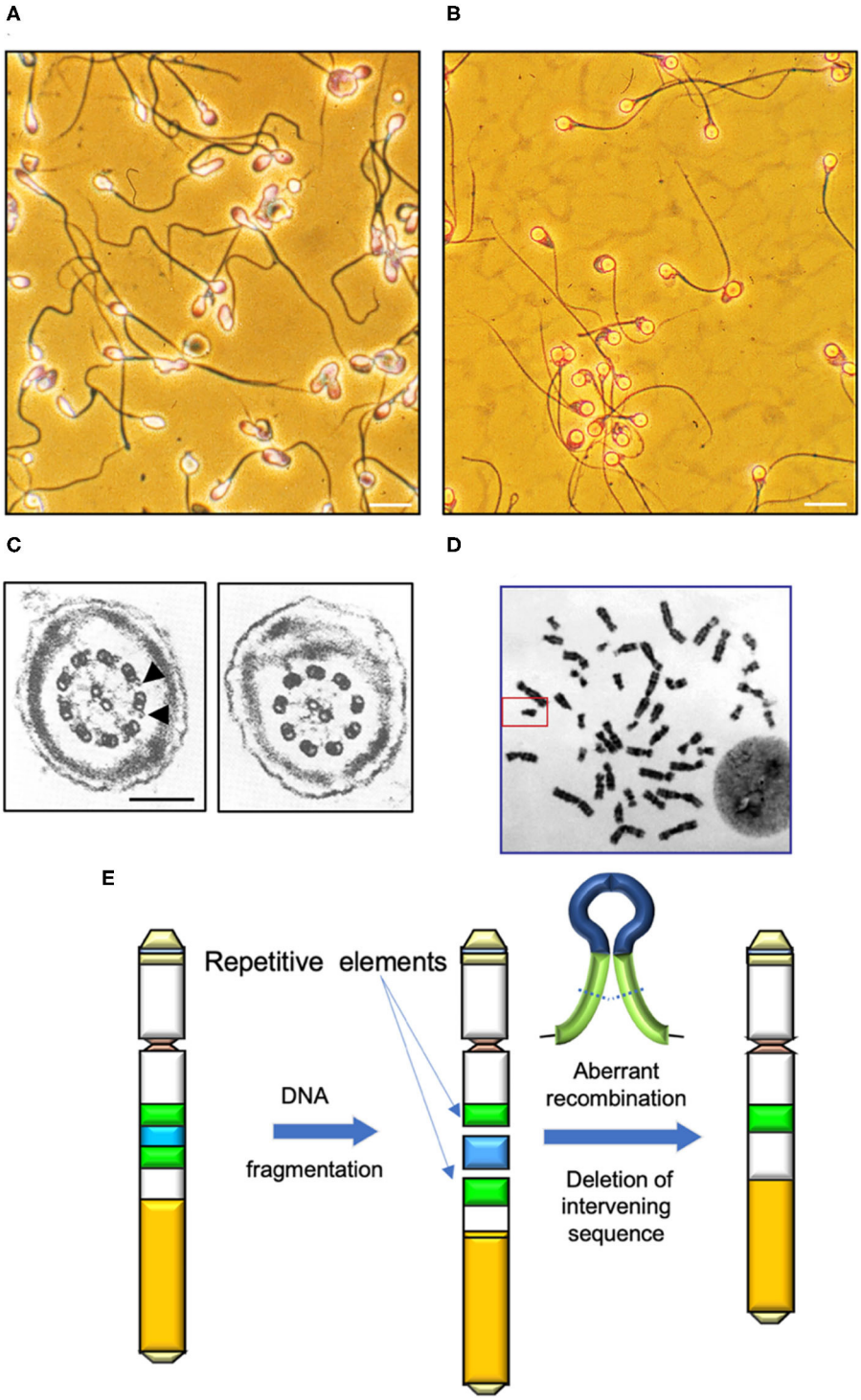


**FIGURE 1 |** Determinants of human sperm quality. **(A)** Micrograph of a typical human sperm population. Note the significant inter-cell variation in morphological appearance of the sperm head, midpiece and tail. Morphological assessments are generally couched in terms of percentage normal cells, however defining the precise attributes of "normal" in this context is difficult. Scale bar = 10 μm. **(B)** A clearly abnormal condition is "globozoospermia" characterized by round headed, acrosome-less cells that are incapable of fertilization even though their motility is normal (10). Scale bar = 10 μm. **(C)** In contrast, Kartgener syndrome is a genetically-determined condition associated with major defects in the axoneme and complete immotility. Left panel cross section through a normal sperm tail highlighting the dynein arms (arrowed); right panel shows a section through the axoneme of a Kartagener syndrome patient, showing the complete absence of dynein arms (11). Scale bar = 200 nm **(D)** Human karyotype with the Y-chromosome, framed. **(E)** Major mechanisms by which DNA fragmentation on the Y is induced is through the aberrant recombination of repetitive elements (green) and deletion of the intervening DNA (blue).

with ICSI can enhance fertilization rates with globozoospermic spermatozoa, but even with this intervention success rates are still significantly lower than control levels (12). One possible explanation for the poor fertilization rates observed with such spermatozoa is that they apparently exhibit high levels of DNA fragmentation in association with poor chromatin packaging and protamine deficiency (13). A related syndrome is "partial globozoospermia" where >50% of cells exhibit the round-headed acrosomeless morphology (14). Although the genetic basis of this partial condition remains uncertain at the present time, the presence of apparently normal cells in the ejaculate means that it can be very effectively treated with ICSI (15).

The causes of globozoospermia are genetic and, as far as we are currently aware, involve mutations in three genes, *SPATA16* (spermatogenesis associated 16), *PICK1* (protein interacting with PRKCA 1) and *DPY19L2* (DPY-19 like 2) with the latter being dominant (16). Homozygous deletions in *DPY19L2* are caused by non-allelic homologous recombination between flanking LCR (low copy repeat) sequences in around 70% of cases (17). Like most genetic conditions responsible for male infertility these causative mutations are rare (<1%). The major *DPY19L2* deletion is thought to have stabilized at a low level within the population because the *de novo* production of mutant alleles balances the negative selection incurred by sterile homozygous males (18).

A second sperm defect with a recognized genetic cause is macrozoospermia, characterized by the presence of a very high percentage of spermatozoa with enlarged heads and multiple flagella. A majority of such cells are diploid and the etiology involves mutations in the *AURKC* (aurora kinase C) gene (16). The incidence of these mutations varies from population to population but a recent analysis of North African males suggested that AURKC mutations were the most common, comprising 2.7% of the infertile male population compared with 1.2% exhibiting *DPY19L2*-dependent globozoospermia and anticipated rates of 1.6% exhibiting Klinefelter syndrome and 0.23% with Y-chromosome deletion (19). Macrozoospermia is observed in patients who are homozygous recessive for AURKC mutations. Two mutations in AURKC have been described: A p.Y248* non-sense mutation which arose 925–1,325 years ago and the c.144delC homozygous frameshift mutation, dated as having occurred 250–650 years ago. In order to account for the retention of these mutations in the population over so many generations, it has been suggested that the heterozygote must have some selective reproductive advantage possibly related to sperm production (20); however, there is no evidence to support this suggestion at present.

Sperm head morphology is also radically altered in a condition known as acephalic spermatozoa. Mutations in several different genes are known to generate this phenotype. Thus, polyamine modulated factor 1 binding protein 1 (*PMFBP1*), testis specific 10 (*TSGA10*), Sad1 and UNC84 domain containing 5 (*SUN5*), bromodomain testis associated (*BRDT*) and centrosomal protein 112 (*CEP112*) have all recently been implicated in the etiology of this condition (21–23). The reason why so many different mutations appear to be involved in the creation of acephalic spermatozoa is a reflection of the inordinate complexity of the head-tail coupling apparatus in these cells (24). Mutations in *SUN5* account for about half of all cases of acephalic spermatozoa in human patients. In some ways this is fortunate, because patients with *SUN5* mutations can be successfully treated using ICSI (25), reflecting the very specific role this protein plays in anchoring the sperm head to the tail (26). However, other mutations associated with the acephalic syndrome such as *TSGA10* and *CEP112* are not readily treatable with assisted conception therapy because the damage is centered on the sperm centriole and, in humans, the paternal centrioles are responsible for orchestrating cell division in the embryo. In the absence of functional centrioles in the spermatozoa, fertilization may be achievable with ICSI but any embryo created will exhibit arrested embryonic development (22).

Just as the complexity of sperm head attachment means that the acephalic condition is potentially associated with mutations in any one of a number of key genes, exactly that same is true of defects in the flagellum in the etiology of asthenozoospermia. We have known about defects in the axoneme in the pathological suppression of sperm motility since the pioneering work of Afzelius (27, 28) recorded defects in several components of the axoneme of men exhibiting either a complete lack of sperm motility or severe asthenozoospermia. These diseases are grouped under the heading of primary ciliary dyskinesias (PCDs) because they affect all ciliary structures in the body, not just the sperm flagella. A classic example of this condition is Kartegener syndrome (**Figure 1C**), which is associated with a complete lack of sperm motility due to the absence of dynein arms, in association with chronic sinusitis, bronchiectasis and, in 50% of cases, situs inversus caused by an inability of the embryonic cilia to shift the heart to the left hand side. The condition is inherited in an autosomal recessive manner as a result of biallelic homozygous or compound heterozygous mutations in several candidate genes including coiled-coil domain containing 40 (*CCDC40*), dynein axonemal heavy chain 1, 5, and 11 (*DNAH1, DNAH5, DNAH7 DNAH11*) dynein axonemal intermediate chain 1 (*DNAI1*), leucine rich repeat containing 6 (*LRRC6*), Zinc finger MYND-type containing 10 (*ZMYND10*) armadillo repeat containing 4 (*ARMC4*) and tetratricopeptide repeat domain 12 (*TTC12*) (29–33). Overall, more than 40 genes have been implicated in this heterogenous disease to date and the list of genes involved is expanding rapidly in concert with improvements in our understanding of ciliary structure and function. In theory, any gene involved in the assembly, structure and function of ciliary/flagellar structures could contribute to the male infertility associated with PCD. The condition is rare (prevalence 1:10,000 to 1:40,000 births) and the ultrastructural phenotypes are variable involving no outer and inner dynein arms (DAs), outer DAs alone, inner DAs with microtubular disorganization or defects yielding an abnormal central complex. Because of this heterogeneity, not all patients exhibiting PCD are infertile. Importantly apart from the loss of motility, spermatozoa obtained from Kartagener syndrome have been shown to be functionally normal in that they will engage in the process of capacitation, will acrosome react and, if physically

manipulated to lie close to the plasma membrane of the oocyte will achieve sperm-oocyte fusion (11). So, even if motility loss is total, conceptions can still be achieved using either sub-zonal insemination (SUZI) techniques that place immotile, but acrosome reacted, spermatozoa adjacent to the vitelline membrane of the oocyte or ICSI (34). Outside of such assisted conception procedures, spontaneous pregnancies are possible with PCD patients providing the loss of motility is partial; however, spontaneous fertility is unlikely with mutations in certain genes including *CCDC39, CCDC40,* dynein axonemal assembly factor 1 (*DNAAF1)* and *LRRC6* (35).

In addition to PCD, where multiple ciliopathies are observed in different organ systems, male infertility is also associated with sperm motility defects involving genes that specifically impact the development of the sperm tail, its detailed architecture and its physiological regulation. These mutations generate isolated male infertility associated with severe asthenozoospermia in the absence of any other pathology and are known collectively as Multiple Morphological Abnormalities of the sperm Flagella (MMAF). The list of mutations responsible for MMAF is expanding rapidly but currently includes mutations in adenylate kinase 7 (*AK7*) (36), glutamine rich 2 (*QRICH2)* (37), cilia and flagella associated proteins (*CFAP43, CFAP44, CFAP65, CFAP69, CFAP70, CFAP91, CFAP251*) (38–44) WD repeat domain 19 (*WDR19*) (45), DAZ interacting zinc finger protein (*DZIP1*) (46), *DNAH1* (also implicated in PCD) (47) *DNAH2* (48), *DNAH6* (49), *DNAH17* (50), *TTC29* (51), *TTC21A* (52), armadillo repeat containing 2 (*ARMC2*) (53) *CEP135* (centrosomal protein 135) (54). fibrous sheath interacting protein 2 (*FSIP2*) (55), ADP ribosylation factor like GTPase 2 binding protein (*ARL2BP*) (56), sperm flagellar 2 (*SPEF2*) (57), and DnaJ heat shock protein family (*Hsp40*) member B13 (*DNAJB13*) (58).

Of course, motility is not the only attribute of sperm function susceptible to interference by genetic and epigenetic mutations. For example, we know that genetic defects in sperm PLCζ, which activates the generation of calcium transients in the fertilized oocyte, impair human oocyte activation and fertilization (59). Fertilization failure has also been associated with mutations in *CATPSERE* (CatSper-epsilon), a component of the sperm calcium channel (60) as well as polymorphisms in the mitochondrial genes MT-ATP6 and MT-CYB (61).

It must be evident from the information presented above that there are a great number of mutations potentially capable of suppressing the fertilizing potential of human spermatozoa. This may not be surprising given the biochemical sophistication underpinning such complex sperm functions as sperm motility (which accommodates a variety of sophisticated behaviors including rheotaxis, chemotaxis and thermotaxis as well as the switch to hyperactivation), sperm transport to the site of fertilization, the ability of a capacitated spermatozoon to recognize just one other cell type in the body (the egg), the induction of acrosomal exocytosis, sperm-oocyte fusion and oocyte activation. Moreover, these are just the mutations affecting the structure and function of spermatozoa, there are a great many more mutations influencing the primary production of spermatozoa at a testicular level.

## Mutations Affecting the Testes and Excurrent Ducts

Obstructive azoospermia is present in around 30% of azoospermia cases and is often due to occlusion of the excurrent duct system subsequent to infection, trauma or surgery. The major genetic cause involves CFTR (Cystic fibrosis transmembrane conductance regulator) mutations that are known to induce abnormal formation or bilateral absence of the vas deferens (62). The remaining 70% of azoospermia cases are non-obstructive and relate to primary failure of spermatogenesis. Primary testicular failure may be observed with different forms of testicular cancer, the genetic origins of which are as complex as their histopathology, including germ cell tumors (seminoma, embryonal carcinoma, yolk sac tumor, and teratoma) and, in older men, testicular lymphomas (63, 64). Gains of chromosome arm 12p and aneuploidy are nearly universal in germ cell tumors (65) while primary testicular lymphomas involve near-uniform loss of CDKN2A (cyclin dependent kinase inhibitor 2A) with rare TP53 (tumor protein p53) mutations as well as 9p24.1/PD-L1/PD-L2 copy number alterations and additional translocations of these loci (66).

Cancer aside, an increasing list of monogenic gene mutations are being associated with non-obstructive azoospermia. Just as we saw with mutations affecting sperm structure and function, gene mutations leading to primary testicular failure are many, varied and infrequent, reflecting the underlying complexity of the spermatogenic process and the inability of any particular mutation to become anything other than rare, given: (1) the negative selection pressure associated with male infertility, (2) the fact that many of these mutations also cause infertility in women and so cannot find refuge in the female germ line (67), and (3) the absence of any particular reproductive advantage in the heterozygous form. These mutations are generally autosomal recessive and inherited from fertile parents in homozygous, compound heterozygous or hemizygous form. However, autosomal dominant monogenic mutations can also precipitate a state of infertility including *SYCP3* (Synaptonemal complex protein 3), *NR5A1* (nuclear receptor subfamily 5 group A member 1) and the WT1 (Wilms' tumor 1) gene (68). A recent detailed analysis of the genetic causes of non-obstructive azoospermia (NOA) concluded that the largest single category of monogenic defects detected in NOA patients comprises genes involved in different stages of spermatogenesis, mostly functioning in the prophase of the first meiotic division as well as transcriptional and endocrine regulators of reproduction (67).

Of course, not all mutations causing non-obstructive azoospermia are expressed in the testes. There are several genes involved in the etiology of hypogonadotrophic hypogonadism that are key components of the hypothalamic-pituitary-gonadal axis involved in the endocrine regulation of spermatogenesis. The human *GNRHR* (gonadotropin-releasing hormone receptor) gene is a case in point. This protein is a G-protein coupled receptor expressed on the surface of pituitary gonadotrophs that respond to pulsatile GnRH stimulation by promoting the secretion of gonadotrophins FSH and LH. Several (at least 19) mutations have been identified in this gene that often exert

their pathological action as compound heterozygotes (68). There are also mutations that interfere with the differentiation of the male reproductive system including cytochrome b5 type A (*CYB5A*) which selectively disrupts 17,20-lyase activity leading to disordered sexual development (69) and deletions on chromosome 21 that also cause defects in male sexual development (70). Mutations on the X chromosome are also linked with male infertility including: anosmin 1 (*ANOS1*), a gene linked to Kallmann syndrome (71), testis expressed 11 (*TEX11*), linked to meiotic arrest (72) and nuclear receptor subfamily 0 group B member 1 (NR0B1) associated with adrenal hyperplasia and hypogonadotropic hypogonadism (73). Mutations in the X-linked androgen receptor gene are also known to induce infertility in around 2% of male patients (74).

Overall, the foregoing summary reveals a bewildering array of monogenic defects involved in male infertility exerting their action at all stages of gonadal development and function, from the initial morphogenesis of the male genital tract in the fetus to the differentiation and maturation of fully functional spermatozoa. However, interestingly, the most common genetic causes of male infertility are not single gene mutations at all, but aneuploidies of which Klinefelter syndrome (XXY) is the most common, accounting for around 10% of patients with non-obstructive azoospermia/severe oligozoospermia (75, 76).

In view of the strong negative selection pressure associated with infertility, the maintenance of such an impressive range of infertility-inducing chromosomal or genetic mutations in the general population must involve the steady spontaneous generation of *de novo* lesions affecting genes and chromosomes involved in the spermatogenic process (77). The importance of such *de novo* mutations in the etiology of male infertility is beautifully illustrated by the presence of Y-chromosome deletions in males exhibiting spontaneous severe oligozoospermia on non-obstructive azoospermia.

## THE Y CHROMOSOME

The Y chromosome is unusual because it lives in isolation (**Figure 1D**). One detrimental consequence of such a solitary existence is that the Y chromosome has limited options when it comes to the repair of DNA damage. All other chromosomes have a homologous chromosome, inherited from the other parent, that can assist in the repair of damage DNA via homologous recombination. The Y chromosome has no homolog to recombine with and so it has reverted to an intrachromosomal form of recombination in order to maintain a modicum of stability. Typically, genes on the Y-chromosome make multiple copies of themselves as a buffer against the chaos that would be introduced by the accumulation of deleterious spontaneous mutations, a lack of selection pressure, the presence of unwanted genetic hitchhikers and genetic drift. Some of these repetitive elements have become inverted to create palindromic sequences that facilitate recombination events in an area of the genome where inter-chromosomal recombination is otherwise suppressed. As a consequence of this strategy, damage to key genes on the Y-chromosome can be repaired by the affected gene recombining with a palindromic copy of itself in a process known as gene conversion. This capacity for intra-chromosomal recombination has enabled the Y chromosome to stabilize after an initial period of exponential decay such that no genes have been lost since the divergence of humans and chimpanzees between 6 and 7 million years ago while only one gene had been lost since humans diverged from the rhesus macaque 25 million years ago (78, 79).

Unfortunately, the presence of such palindromic sequences, as well as repetitive retroviral elements on the human Y chromosome, facilitates the creation of chromosome deletions as a consequence of aberrant recombination events (**Figure 1E**). Three common Yq deletions that recur in infertile males are termed AZF (Azoospermia Factor) microdeletions–AZFa, AZFb and AZFc. In addition, the combinations, AZFbc, AZFabc, and a partial AZFc, called AZFc/gr/gr are also observed (80, 81). AZFa and AZFb deletions usually result in complete azoospermia, with no current potential for treatment. However, patients with AZFc deletions are typically characterized by severe oligozoospermia, with small numbers of spermatozoa recoverable from the ejaculate or from testicular biopsy material. These cells are sufficiently normal to permit treatment options involving ICSI. Of course, an inevitable consequence of the Y-chromosome's genetic isolation, is that any son generated as a consequence of ICSI will inherit his father's microdeletion and, thus, his infertility (80).

The prevalence of Y chromosome deletions and microdeletions is estimated at 1:2,000 to 1:3,000 males while the frequency of Yq microdeletions in males with azoospermia is 15% and with severe oligozoospermia about 5% (82). Unlike the monogenic gene mutations responsible for defective sperm production or primary testicular failure reviewed above, Y-chromosome mutations cannot be maintained in the population as heterozygotes or via passage through the female germ line. With very few exceptions, every new case of Y-chromosome deletion has been spontaneously created in the fertile father's germ line. This tells us that DNA damage and repair must be a major feature of male reproduction. Indeed, a recent analysis has found that 75% of all *de novo* mutations arise in the male germ line via mechanisms that have little to do with replication error, as commonly supposed (83). Rather, the high rate of mutations observed in the male germ line, including common C-to-G transversions and CpG transitions show genomic distributions and sex-specific age dependencies indicative of double-strand break repair and methylation-associated damage, respectively (83). The Y-chromosome's strategy of gene amplification and intra-chromosomal recombination in order to stabilize has, undeniably, been effective in slowing the rate of gene attrition on this chromosome. However, the presence of so many repetitive palindromic elements also creates a measure of vulnerability. If DNA fragmentation rates are high, then there is the potential for distant palindromic sequences to recombine, resulting in deletion of the intervening genetic information. Such deletions could either occur during spermatogenesis or, in principle, in the oocyte as a result of aberrant DNA repair prior to the S-phase that precedes the first cleavage division. Thus, Y-chromosome deletions, like many mutations that arise in our species may

be seen as a consequence of the high rates of DNA damage and fragmentation that characterize the male germline. In this context, there is a significant body of evidence indicating that DNA fragmentation is a consistent feature of human spermatozoa and that the induction of such damage is oxidative.

## OXIDATIVE STRESS

The importance of oxidative stress in the etiology of defective sperm function has been recognized since the pioneering studies of Thaddeus Mann and colleagues at the University of Cambridge demonstrated that mammalian spermatozoa were vulnerable to a lipid peroxidation process that attacks the unsaturated fatty acids in these cells, destroying the plasma membrane and compromising their functional competence (84). The induction of such stress may involve the enhanced generation of reactive oxygen species (ROS) by these cells and/or a deficiency in the levels of antioxidant protection they are afforded (85–87). The net impacts of oxidative stress include a loss of motility, a decrease in the ability of the spermatozoa to undergo the acrosome reaction, an impaired capacity to fuse with the vitelline membrane of the oocyte—and DNA damage (85, 88–90).

### Leukocyte Infiltration and ROS Generation

The sources of ROS that create this oxidative stress are complex and may be due to either intrinsic or extrinsic factors. The major extrinsic factor are leukocytes that enter the semen at the moment of ejaculation from the secondary sexual glands. The major leukocyte species in this context are neutrophils (**Figure 2A**) that arrive in the seminal compartment in an activated, free radical-generating state (**Figure 2B**). The presence of these cells is thought to reflect an underlying reproductive tract infection (92, 93), although other factors such as trauma, surgery, and autoimmunity could also be involved. As long as leukocyte numbers are relatively low (less than the leukocytospermic threshold of 1 million/ml), the ROS generated by infiltrating leukocytes have no effect on the functionality of the spermatozoa because these cells are adequately protected by the powerful antioxidants present in seminal plasma (94). However, if the leukocyte numbers exceed this leukocytospermic threshold, or are just below it, then a state of oxidative stress prevails, and sperm function is compromised (95). Furthermore, if leukocytes are still present in the washed sperm suspensions used in assisted conception procedures then significant oxidative stress will again be created due to the absence of significant antioxidant protection in conventional IVF culture media. The existence of low-level leukocyte contamination in washed human sperm suspensions is a significant issue for IVF therapy because it negatively impacts the fertilization rates subsequently observed (96, 97). This problem can either be addressed through the incorporation of antioxidants such as N-acetylcysteine or hypotaurine in the culture medium (98) and/or through the selective removal of contaminating leukocytes using magnetic particles coated with a monoclonal antibody against the common leukocyte antigen (**Figure 2C**) (91). Such treatments effectively reduce levels of oxidative stress in the sperm suspensions and significantly enhance the fertilization rates subsequently observed.

### Spermatozoa and ROS Generation

Outside of leukocyte contamination, a majority of the ROS that compromise sperm function are generated endogenously via a variety of pathways, in response to a variety of stimuli. It is important to point out that the levels of ROS generated by spermatozoa are orders of magnitude lower than leukocytes (99). This is the reason why flow cytometry is such a useful technique for monitoring seminal ROS production because it enables the separation of spermatozoa from other cell types and simplifies interpretation of the data. By contrast, techniques such as luminometry, always run the risk of generating data that is heavily influenced by the presence of contaminating leukocytes (100, 101). It should also be recognized that a vast majority of the probes used with flow cytometry are redox active agents that do not measure ROS directly but rather, oxidative activity. Nevertheless, by using dihydroethidium as a probe and separating out the reaction product specifically generated by superoxide anion, 2-hydroxyethidium, it has been possible to generate evidence of superoxide production by mammalian spermatozoa (102, 103). Moreover, these data have been confirmed with definitive techniques such as electron paramagnetic resonance spectroscopy (104–106).

There has also been discussion as to which ROS is the more important in the determination of sperm function, superoxide (107) hydrogen peroxide (86, 108–110) nitric oxide (111) or peroxynitrite (112). In reality, all these oxidants and free radical species are so reactive that they are constantly interconverting and contributing to the oxidative stress experienced by the male gamete. It is doubtful whether any particular species actually pre-dominates.

### Sperm Mitochondria as a Source of ROS

One of the major sources of superoxide anion within spermatozoa are the mitochondria (113). These organelles generate ROS as a normal by-product of aerobic metabolism due to the leakage of electrons from the mitochondrial electron transport chain, which are then swept up by the universal electron acceptor, oxygen, to generate superoxide anion. Mitochondrial ROS are also produced as part of the intrinsic apoptotic cascade that becomes activated whenever the phosphoinositide signaling pathway is compromised (114). Under physiological circumstances, a variety of pro-survival factors, including as insulin, prolactin or angiotensin 1–7 (115, 116) stimulate phosphorylation and activation of phosphoinositide-3 kinase (PI3K). The latter in turn phosphorylates another kinase, AKT. As long as AKT is phosphorylated, downstream targets of this kinase such as the apoptosis regulator, BCL2-associated-agonist-of-cell-death (BAD) are also phosphorylated. Phospho-BAD forms a heterodimer with its 14-3-3 keeper protein, leaving Bcl-2 free to inhibit Bax-triggered apoptosis, thereby maintaining spermatozoa a viable motile state (114). However, if PI3K activity is disrupted, AKT and its downstream target, BAD, become dephosphorylated allowing the latter to escape from the grip of its 14-3-3 keeper to form a heterodimer with Bcl-2 and Bcl-xL, inactivating these regulators and thus allowing Bax/Bak-triggered apoptosis. The intrinsic apoptotic cascade is associated with rapid motility loss, mitochondrial ROS

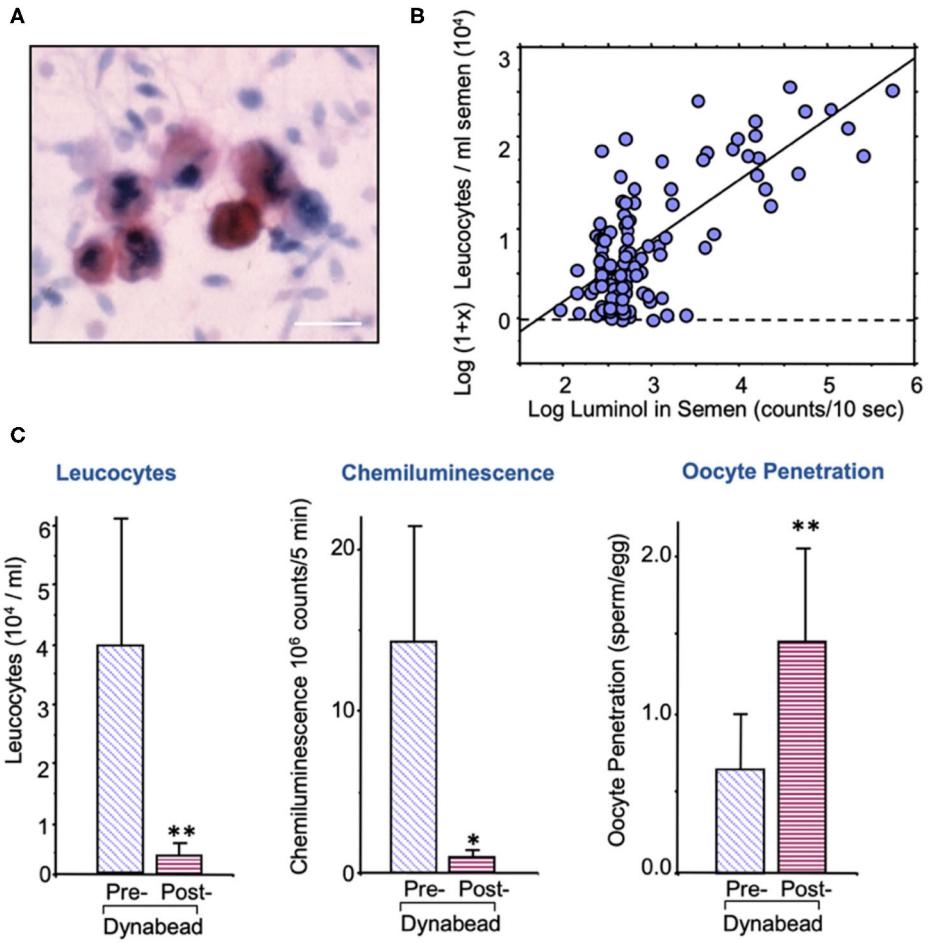


**FIGURE 2 |** Leukocyte contamination of human semen samples. **(A)** All human semen samples are contaminated with leukocytes, largely neutrophils, and macrophages; sample stained with an antibody against the common leukocyte antigen, CD45. Sale bar = 10 μm. **(B)** The spontaneous generation of reactive oxygen species by human semen samples is highly correlated with the level of leukocyte contamination. **(C)** Treatment of washed human sperm suspensions with magnetic beads coated in anti-DC45 removes a majority of the leukocytes, reduces the levels of oxidative stress as determined by luminol dependent chemiluminescence and increases fertilization rate in the heterologous sperm-oocyte fusion assay (91). *$P < 0.05$; **$P < 0.01$.

generation, caspase activation in the cytosol, annexin V binding to the cell surface, cytoplasmic vacuolization and oxidative DNA damage (**Figure 3**) (114). So, any stress factor that will induce an apoptotic response in human spermatozoa will trigger mitochondrial ROS generation and a loss of sperm function.

Mitochondrial ROS generation and apoptosis may also be important in the mechanisms underpinning sperm senescence. All mammalian spermatozoa have a finite life span and after a few days (depending on species) will become senescent *in vivo* and *in vitro*, losing their vitality, motility, tyrosine phosphorylation status and DNA integrity with the passage of time (117, 118). Oxidative stress appears to be one component of the senescence process judging from the fact that sperm motility and DNA integrity can be significantly improved *in vitro* if oxygen tensions are reduced and/or antioxidants are incorporated into the medium (119–121). Furthermore, the generation of ROS on prolonged incubation has been found to increase with time and a majority of this ROS appears to be mitochondrial, possibly reflecting the progressive entry of senescent cells into the intrinsic apoptotic pathway (121, 122). Accordingly, antioxidants that target mitochondrial ROS generation such as co-enzyme Q10 and pyrroloquinoline quinone as well as thiols such as N-acetylcysteine and penicillamine have been found to extend sperm motility *in vitro* (121, 123, 124). Similarly, treatments that divert energy generation away from mitochondrial oxidative phosphorylation and toward glycolysis, such as exposure to rosiglitazone, decrease mitochondrial ROS generation, and allow spermatozoa to maintain high levels of motility *in vitro* for at least 6 days (125). However, there is still some conjecture as to how many other factors are involved in sperm motility loss *in vitro*. In a recent study of human spermatozoa incubated over a 5 day period, the loss of virtually all motility was not accompanied by a corresponding increase in 4-HNE levels by Western blot (102). In light of these data, we have to conclude that there may be multiple

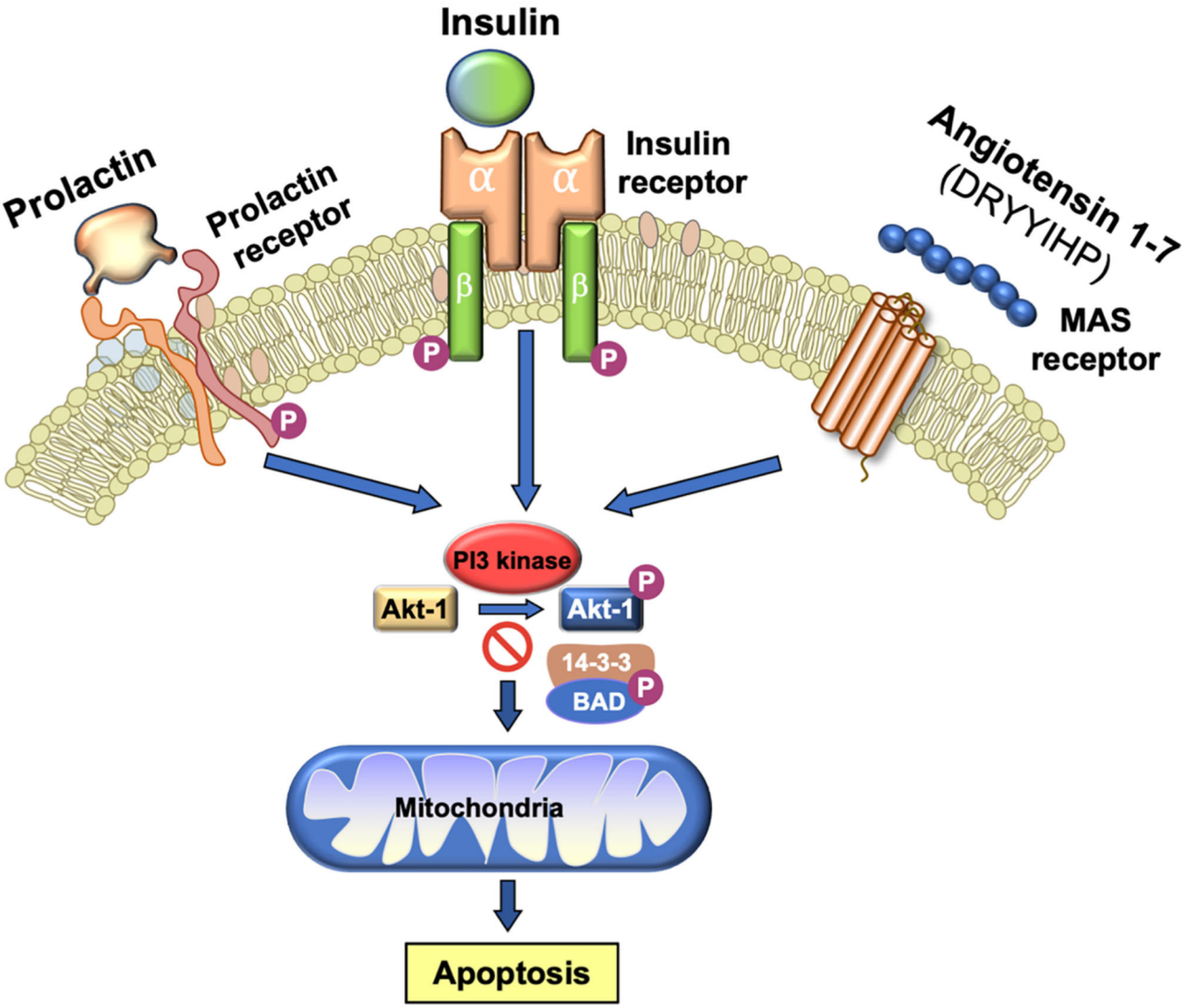


**FIGURE 3 |** Importance of PI3 kinase in the maintenance of sperm viability**.** As long as PI3 kinase is phosphorylated, its downstream target AKT1 is also phosphorylated. Phosphorylated AKT1 in turn phosphorylates BAD which, in this state, remains bound to its 14-3-3 keeper protein. This system is driven by a series of prosurvival factors such as prolactin, insulin and angiotensin 1–7 that act through their cognate receptors to maintain PI3 kinase in a phosphorylated state. If this system is perturbed and PI3 kinase becomes dephosphorylated, then BAD reverts to its dephosphorylated state, releases 14-3-3 and moves to the mitochondria where it forms heterodimers with Bcl-2 and Bcl-xL, inactivating these regulators and allowing Bax/Bak-triggered apoptosis. The induction of PI3 kinase phosphorylation is therefore essential for sperm survival.

reasons why spermatozoa become senescent *in vitro* and we are yet to resolve the full complexities of this process.

Mitochondrial ROS can also be triggered by a range of amphiphilic compounds in human spermatozoa including cis-unsaturated fatty acids; the polar nature of these compounds favoring their corporation into mitochondrial membranes, altering membrane fluidity and facilitating electron leakage (126). Since the free unsaturated fatty acid content of defective human sperm populations is positively correlated with the induction of mitochondrial superoxide generation, we can conclude that the pathophysiology of defective sperm function is at least partly dependent on changes to the lipid composition of these cells (127). Mitochondrial ROS can also be stimulated by toxicants that can perturb the flow of electrons along the mitochondrial electron transport chain. For example, the common preservative, parabens (a mixture of parabenzoic esters), has been shown to stimulate mitochondrial ROS in spermatozoa in a manner which is correlated with alkyl chain length (128). Similarly, the xenoestrogen bisphenol A stimulates mitochondrial ROS generation by human sperm mitochondria (129) as do certain polyphenols (epigallocatechin gallate, genistein, didox gossypol) several of which are traditionally regarded as antioxidants (130).

The cryopreservation of spermatozoa is another situation in which sperm function is compromised partly as a consequence

of oxidative stress created by enhanced mitochondrial ROS generation. Consequently, a large number of studies, conducted in a range of different species, have examined the impact of antioxidants on post-thaw functionality. Since antioxidants such as L-carnitine and Mito Tempo have already proven effective in this regard (131, 132), a systematic comparison of antioxidants targeting the mitochondria is now warranted, to determine the optimal formulation for protecting spermatozoa against cryostorage injury.

Another extraneous factor which is thought to enhance mitochondrial ROS generation in mammalian spermatozoa is radiofrequency electromagnetic radiation (RFEMR) (133). The concept that RFMR induces electron leakage from the mitochondrial electron transport chain, and thus promotes superoxide anion generation (134), is controversial but has received support from numerous independent studies (135). Mitochondrial ROS generation activated by RFEMR has, in turn, been associated with the suppression of sperm motility, the induction of apoptosis, the loss of local antioxidant protection and the stimulation of sperm oxidative DNA damage both *in vivo* and *in vitro* (136–140).

*In vitro* exposure to electromagnetic radiation in the form of UVB light will also trigger mitochondrial ROS generation in human spermatozoa (141) as will the *in vitro* exposure of these cells to temperatures above 40°C (134). Mild heat stress (35°C) has also been found to activate mitochondrial ROS generation *in vivo*, with round spermatids being particularly vulnerable to this form of stress (135).

Mitochondrial ROS generation can also be stimulated by the electrophilic lipid aldehydes generated as an end-product of lipid peroxidation. Not all lipid aldehydes are equivalent in this respect, the differences generally correlating with the second order rate constants describing their interaction with the model nucleophile, glutathione. Acrolein and 4-hydroxynonenal (4-HNE) are the most active, stimulating mitochondrial ROS generation by binding to components of the mitochondrial electron transport chain, particularly succinic acid dehydrogenase (142, 143). The capacity of lipid aldehydes generated as a result of oxidative stress to bind to components of the mitochondrial electron transport chain and stimulate yet more ROS generation means that once this process is initiated, it becomes a self-perpetuating process unless a chain breaking antioxidant intervenes.

## NAD(P)H Oxidase

Another potential source of ROS generation in human spermatozoa are NAD(P)H oxidases reminiscent of the enzyme responsible for the oxidative burst in phagocytic leukocytes. Several authors (144, 145) have suggested that spermatozoa generate ROS via such enzymes based on the inhibitory action of diphenylene iodonium (DPI). However, DPI is a generalized flavoprotein inhibitor and so also suppresses ROS generation by oxidoreductases in the mitochondrial electron transport chain. This, and other factors, has, in some minds, cast doubt on the existence of such an enzyme in human spermatozoa and its possible contribution to oxidative stress in the male germ line (146, 147). However, in 2002, Banfi et al. (148) described the existence of an NADPH oxidase (NOX5) in several cell types, including human spermatozoa. Musset et al. (149) subsequently confirmed immunocytochemically that this oxidase was not only present in the neck or acrosomal region of these cells but generated superoxide anion in a calcium-dependent manner. They also identified cAbl as a tyrosine kinase associated with the activation of this oxidase. In addition, exposure to hydrogen peroxide was found to activate ROS generation by NOX5. These findings explain why several authors (150–152) have found that sustained ROS generation by human spermatozoa can be triggered by transient exposure to an oxidizing agent such as hydrogen peroxide—it also provides a mechanism. ROS generation is known to be associated with the stimulation of tyrosine phosphorylation in mammalian spermatozoa through the suppression of tyrosine phosphatase activity and the activation of adenylyl cyclase (153–155). cAMP is, in turn, known to activate cAbl in spermatozoa (156), and this kinase would then be expected to activate NOX5 leading to yet more ROS generation in concert with sperm capacitation, which is also cAMP driven (**Figure 4**).

The clinical significance of NOX5 is indicated by its high level of expression in the spermatozoa of asthenozoospermic males in concert with increases in superoxide and hydrogen peroxide generation and DNA damage (157). NOX5 expression has also been shown to be elevated in cases of teratozoospermia (158). Physiologically, the H(V)1 proton channel, which has been implicated in the regulation of sperm motility, is required for optimal superoxide production by spermatozoa via NOX5 (149), presumably by preventing cytoplasmic acidification that inevitably follows NADPH oxidation:

$$\underset{\text{NADPH oxidase}}{\text{NADPH} + 2O_2} \Rightarrow \underset{\text{superoxide}}{\text{NADP}^+ + 2O_2^{-\bullet}} + \underset{\text{proton}}{H^+}$$

Moreover, there is evidence that NOX5 and H(V)1 are involved in the induction of calcium signaling via Catsper in response to progesterone stimulation (159), again reflecting the fact that the activity of this calcium channel is highly sensitive to changes in pH (160) and that NOX 5 achieves intracellular alkalinization by activating the H(V)1 proton channel. Thus, it is possible that that NOX5 is a key regulator of sperm function as well as a potential mediator of oxidative stress and sperm pathology (**Figure 4**). However, we do not yet know why NOX5 activity would be elevated in the spermatozoa of infertile males. It is possible that the supply of NADPH is rate limiting in this situation and that cells possessing a large amount of residual cytoplasm (a characteristic feature of defective human spermatozoa suffering from oxidative stress) could fuel oxidase activity because they are over-endowed with glucose-6-phosphate dehydrogenase, a key regulator of the hexose monophosphate shunt responsible for regulating NADPH generation (161). We also do not understand how mouse spermatozoa can exhibit the same relationship between cAMP, ROS generation and sperm capacitation (162) but do not possess NOX5—possibly other oxidases are active in this species.

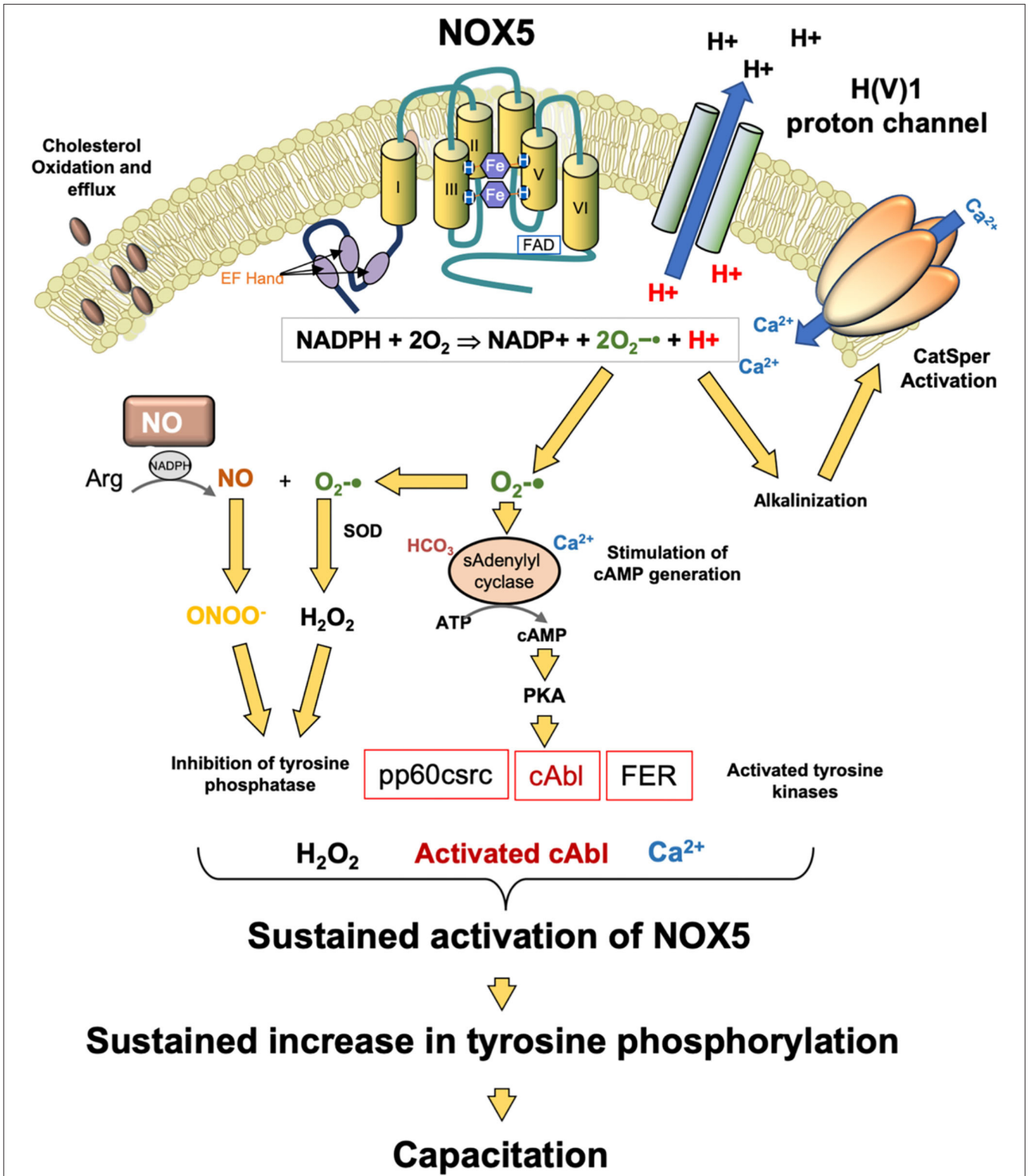


**FIGURE 4 |** Potential role for NOX5 in the regulation of sperm capacitation. NOX5 catalyzes the generation of superoxide anion and protons. The latter activate the H(V)1 proton channel leading to proton extrusion and alkalinization of the cytoplasm. This pH change activates the CatSperm channel leading to calcium influx which in turn further promotes NOX5 activity. The other product of NOX5 action, superoxide anion participates tin the activation of soluble adenylyl cyclase and the generation of cAMP. cAMP activates PKA which then phosphorylates as number of tyrosine kinases. At the same time, hydrogen peroxide and peroxynitrite ($ONOO^-$) co-operate to silence tyrosine phosphatase activity leading to a global increased in phosphotyrosie expression. One of the kinases activated in this process, cAbl, as well as hydrogen peroxide, are both known to stimulate NOX5 activity. As a consequence of these activities, NOX5 is maintained in an activated state, driving the tyrosine phosphorylation cascades that culminate in the attainment of a capacitated state.

## Lipoxygenase

Another factor in the oxidative stress equation that may be upregulated in spermatozoa possessing excess residual cytoplasm is lipoxygenase. This enzyme is present in cytoplasmic droplets of defective spermatozoa (163) and catalyzes the dioxygenation of polyunsaturated fatty acids to the corresponding hydroperoxide. The enzyme facilitates the initial hydrogen abstraction step in the peroxidation process, creating a lipid radical that then combines with molecular oxygen to create a peroxyl radical that on protonation generates the corresponding hydroperoxide. There are two ways in which this chemistry could be associated with oxidative stress. The first is that in the presence of NADPH, lipoxygenases can generate superoxide anion directly (164). Pharmacological inhibition of the lipoxygenase, ALOX15, in precursor male germ cells and spermatozoa results in a significant reduction in both mitochondrial and cytoplasmic ROS generation, as well as a dramatic reduction in 4-HNE accumulation (165, 166). These findings are consistent with the ability of polyunsaturated fatty acids to stimulate ROS generation in human spermatozoa and thereby compromise sperm function (126). The second means by which excess lipoxygenase activity could create oxidative stress in these cells is by fueling the generation of lipid hydroperoxides in the sperm plasma membrane. These peroxides have the potential to initiate a lipid peroxidation cascade if sufficient transition metals (e.g., iron, copper) are present in the immediate vicinity (167). If left unbroken, such peroxidative chain reactions culminate in the generation of electrophilic lipid aldehydes such as 4-HNE which can have a devastating impact on sperm function by binding to key proteins involved in the execution of sperm function. In this context, incubating human spermatozoa in the presence of a lipoxygenase inhibitor has been shown to both reduce the levels of 4-HNE accumulation in human spermatozoa and promote the functional competence of these cells (166).

## L-amino Acid Oxidase

The first enzyme that was ever shown to generate ROS in mammalian spermatozoa was an L-amino acid oxidase. In their landmark papers published more than 70 years ago, Tosic and Walton (168, 169) described the presence of an enzyme in bovine spermatozoa that generated significant quantities of hydrogen peroxide using aromatic acids, such as phenylalanine. Interestingly dead cells were more responsive to phenylalanine stimulation because the disrupted plasma membrane allowed this amino acid substrate access to the oxidase. So, when bovine spermatozoa were exposed to high concentrations of aromatic amino acids (as happens when these cells are suspended in cryostorage media supplemented with egg yolk) the dead cells generated high amounts of hydrogen peroxide which then impeded the motility and fertilizing potential of live cells in the immediate vicinity. The same oxidase has been detected in equine (170), ovine (171), and human spermatozoa (172). In the latter, oxidase activity was lost from non-viable cells, so the "dead cell-influencing-live cell" scenario established for ungulate spermatozoa does not apply in the human situation. The physiological purpose of the oxidase is currently unclear, although in the presence of phenylalanine human spermatozoa develop many of the hallmarks of capacitation, including increased tyrosine phosphorylation and enhanced acrosome rates, via mechanisms that could be reversed by the concomitant presence of catalase. It is therefore possible that this enzyme contributes to the redox regulation of capacitation as spermatozoa ascend the female reproductive tract (172)-always assuming the bioavailability of adequate quantities of aromatic amino acids in this location.

## Antioxidant Protection

Spermatozoa possess very little cytoplasm and, as a consequence, they are deficient in the antioxidant enzymes that protect most cells from oxidative stress. This does not mean that these cells are totally lacking in any form of intracellular protection because they do possess a number of important antioxidant enzymes such as peroxiredoxin 6, superoxide dismutase, the glutathione peroxidase-reductase couple and limited catalase (86, 173–175). However, the level of protection afforded by these systems has finite limits with the result that spermatozoa are heavily dependent on extracellular antioxidants present the seminiferous tubule fluid (176) epididymal plasma (177), seminal plasma (178) and uterotubal fluid (179) to provide them with complete protection during their voyage from the seminiferous tubules to the vitelline membrane of the oocyte. As a result, any factors that impact the overall antioxidant status of an individual, and thus the bioavailability of extracellular antioxidants, can have an impact on the levels of oxidative stress suffered by the male germ line and thence fertility (180, 181). Examples of such conditions include diet (182), varicocele (183) smoking (184), obesity (185), heat stress (186), and environmental toxicants such as bisphenol A (187) all of which can impact systemic antioxidant tone and thus the vulnerability of spermatozoa to oxidative stress. Inadequate intracellular and extracellular antioxidant protection could therefore be a major factor in the etiology of both male infertility and oxidative DNA damage to the paternal genome (188). Given this background it would seem axiomatic that if oxidative stress is such a powerful cause of reproductive dysfunction in the male, then antioxidants should be part of the cure (189, 190). Unfortunately, the clinical trials needed to demonstrate that the administration of antioxidants to patients suffering demonstrable oxidative stress in their germ line will reap a therapeutic reward, have not yet been conducted at scale. Some promising pilot studies have been conducted with positive results (191) and efficacy has clearly been demonstrated in animal models (190). However, the gold standard randomized, double-blind, cross-over, placebo-controlled trial has yet to be conducted, partly because there is currently no consensus over the oxidative stress markers that should be used to identify appropriate patients for antioxidant therapy.

## Other Therapeutic Interventions

In addition to antioxidant administration we should also recognize that a variety of lifestyle interventions might also influence the oxidative stress underpinning male infertility including, increased exercise, improved diet as well as the cessation of alcohol consumption and cigarette smoking. Interestingly, the practice of yoga has been found to have a

positive impact on the mRNA profile of human spermatozoa, their epigenetic status, the generation of ROS, the levels of DNA damage in the sperm nucleus and various attributes of the conventional semen profile (192, 193). Such results suggest that male infertility may have a strong psychogenic component which can be ameliorated by yoga-based lifestyle interventions aimed at reducing cognitive, as well as oxidative, stress.

## Oxidative Stress and Embryo Development

While we have traditionally viewed male reproductive competence in terms of fertilization capacity, it is now clear that the definition of "competence" should extend beyond conception to encompass the establishment of a normal viable pregnancy as well as the health and well-being of the offspring. It is therefore important to acknowledge that oxidative damage to human spermatozoa does not just influence their capacity for fertilization but also has a major impact on the developmental potential of the embryo (194, 195). Importantly, when spermatozoa are subjected to increasing levels of oxidative stress, the induction of significant DNA damage precedes the loss of fertilizing potential (196). As a consequence, it is perfectly possible for a DNA damaged spermatozoon to achieve fertilization of the oocyte. When this happens, the oocyte immediately launches into a round of DNA repair in order to address any damage in the paternal genome prior to the initiation of S-phase of the first mitotic cell division. If the oocyte makes a mistake at this point or is overwhelmed by the levels of DNA damage brought in by the fertilizing spermatozoon, it has the potential to increase mutations in the offspring that may significantly impact the latter's developmental potential and long-term health trajectory.

Oocytes are particularly vulnerable to the introduction of oxidative DNA damage by the fertilizing spermatozoon because they are deficient in the first enzyme of the base excision repair pathway, OGG-1 (197). Typically, defective human spermatozoa carry extremely high levels of oxidative DNA damage (198, 199) so the chances are, that fertilization will force the oocyte to the very limits of its repair capacity. Persistence of paternally-derived oxidized DNA base adducts into S-phase of the first mitotic division will enhance the risk of *de novo* mutations being created as the embryos enter the cleavage stage of development. Experimentally, if gametes are generated expressing high levels of oxidative DNA damage by genetically inactivating the major base excision DNA repair pathways, then the offspring exhibit high levels of *de novo* mutations, particularly G to T transversions, and live lives shortened by birth defects and disease, including cancer (200). Oxidative stress in the male germ line can therefore not only create *de novo* mutations directly; it can also generate pre-mutational damage that becomes fixed as a genetic mutation following fertilization, as a result of deficient or aberrant repair in the oocyte prior to the first cleavage division (82). It has recently been recognized that while most age-related *de novo* mutations are paternal in origin, there is also a powerful maternal contribution (83). We hypothesize that this maternal factor could be the negative impact of age on the DNA repair capacity in the oocyte (201). Viewed in this light, the creation of *de novo* mutations could be regarded, at least in part, as a collusion between the male and female germ lines. In the male germ line, we see extensive evidence of oxidative DNA damage, the incidence of damage increasing with age as the DNA repair capacity of the germ line declines. This oxidatively damaged DNA is then brought into the oocyte by the fertilizing sperm, overwhelming the latter's capacity for effective DNA repair and stimulating the creation of *de novo* mutations that ultimately impact the health and well-being, and potentially the fertility, of the offspring.

## A Unifying Concept: Oxidative DNA Damage, Infertility and Offspring Health

So, we have a male infertility landscape which, until recently has been largely unexplained. However, in the past decade or so, it has become clear that both genetics and oxidative stress play important roles in the definition of semen quality. In the final section of this review we ask whether these two epidemiological pathways might be causally linked.

Clearly oxidative stress in the male germ line can be driven by a great many environmental, lifestyle and clinical factors ranging from smoking, obesity, electromagnetic radiation including heat, a variety of xenobiotic toxicants including the omnipresent phthalate esters and bisphenol A, varicocele, infection and autoimmunity [(202, 203); **Figure 5**]. As germ cells differentiate, they become progressively less adept at DNA repair and this ineptness only increases with paternal age (204). The oxidative damage sustained by these cells will, if it is sufficiently intense, suppress the fertilizing capacity of the spermatozoa by impairing their motility and their capacity for interaction with the oocyte (85). If the damage is less intense, these cells may still retain their capacity for fertilization and thus have the ability to carry oxidatively damaged DNA (194) and, potentially, oxidatively damaged centrioles (205) and telomeres (206) (guanine rich structures at the end of chromosomes that are known to be paternally inherited and vulnerable to oxidative stress), into the oocyte, all of which could disrupt embryonic development, implantation and the progress of pregnancy to term. Genetic and epigenetic mutations induced via this mechanism have the potential to impact the fertility, health and well-being of the offspring.

In terms of infertility, the DNA fragmentation precipitated by the induction of oxidative stress has the potential to create Y chromosome deletions through aberrant recombination of the repetitive sequences that abound on this chromosome. Stochastic point mutations introduced by such means also have the potential to induce infertility if they interfere with expression of key genes involved in orchestrating the production and differentiation of spermatozoa. The complex nature of these cells ensures that there are plenty of genes to choose from.

We have recently reported that when spermatozoa are exposed to an oxidative stress, DNA damage is generated across the genome as one might have anticipated, although the sex chromosomes are to some degree protected. Against this background there is, however, one area of the human sperm genome that is particularly vulnerable to oxidative attack on chromosome 15 (207). Such susceptible genomic sites

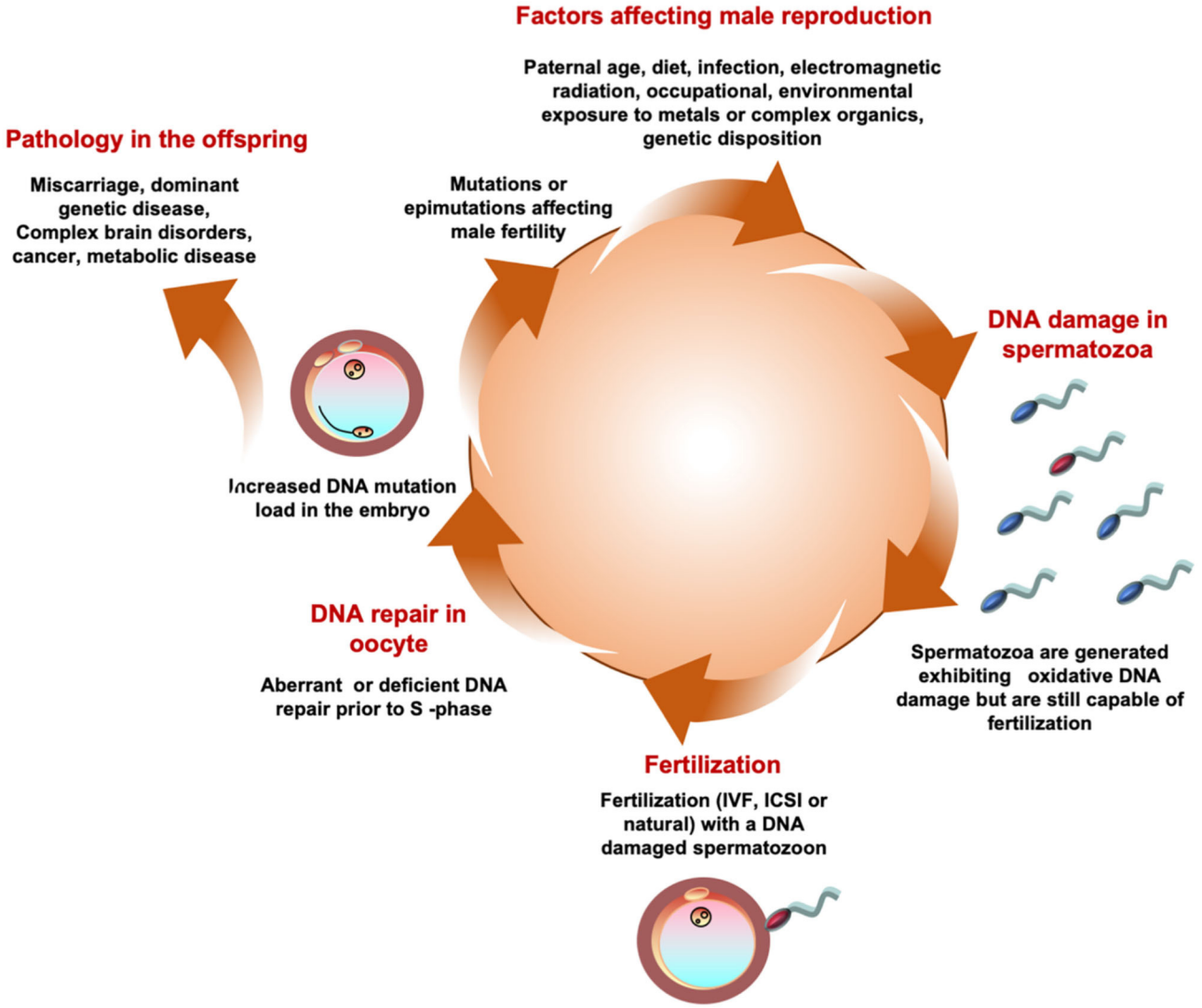


**FIGURE 5 |** A possible unifying hypothesis. According to this model a variety of environmental, lifestyle, and medical factors conspire to generate a state of oxidative stress in the male germ. Oxidaitve stress then induces DNA damage in human spermatozoa. A spermatozoon carrying high levels of oxidative DNA damage then fertilizes the egg and in the hours between fertilization and the initiation of S-phase of the first mitotic cell division the oocyte attempts to repair the DNA damage brought in by the fertilizing spermatozoon. If the oocyte makes a mistake at this point it may fix the paternal oxidative DNA damage as a mutation in the embryo. Such mutations may induce infertility in the offspring as a result of, for example, point mutations in genes that are critical for the reproductive process or Y-chromosome deletions. Alternatively, the oxidative DNA damage may occur in an area of the genome, such as chromosome 15, that does not frequently affect fertility but influences the health and well-being of the offspring inducing pathologies, including brain disorders, imprinting errors and cancers, that are known to have a powerful paternal component.

experienced a dramatic (2–15 fold) increase in their burden of oxidative DNA damage in patients undergoing infertility evaluation compared to normal healthy donors. Translocations affecting this area of the genome are known to cause male infertility (208) as are point mutations affecting genes on chromosome 15 such as SNRPA1. The latter is involved in establishing the spliceosome, a dynamic ribonucleoprotein complex responsible for orchestrating the processing of RNA during spermatogenesis, defects in which cause non-obstructive azoospermia (209). Catsper is also located in this area of the genome and defects in this critical sperm component are also associated with male infertility (210). The presence of small supernumerary marker chromosomes (sSMCs) is similarly associated with severe male infertility involving oligoasthenoteratozoospermia and most commonly involves chromosome 15 (211). Large deletions on chromosome 15 including the Catsper gene, and another gene also associated with spermatozoa, STRC (stereocilin), have also been linked to a rare condition characterized by male infertility and deafness (212). Interestingly, none of the other point mutations

associated with male infertility that were covered in the first part of this review, involve chromosome 15. Whether the observed mutations are involved in the central endocrine drive to spermatogenesis, the process of spermatogenesis itself or the production of morphologically normal motile spermatozoa capable of fertilization, they occur in virtually every part of the genome but not chromosome 15. Perhaps this is too vulnerable an area of the genome to sequester genes that are critical to reproductive success.

However, chromosome 15 does encode a number of genes involved neurological development. The very area of the genome that we have found to be vulnerable to oxidative attack is also the site of genetic perturbations associated with a variety of brain disorders including Marfan syndrome, epilepsy, spontaneous schizophrenia, bipolar disease, attention deficit hyperactivity disorder and, critically, autism (213). Interestingly, all of these conditions are highly correlated with the age of the father at the moment of conception. Since paternal age is associated with oxidative stress in the male germ line, we hypothesize that an age-related oxidative attack on the paternal germ line precipitated the mutations that ultimately led to these conditions appearing in the offspring (**Figure 5**).

Of course, aging is just one way of creating oxidative stress in germ cells, there are many others not least of which is infertility itself. Since male infertility is commonly associated with the kind of oxidative stress we associate with aging, we might anticipate an increase in conditions such as autism in the offspring of ART patients, particularly when ICSI is used as the insemination procedure. In keeping with this proposition, a report from the USA did indeed find an increased risk of autism in the progeny when ICSI, not IVF, was used to inseminate the oocytes (214). Similarly, the area of chromosome 15 which is vulnerable to oxidative attack also houses the imprinted genes responsible for Prader-Willi and Angelman syndromes and evidence exists to suggest that both of these conditions may elevated in the offspring of subfertile couples and may be exacerbated by assisted conception therapy (215). Although this association between assisted conception therapy and imprinting disorders is not consistently observed across all datasets, the 3.44-fold increase in Prader Willi syndrome observed by Hattori et al. (215) is particularly striking and could be explained by oxidative destruction of the paternal allele, allowing the maternal allele to dominate. Interestingly, deletions in this area of the genome are thought to involve DNA fragmentation followed by aberrant recombination of flanking repeat elements (END-repeats), in much the same way as deletions are induced on the Y-chromosome (216).

Smoking is another condition associated with oxidative stress in the male germ line, that is independent of age and generates 8OHdG lesions in human spermatozoa via mechanisms that can be exacerbated by antioxidant deficient diets and OGG1 Ser326Cys polymorphisms (217, 218). There are also data to indicate a significant association between paternal smoking and cancer in the offspring, particularly leukemias including acute lymphoblastic leukemia (219). Consistent with the data presented above, it is of interest that one of the loci associated with childhood leukemia is on chromosome 15 (15q13–15) (220). Fundamentally, oxidative DNA damage induced in human spermatozoa as a result of heavy smoking is probably responsible of introducing millions of *de novo* mutations into our species (221), with implications for the future incidence of childhood leukemias (and possibly other conditions) in affected lineages.

The genomic domain we have identified as being particularly susceptible to oxidative damage is known to be a hot spot for copy number variation (222) and microdeletions in 15q11.2 BP1-BP2 (the Burnside-Butler susceptibility locus) are known to associated with intellectual impairment (223). Thus, oxidative damage to this area of the genome in human spermatozoa may be associated with a range of phenotypes in the offspring including male infertility, imprinting disorders and a plethora of behavioral/intellectual defects. Any conditions that promote oxidative damage in human spermatozoa may increase the incidence of such conditions in the offspring depending on the severity and location of the damage and the efficiency of DNA repair.

## CLINICAL SIGNIFICANCE

What is the clinical significance of all this information? Fundamentally, this review is intended to highlight the potential significance of oxidative stress in both the etiology of male infertility and the mutational load subsequently carried by the offspring. The management of male infertility patients should therefore involve an assessment by the levels of oxidative stress and DNA damage suffered by the spermatozoa. If these parameters are elevated, then, and only then, should interventions be considered. The most obvious therapeutic intervention would be to give affected patients antioxidants and then monitor the levels of oxidative stress over time to ensure that this approach (or the particular antioxidant product selected) is being effective. It is important not to give antioxidants in the absence of a diagnosis of oxidative stress in order to avoid the induction of reductive stress, which can be just as damaging as its oxidative counterpart. From a preventative standpoint, it would also be important to carefully review the patient's medical history, occupation and lifestyle in an attempt to identify potential causes of the oxidative stress which could be addressed. Thus, the presence of a varicocele, infection, sedentary lifestyle, obesity, lack of exercise, poor diet, high scrotal temperatures, excessive consumption of recreational drugs including alcohol and tobacco and psychological stress, are all possible contributors to oxidative stress in infertility patients that might be addressed by simple lifestyle interventions. As far as genetic factors are concerned, the management options are more challenging because the damage has already been done in the germlines of the patients' forebearers and, in the absence of gene therapy, the only realistic option is IVF/ICSI. While this form treatment has enjoyed some success, knowing the genetic basis of the infertility (particularly for the most common genetic conditions such as cystic fibrosis gene mutations, Klinefelter syndrome, Y chromosome microdeletions, Noonan syndrome, and chromosomal translocations) may be important in counseling the patients on the likely health trajectory of their offspring and the possible benefits of using

donor spermatozoa. See Ferlin and Foresta (224) for further detailed discussion of these issues from a clinical perspective.

## CONCLUSION

In conclusion. a variety of environmental and lifestyle factors including age, smoking, infertility, obesity, exposure to a range of xenobiotic toxicants, radiofrequency electromagnetic radiation, heat and cryopreservation (213, 225, 226) conspire to generate oxidative DNA damage and fragmentation in the male germline. Inefficient or aberrant repair then fixes this damage as a mutation, either in the germline itself or in the newly fertilized oocyte. Mutations generated in this way can cause infertility in male offspring as a consequence of Y-chromosome deletions or a wide range of mutations in other parts of the genome that influence the production or functionality of the spermatozoa. In addition, these mutations can cause a range of other diseases particularly cancer and brain disorders, including autism and spontaneous schizophrenia. This tentative unifying hypothesis is summarized in **Figure 5** and provides us with a model we can test in future studies.

## AUTHOR CONTRIBUTIONS

RJA prepared the first draft of the manuscript which was then critically reviewed by MB. Both authors approve the final version and submission of this article.

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## REFERENCES

1. Aitken RJ. The male is significantly implicated as the cause of unexplained infertility. *Sem Reprod Med.* (2020). [Epub ahead of print].
2. Aitken RJ, Irvine DS, Wu FC. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol.* (1991) 164:542–51. doi: 10.1016/S0002-9378(11)80017-7
3. Kini S, Morrell D, Thong KJ, Kopakaki A, Hillier S, Irvine DS. Lack of impact of semen quality on fertilization in assisted conception. *Scott Med J.* (2010) 55:20–3. doi: 10.1258/RSMSMJ.55.1.20
4. Irvine DS, Aitken RJ. Predictive value of *in-vitro* sperm function tests in the context of an AID service. *Hum Reprod.* (1986) 1:539–45. doi: 10.1093/oxfordjournals.humrep.a136470
5. Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Prediction of the *in-vitro* fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Hum Reprod.* (1996) 11:1030–4. doi: 10.1093/oxfordjournals.humrep.a019291
6. Bostofte E, Serup J, Rebbe H. Relation between sperm count and semen volume, and pregnancies obtained during a twenty-year follow-up period. *Int J Androl.* (1982) 5:267–75. doi: 10.1111/j.1365-2605.1982.tb00255.x
7. Wallace EM, Aitken RJ, Wu FC. Residual sperm function in oligozoospermia induced by testosterone enanthate administered as a potential steroid male contraceptive. *Int J Androl.* (1992) 15:416–24. doi: 10.1111/j.1365-2605.1992.tb01356.x
8. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, et al. World health organization reference values for human semen characteristics. *Hum Reprod Update.* (2010) 16:231–45. doi: 10.1093/humupd/dmp048
9. Zinaman MJ, Brown CC, Selevan SG, Clegg ED. Semen quality and human fertility: a prospective study with healthy couples. *J Androl.* (2000) 21:145–53.
10. Aitken RJ, Kerr L, Bolton V, Hargreave T. Analysis of sperm function in globozoospermia: implications for the mechanism of sperm-zona interaction. *Fertil Steril.* (1990) 54:701–7. doi: 10.1016/S0015-0282(16)53833-3
11. Aitken RJ, Ross A, Lees MM. Analysis of sperm function in kartagener's syndrome. *Fertil Steril.* (1983) 40:696–8. doi: 10.1016/S0015-0282(16)47434-0
12. Tavalaee M, Nomikos M, Lai FA, Nasr-Esfahani MH. Expression of sperm PLCζ and clinical outcomes of ICSI-AOA in men affected by globozoospermia due to DPY19L2 deletion. *Reprod Biomed Online.* (2018) 36:348–355. doi: 10.1016/j.rbmo.2017.12.013
13. Talebi AR, Ghasemzadeh J, Khalili MA, Halvaei I, Fesahat F. Sperm chromatin quality and DNA integrity in partial versus total globozoospermia. *Andrologia.* (2018) 50:1. doi: 10.1111/and.12823
14. Dam AH, Ramos L, Dijkman HB, Woestenenk R, Robben H, van den Hoven L, et al. Morphology of partial globozoospermia. *J Andro.* (2011) 32:199–206. doi: 10.2164/jandrol.109.009530
15. Dam AH, Pijnenburg AJ, Hendriks JC, Westphal H, Ramos L, Kremer JA. Intracytoplasmic sperm injection in partial globozoospermia. *Fertil Steril.* (2012) 97:60–6. doi: 10.1016/j.fertnstert.2011.10.003
16. De Braekeleer M, Nguyen MH, Morel F, Perrin A. Genetic aspects of monomorphic teratozoospermia: a review. *J Assist Reprod Genet.* (2015) 32:615–23. doi: 10.1007/s10815-015-0433-2
17. Elinati E, Kuentz P, Redin C, Jaber S, Vanden Meerschaut F, Makarian J, et al. Globozoospermia is mainly due to DPY19L2 deletion via non-allelic homologous recombination involving two recombination hotspots. *Hum Mol Genet.* (2012) 21:3695–702. doi: 10.1093/hmg/dds200
18. Coutton C, Abada F, Karaouzene T, Sanlaville D, Satre V, Lunardi J, et al. Fine characterisation of a recombination hotspot at the DPY19L2 locus and resolution of the paradoxical excess of duplications over deletions in the general population. *PLoS Genet.* (2013) 9:e1003363. doi: 10.1371/journal.pgen.1003363
19. Ounis L, Zoghmar A, Coutton C, Rouabah L, Hachemi M, Martinez D, et al. Mutations of the aurora kinase C gene causing macrozoospermia are the most frequent genetic cause of male infertility in Algerian men. *Asian J Androl.* (2015) 17:68–73. doi: 10.4103/1008-682X.136441
20. Ben Khelifa M, Coutton C, Blum MG, Abada F, Harbuz R, Zouari R, et al. Identification of a new recurrent aurora kinase C mutation in both European and African men with macrozoospermia. *Hum Reprod.* (2012) 27:3337–46. doi: 10.1093/humrep/des296
21. Liu G, Wang N, Zhang H, Yin S, Dai H, Lin G, et al. Novel mutations in PMFBP1, TSGA10 and SUN5: expanding the spectrum of mutations that may cause acephalic spermatozoa. *Clin Genet.* (2020) 97:938–9. doi: 10.1111/cge.13747
22. Nie H, Tang Y, Qin W. Beyond acephalic spermatozoa: the complexity of intracytoplasmic sperm injection outcomes. *Biomed Res Int.* (2020) 2020:6279795. doi: 10.1155/2020/6279795
23. Sha Y, Wang X, Yuan J, Zhu X, Su Z, Zhang X, et al. Loss-of-function mutations in centrosomal protein 112 is associated with human acephalic spermatozoa phenotype. *Clin Genet.* (2020) 97:321–8. doi: 10.1111/cge.13662
24. Wu B, Gao H, Liu C, Li W. The coupling apparatus of the sperm head and tail. *Biol Reprod.* (2020) 102:988–98. doi: 10.1093/biolre/ioaa016
25. Fang J, Zhang J, Zhu F, Yang X, Cui Y, Liu J. Patients with acephalic spermatozoa syndrome linked to SUN5 mutations have

a favorable pregnancy outcome from ICSI. *Hum Reprod.* (2018) 33:372–7. doi: 10.1093/humrep/dex382
26. Shang Y, Zhu F, Wang L, Ouyang YC, Dong MZ, Liu C, et al. Essential role for SUN5 in anchoring sperm head to the tail. *eLife.* (2017) 6:e28199. doi: 10.7554/eLife.28199
27. Afzelius BA. The immotile-cilia syndrome and other ciliary diseases. *Int Rev Exp Pathol.* (1979) 19:1–43. doi: 10.1016/S0022-5320(79)80041-6
28. Afzelius BA. Genetical and ultrastructural aspects of the immotile-cilia syndrome. *Am J Hum Genet.* (1981) 33:852–64.
29. Zariwala MA, Gee HY, Kurkowiak M, Al-Mutairi DA, Leigh MW, Hurd TW, et al. ZMYND10 is mutated in primary ciliary dyskinesia and interacts with LRRC6. *Am J Hum Genet.* (2013) 93:336–45. doi: 10.1016/j.ajhg.2013.06.007
30. Onoufriadis A, Shoemark A, Munye MM, James CT, Schmidts M, Patel M, et al. Combined exome and whole-genome sequencing identifies mutations in ARMC4 as a cause of primary ciliary dyskinesia with defects in the outer dynein arm. *J Med Genet.* (2014) 51:61–7. doi: 10.1136/jmedgenet-2013-101938
31. Pereira R, Barbosa T, Gales L, Oliveira E, Santos R, Oliveira J, et al. Clinical and genetic analysis of children with Kartagener syndrome. *Cells.* (2019) 8:E900. doi: 10.3390/cells8080900
32. Yue Y, Huang Q, Zhu P, Zhao P, Tan X, Liu S, et al. Identification of pathogenic mutations and investigation of the notch pathway activation in Kartagener syndrome. *Front Genet.* (2019) 10:749. doi: 10.3389/fgene.2019.00749
33. Thomas L, Bouhouche K, Whitfield M, Thouvenin G, Coste A, Louis B, et al. TTC12 loss-of-function mutations cause primary ciliary dyskinesia and unveil distinct dynein assembly mechanisms in motile cilia versus flagella. *Am J Hum Genet.* (2020) 106:153–69. doi: 10.1016/j.ajhg.2019.12.010
34. Nijs M, Vanderzwalmen P, Vandamme B, Segal-Bertin G, Lejeune B, Segal L, et al. Fertilizing ability of immotile spermatozoa after intracytoplasmic sperm injection. *Hum Reprod.* (1996) 11:2180–5. doi: 10.1093/oxfordjournals.humrep.a019073
35. Vanaken GJ, Bassinet L, Boon M, Mani R, Honoré I, Papon JF, et al. Infertility in an adult cohort with primary ciliary dyskinesia: phenotype-gene association *Eur Respir J.* (2017) 50:1700314. doi: 10.1183/13993003.00314-2017
36. Lorès P, Coutton C, El Khouri E, Stouvenel L, Givelet M, Thomas L, et al. Homozygous missense mutation L673P in adenylate kinase 7 (AK7) leads to primary male infertility and multiple morphological anomalies of the flagella but not to primary ciliary dyskinesia. *Hum Mol Genet.* (2018) 27:1196–211. doi: 10.1093/hmg/ddy034
37. Kherraf ZE, Cazin C, Coutton C, Amiri-Yekta A, Martinez G, Boguenet M, et al. Whole exome sequencing of men with multiple morphological abnormalities of the sperm flagella reveals novel homozygous QRICH2 mutations. *Clin Genet.* (2019) 96:394–401. doi: 10.1111/cge.13604
38. Li W, He X, Yang S, Liu C, Wu H, Liu W, et al. Biallelic mutations of CFAP251 cause sperm flagellar defects and human male infertility. *J Hum Genet.* (2019) 64:49–54. doi: 10.1038/s10038-018-0520-1
39. He X, Li W, Wu H, Lv M, Liu W, Liu C, et al. Novel homozygous CFAP69 mutations in humans and mice cause severe asthenoteratospermia with multiple morphological abnormalities of the sperm flagella. *J Med Genet.* (2019) 56:96–103. doi: 10.1136/jmedgenet-2018-105486
40. Beurois J, Martinez G, Cazin C, Kherraf ZE, Amiri-Yekta A, Thierry-Mieg N, et al. CFAP70 mutations lead to male infertility due to severe astheno-teratozoospermia. A case report. *Hum Reprod.* (2019) 34:2071–9. doi: 10.1093/humrep/dez166
41. Zhang X, Shen Y, Wang X, Yuan G, Zhang C, Yang Y. A novel homozygous CFAP65 mutation in humans causes male infertility with multiple morphological abnormalities of the sperm flagella. *Clin Genet.* (2019) 96:541–8. doi: 10.1111/cge.13644
42. Wu H, Li W, He X, Liu C, Fang Y, Zhu F, et al. Novel CFAP43 and CFAP44 mutations cause male infertility with multiple morphological abnormalities of the sperm flagella (MMAF). *Reprod Biomed Online.* (2019) 38:769–78. doi: 10.1016/j.rbmo.2018.12.037
43. Li L, Feng F, Wang Y, Guo J, Yue W. Mutational effect of human CFAP43 splice-site variant causing multiple morphological abnormalities of the sperm flagella. *Andrologia.* (2020) 52:e13575. doi: 10.1111/and.13575
44. Martinez G, Beurois J, Dacheux D, Cazin C, Bidart M, Kherraf ZE, et al. Biallelic variants in MAATS1 encoding CFAP91, a calmodulin-associated and spoke-associated complex protein, cause severe astheno-teratozoospermia and male infertility. *J Med Genet.* (2020) S1698-031X(19)30081-0. doi: 10.1136/jmedgenet-2019-106775
45. Ni X, Wang J, Lv M, Liu C, Zhong Y, Tian S, et al. A novel homozygous mutation in WDR19 induces disorganization of microtubules in sperm flagella and nonsyndromic asthenoteratospermia. *J Assist Reprod Genet.* (2020) 37:1431–9. doi: 10.1007/s10815-020-01770-1
46. Lv M, Liu W, Chi W, Ni X, Wang J, Cheng H, et al. Homozygous mutations in DZIP1 can induce asthenoteratospermia with severe MMAF. *J Med Genet.* (2020) 57:445–53. doi: 10.1136/jmedgenet-2019-106479
47. Sha Y, Yang X, Mei L, Ji Z, Wang X, Ding L, et al. DNAH1 gene mutations and their potential association with dysplasia of the sperm fibrous sheath and infertility in the Han Chinese population. *Fertil Steril.* (2017) 107:1312–8.e2. doi: 10.1016/j.fertnstert.2017.04.007
48. Li Y, Sha Y, Wang X, Ding L, Liu W, Ji Z, et al. DNAH2 is a novel candidate gene associated with multiple morphological abnormalities of the sperm flagella. *Clin Genet.* (2019) 95:590–600. doi: 10.1111/cge.13525
49. Tu C, Nie H, Meng L, Yuan S, He W, Luo A, et al. Identification of DNAH6 mutations in infertile men with multiple morphological abnormalities of the sperm flagella. *Sci Rep.* (2019) 9:15864. doi: 10.1038/s41598-019-52436-7
50. Sha Y, Wei X, Ding L, Mei L, Huang X, Lin S, et al. DNAH17 is associated with asthenozoospermia and multiple morphological abnormalities of sperm flagella. *Ann Hum Genet.* (2020) 84:271–9. doi: 10.1111/ahg.12369
51. Liu C, He X, Liu W, Yang S, Wang L, Li W, et al. Bi-allelic mutations in TTC29 cause male subfertility with asthenoteratospermia in humans and mice. *Am J Hum Genet.* (2019) 105:1168–81. doi: 10.1016/j.ajhg.2019.10.010
52. Liu W, He X, Yang S, Zouari R, Wang J, Wu H, et al. Bi-allelic mutations in TTC21A induce asthenoteratospermia in humans and mice. *Am J Hum Genet.* (2019) 104:738–48. doi: 10.1016/j.ajhg.2019.02.020
53. Coutton C, Martinez G, Kherraf ZE, Amiri-Yekta A, Boguenet M, Saut A, et al. Bi-allelic mutations in ARMC2 lead to severe astheno-teratozoospermia due to sperm flagellum malformations in humans and mice. *Am J Hum Genet.* (2019) 104:331–40. doi: 10.1016/j.ajhg.2018.12.013
54. Sha YW, Xu X, Mei LB, Li P, Su ZY, He XQ, et al. A homozygous CEP135 mutation is associated with multiple morphological abnormalities of the sperm flagella (MMAF). *Gene.* (2017) 633:48–53. doi: 10.1016/j.gene.2017.08.033
55. Martinez G, Kherraf ZE, Zouari R, Fourati Ben Mustapha S, Saut A, Pernet-Gallay K, et al. Whole-exome sequencing identifies mutations in FSIP2 as a recurrent cause of multiple morphological abnormalities of the sperm flagella. *Hum Reprod.* (2018) 33:1973–84. doi: 10.1093/humrep/dey264
56. Moye AR, Bedoni N, Cunningham JG, Sanzhaeva U, Tucker ES, Mathers P, et al. Mutations in ARL2BP, a protein required for ciliary microtubule structure, cause syndromic male infertility in humans and mice. *PLoS Genet.* (2019) 15:e1008315. doi: 10.1371/journal.pgen.1008315
57. Tu C, Nie H, Meng L, Wang W, Li H, Yuan S, et al. Novel mutations in SPEF2 causing different defects between flagella and cilia bridge: the phenotypic link between MMAF and PCD. *Hum Genet.* (2020) 139:257–71. doi: 10.1007/s00439-020-02110-0
58. Li WN, Zhu L, Jia MM, Yin SL, Lu GX, Liu G. Missense mutation in DNAJB13 gene correlated with male fertility in asthenozoospermia. *Andrology.* (2020) 8:299–306. doi: 10.1111/andr.12685
59. Yuan P, Yang C, Ren Y, Yan J, Nie Y, Yan L, et al. A novel homozygous mutation of phospholipase C zeta leading to defective human oocyte activation and fertilization failure. *Hum Reprod.* (2020) 35:977–85. doi: 10.1093/humrep/dez293
60. Brown SG, Miller MR, Lishko PV, Lester DH, Publicover SJ, Barratt CLR, et al. Homozygous in-frame deletion in CATSPERE in a man producing spermatozoa with loss of CatSper function and compromised fertilizing capacity. *Hum Reprod.* (2018) 33:1812–6. doi: 10.1093/humrep/dey278
61. Mao GH, Wang YN, Xu M, Wang WL, Tan L, Tao SB. Polymorphisms in the MT-ATP6 and MT-CYB genes in *in vitro* fertilization failure. *Mitochondrial DNA.* (2015) 26:20–4. doi: 10.3109/19401736.2013.840612
62. Lissens W, Mercier B, Tournaye H, Bonduelle M, Férec C, Seneca S, et al. Cystic fibrosis and infertility caused by congenital bilateral absence of

the vas deferens and related clinical entities. *Hum Reprod.* (1996) 11(Suppl. 4):55–80. doi: 10.1093/humrep/11.suppl_4.55
63. Twa DDW, Mottok A, Savage KJ, Steidl C. The pathobiology of primary testicular diffuse large B-cell lymphoma: implications for novel therapies. *Blood Rev.* (2018) 32:249–55. doi: 10.1016/j.blre.2017.12.001
64. Shen H, Shih J, Hollern DP, Wang L, Bowlby R, Tickoo SK, et al. Integrated molecular characterization of testicular germ cell tumors. *Cell Rep.* (2018) 23:3392–406. doi: 10.1016/j.celrep.2018.05.039
65. Taylor-Weiner A, Zack T, O'Donnell E, Guerriero JL, Bernard B, Reddy A, et al. Genomic evolution and chemoresistance in germ-cell tumours. *Nature.* (2016) 540:114–8. doi: 10.1038/nature20596
66. Chapuy B, Roemer MG, Stewart C, Tan Y, Abo RP, Zhang L, et al. Targetable genetic features of primary testicular and primary central nervous system lymphomas. *Blood.* (2016) 127:869–81. doi: 10.1182/blood-2015-10-673236
67. Kasak L, Laan M. Monogenic causes of non-obstructive azoospermia: challenges, established knowledge, limitations and perspectives. *Hum Genet.* (2020). doi: 10.1007/s00439-020-02112-y
68. Jedidi I, Ouchari M, Yin Q. Autosomal single-gene disorders involved in human infertility. *Saudi J Biol Sci.* (2018) 25:881–7. doi: 10.1016/j.sjbs.2017.12.005
69. Auchus RJ. Steroid 17-hydroxylase and 17,20-lyase deficiencies, genetic and pharmacologic. *J Steroid Biochem Mol Biol.* (2017) 165:71–8. doi: 10.1016/j.jsbmb.2016.02.002
70. Alkhalaf M, Verghese L, Muharib N. A cytogenetic study of Kuwaiti couples with infertility and reproductive disorders: short arm deletion of chromosome 21 is associated with male infertility. *Ann Genet.* (2002) 45:147–9. doi: 10.1016/S0003-3995(02)01110-3
71. Gonçalves CI, Fonseca F, Borges T, Cunha F, Lemos MC. Expanding the genetic spectrum of ANOS1 mutations in patients with congenital hypogonadotropic hypogonadism. *Hum Reprod.* (2017) 32:704–711. doi: 10.1093/humrep/dew354
72. Yatsenko AN, Georgiadis AP, Röpke A, Berman AJ, Jaffe T, Olszewska M, et al. X-linked TEX11 mutations, meiotic arrest, and azoospermia in infertile men. *N Engl J Med.* (2015) 372:2097–107. doi: 10.1056/NEJMoa1406192
73. Vargas MCC, Moura FS, Elias CP, Carvalho SR, Rassi N, Kunii IS, et al. Spontaneous fertility and variable spectrum of reproductive phenotype in a family with adult-onset X-linked adrenal insufficiency harboring a novel DAX-1/NR0B1 mutation. *BMC Endocr Disord.* (2020) 20:21. doi: 10.1186/s12902-020-0500-2
74. Ferlin A, Vinanzi C, Garolla A, Selice R, Zuccarello D, Cazzadore C, et al. Male infertility and androgen receptor gene mutations: clinical features and identification of seven novel mutations. *Clin Endocrinol.* (2006) 65:606–10. doi: 10.1111/j.1365-2265.2006.02635.x
75. Kim SY, Lee BY, Oh AR, Park SY, Lee HS, Seo JT. Clinical, hormonal, and genetic evaluation of idiopathic nonobstructive azoospermia and Klinefelter syndrome patients. *Cytogenet Genome Res.* (2017) 153:190–7. doi: 10.1159/000487039
76. Gumus E, Kati B, Pelit ES, Ordek E, Ciftci H. A different look at genetic factors in individuals with non-obstructive azoospermia or oligospermia in our research study: to whom, which threshold, when, in what way? *Rev Int Androl.* (2020). doi: 10.1016/j.androl.2019.08.001
77. Salahshourifar I, Gilani MA, Vosough A, Tavakolzadeh T, Tahsili M, Mansori Z, et al. *De novo* complex chromosomal rearrangement of 46, XY, t (3; 16; 8) (p26; q13; q21.2) in a non-obstructive azoospermic male. *J Appl Genet.* (2007) 48:93–4. doi: 10.1007/BF03194664
78. Hughes JF, Skaletsky H, Pyntikova T, Minx PJ, Graves T, Rozen S, et al. Conservation of Y-linked genes during human evolution revealed by comparative sequencing in chimpanzee. *Nature.* (2005) 437:100–3. doi: 10.1038/nature04101
79. Bachtrog D. Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. *Nat Rev Genet.* (2013) 14:113–24. doi: 10.1038/nrg3366
80. Colaco S, Modi D. Genetics of the human Y chromosome and its association with male infertility. *Reprod Biol Endocrinol.* (2018) 16:14. doi: 10.1186/s12958-018-0330-5
81. Li Z, Haines CJ, Han Y. Micro-deletions of the human Y chromosome and their relationship with male infertility. *J Genet Genomics.* (2008) 35:193–9. doi: 10.1016/S1673-8527(08)60027-2
82. Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction.* (2001) 122:497–506. doi: 10.1530/reprod/122.4.497
83. Gao Z, Moorjani P, Sasani TA, Pedersen BS, Quinlan AR, Jorde LB, et al. Overlooked roles of DNA damage and maternal age in generating human germline mutations. *Proc Natl Acad Sci USA.* (2019) 116:9491–500. doi: 10.1073/pnas.1901259116
84. Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril.* (1979) 31:531–7. doi: 10.1016/S0015-0282(16)43999-3
85. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod.* (1989) 41:183–97. doi: 10.1095/biolreprod41.1.183
86. Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. *J Androl.* (1987) 8:338–48. doi: 10.1002/j.1939-4640.1987.tb00973.x
87. Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertil Steril.* (2000) 73:459–64. doi: 10.1016/S0015-0282(99)00567-1
88. Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, Wu FC. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl.* (1989) 10:214–20. doi: 10.1002/j.1939-4640.1989.tb00091.x
89. Griveau JF, Dumont E, Renard P, Callegari JP, Le Lannou D. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *J Reprod Fertil.* (1995) 103:17–26. doi: 10.1530/jrf.0.1030017
90. Aitken RJ, De Iuliis GN. Origins and consequences of DNA damage in male germ cells. *Reprod Biomed Online.* (2007) 14:727–33. doi: 10.1016/S1472-6483(10)60676-1
91. Aitken RJ, Buckingham DW, West K, Brindle J. On the use of paramagnetic beads and ferrofluids to assess and eliminate the leukocytic contribution to oxygen radical generation by human sperm suspensions. *Am J Reprod Immunol.* (1996) 35:541–51. doi: 10.1111/j.1600-0897.1996.tb00055.x
92. Aitken RJ, Baker MA. Oxidative stress, spermatozoa and leukocytic infiltration: relationships forged by the opposing forces of microbial invasion and the search for perfection. *J Reprod Immunol.* (2013) 100:11–9. doi: 10.1016/j.jri.2013.06.005
93. Fraczek M, Kurpisz M. Mechanisms of the harmful effects of bacterial semen infection on ejaculated human spermatozoa: potential inflammatory markers in semen. *Folia Histochem Cytobiol.* (2015) 53:201–17. doi: 10.5603/fhc.a2015.0019
94. Aitken RJ, West K, Buckingham D. Leukocytic infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function. *J Androl.* (1994) 15:343–52.
95. Saleh RA, Agarwal A, Kandirali E, et al. Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fertil Steril.* (2002) 78:1215–24. doi: 10.1016/S0015-0282(02)04237-1
96. Krausz C, Mills C, Rogers S, Tan SL, Aitken RJ. Stimulation of oxidant generation by human sperm suspensions using phorbol esters and formyl peptides: relationships with motility and fertilization *in vitro*. *Fertil Steril.* (1994) 62:599–605. doi: 10.1016/S0015-0282(16)56952-0
97. Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Predicting the fertilizing potential of human sperm suspensions *in vitro*: importance of sperm morphology and leukocyte contamination. *Fertil Steril.* (1995) 63:1293–300. doi: 10.1016/S0015-0282(16)57614-6
98. Baker HW, Brindle J, Irvine DS, Aitken RJ. Protective effect of antioxidants on the impairment of sperm motility by activated polymorphonuclear leukocytes. *Fertil Steril.* (1996) 65:411–9. doi: 10.1016/S0015-0282(16)58109-6
99. Armstrong JS, Bivalacqua TJ, Chamulitrat W, Sikka S, Hellstrom WJ. A comparison of the NADPH oxidase in human sperm and white blood cells. *Int J Androl.* (2002) 25:223–9. doi: 10.1046/j.1365-2605.2002.00351.x

100. Aitken RJ, West KM. Analysis of the relationship between reactive oxygen species production and leucocyte infiltration in fractions of human semen separated on percoll gradients. *Int J Androl.* (1990) 13:433–51. doi: 10.1111/j.1365-2605.1990.tb01051.x
101. Aitken RJ, Baker MA, O'Bryan M. Shedding light on chemiluminescence: the application of chemiluminescence in diagnostic andrology. *J Androl.* (2004) 25:455–65. doi: 10.1002/j.1939-4640.2004.tb02815.x
102. Netherton JK, Hetherington L, Ogle RA, Gavgani MM, Velkov T, Villaverde AIB, et al. Mass spectrometry reveals new insights into the production of superoxide anions and 4-hydroxynonenal adducted proteins in human sperm. *Proteomics.* (2020) 20:e1900205. doi: 10.1002/pmic.201900205
103. Zielonka J, Vasquez-Vivar J, Kalyanaraman B. Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nat Protoc.* (2008) 3:8–21. doi: 10.1038/nprot.2007.473
104. Lavi R, Shainberg A, Shneyvays V, Hochauser E, Isaac A, Zinman T, et al. Detailed analysis of reactive oxygen species induced by visible light in various cell types. *Lasers Surg Med.* (2010) 42:473–80. doi: 10.1002/lsm.20919
105. Kumar PG, Laloraya M, Laloraya MM. Superoxide radical level and superoxide dismutase activity changes in maturing mammalian spermatozoa. *Andrologia.* (1991) 23:171–5. doi: 10.1111/j.1439-0272.1991.tb02528.x
106. Zhang H, Zheng RL. Promotion of human sperm capacitation by superoxide anion. *Free Radic Res.* (1996) 24:261–8. doi: 10.3109/10715769609088023
107. de Lamirande E, Gagnon C. Capacitation-associated production of superoxide anion by human spermatozoa. *Free Radic Biol Med.* (1995) 18:487–95. doi: 10.1016/0891-5849(94)00169-K
108. Sakamoto T, Imai H. Hydrogen peroxide produced by superoxide dismutase SOD-2 activates sperm in *Caenorhabditis elegans*. *J Biol Chem.* (2017) 292:14804–13. doi: 10.1074/jbc.M117.788901
109. Bize I, Santander G, Cabello P, Driscoll D, Sharpe C. Hydrogen peroxide is involved in hamster sperm capacitation *in vitro*. *Biol Reprod.* (1991) 44:398–403. doi: 10.1095/biolreprod44.3.398
110. Purdey MS, Connaughton HS, Whiting S, Schartnera EP, Monroa TM, Thompson JG, et al. Boronate probes for the detection of hydrogen peroxide release from human spermatozoa. *Free Radic Biol Med.* (2015) 81:69–76. doi: 10.1016/j.freeradbiomed.2015.01.015
111. Herrero MB, de Lamirande E, Gagnon C. Nitric oxide is a signaling molecule in spermatozoa. *Curr Pharm Des.* (2003) 9:419–25. doi: 10.2174/1381612033391720
112. Herrero MB, de Lamirande E, Gagnon C. Tyrosine nitration in human spermatozoa: a physiological function of peroxynitrite, the reaction product of nitric oxide and superoxide. *Mol Hum Reprod.* (2001) 7:913–21. doi: 10.1093/molehr/7.10.913
113. Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab.* (2008) 93:3199–207. doi: 10.1210/jc.2007-2616
114. Koppers AJ, Mitchell LA, Wang P, Lin M, Aitken RJ. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. *Biochem J.* (2011) 436:687–98. doi: 10.1042/BJ20110114
115. Pujianto DA, Curry BJ, Aitken RJ. Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. *Endocrinology.* (2010) 151:1269–79. doi: 10.1210/en.2009-0964
116. Valdivia A, Cortés L, Beitia M, Totorikaguena L, Agirregoitia N, Corcostegui B, et al. Role of angiotensin-(1–7) via MAS receptor in human sperm motility and acrosome reaction. *Reproduction.* (2020) 159:241–9. doi: 10.1530/REP-19-0274
117. Matsuura R, Takeuchi T, Yoshida A. Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. *Asian J Androl.* (2010) 12:753–9. doi: 10.1038/aja.2010.46
118. Krzyzosiak J, McMillan G, Molan P, Vishwanath R. Protein tyrosine phosphorylation during prolonged *in vitro* incubation of ejaculated bovine spermatozoa is regulated by the oxidative state of the medium. *Biol Reprod.* (2000) 62:1615–23. doi: 10.1095/biolreprod62.6.1615
119. Vishwanath R, Shannon P. Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. *Reprod Fertil Dev.* (1997) 9:321–31. doi: 10.1071/R96088
120. Chi HJ, Kim JH, Ryu CS, et al. Protective effect of antioxidant supplementation in sperm-preparation medium against oxidative stress in human spermatozoa. *Hum Reprod.* (2008) 23:1023–8. doi: 10.1093/humrep/den060
121. Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS, De Iuliis GN. Sperm motility is lost *in vitro* as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biol Reprod.* (2012) 87:110. doi: 10.1095/biolreprod.112.102020
122. Calamera JC, Fernandez PJ, Buffone MG, Acosta AA, Doncel GF. Effects of long-term *in vitro* incubation of human spermatozoa: functional parameters and catalase effect. *Andrologia.* (2001) 33:79–86. doi: 10.1046/j.1439-0272.2001.00409.x
123. Liu T, Han Y, Zhou T, et al. Mechanisms of ROS-induced mitochondria-dependent apoptosis underlying liquid storage of goat spermatozoa. *Aging.* (2019) 11:7880–98. doi: 10.18632/aging.102295
124. Zhu Z, Kawai T, Umehara T, Hoque SAM, Zeng W, Shimada M. Negative effects of ROS generated during linear sperm motility on gene expression and ATP generation in boar sperm mitochondria. *Free Radic Biol Med.* (2019) 141:159–71. doi: 10.1016/j.freeradbiomed.2019.06.018
125. Swegen A, Lambourne SR, Aitken RJ, Gibb Z. Rosiglitazone improves stallion sperm motility, ATP content, and mitochondrial function. *Biol Reprod.* (2016) 95:107. doi: 10.1095/biolreprod.116.142687
126. Aitken RJ, Wingate JK, De Iuliis GN, Koppers AJ, McLaughlin EA. Cis-unsaturated fatty acids stimulate reactive oxygen species generation and lipid peroxidation in human spermatozoa. *J Clin Endocrinol Metab.* (2006) 91:4154–63. doi: 10.1210/jc.2006-1309
127. Koppers AJ, Garg ML, Aitken RJ. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radic Biol Med.* (2010) 48:112–9. doi: 10.1016/j.freeradbiomed.2009.10.033
128. Samarasinghe SVAC, Krishnan K, Naidu R, Megharaj M, Miller K, Fraser B, et al. Parabens generate reactive oxygen species in human spermatozoa. *Andrology.* (2018) 6:532–41. doi: 10.1111/andr.12499
129. Barbonetti A, Castellini C, Di Giammarco N, Santilli G, Francavilla S, Francavilla F. *In vitro* exposure of human spermatozoa to bisphenol A induces pro-oxidative/apoptotic mitochondrial dysfunction. *Reprod Toxicol.* (2016) 66:61–7. doi: 10.1016/j.reprotox.2016.09.014
130. Aitken RJ, Muscio L, Whiting S, Connaughton HS, Fraser BA, Nixon B, et al. Analysis of the effects of polyphenols on human spermatozoa reveals unexpected impacts on mitochondrial membrane potential, oxidative stress and DNA integrity; implications for assisted reproductive technology. *Biochem Pharmacol.* (2016) 121:78–96. doi: 10.1016/j.bcp.2016.09.015
131. Fu LL, Zhang LY, An Q, Zhou F, Tiong Y, Guo Y, et al. L-carnitine protects the motion parameters and mitochondrial function of human sperm in cryopreservation. *Zhonghua Nan Ke Xue.* (2018) 24:1059-63.
132. Zhang X, Lu X, Li J, Xia Q, Gao J, Wu B. Mito-tempo alleviates cryodamage by regulating intracellular oxidative metabolism in spermatozoa from asthenozoospermic patients. *Cryobiology.* (2019) 91:18–22. doi: 10.1016/j.cryobiol.2019.11.005
133. Houston BJ, Nixon B, King BV, De Iuliis GN, Aitken RJ. The effects of radiofrequency electromagnetic radiation on sperm function. *Reproduction.* (2016) 152:R263–76. doi: 10.1530/REP-16-0126
134. De Iuliis GN, Newey RJ, King BV, Aitken RJ. Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa *in vitro*. *PLoS ONE.* (2009) 4:e6446. doi: 10.1371/journal.pone.0006446
135. Santini SJ, Cordone V, Falone S, Mijit M, Tatone C, Amicarelli F, et al. Role of mitochondria in the oxidative stress induced by electromagnetic fields: focus on reproductive systems. *Oxid Med Cell Longev.* (2018) 2018:5076271. doi: 10.1155/2018/5076271
136. Agarwal A, Desai NR, Makker K, Varghese A, Mouradi R, Sabanegh E, et al. Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: an *in vitro* pilot study. *Fertil Steril.* (2009) 92:1318–25. doi: 10.1016/j.fertnstert.2008.08.022

137. Liu C, Duan W, Xu S, Chen C, He M, Zhang L, et al. Exposure to 1800 MHz radiofrequency electromagnetic radiation induces oxidative DNA base damage in a mouse spermatocyte-derived cell line. *Toxicol Lett.* (2013) 218:2–9. doi: 10.1016/j.toxlet.2013.01.003
138. Liu Q, Si T, Xu X, Liang F, Wang L, Pan S. Electromagnetic radiation at 900 MHz induces sperm apoptosis through bcl-2, bax and caspase-3 signaling pathways in rats. *Reprod Health.* (2015) 12:65. doi: 10.1186/s12978-015-0062-3
139. Houston BJ, Nixon B, King BV, Aitken RJ, De Iuliis GN. Probing the origins of 1,800 MHz radio frequency electromagnetic radiation induced damage in mouse immortalized germ cells and spermatozoa *in vitro*. *Front Public Health.* (2018) 6:270. doi: 10.3389/fpubh.2018.00270
140. Gautam R, Singh KV, Nirala J, Murmu NN, Meena R, Rajamani P. Oxidative stress-mediated alterations on sperm parameters in male wistar rats exposed to 3G mobile phone radiation. *Andrologia.* (2019) 51:e13201. doi: 10.1111/and.13201
141. Amaral S, Redmann K, Sanchez V, Mallidis C, Ramalho-Santos J, Schlatt S. UVB irradiation as a tool to assess ROS-induced damage in human spermatozoa. *Andrology.* (2013) 1:707–14. doi: 10.1111/j.2047-2927.2013.00098.x
142. Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P, et al. Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol Hum Reprod.* (2015) 21:502–15. doi: 10.1093/molehr/gav014
143. Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J Biol Chem*. (2012) 287:33048–60. doi: 10.1074/jbc.M112.366690
144. Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, et al. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev*. (1997) 47:468–82. doi: 10.1002/(SICI)1098-2795(199708)47:4<468::AID-MRD14>3.0.CO;2-S
145. Miraglia E, Lussiana C, Viarisio D, Racca C, Cipriani A, Gazzano E, et al. The pentose phosphate pathway plays an essential role in supporting human sperm capacitation. *Fertil Steril.* (2010) 93:2437–40. doi: 10.1016/j.fertnstert.2009.09.005
146. Richer SC, Ford WC. A critical investigation of NADPH oxidase activity in human spermatozoa. *Mol Hum Reprod.* (2001) 7:237–44. doi: 10.1093/molehr/7.3.237
147. Ford WC. Regulation of sperm function by reactive oxygen species. *Hum Reprod Update.* (2004) 10:387–99. doi: 10.1093/humupd/dmh034
148. Bánfi B, Molnár G, Maturana A, Steger K, Hegedûs B, Demaurex N, et al. A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem.* (2001) 276:37594–601. doi: 10.1074/jbc.M103034200
149. Musset B, Clark RA, DeCoursey TE, Petheo GL, Geiszt M, Chen Y, et al. NOX5 in human spermatozoa: expression, function, and regulation. *J Biol Chem.* (2012) 287:9376–88. doi: 10.1074/jbc.M111.314955
150. du Plessis SS, McAllister DA, Luu A, Savia J, Agarwal A, Lampiao F. Effects of H(2)O(2) exposure on human sperm motility parameters, reactive oxygen species levels and nitric oxide levels. *Andrologia.* (2010) 42:206–10. doi: 10.1111/j.1439-0272.2009.00980.x
151. Mahfouz RZ, du Plessis SS, Aziz N, Sharma R, Sabanegh E, Agarwal A. Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress. *Fertil Steril.* (2010) 93:814–21. doi: 10.1016/j.fertnstert.2008.10.068
152. de Lamirande E, Lamothe G. Reactive oxygen-induced reactive oxygen formation during human sperm capacitation. *Free Radic Biol Med.* (2009) 46:502–10. doi: 10.1016/j.freeradbiomed.2008.11.004
153. Rivlin J, Mendel J, Rubinstein S, Etkovitz N, Breitbart H. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biol Reprod.* (2004) 70:518–22. doi: 10.1095/biolreprod.103.020487
154. Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. *J Cell Sci.* (1998) 111:645–56.
155. Lewis B, Aitken RJ. A redox-regulated tyrosine phosphorylation cascade in rat spermatozoa. *J Androl.* (2001) 22:611–22.
156. Baker MA, Hetherington L, Curry B, Aitken RJ. Phosphorylation and consequent stimulation of the tyrosine kinase c-Abl by PKA in mouse spermatozoa; its implications during capacitation. *Dev Biol.* (2009) 333:57–66. doi: 10.1016/j.ydbio.2009.06.022
157. Vatannejad A, Tavilani H, Sadeghi MR, Karimi M, Lakpour N, Amanpour S, et al. Evaluation of the NOX5 protein expression and oxidative stress in sperm from asthenozoospermic men compared to normozoospermic men. *J Endocrinol Invest.* (2019) 42:1181–9. doi: 10.1007/s40618-019-01035-4
158. Ghani E, Keshtgar S, Habibagahi M, Ghannadi A, Kazeroni M. Expression of NOX5 in human teratozoospermia compared to normozoospermia. *Andrologia.* (2013) 45:351–6. doi: 10.1111/and.12023
159. Ghanbari H, Keshtgar S, Zare HR, Gharesi-Fard B. Inhibition of CatSper and Hv1 channels and NOX5 enzyme affect progesterone-induced increase of intracellular calcium concentration and ROS generation in human sperm. *Iran J Med Sci.* (2019) 44:127–34.
160. Kirichok Y, Navarro B, Clapham DE. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. *Nature.* (2006) 439:737–40. doi: 10.1038/nature04417
161. Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl.* (1996) 17:276–87.
162. Ecroyd HW, Jones RC, Aitken RJ. Endogenous redox activity in mouse spermatozoa and its role in regulating the tyrosine phosphorylation events associated with sperm capacitation. *Biol Reprod.* (2003) 69:347–54. doi: 10.1095/biolreprod.102.012716
163. Fischer KA, Van Leyen K, Lovercamp KW, Manandhar G, Sutovsky M, Feng D, et al. 15-Lipoxygenase is a component of the mammalian sperm cytoplasmic droplet. *Reproduction*. (2005) 130:213–22. doi: 10.1530/rep.1.00646
164. Roy P, Roy SK, Mitra A, Kulkarni AP. Superoxide generation by lipoxygenase in the presence of NADH and NADPH. *Biochim Biophys Acta*. (1994) 1214:171–9. doi: 10.1016/0005-2760(94)90041-8
165. Bromfield EG, Mihalas BP, Dun MD, Aitken RJ, McLaughlin EA, Walters JLH, et al. Inhibition of arachidonate 15-lipoxygenase prevents 4-hydroxynonenal-induced protein damage in male germ cells. *Biol Reprod.* (2017) 96:598–609. doi: 10.1093/biolre/iox005
166. Walters JLH, De Iuliis GN, Dun MD, Aitken RJ, McLaughlin EA, Nixon B, et al. Pharmacological inhibition of arachidonate 15-lipoxygenase protects human spermatozoa against oxidative stress. *Biol Reprod.* (2018) 98:784–94. doi: 10.1093/biolre/ioy058
167. Aitken RJ, Harkiss D, Buckingham DW. Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol Reprod Dev*. (1993) 35:302–15. doi: 10.1002/mrd.1080350313
168. Tosic J, Walton A. Formation of hydrogen peroxide by spermatozoa and its inhibitory effect of respiration. *Nature.* (1946) 158:485. doi: 10.1038/158485a0
169. Tosic J, Walton A. Metabolism of spermatozoa. The formation and elimination of hydrogen peroxide by spermatozoa and effects on motility and survival. *Biochem J.* (1950) 47:199–212. doi: 10.1042/bj0470199
170. Aitken JB, Naumovski N, Curry B, Grupen CG, Gibb Z, Aitken RJ. Characterization of an L-amino acid oxidase in equine spermatozoa. *Biol Reprod.* (2015) 92:125. doi: 10.1095/biolreprod.114.126052
171. Upreti GC, Jensen K, Munday R, Duganzich DM, Vishwanath R, Smith JF. Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. *Anim Reprod Sci.* (1998) 51:275–87. doi: 10.1016/S0378-4320(98)00082-7
172. Houston B, Curry B, Aitken RJ. Human spermatozoa possess an IL4I1 l-amino acid oxidase with a potential role in sperm function. *Reproduction.* (2015) 149:587–96. doi: 10.1530/REP-14-0621
173. Fernandez MC, Yu A, Moawad AR, O'Flaherty C. Peroxiredoxin 6 regulates the phosphoinositide 3-kinase/AKT pathway to maintain human sperm viability. *Mol Hum Reprod*. (2019) 25:787–96. doi: 10.1093/molehr/gaz060

174. Williams AC, Ford WC. Functional significance of the pentose phosphate pathway and glutathione reductase in the antioxidant defenses of human sperm. *Biol Reprod*. (2004) 71:1309–16. doi: 10.1095/biolreprod.104.028407
175. Jeulin C, Soufir JC, Weber P, Laval-Martin D, Calvayrac R. Catalase activity in human spermatozoa and seminal plasma. *Gamete Res*. (1989) 24:185–96. doi: 10.1002/mrd.1120240206
176. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev*. (2008) 1:15–24. doi: 10.4161/oxim.1.1.6843
177. Koziorowska-Gilun M, Koziorowski M, Fraser L, Strzezek J. Antioxidant defence system of boar cauda epididymidal spermatozoa and reproductive tract fluids. *Reprod Domest Anim*. (2011) 46:527–33. doi: 10.1111/j.1439-0531.2010.01701.x
178. Rhemrev JP, van Overveld FW, Haenen GR, Teerlink T, Bast A, Vermeiden JP. Quantification of the nonenzymatic fast and slow TRAP in a postaddition assay in human seminal plasma and the antioxidant contributions of various seminal compounds. *J Androl*. (2000) 21:913–20.
179. Faulkner S, Elia G, Mullen MP, O'Boyle P, Dunn MJ, Morris D. A comparison of the bovine uterine and plasma proteome using iTRAQ proteomics. *Proteomics*. (2012) 12:2014–23. doi: 10.1002/pmic.201100609
180. Benedetti S, Tagliamonte MC, Catalani S, Primiterra M, Canestrari F, De Stefani S, et al. Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. *Reprod Biomed Online*. (2012) 25:300–6. doi: 10.1016/j.rbmo.2012.05.011
181. Gupta S, Finelli R, Agarwal A, Henkel R. Total antioxidant capacity-relevance, methods and clinical implications *Andrologia*. (2020) 12:e13624. doi: 10.1111/and.13624
182. Nassan FL, Chavarro JE, Tanrikut C. Diet and men's fertility: does diet affect sperm quality? *Fertil Steril*. (2018) 110:570–7. doi: 10.1016/j.fertnstert.2018.05.025
183. Pasqualotto FF, Sundaram A, Sharma RK, Borges E, Jr, Pasqualotto EB, Agarwal A. Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. *Fertil Steril*. (2008) 89:602–7. doi: 10.1016/j.fertnstert.2007.03.057
184. Mostafa T, Tawadrous G, Roaia MM, Amer MK, Kader RA, Aziz A. Effect of smoking on seminal plasma ascorbic acid in infertile and fertile males. *Andrologia*. (2006) 38:221–4. doi: 10.1111/j.1439-0272.2006.00744.x
185. Ferigolo PC, Ribeiro de Andrade MB, Camargo M, Carvalho VM, Cardozo KHM, Bertolla RP, et al. Sperm functional aspects and enriched proteomic pathways of seminal plasma of adult men with obesity. *Andrology*. (2019) 7:341–9. doi: 10.1111/andr.12606
186. Zhang MH, Zhai LP, Fang ZY, Li AN, Xiao W, Qiu Y. Effect of scrotal heating on sperm quality, seminal biochemical substances, and reproductive hormones in human fertile men. *J Cell Biochem*. (2018) 119:10228–38. doi: 10.1002/jcb.27365
187. Samova S, Doctor H, Verma R. *In vivo* analysis of bisphenol A induced dose-dependent adverse effects in cauda epididymis of mice. *Interdiscip Toxicol*. (2018) 11:209–16. doi: 10.2478/intox-2018-0019
188. Storey BT. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol Hum Reprod*. (1997) 3:203–13. doi: 10.1093/molehr/3.3.203
189. Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod*. (2011) 26:1628–40. doi: 10.1093/humrep/der132
190. Gharagozloo P, Gutiérrez-Adán A, Champroux A, Noblanc A, Kocer A, Calle A, et al. A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: promising preclinical evidence from animal models. *Hum Reprod*. (2016) 31:252–62. doi: 10.1093/humrep/dev302
191. Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl*. (1996) 17:530–7.
192. Dhawan V, Kumar M, Deka D, Malhotra N, Dadhwal V, Singh N, et al. Meditation & yoga: impact on oxidative DNA damage and dysregulated sperm transcripts in male partners of couples with recurrent pregnancy loss. *Indian J Med Res*. (2018) 148(Suppl):S134–9. doi: 10.4103/ijmr.IJMR_1988_17
193. Bisht S, Banu S, Srivastava S, Pathak RU, Kumar R, Dada R, et al. Sperm methylome alterations following yoga-based lifestyle intervention in patients of primary male infertility: a pilot study. *Andrologia*. (2020) 52:e13551. doi: 10.1111/and.13551
194. Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi PG, Shoukir Y, et al. Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. *Hum Reprod*. (1998) 13(Suppl 4):11–9. doi: 10.1093/humrep/13.suppl_4.11
195. Casanovas A, Ribas-Maynou J, Lara-Cerrillo S, Jimenez-Macedo R, Hortal O, Benet J, et al. Double-stranded sperm DNA damage is a cause of delay in embryo development and can impair implantation rates. *Fertil Steril*. (2019) 111:699–707.e1. doi: 10.1016/j.fertnstert.2018.11.035
196. Aitken RJ, Gordon E, Harkiss D, Twigg J, Milne P, Jennings Z, et al. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod*. (1998) 59:1037–46. doi: 10.1095/biolreprod59.5.1037
197. Lord T, Aitken RJ. Fertilization stimulates 8-hydroxy-2'-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Dev Biol*. (2015) 406:1–13. doi: 10.1016/j.ydbio.2015.07.024
198. Vorilhon S, Brugnon F, Kocer A, Dollet S, Bourgne C, Berger M, et al. Accuracy of human sperm DNA oxidation quantification and threshold determination using an 8-OHdG immuno-detection assay. *Hum Reprod*. (2018) 33:553–62. doi: 10.1093/humrep/dey038
199. Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod*. (2010) 25:2415–26. doi: 10.1093/humrep/deq214
200. Ohno M, Sakumi K, Fukumura R, Furuichi M, Iwasaki Y, Hokama M, et al. 8-oxoguanine causes spontaneous *de novo* germline mutations in mice. *Sci Rep*. (2014) 4:4689. doi: 10.1038/srep04689
201. Horta F, Catt S, Ramachandran P, Vollenhoven B, Temple-Smith P. Female ageing affects the DNA repair capacity of oocytes in IVF using a controlled model of sperm DNA damage in mice. *Hum Reprod*. (2020) 35:529–44. doi: 10.1093/humrep/dez308
202. Sedha S, Kumar S, Shukla S. Role of oxidative stress in male reproductive dysfunctions with reference to phthalate compounds. *Urol J*. (2015) 12:2304–16.
203. Aitken RJ, Drevet JR. The importance of oxidative stress in determining the functionality of mammalian spermatozoa: a two-edged sword. *Antioxidants*. (2020) 9:111. doi: 10.3390/antiox9020111
204. Taylor JD, Baumgartner A, Schmid TE, Brinkworth MH. Responses to genotoxicity in mouse testicular germ cells and epididymal spermatozoa are affected by increased age. *Toxicol Lett*. (2019) 310:1–6. doi: 10.1016/j.toxlet.2019.04.013
205. Lu Y, Lin M, Aitken RJ. Exposure of spermatozoa to dibutyl phthalate induces abnormal embryonic development in a marine invertebrate *Galeolaria caespitosa* (Polychaeta: Serpulidae). *Aquat Toxicol*. (2017) 191:189–200. doi: 10.1016/j.aquatox.2017.08.008
206. Darmishonnejad Z, Zarei-Kheirabadi F, Tavalaee M, Zarei-Kheirabadi M, Zohrabi D, Nasr-Esfahani MH. Relationship between sperm telomere length and sperm quality in infertile men. *Andrologia*. (2020) 52:e13546. doi: 10.1111/and.13546
207. Xavier MJ, Nixon B, Roman SD, Scott RJ, Drevet JR, Aitken RJ. Paternal impacts on development: identification of genomic regions vulnerable to oxidative DNA damage in human spermatozoa. *Hum Reprod*. (2019) 34:1876–90. doi: 10.1093/humrep/dez153
208. Zhang H, Wang R, Yu Y, Zhu H, Li L, Yang X, et al. Non-robertsonian translocations involving chromosomes 13, 14, or 15 in male infertility: 28 cases and a review of the literature. *Medicine*. (2019) 98:e14730. doi: 10.1097/MD.0000000000014730
209. Wu H, Sun L, Wen Y, Liu Y, Yu J, Mao F, et al. Major spliceosome defects cause male infertility and are associated with nonobstructive azoospermia in humans. *Proc Natl Acad Sci USA*. (2016) 113:4134–9. doi: 10.1073/pnas.1513682113
210. Jaiswal D, Singh V, Dwivedi US, Trivedi S, Singh K. Chromosome microarray analysis: a case report of infertile brothers with CATSPER gene deletion. *Gene*. (2014) 542:263–5. doi: 10.1016/j.gene.2014.03.055

211. Sun M, Wang R, Zhang H, Jiang Y, He J, Li S, et al. Molecular cytogenetic characterization of small supernumerary marker 15 in infertile male: a case report. *Exp Ther Med.* (2020) 19:2927–32. doi: 10.3892/etm.2020.8542
212. Zhang Y, Malekpour M, Al-Madani N, Kahrizi ZK, Zanganeh M, Mohseni M, et al. Sensorineural deafness and male infertility: a contiguous gene deletion syndrome. *J Med Genet.* (2007) 44:233–40. doi: 10.1136/jmg.2006.045765
213. Aitken RJ, De Iuliis GN, Nixon B. The sins of our forefathers: paternal impacts on *de novo* mutation rate and development. *Ann Rev Genet.* (2020). doi: 10.1146/annurev-genet-112618-043617
214. Kissin DM, Zhang Y, Boulet SL, Fountain C, Bearman P, Schieve L, et al. Association of assisted reproductive technology (ART) treatment and parental infertility diagnosis with autism in ART-conceived children. *Hum Reprod.* (2015) 30:454–65. doi: 10.1093/humrep/deu338
215. Hattori H, Hiura H, Kitamura A, Miyauchi N, Kobayashi N, Takahashi S, et al. Association of four imprinting disorders and ART. *Clin Epigenetics.* (2019) 11:21. doi: 10.1186/s13148-019-0623-3
216. Amos-Landgraf JM, Ji Y, Gottlieb W, Wandstrat AE, Cassidy SB, Driscoll DJ, et al. Chromosome breakage in the prader-willi and angelman syndromes involves recombination between large, transcribed repeats at proximal and distal breakpoints. *Am J Hum Genet.* (1999) 65:370–86. doi: 10.1086/302510
217. Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci USA.* (1991) 88:11003–6. doi: 10.1073/pnas.88.24.11003
218. Ji G, Yan L, Liu W, Qu J, Gu A. OGG1 Ser326Cys polymorphism interacts with cigarette smoking to increase oxidative DNA damage in human sperm and the risk of male infertility. *Toxicol Lett.* (2013) 218:144–9. doi: 10.1016/j.toxlet.2013.01.017
219. Lee KM, Ward MH, Han S, Ahn HS, Kang HJ, Choi HS, et al. Paternal smoking, genetic polymorphisms in CYP1A1 and childhood leukemia risk. *Leuk Res.* (2009) 33:250–8. doi: 10.1016/j.leukres.2008.06.031
220. Heerema NA, Sather HN, Sensel MG, La MK, Hutchinson RJ, Nachman JB, et al. Abnormalities of chromosome bands 15q13–15 in childhood acute lymphoblastic leukemia. *Cancer.* (2002) 94:1102–10. doi: 10.1002/cncr.10325
221. Beal MA, Yauk CL, Marchetti F. From sperm to offspring: assessing the heritable genetic consequences of paternal smoking and potential public health impacts. *Mutat Res.* (2017) 773:26–50. doi: 10.1016/j.mrrev.2017.04.001
222. Kinoshita T, Mikami M, Ayabe T, Matsubara K, Ono H, Ohki K, et al. Frequency of common copy-number variations at 15q11.2q13 in sperm of healthy men. *Cytogenet Genome Res.* (2019) 159:66–73. doi: 10.1159/000503267
223. Butler MG. Clinical and genetic aspects of the 15q11.2 BP1-BP2 microdeletion disorder. *J Intellect Disabil Res.* (2017) 61:568–79. doi: 10.1111/jir.12382
224. Ferlin A, Foresta C. Infertility: practical clinical issues for routine investigation of the male partner. *J Clin Med.* (2020) 9:1644. doi: 10.3390/jcm9061644
225. Khosravizadeh Z, Hassanzadeh G, Tavakkoly Bazzaz J, Alizadeh F, Totonchi M, Salehi E, et al. The effect of cryopreservation on DNA methylation patterns of the chromosome 15q11-q13 region in human spermatozoa. *Cell Tissue Bank.* (2020) 21:433–45. doi: 10.1007/s10561-020-09828-1
226. Houston BJ, Nixon B, Martin JH, De Iuliis GN, Trigg NA, Bromfield EG, et al. Heat exposure induces oxidative stress and DNA damage in the male germ line. *Biol Reprod.* (2018) 98:593–606. doi: 10.1093/biolre/ioy009


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# *In "Vitro"* Lps-Stimulated Sertoli Cells Pre-Loaded With Microparticles: Intracellular Activation Pathways

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Sertoli cells (SC) are immune privileged cells with the capacity of modulating the immune response by expressing several immune-regulatory factors. SC have the capacity to respond to external stimuli through innate phagocytic and antibacterial activities. This evidence evoked a potential role of SC as drug carriers and therapeutic agents. Such stimuli drive SC towards a still unknown evolution, the clinical relevance of which as yet remains undisclosed. This study sought to investigate the effects of external stimuli in the form of polymeric microparticles (MP) and bacteria derived endotoxins, such as lipopolysaccharides (LPS), in order to identify the pathways potentially involved in cell phenotype modifications. Compared to single stimulation, when combined, MP and LPS provoked a significant increase in the gene expression of IDO, PD-L1, FAS-L, TLR-3, TLR-4, MHC-II, ICAM-1, TFGβ1, BDF123, BDF129, BDF3 and pEP2C. Western Blotting analysis demonstrated up-regulation of the ERK 1–2 and NF-kB p65 phosphorylation ratios. Our study, showing the exponential increase of these mediators upon combined MP and LPS stimulation, suggests a "switch" of SC function from typical cells of the blood-testicular barrier to nonprofessional tolerogenic antigen-presenting cells. Further studies should target the clinical and technological implications of such stimuli-induced SC transformation.

**Keywords: Sertoli cells, lipopolysaccharide, microparticles, pro-inflammatory pathways, nonprofessional tolerogenic**

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## INTRODUCTION

Sertoli Cells (SC), located in the seminiferous epithelium, are somatic "nursing" cells that mechanically segregate germ cell autoantigens by means of the blood-testis barrier (BTB) and create a microenvironment that protects the germ cells from the host's immune system attack on their development within the tubules (1, 2).

SC not only serve as a physical barrier, but can enable modulation of the immune response as well by secreting trophic, anti-inflammatory, and immunomodulatory factors (3). In particular, SC produce different hormones, including the anti-Müllerian hormone (AMH), Activin A, and inhibin B that play an important role for the preservation of their function (4).

Additionally, other cytokines and immunomodulatory factors, including the Transforming Growth Factor-β (TGFβ), Interleukin (IL)-1α and IL-6, defensins (α-, β-defensin), Indoleamine 2,3-dioxygenase (IDO), and the presence of several toll-like receptors contribute to SC immune competence (5–8). Due to these properties, SC have been used for different therapeutic applications, in allogeneic and xenogeneic transplant protocols as well as for the management of several chronic diseases (9–15).

The multifaceted SC nature enables them to respond to multiple stimuli and conditions. As a result, SC can accomplish a number of fundamental roles, including scavenger activities during spermatogenesis and infections.

These innate phagocytic and antibacterial aptitudes further make SC therapeutically attractive.

SC provide the first line of response to invading pathogens gaining access *via* the male genital tract (16, 17).

A bacteriostatic action of rat SC was postulated against phagocytized *Staphylococcus aureus* (18) and ascribed to the secretion of defensins in rodents and canines (19, 20) and to the high mobility group box chromosomal protein 1 in rat and human SC (21).

Moreover, SC have been found potentially useful as safe drug carriers against inflammatory conditions and infections. Kumar et al., transplanted rat SC pre-loaded with curcumin containing chitosan nanoparticles, in a mouse model of acute pulmonary inflammation.

SC accumulated preferentially in the lungs with no immune complications or other observed side effects (22). In another study, the SC phagocytic and antibacterial activities were exploited to formulate a SC-based drug delivery system (23). The SC loaded with a microencapsulated antibiotic complex showed good antibacterial activity over time and storage potential, posing the base for the development of novel cell-based drug delivery systems.

Nevertheless, even though most of SC functions were preserved, it is conceivable to suppose that such a manipulation may induce significant changes in cell characteristics. Moreover, whether such changes may occur even upon external stimulation by toxicants during infection or exposure to non-toxic contaminants in the testis is not known.

Being the whole picture rather unclear and, to the best of our knowledge, not been explored so far, this work was focused on the understanding of how external stimuli may alter SC features and induce SC evolution towards a mutated phenotype, which may suggest potential clinically relevant health consequences.

On this purpose, lipopolysaccharides (LPS), potent pro-inflammatory bacterial endotoxins from the cell wall of Gram-negative bacteria, were chosen as an infection-related stimulus, since they are generally recognized as a standard model for investigating inflammation and response to infection *in vivo* and *in vitro* (24, 25). In fact, LPS can initiate a strong immune response and serve as an early warning signal of bacterial infection. Circulating LPS are intercepted by the LPS binding protein (LBP) in the serum (26), which then transfers LPS to CD14 that splits LPS aggregates into monomeric molecules and presents them to the TLR4–MD-2 complex. Aggregation of the TLR4–MD-2 complex after LPS binding leads to the activation of multiple signaling components, including NF-kB and IRF3, and the subsequent production of pro-inflammatory cytokines (27, 28).

As a non-toxic contaminant stimulus, blank poly(lactide-co-glycolide) microparticles (MP) were employed based on previous evidence of safety and the capacity to trigger endocytic/phagocytic processes (23).

Upon exposure to such stimuli, SC were characterized by addressing the activated mediators and identifying the underlying signalling pathways.

## MATERIALS AND METHODS

### Primary Cultures of Neonatal Porcine Sertoli Cells

Animal studies were conducted in agreement with the guidelines adopted by the Italian Approved Animal Welfare Assurance (A-3143-01) and European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocols were approved by the University of Perugia. Number 2 large white neonatal pigs (15 to 20 days old) underwent bilateral orchidectomy after general anesthesia with ketamine (Ketavet 100; Intervet, Milan, Italy), at a dose of 40 mg/kg, and dexmedetomidine (Dexdomitor, Orion Corporation, Finland), at a dose of 40 g/kg, and were used as SC donors. Specifically, pure porcine neonatal SC were isolated, characterized and tested for functional competence according to previously established methods (29, 30).

### Microparticle Preparation-Size and Uptake Process

MP were prepared by spray-drying of an acetonitrile polymer solution by using a Buchi Mini spray-dryer B290 (Buchi, Italy). The polymer employed was poly (DL-lactide) (PLA)

R203H (MW 20–30 kDa, Boehringer Ingelheim, Germany). The instrumental parameters were set according to previously published experiments (23). Additionally, particle size analysis was performed by an Accusizer C770 particle counter (PSS, Santa Barbara, CA) equipped with an autodilution system. MP were dispersed in 1% w/v Tween 80 solution, slightly sonicated and immediately analyzed. MP size was expressed as volume

mean diameter and population spread as span [Eq. (1)]:

$$span = (d_{90} - d_{10})/d_{50} \quad (1)$$

where $d_{90}$, $d_{10}$ and $d_{50}$ are the diameters ≤90%, 10% and 50% of the population distribution, respectively (23).

Subsequently, the MP were morphologically characterized by Scanning Electron Microscopy (SEM) using a FEG LEO 1525 microscope (LEO Electron Microscopy Inc., NY). The acceleration potential voltage was maintained at 10 keV. Samples were suspended in water and placed onto carbon tape coated aluminum stubs. After complete water evaporation the stubs were sputter coated with chromium prior to imaging by a high resolution sputter (Quorum Technologies, East Essex, UK). Coating was performed at 20 mA for 30 s (23).

MP loading into cells was accomplished by exploiting the innate phagocytic capacity of SC.

MP were properly weighed and dispersed into HAMF12 medium by gentle bath sonication in order to avoid foaming and MP flotation. SC were plated at a density of 0.5–1 × $10^5$ cells/$cm^2$ and incubated in standard conditions (37°C, 5% CO2) with MP at a concentration of 30μg/$cm^2$ for 5 h according to Giovagnoli et al. (23), obtaining the MP-SC.

## Experimental Design

The experimental groups were assigned as follow:

- Control: untreated SC
- MP-SC
- SC plus LPS (LPS) at concentration of 1μg/ml for 5 h (31).
- MP-SC plus LPS at concentration of 1μg/ml for 5 h.

## Quantitative, Real–Time PCR

Real-time PCR analyses for IDO, PD-L1, TLR-3, TLR-4, MHC-II, ICAM-1, TFGβ1, BDF123, BDF129, BDF3 and pEP2C were conducted as previously described (32) by employing the primers listed in **Table 1**. Briefly, total RNA was extracted, using Trizol reagent (Sigma-Aldrich, Milan, Italy) and quantified by reading the optical density at 260 nm. In particular, 2.5μg of total RNA was subjected to reverse transcription (RT Thermo Scientific, Waltham, MA, USA) in a final volume of 20 μL. Real-time PCR was performed using 25 ng of cDNA prepared by the RT reaction and SYBR Green master mix (Stratagene, Amsterdam, the Netherlands). This procedure was performed in an Mx3000P cycler (Stratagene), using FAM for detection and ROX as reference dye. The mRNA level of each sample was normalized by β-actin mRNA and expressed as fold changes vs the level of the control group.

## Protein Extraction and Western Blot Analysis

Total protein extracts were prepared by lysing cells in 100 μl of radio-immunoprecipitation assay lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

After centrifuging the mixture at 1,000×g (Eppendorf, NY, USA) for 10 min, the supernatant was collected, and total protein content was assayed by the Bradford method (33). Sample aliquots were stored at −20°C for WB analysis. The cell extracts were separated by 4–12% SDS-PAGE, and equal amounts of protein (70 μg protein/lane) were run and blotted on nitrocellulose membranes (BioRad, Hercules, CA, USA). The membranes were incubated overnight in a buffer containing 10 mM TRIS, 0.5 M NaCl, 1% (v/v) Tween 20 (Sigma-Aldrich), rabbit anti-ERK1/2 (Millipore, MA, USA; dilution factor, 1:2,000), mouse anti-phospho- ERK1/2 (Millipore; dilution factor, 1:100), rabbit anti-NF-kB p65 antibody (AbCam, Cambridge, UK; dilution factor, 1:1,000).

Primary antibody binding was then detected by incubating the membranes for an additional 60 min in a buffer containing horseradish peroxidase conjugated anti-rabbit (Sigma-Aldrich; dilution factor, 1:5,000) and/or anti-mouse (Santa Cruz Biotechnology Inc.; dilution factor, 1:5,000) IgG secondary antibodies. The bands were detected by enhanced chemiluminescence.

## Statistical Analysis

Values reported in the figures are the mean ± S.D. of three independent experiments, each one performed in triplicate. Statistical analysis was performed by the paired Student's t-test

**TABLE 1** | Primer sequences for PCR analyses.

| Gene | Forward | Reverse | $T_a$ |
|---|---|---|---|
| β-actina | ATGGTGGGTATGGGTCAGAA | CTTCTCCATGTCGTCCCAGT | 56°C |
| IDO | ATGAAGGCGTTTGGGACACC | GAGGAATCCAGCAGCAGAGC | 56°C |
| PDL-1 | AAACAATTAGACCTGGCTG | TCTTACCACTCAGGACTTG | 56°C |
| FAS-L | GCAGAAGGAACTGGCAGAAC | TTAAATGGGCCACACTCCTC | 56°C |
| MHCII | GACCAGATGAGGTTATTGG | GGTCCTGTAGTTGTGTCT | 56°C |
| ICAM-1 | AGGGAAACCAGACACAAG | ACGACAAGTTAGCCAGTT | 56°C |
| TLR-3 | CACTATGCTCGATCTTTCCTAC | CAATTCAGGTACCTCACATTG | 56°C |
| TLR-4 | CTTCACTACAGAGACTTCA | ACAATAACCTTCCGACTT | 56°C |
| TGF1β | GCCCTGGACACCAACTATTGC | GCTGCACTTGCAGGAGCGCAC | 56°C |
| TGF3β | GCACTTGCAAAGGGCTC | TTGGCATAGTATTCCGA | 56°C |
| BDF123 | GAGTGCGTTGGGAAGATG | TCGGTATGTACTTGGGATGT | 56°C |
| BDF129 | TGAAGAGGTCGCCAAGAA | GGATGATGGTGGTGTTGATG | 56°C |
| BDF3 | GCCTTGCTCTTCTTGTTC | GCTACCTATCTGTTCCTCTT | 56°C |
| BDF4 | GCTACCTATCTGTTCCTCTT | GCATCAAGGTCATTTCTCA | 56°C |
| pEP2C | CAAGTCTCACCTGTTACG | ATCTGCCTTCACTTCTCT | 56°C |

using SigmaStat 4.0 software (Systat Software Inc., CA, USA). All tests were performed in triplicate, and statistically significance was assigned to $p < 0.05$.

# RESULTS

## Sertoli Cell Purification, Characterization, and Function

SC isolated from testes of Large White neonatal pigs were comprised of highly purified tissue (95%) as indicated by the immunostaining for AMH, a specific and unique neonatal SC marker.

The presence of "contaminating" non-SC cells was extremely low (<5%) according to Arato et al. (34).

## Microparticle Dimensional Analysis and Uptake Process

The average size of MP was consistent with possible uptake by SC.

The average volume mean diameter of MP was 8.8 μm with a span value of 1.2, which indicates a good homogeneity of the particle size distribution as also shown by SEM analysis (**Figure 1**).

## RT-PCR Analysis

As revealed by real-time PCR analyses, a general major increase of gene expression was observed for nearly all the selected mediators mainly when LPS stimulus was employed with MP loading.

In fact, *IDO* gene expression did not changes in MP-SC, while it was significantly higher compared with the control after LPS and MP-SC plus LPS stimulation (**Figure 2A**, $p < 0.001$). A similar trend was recorded in Programmed Death-Ligand 1 (*PD-L1*) expression that increased with LPS and, in particular upon MP-SC plus LPS stimulation, while it resulted even down-regulated in MP-SC (**Figure 2A**, $p < 0.05$ and $p < 0.001$). On the other hand, the Fas ligand (*FAS-L*) was slightly more expressed only when coupling the MP-SC and LPS treatment (**Figure 2A**, $p < 0.05$). Likewise, the measurement of the Toll-Like Receptor (*TLR-3*) and (*TLR-4*) expression confirmed a major effect of LPS

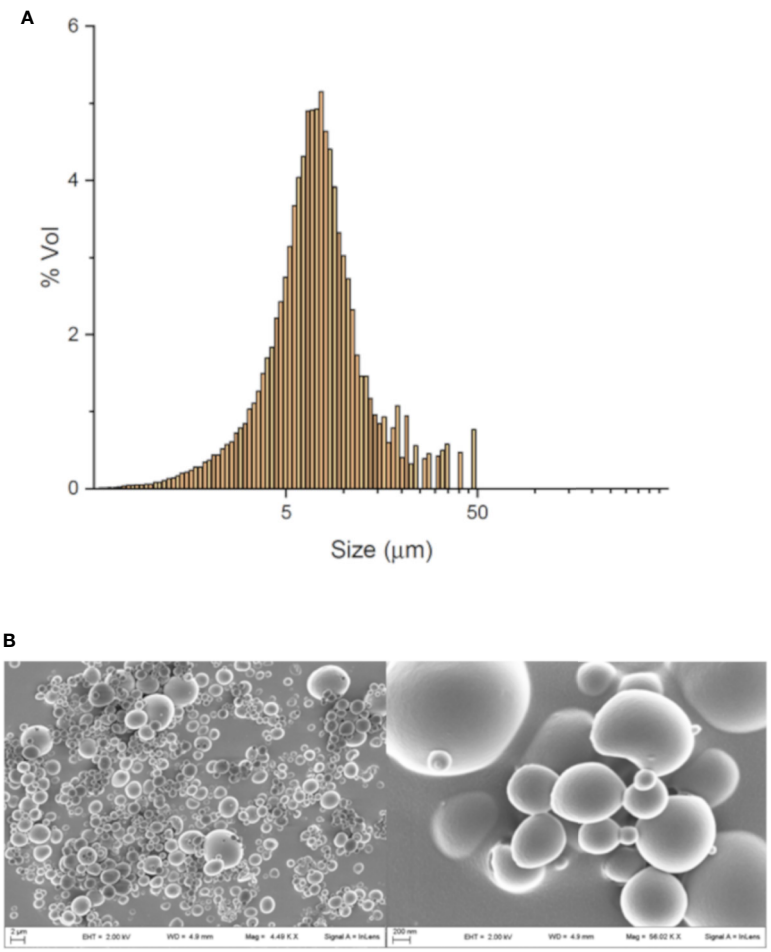


**FIGURE 1** | MP characterization and uptake. Dimensional analysis obtained by granulometer **(A)** and SEM image of MP **(B)**.

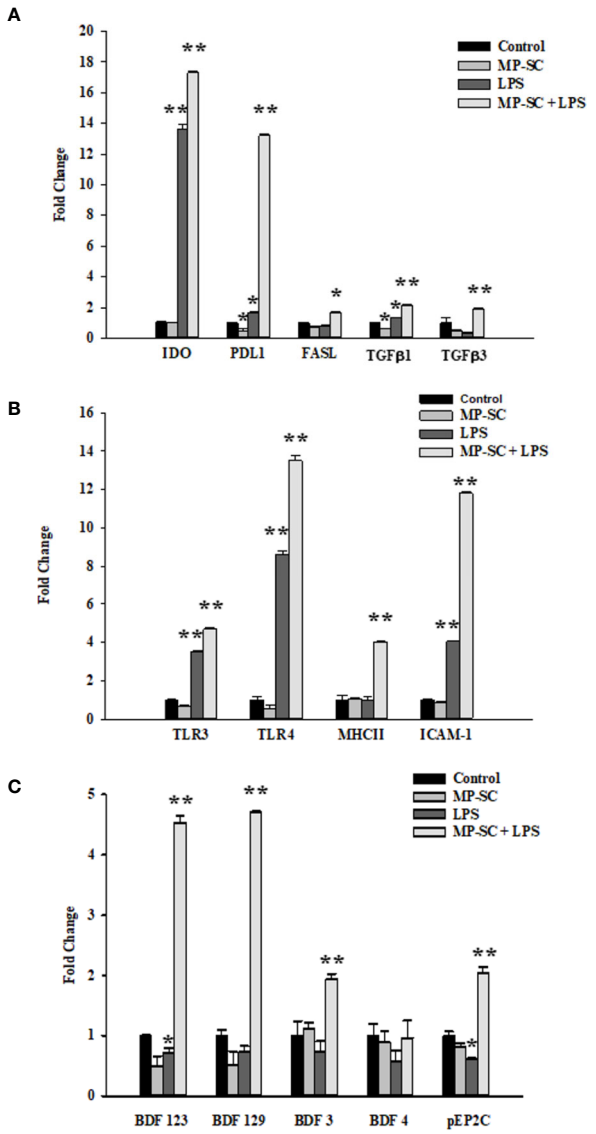


**FIGURE 2** | Real-Time PCR. Gene expressions of IDO, PD-L1, FAS-L, TFGβ1, TFGβ3 **(A)** TLR-3, TLR-4, MHC-II, ICAM-1 **(B)** BDF123, BDF129, BDF3, BDF4 and pEP2C **(C)** were evaluated by Real Time PCR. See text for more details. Data represent the mean ± S.E.M. (*$p < 0.05$ and **$p < 0.001$ respect to untreated SC) of three independent experiments, each performed in triplicate.

and MP-SC plus LPS treatments as compared with the control and no changes in MP-SC (**Figure 2B**, $p < 0.001$). Moreover, we observed a significant increase in the expression of the Major Histocompatibility Complex (*MHC-II*) in MP-SC plus LPS-stimulated SC and not in the other experimental groups. (**Figure 2B**, $p < 0.001$). The Intercellular Adhesion Molecule 1 (*ICAM-1*) and *TGFβ1* expression were upregulated following LPS and MP-SC plus LPS treatments, while only TGFβ1 was down-regulated in MP-SC (**Figures 2A, B**, $p < 0.05$ and $p < 0.001$).

The same behavior was observed for BDF123, BDF129, BDF3 and pEP2Cexpression following LPS and MP-SC plus LPS treatment with no changes in MP-SC. The only exception was BDF123 and pEP2C that were down-regulated by LPS treatment (**Figure 2C**, $p < 0.05$ and $p < 0.001$).

## Western Blot Analysis

The WB recorded showed a general increase of the phosphorylation ratio of ERK1-2 compared to the control by MP plus LPS treatment, while we observed a significant reduction after MP-SC and LPS treatment (**Figures 3A, B**, $p < 0.05$ and $p < 0.001$).

On the other hand, the Nf-kB p65 level was significantly up-regulated only for MP-SC plus LPS treatment, with a statistically significant reduction after MP-SC treatment and not changes after LPS stimulus compared to the control (**Figures 3A, B**, $p < 0.05$ and $p < 0.001$).

# DISCUSSION

In the present study, we focused on the effect of the exposure of SC to MP and LPS as models of non-toxic and infection-released stimuli, respectively, to understand changes in SC signatures due to the activation of selected mediators and signalling pathways.

In particular, our experimental design consisted in a first step in which SC were loaded with empty MP mimicking what reported by Giovagnoli et al. (23). that could be followed or not by a second step of stimulation with LPS to create a useful high mammalian *in vitro* model to assess the cell responses at an early stage of infection by i.e. Gram-negative bacteria or mycobacteria (31). In fact, LPS is a standard agent employed in *in vivo* and *in vitro* protocols acting as a potent stimulator of the innate immune response (28). This setup was intended to simulate cell activation against pro-inflammatory and immunostimulatory agents (23).

MP loading was easily achieved by exploiting the natural scavenging role of SC in the testis. In fact, it is well known that, under physiologic conditions, SC internalize residual cytoplasmic bodies as an important function to maintain the homeostasis of the testis (35, 36).

In response to LPS stimulation of MP-SC, we observed up-regulation of gene expression for important markers involved in the innate immune response such as IDO, PD-L1, FAS-L, TLR-3, TLR-4, MHC-II, ICAM-1, TFGβ1, BDF123, BDF129, BDF3 and pEP2C.

*IDO* interferes with immunity as a result of its involvement in the kynurenine pathway (the $O_2$-dependent oxidation of L-tryptophan) and it increases the production of Treg, involved in the surveillance of self-tolerance to auto-antigens, thus preventing autoimmunity (37).

Fallarino et al., 2009, for the first time, demonstrated IDO's expression in SC (9). Herein, the up-regulation of IDO gene expression, after subsequent stimulation with LPS, would explain a possible role of SC in immuno-regulation, within a TGFβ

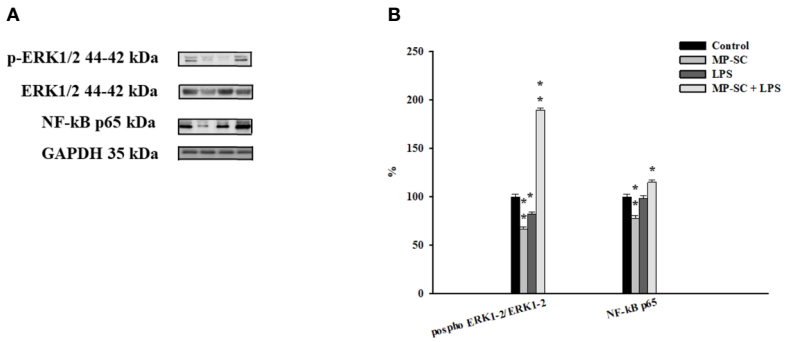


**FIGURE 3** | WB analysis. Immunoblots of phospho-ERK1-2/ERK1-2 and NF-kB p65 **(A)**. Densitometric analysis of the protein bands of phospho- ERK1-2/ERK1-2 and NF-kB p65 **(B)**. Data represent the mean ± S.E.M. (*p < 0.05 and **p < 0.001, respect to untreated SC) of three independent experiments, each performed in triplicate.

mediated IDO-dependent mechanism as demonstrated by the further up-regulation of *TGFβ1* gene expressions (9).

*PD-L1*, also known as CD274 or B7-H1, is a fundamental trans-membrane protein involved in the repression of the immune response during pregnancy, allograft, autoimmune diseases and other diseases such as hepatitis (38). The expression of B7-H1 contributes to the inhibition of immune responses by negatively interfering with CD8+ T cell proliferation, while inducing the expression of MHC class II, which mediates the increase of Tregs (39), as also demonstrated by the observed up regulation of MHC-II and ICAM1 gene expression, confirming previous published reports (39). In fact, *MHC-II* is a well-known trans-membrane protein and its main function is to present processed antigens, which are derived primarily from exogenous sources, to CD4(+) T-lymphocytes, playing an essential role in innate as well as acquired immunity (40). *ICAM-1*, also known as CD54, is a cell surface glycoprotein expressed on a wide variety of cell types, with distinct patterns of gene regulation and effector functions. ICAM-1 is expressed constitutively at low levels in endothelial cells and some lymphocytes and monocytes and its expression can be significantly increased, in the presence of cytokines, even in nonvascular cells such as SC (5, 31).

In our model, the increased expression of these mediators seems to confirm the data reported by Dal Secco et al, showing how SC could function as nonprofessional tolerogenic antigen-presenting cells by inducing enrichment in regulatory T cells (Tregs) in a mixed T lymphocyte population (39).

All these data were further corroborated by upregulation of *FAS-L* gene expression that is a trans-membrane protein belonging to the TNF (Tumor necrosis factor) family regulating the immune response by formation of the immunological synapse (41).

Additionally, our experimental setup confirmed the involvement of TLR-4 in LPS stimulation of MP-SC, where *TLR-4* is a transmembrane protein capable of recognizing specific pathogen molecules such as LPS of mycobacteria wall and Gram-negative bacteria. Specifically, LPS exerts its effects exclusively through the TLR-4 receptor complex (28) leading to activation of the intracellular signaling pathway of NFkB, responsible for the activation of the innate immune system, as confirmed by our WB data analysis where we observed the up-regulation of *NF-kB p65*, an important effector of canonical activation of the NF-kB pathway (5, 28).

Furthermore, we observed an increased gene expression of *TLR-3*, another member of the TLR family that plays a fundamental role in the recognition of non-self (DAMP/PAMP) molecules, by activating the innate immune response (5). These observations confirmed the data of Qin et al. showing how TLR3 upregulation contributed on the modulation of inflammatory cytokine generation during orchitis in testicular cells (42).

In the present study, we focused, additionally, on analyzing the expression of β-defensins (BDF) by MP-SC after LPS treatment.

*β-defensins*, a major group of mammalian antimicrobial peptides, are expressed in SC and represent one of the earliest mediators of the host's defense in human and animals (19). The expression of β-defensins in the testis and different regions of the epididymis provides an innate defense immune mechanism in the male reproductive tract (43). In European wild boar naturally infected with *Mycobacterium bovis*, Galindo et al. (40) observed an overexpression of BDF129 suggesting its protective role against mycobacteria and confirming the data of Riva-Santiago et al. (44). who observed the overexpression of BDF3 and BDF4 mRNA, in a mouse model of mycobacterial infection, thus hypothesizing their role in the control of mycobacterial growth.

In our study, we observed upregulation of *BDF123, BDF129, BDF3* and *pEP2C*, confirming their role in the innate defense immune mechanisms in the male reproductive tract (44).

Finally, our WB data, demonstrated un up-regulation of phosphorylation ratio of ERK 1-2.

The increase of ERK, member of the mitogen-activated protein kinases (MAPK) pathway, suggest their involvement in LPS stimulation.

There are several studies showing that MAPK play an important role in numerous male reproductive processes, including BTB dynamics, germ cell-cycle progression and

differentiation, and germ cell apoptosis in the seminiferous epithelium (45, 46).

In particular, previous studies showed that BTB dysfunction is closely associated with the activation of the MAPK and NFκB pathways in some infections (5, 47, 48).

In conclusion, our data confirmed that SC are able to uptake MP, as previously described (23), and may be subsequently activated by LPS stimulation (31), suggesting that this experimental procedure seems to be the best strategy to activate SC against a microbial agent (synergistic effect).

Moreover, the ability of SC to internalize MP could be exploited by using them as carriers to deliver drugs to the target area, with no systemic side effects.

The present work, by pinpointing the exponential increase of pro-inflammatory pathways in MP-SC, after LPS stimulation, suggests a hypothetical "role" of SC, additional to that of cells of the blood-testicular barrier, as nonprofessional tolerogenic antigen-presenting cells.

All these data strengthen the concept that SC can be considered a complex "micro-laboratory" secreting a cocktail of immunomodulatory factors.

These findings warrant further investigation to understand how the SC respond to infections by activating several pro-inflammatory pathways.

A prospective impact of the present study is the possibility to transform SC into potentially valuable therapeutic tools by exploring their native and induced competences. Unraveling the effect of external stimuli on SC competence is crucial to depict the underlying response mechanisms that may be useful to tackle diseases with inflammatory or immunological signatures even of infective origin. The dual capacity of SC to function as carries as well as immunomodulatory/antibacterial agents could be exploited to develop novel cell-based treatments for pathologies otherwise difficult to treat. Such an ambitious goal cannot be sought without knowing how SC manipulation alters their natural features and without a full disclosure of the clinical relevance of such observations.

This study represents a first attempt to comprehend the intricate pattern of SC stimulation that naturally occurs when cells are exposed to non-physiological conditions or are manipulated in laboratory settings. As such, this study shows several limitations as it lacks of insightful quantification of cell signaling pathways and secretome analysis. In this regard, future studies are desirable implying the application of advanced molecular techniques, including proteomics, single-cell RNA analysis and macroarray investigations to identify the possible involvement of secondary factors and pathways to broaden the picture on induced sertolian functions.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Italian Approved Animal Welfare Assurance (A-3143-01).

## AUTHOR CONTRIBUTIONS

All authors had critically revised and approved the final version of the manuscript. IA and DM designed and drafted the manuscript. The experimental procedures and data analysis were performed by CB, CL, SB, SC, PM, MC, and TB, SG, GG, and RC gave experimental guidance. FM and GL supervised and revised the manuscript. All authors contributed to the article and approved the submitted version.

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## REFERENCES

1. MK Skinner, MD Griswold eds. *Sertoli Cell Biology* Vol. 1. San Diego, CA, USA: Elsevier Academic Press (2005).
2. Mital P, Hinton BT, Dufour JM. The blood-testis and blood epididymis barriers are more than just their tight junctions. *Biol Reprod* (2011) 84:851–58. doi: 10.1095/biolreprod.110.087452
3. Doyle TJ, Kaur G, Putrevu SM, Dyson EL, Dyson M, McCunniff WT, et al. Immunoprotective properties of primary Sertoli cells in mice: potential functional pathways that confer immune privilege. *Biol Reprod* (2012) 86:1–14. doi: 10.1095/biolreprod.110.089425
4. Griswold MD. Protein secretion by Sertoli cells: general considerations. In: LD Russell, MD Griswold, editors. *The Sertoli Cell*. Clearwater, FL: Cache River Press (1993). p. 195–200.
5. Riccioli A, Starace D, Galli R, Fuso A, Scarpa S, Palombi F, et al. Sertoli Cells Initiate Testicular Innate Immune Responses through TLR Activation. *J Immunol* (2006) 177:7122–30. doi: 10.4049/jimmunol.177.10.7122
6. Mital P, Kaur G, Dufour JM. Immunoprotective Sertoli cells: making allogeneic and xenogeneic transplantation feasible. *Reproduction* (2010) 139:495–504. doi: 10.1530/REP-09-0384
7. França LR, Hess RA, Dufour JM, Hofmann MC, Griswold MD. The Sertoli cell: one hundred fifty years of beauty and plasticity. *Andrology* (2016) 4:189–212. doi: 10.1111/andr.12165
8. Luca G, Baroni T, Arato I, Hansen BC, Cameron DF, Calafiore R. Role of Sertoli Cell Proteins in Immunomodulation. *Protein Pept Lett* (2018) 25:440–45. doi: 10.2174/0929866525666180412163151
9. Fallarino F, Luca G, Calvitti M, Mancuso F, Nastruzzi C, Fioretti MC, et al. Therapy of experimental type 1 diabetes by isolated Sertoli cell xenografts alone. *J Exp Med* (2009) 206:2511–26. doi: 10.1084/jem.20090134
10. Luca G, Arato I, Mancuso F, Calvitti M, Falabella G, Murdolo G, et al. Xenograft of microencapsulated Sertoli cells restores glucose homeostasis in db/db mice with spontaneous diabetes mellitus. *Xenotransplantation* (2016) 23:1–11. doi: 10.1111/xen.12274

11. Shamekh R, El-Badri NS, Saporta S, Pascual C, Sanberg PR, Cameron DF. Sertoli cells induce systemic donor-specific tolerance in xenogenic transplantation model. *Cell Transplant* (2006) 15:45e53. doi: 10.3727/000000006783982205
12. Chiappalupi S, Luca G, Mancuso F, Madaro L, Fallarino F, Nicoletti C, et al. Intraperitoneal injection of microencapsulated Sertoli cells restoresmuscle morphology and performance in dystrophic mice. *Biomaterials* (2016) 75:313–26. doi: 10.1016/j.biomaterials.2015.10.029
13. Luca G, Bellezza I, Arato I, Di Pardo A, Mancuso F, Calvitti M, et al. Terapeutic Potential of Microencapsulated Sertoli Cells in Huntington Disease. *CNS Neurosci Ther* (2016) 22:686–90. doi: 10.1111/cns.12569
14. Emerich DF, Hemendinger R, Halberstadt CR. The testicular-derived Sertoli cell: cellular immunoscience to enable transplantation. *Cell Transplant* (2003) 12:335–49. doi: 10.3727/000000003108746894
15. Bistoni G, Calvitti M, Mancuso F, Arato I, Falabella G, Cucchia R, et al. Prolongation of skin allograft survival in rats by the transplantation of microencapsulated xenogeneicneonatal porcine Sertolicells. *Biomaterials* (2012) 33:5333e40. doi: 10.1016/j.biomaterials.2012.04.020
16. Nagaosa K, Nakashima C, Kishimoto A, Nakanishi Y. Immune response to bacteria in seminiferous epithelium. *Reproduction* (2009) 137:879–88. doi: 10.1530/rep-08-0460
17. Winnall WR, Muir JA, Hedger MP. Differential responses of epithelial Sertoli cells of the rat testis to Toll-like receptor 2 and 4 ligands: Implications for studies of testicular inflammation using bacterial lipopolysaccharides. *Innate Immun* (2011) 17:123–36. doi: 10.1177/1753425909354764
18. Shiratsuchi A, Osada Y, Nakanishi Y. Differences in the mode of phagocytosis of bacteria between macrophages and testicular Sertoli cells. *Drug Discov Ther* (2013) 7:73–7. doi: 10.5582/DDT.2013.V7.2.73
19. Sang Y, Ortega MT, Blecha F, Prakash O, Melgarejo T. Molecular cloning and characterization of three β-defensins from canine testes. *Infect Immun* (2005) 73:2611–20. doi: 10.1128/IAI.73.5.2611-2620.2005
20. Grandjean V, Vincent S, Martin L, Rassoulzadegan M, Cuzin F. Antimicrobial protection of the mouse testis: synthesis of defensins of the cryptdin family. *Biol Reprod* (1997) 57:1115–22. doi: 10.1095/biolreprod57.5.1115
21. Zetterström CK, Strand ML, Söder O. The high mobility group box chromosomal protein 1 is expressed in the human and rat testis where it may function as an antibacterial factor. *Hum Reprod* (2006) 21:2801–9. doi: 10.1093/humrep/del256
22. Kumar A, Glaum M, El-Badri N, Mohapatra S, Haller E, Park S, et al. Initial observation of cells mediated drug delivery to the deep lung. *Cell Transplant* (2011) 20:609–18. doi: 10.3727/096368910X536491
23. Giovagnoli S, Mancuso F, Vannini S, Calvitti M, Piroddi M, Pietrella D, et al. Microparticle-loaded neonatal porcine Sertoli cells for cell-based therapeutic and drug delivery system. *J Controlled Release* (2014) 192:249–61. doi: 10.1016/j.jconrel.2014.08.001
24. O'Bryan MK, Schlatt S, Phillips DJ, de Kretser DM, Hedger MP. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo. *Endocrinology* (2000) 141:238–46. doi: 10.1210/endo.141.1.7240
25. O'Bryan MK, Gerdprasert O, Nikolic-Paterson DJ, Meinhardt A, Muir JA, Foulds LM, et al. Cytokine profiles in the testes of rats treated withlipopolysaccharide reveal localized suppression of inflammatoryresponses. *Am J Physiol Regul Integr Comp Physiol* (2005) 288:R1744–55. doi: 10.1152/ajpregu.00651.2004
26. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* (2002) 71:635–700. doi: 10.1146/annurev.biochem.71.110601.135414
27. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* (2004) 4:499–511. doi: 10.1038/nri1391
28. Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med* (2013) 45:e66. doi: 10.1038/emm.2013.97
29. Luca G, Fallarino F, Calvitti M, Mancuso F, Nastruzzi C, Arato I, et al. Xenograft of Microencapsulated Sertoli CellsReverses T1DM in NOD Mice by Inducing Neogenesisof Beta-Cells. *Transplantation* (2010) 90:1352–57. doi: 10.1097/tp.0b013e3181ffb9d2
30. Arato I, Luca G, Mancuso F, Bellucci C, Lilli C, Calvitti M, et al. An in vitro prototype of a porcine biomimetictestis–like cell culture system: a novel tool for thestudy of reassembled Sertoli and Leydig cells. *Asian J Androl* (2018) 20:160–65. doi: 10.4103/aja.ja_47_17
31. Riccioli A, Filippini A, de Cesaris P, Barbacci E, Stefanini M, Starace G, et al. Inflammatory mediators increase surface expression of integrin ligands, adhesion to lymphocytes, and secretion of interleukin 6 in mouse Sertoli cells. *Proc Natl Acad Sci USA* (1995) 92:5808–12. doi: 10.1073/pnas.92.13.5808
32. Mancuso F, Arato I, Lilli C, Bellucci C, Bodo M, Calvitti M, et al. Acute effects of lead on porcine neonatal Sertoli cells in vitro. *Toxicol Vitro* (2018) 48:45–52. doi: 10.1016/j.tiv.2017.12.013
33. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* (1976) 72:248–54. doi: 10.1006/abio.1976.9999
34. Arato I, Grande G, Barrachina F, Bellucci C, Lilli C, Jodar M, et al. "In vitro" Effect of Different Follicle—Stimulating Hormone Preparations on Sertoli Cells: Toward a Personalized Treatment for Male Infertility. *Front Endocrinol (Lausanne)* (2020) 11:401. doi: 10.3389/fendo.2020.00401
35. Griswold MD. *Sertoli Cell Biology*, *2nd Edition*. ed. M Griswold. Elsevier (2014).
36. Griswold MD. *Sertoli Cell Biology*. Oxford: Elsevier Academic Press (2015).
37. Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* (2004) 4:762–74. doi: 10.1038/nri1457
38. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. 'SHP-1 and SHP-2 Associate with Immunoreceptor Tyrosine-Based Switch Motif of Programmed Death 1 upon Primary Human T Cell Stimulation, but Only Receptor Ligation Prevents T Cell Activation'. *J Immunol (1950)* (2004) 173:945–54. doi: 10.4049/jimmunol.173.2.945
39. Dal Secco V, Riccioli A, Padula F, Ziparo E, Filippini A. Mouse Sertoli Cells Display Phenotypical and Functional Traits of Antigen-Presenting Cells in Response to Interferon Gamma. *Biol Reprod* (2008) 78:234–42. doi: 10.1095/biolreprod.107.063578
40. Galindo RC, Ayoubi P, Naranjo V, Gortazar C, Kocan KM, de la Fuente J. Gene expression profiles of European wild boar naturally infected with Mycobacterium bovis. *Vet Immunol Immunopathol* (2009) 129:119–25. doi: 10.1016/j.vetimm.2008.12.012
41. Lynch DH, Ramsdell F, Alderson MR. Fas and FasL in the homeostatic regulation of immune responses. *Immunol Today* (1995) 16:569–74. doi: 10.1016/0167-5699(95)80079-4
42. Qin L, Lin J, Xie X. CircRNA-9119 suppresses poly I:C induced inflammation in Leydig and Sertoli cells via TLR3 and RIG-I signal pathways. *Mol Med* (2019) 25:28. doi: 10.1186/s10020-019-0094-1
43. Sang Y, Patil AA, Zhang G, Ross CR, Blecha F. Bioinformatic and Expression Analysis of Novel Porcine Beta-Defensins. *Mamm Genome* (2006) 17:332–39. doi: 10.1007/s00335-005-0158-0
44. Rivas-Santiago B, Sada E, Tsutsumi V, Aguilar-Leon D, Contreras JL, Hernandez-Pando R. β-Defensin Gene Expression during the Course of Experimental Tuberculosis Infection. *J Infect Dis* (2006) 194:697–701. doi: 10.1086/506454
45. Li MW, Mruk DD, Cheng CY. Mitogen-activated protein kinases in male reproductive function. *Trends Mol Med* (2009) 15:159–68. doi: 10.1016/j.molmed.2009.02.002
46. Wong CH, Cheng CY. Mitogen-activated protein kinases, adherens junction dynamics, and spermatogenesis: a review of recent data. *Dev Biol* (2005) 286:1–15. doi: 10.1016/j.ydbio.2005.08.001
47. Lie PP, Cheng CY, Mruk DD. Signalling pathways regulating the blood-testis barrier. *Int J Biochem Cell Biol* (2013) 45:621–5. doi: 10.1016/j.biocel.2012.12.009
48. Haas A. Reprogramming the phagocytic pathway—intracellular pathogens and their vacuoles (Review). *Mol Membr Biol* (1998) 15(3):103–121. doi: 10.3109/09687689809074522


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# Male Infertility Diagnosis: Improvement of Genetic Analysis Performance by the Introduction of Pre-Diagnostic Genes in a Next-Generation Sequencing Custom-Made Panel

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**Background:** Infertility affects about 7% of the general male population. The underlying cause of male infertility is undefined in about 50% of cases (idiopathic infertility). The number of genes involved in human spermatogenesis is over two thousand. Therefore, it is essential to analyze a large number of genes that may be involved in male infertility. This study aimed to test idiopathic male infertile patients negative for a validated panel of "diagnostic" genes, for a wide panel of genes that we have defined as "pre-diagnostic."

**Methods:** We developed a next-generation sequencing (NGS) gene panel including 65 pre-diagnostic genes that were used in 12 patients who were negative to a diagnostic genetic test for male infertility disorders, including primary spermatogenic failure and central hypogonadism, consisting of 110 genes.

**Results:** After NGS sequencing, variants in pre-diagnostic genes were identified in 10/12 patients who were negative to a diagnostic test for primary spermatogenic failure (n = 9) or central hypogonadism (n = 1) due to mutations of single genes. Two pathogenic variants of *DNAH5* and *CFTR* genes and three uncertain significance variants of *DNAI1*, *DNAH11*, and *CCDC40* genes were found. Moreover, three variants with high impact were found in *AMELY*, *CATSPER 2*, and *ADCY10* genes.

**Conclusion:** This study suggests that searching for pre-diagnostic genes may be of relevance to find the cause of infertility in patients with apparently idiopathic primary spermatogenic failure due to mutations of single genes and central hypogonadism.

**Keywords: male infertility, next-generation sequencing, genetic test, spermatogenesis defects, azoospermia, oligozoospermia**

## INTRODUCTION

The increasing knowledge of male reproduction physiology, of fertilization, and the advent of increasingly effective assisted reproductive techniques, have led to a profound change in the management of male infertility. Currently, the diagnostic workflow offered to male infertile patients includes medical history collection and physical examination, followed by a combination of laboratory testing tailored to each case, including an in-depth genetic laboratory analysis (1–3). Diagnostic tests should be performed after at least 1 year of infertility. Accordingly, a couple can be defined infertile if they do not reach pregnancy after a year of unprotected and regular sexual intercourses (4).

Genetic factors are found in about 15% of male infertile patients. They include chromosomal abnormalities or single-gene mutations (5, 6). Over 200 genetic disorders related to male infertility are reported in the Online Mendelian Inheritance in Man (OMIM) database (7, 8). The genetic of male infertility is greatly complex because semen and testis histological phenotypes are very heterogeneous and up to 2,300 genes are involved in spermatogenesis (1, 9). Moreover, studies in male infertility are challenging. Accordingly, genetic infertility results in an elimination of these mutations from the gene pool, since these are not transmitted. Furthermore, genetic and epigenetic changes accumulate in spermatozoa with aging, and rare single nucleotide polymorphisms and copy number variants can contribute to idiopathic male infertility (1). It is important to trace the non-genetic and genetic causes of male infertility since the latter are the cause of half of the cases of non-conception (4). Notably, to identify new genetic biomarkers of genetic infertility deserve investigation, because the standard clinical evaluation of infertile patients and karyotype analysis can identify the cause of infertility only in about 50% of the cases (10). The combination of genetic and epigenetic testing seems to identify genetic variations and differential expression of specific genes, providing information on the true ability of a man to reproduce. In contrast, a semen analysis may fail to evidence even a partial impairment of sperm parameters (9).

There are two general approaches for finding genes involved in infertility: the candidate gene approach in model animals, and the whole genome studies such as single-nucleotide polymorphism microarray and next-generation sequencing (NGS) technologies, such as exome or whole-genome sequencing (11, 12). Despite a throughout diagnostic workup, conventional genetic tests largely fail to reach a diagnosis (13) and the cause of male infertility remains elusive in up to ∼70% of cases (14). Recent research seems to address the role of NGS technology in raising the rate of diagnosis in male infertility (15, 16). Accordingly, several diagnostic genes have already been shown to be involved in the pathogenesis of male infertility (15). Pre-diagnostic genes, including those reported in association with male infertility but with no definitive evidence of a causative role, may help to reach a diagnosis. To this end, the present study was undertaken to evaluate a series of pre-diagnostic genes by comparing the results with those obtained with our usual NGS custom-made gene panel for the diagnosis of male infertility, including 110 genes.

## METHODS

### Patients and Samples

Twelve patients with a clinical diagnosis of male infertility and negative to diagnostic genetic testing were selected for this study. Eleven were suspected to have primary spermatogenic failure and one was suspected to have central hypogonadism. More in detail, primary spermatogenic failure was suspected for a history of couple infertility longer than 2 years, after the exclusion of the female factor infertility and of acquired causes of male infertility (e.g. male accessory gland infection, varicocele, testicular trauma, etc.). Also, patients enrolled in this study were negative for first step genetic analysis, such as karyotype abnormalities, Y chromosome AZF microdeletions, or *CFTR* conventional gene mutations.

An informed written consent was obtained from each patient. The study was carried out following the tenets of the Declaration of Helsinki and it was approved by the local Ethics Committee. A blood EDTA sample was collected from each subject. Samples of genomic DNA of all subjects were extracted from peripheral blood using a commercial kit (SAMAG 120 BLOOD DNA Extraction Kit). DNA was quantified using Quant-iT Picogreen dsDNA Assay Kit (Life Sciences) and a Varioskan LUX (Thermo Scientific).

### Gene Panel Design

A single NGS panel related to male infertility disorders comprising a total of 175 genes was designed. Then, 110 genes were analyzed in a diagnostic setting, and 65 genes comprising pre-diagnostic or informative genes were analyzed in patients who resulted negative to the diagnostic testing. The genes included in the panel were based on their correlation with male infertility described in Online Mendelian Inheritance in Man (OMIM) (7), GeneReviews (17), and primary literature. Genes were classified as "diagnostic" when they and their genetic variants were clearly correlated to male infertility in literature. Instead, genes were classified as "informative or pre-diagnostic" when they were reported to be associated with male infertility, but the causality link has not been unequivocally established. The list of genes associated with male infertility related to the diagnostic suspect of the considered subjects included in the two NGS panel, is shown in **Table 1**.

The custom Illumina Nextera panel included genomic targets comprising coding exons and 15 bp flanking regions of each gene. The target length of the diagnostic panel was 314,814 bp. Instead, the target length of the pre-diagnostic panel was 188,074 bp. **Figure 1** describes the laboratory and analysis workflow.

### Genetic Analysis and Variant Detection

DNA samples were processed using MiSeq personal sequencer (Illumina, San Diego, CA, USA) using a paired-end protocol and a 150 bp long reads, following the laboratory methods described

**TABLE 1** | Diagnostic and pre-diagnostic genes associated with male infertility included in the custom NGS panels.

| Diagnostic and pre-diagnostic genes (Male condition) | Genes (coverage) | OMIM | REFSEQ |
|---|---|---|---|
| **Diagnostic genes (Defects of primary spermatogenesis)** | *AURKC* | *603495 | NM_001015878 |
| | *CATSPER1* | *606389 | NM_053054 |
| | *CFAP44* | *617559 | NM_018338 |
| | *DPY19L2* | *613893 | NM_173812 |
| | *KLHL10* | *608778 | NM_152467 |
| | *NANOS1* | *608226 | NM_199461 |
| | *PICK1* | *605926 | NM_012407 |
| | *PLK4* | *605031 | NM_014264 |
| | *SEPT12* | *611562 | NM_144605 |
| | *SOHLH1* | *610224 | NM_001012415 |
| | *SUN5* | *613942 | NM_080675 |
| | *SYCP3* | *604759 | NM_001177948 |
| | *TEX11* | *300311 | NM_001003811 |
| | *USP9Y* | *400005 | NM_004654 |
| | *ZPBP* | *608498 | NM_007009 |
| | *BRDT* | *602144 | NM_001726 |
| | *CFAP43* | *617558 | NM_025145 |
| | *DNAH1* | *603332 | NM_015512 |
| | *HSF2* | *140581 | NM_004506 |
| | *MEIOB* | *617670 | NM_152764 |
| | *NR5A1* | *184757 | NM_004959 |
| | *PLCZ1* | *608075 | NM_033123 |
| | *RHOXF2* | *300447 | NM_032498 |
| | *SLC26A8* | *608480 | NM_052961 |
| | *SPATA16* | *609856 | NM_031955 |
| | *SYCE1* | *611486 | NM_130784 |
| | *TAF4B* | *601689 | NM_005640 |
| | *TEX15* | *605795 | NM_001350162 |
| | *ZMYND15* | *614312 | NM_001136046 |
| **diagnostic genes (Hypogonadotropic hypogonadism)** | *ANOS1* | *300836 | NM_000216 |
| | *CCDC141* | *616031 | NM_173648 |
| | *DUSP6* | *602748 | NM_001946 |
| | *FGF17* | *603725 | NM_003867 |
| | *(100.0%)* | *136350 | NM_023110 |
| | *FGFR1* | *136530 | NM_000510 |
| | *(100.0%)* | *138850 | NM_000406 |
| | *FSHB* | *606807 | NM_017563 |
| | *(100.0%)* | *604161 | NM_032551 |
| | *GNRHR* | *608137 | NM_015537 |
| | *(100.0%)* | *607002 | NM_021935 |
| | *IL17RD* | *603961 | NM_006080 |
| | *(100.0%)* | *610224 | NM_001012415 |
| | *KISS1R* | *607984 | NM_030964 |
| | *(84.84%)* | *603819 | NM_001035235 |
| | *NSMF* | *162332 | NM_001059 |
| | *(95.03%)* | *109135 | NM_021913 |
| | *PROK2* | *608892 | NM_017780 |
| | *(97.67%)* | *613301 | NM_001024613 |
| | *SEMA3A* | *600483 | NM_033163 |
| | *(100.0%)* | *604808 | NM_198391 |
| | *SOHLH1* | *152760 | NM_001083111 |
| | *(100.0%)* | *604846 | NM_004807 |
| | *SPRY4* | *603286 | NM_002256 |
| | *(98.25%)* | *152780 | NM_000894 |
| | *SRA1* | *607002 | NM_001126128 |
| | *(100.0%)* | *607123 | NM_144773 |
| | *TACR3* | *608166 | NM_012431 |
| | *(100.0%)* | *602229 | NM_006941 |
| | *AXL* | *607984 | NM_001293290 |
| | *(100.0%)* | *162330 | NM_013251 |
| | *CHD7* | *606417 | NM_018117 |
| | *(99.54%)* | | |
| | *FEZF1* | | |
| | *(96.46%)* | | |
| | *FGF8* | | |
| | *(93.16%)* | | |
| | *FLRT3* | | |
| | *(100.0%)* | | |
| | *GNRH1* | | |
| | *(100.0%)* | | |
| | *HS6ST1* | | |
| | *(96.3%)* | | |
| | *KISS1* | | |
| | *(100.0%)* | | |
| | *LHB* | | |
| | *(100.0%)* | | |
| | *PROK2* | | |
| | *(97.67%)* | | |
| | *PROKR2* | | |
| | *(100.0%)* | | |
| | *SEMA3E* | | |
| | *(100.0%)* | | |
| | *SOX10* | | |
| | *(100.0%)* | | |
| | *SPRY4* | | |
| | *(98.25%)* | | |
| | *TAC3* | | |
| | *(100.0%)* | | |
| | *WDR11* | | |
| | *(100.0%)* | | |
| **Pre-diagnostic genes** | *ADGRG2* | *300572 | NM_001079858 |
| | *CFTR* | *602421 | NM_000492 |
| | *NLRP14* | *609665 | NM_176822 |
| | *RBMXL2* | *605444 | NM_014469 |
| | *INHBB* | *147390 | NM_002193 |
| | *INSL6* | *606414 | NM_007179 |
| | *FKBPL* | *617076 | NM_022110 |
| | *KLK12* | *605539 | NM_019598 |
| | *KLK14* | *606135 | NM_022046 |
| | *KLK15* | *610601 | NM_017509 |
| | *KLK3* | *176820 | NM_145864 |
| | *KLK4* | *603767 | NM_004917 |
| | *KLK6* | *602652 | NM_002774 |
| | *SEMG1* | *182140 | NM_003007 |
| | *TSPY1* | *480100 | NM_003308 |
| | *PRM1* | *182880 | NM_002761 |
| | *PRM2* | *182890 | NM_001286356 |
| | *NPAS2* | *603347 | NM_002518 |
| | *CFAP65* | *614270 | NM_194302 |
| | *DNAH6* | *603336 | NM_001370 |
| | *TDRD9* | *617963 | NM_153046 |
| | *RSPH1* | *609314 | NM_001286506 |
| | *CCDC40* | *613799 | NM_001243342 |
| | *CCDC39* | *613798 | NM_181426 |
| | *SPAG17* | *616554 | NM_206996 |
| | *DNAH10* | *605884 | NM_001372106 |
| | *CCDC103* | *614677 | NM_213607 |
| | *GAS8* | *605178 | NM_001286205 |
| | *DNAH5* | *603335 | NM_001369 |
| | *DNAI1* | *604366 | NM_012144 |
| | *AURKB* | *604970 | NM_004217 |

*(Continued)*

**TABLE 1** | Continued

| Diagnostic and pre-diagnostic genes (Male condition) | Genes (coverage) | OMIM | REFSEQ |
|---|---|---|---|
| | *CAMK4* | *114080 | NM_001744 |
| | *DPP6* | *126141 | NM_130797 |
| | *HORMAD1* | *609824 | NM_032132 |
| | *MAGEB4* | *300153 | NM_002367 |
| | *PIWIL1* | *605571 | NM_001190971 |
| | *PYGO2* | *606903 | NM_138300 |
| | *SPINK2* | *605753 | NM_021114 |
| | *TNP1* | *190231 | NM_003284 |
| | *TSPYL1* | *604714 | NM_003309 |
| | *E2F1* | *189971 | NM_005225 |
| | *USP26* | *300309 | NM_031907 |
| | *FKBP6* | *604839 | NM_003602 |
| | *NR0B1* | *300473 | NM_000475 |
| | *WT1* | *607102 | NM_000378 |
| | *NSUN7* | *617185 | NM_024677 |
| | *DNAH11* | *603339 | NM_003777 |
| | *GALNTL5* | *615133 | NM_145292 |
| | *GAPDHS* | *609169 | NM_014364 |
| | *TEKT2* | *608953 | NM_014466 |
| | *ADCY10* | *605205 | NM_018417 |
| | *PLA2G6* | *603604 | NM_001004426 |
| | *CATSPER2* | *607249 | NM_054020 |
| | *CATSPER4* | *609121 | NM_198137 |
| | *CATSPER3* | *609120 | NM_178019 |
| | *BSCL2* | *606158 | NM_032667 |
| | *NXF3* | *300316 | NM_022052 |
| | *PRMT7* | *610087 | NM_019023 |
| | *ANKS1A* | *608994 | NM_015245 |
| | *TSPAN7* | *300096 | NM_004615 |
| | *SPANXN5* | *300668 | NM_001009616 |
| | *SSX7* | *300542 | NM_173358 |
| | *AMELY* | *410000 | NM_001143 |
| | *EPHA3* | *179611 | NM_005233 |
| | *H2BFWT* | *300507 | NM_001002916 |

elsewhere (18, 19). Fastq (forward-reverse) files were obtained after sequencing. Reads alignment was done by the BWA (0.7.17-r1188) software. Duplicates were removed using the SAMBAMBA (0.6.7) program and GATK (4.0.0.0) were used for re-alignment. We used international databases dbSNP (www.ncbi.nlm.nih.gov/SNP/) and Human Gene Mutation Database professional (HGMD; https://apps.ingenuity.com/ingsso/login) for all nucleotide changes. *In silico* evaluation of the pathogenicity of nucleotide changes in exons was performed using Polymorphism Phenotyping v2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT, https://sift.bii.a-star.edu.sg/), and MutationTaster (http://www.mutationtaster.org). Minor allele frequencies (MAF) were checked in the Genome Aggregation Database gnomAD (http://gnomad.broadinstitute.org/). Sanger sequencing was performed for confirmation when target region coverage was less than 15 reads. Nucleotide alterations were analyzed and validated by Sanger sequencing. After confirmation, each variant was classified as a pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, or benign, according to the American College of Medical Genetics (ACMG) guidelines (20). Coding genomic regions (CDS) that were sequenced with coverage less than 15X were eventually re-sequenced using Sanger technology.

## RESULTS

Twelve infertile patients were analyzed with two NGS custom-made panels. They had a median age of 38 years (range 24–55). Clinical details, including testicular histology and responsiveness to FSH therapy (when available), are reported in **Table 2**.

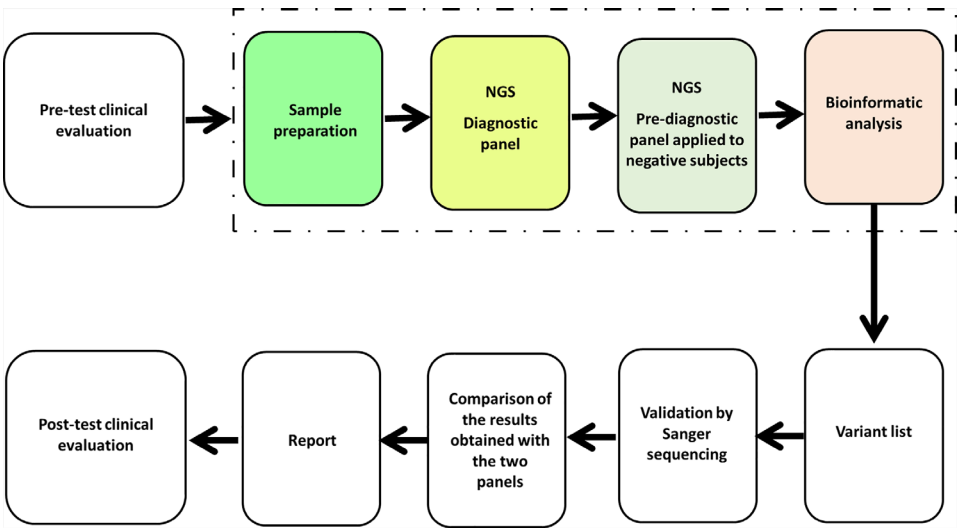


**FIGURE 1** | Laboratory and analysis workflow.

Unpredictably, after genetic testing and a more than a 2 year-long history of couple infertility, patients 5 (despite mild oligozoospermia) and 8 (despite oligozoospermia and testicular hypotrophy) spontaneously impregnated their wives, fathering healthy children.

Our gene panel design generated a mean sequencing depth of 359X, whereas 98% of the target regions had a sequencing depth of at least 25X. Variants in the pre-diagnostic genes were identified in 10/12 subjects negative to diagnostic testing with suspected defects of primary spermatogenesis (83%). Seventeen filtered variants were detected in 12 of the 65 genes analyzed (18%): *DNAH11*, *DNAH10*, *DNAH5*, *DNAI1*, *CCDC40*, *CFTR*, *GALNTL5*, *AMELY*, *KLK4*, *KLK14*, *CATSPER2*, and *ADCY10*. In particular, two heterozygous variants (p.Lys1853*, rs748618094, in *DNAH5* and p.Asp1152His, rs75541969, in *CFTR*) already reported as pathogenic were detected. Three variants with uncertain significance: p.Arg654Cys, rs140820295 in *DNAI1* (heterozygous); p.Pro3935Leu, rs72658814 in *DNAH11* (homozygous); and p.Asp284His, rs201042940 in *CCDC40* (heterozygous) were also found. All of them were predicted to be disease-causing by MutationTaster, Damaging by SIFT, and Probably Damaging by Polyphen-2.

Moreover, three variants with high impact were identified: the hemizygous splice variant c.574-1G>A (rs760519968) in *AMELY* affects the acceptor splice site of the last exon and may cause the activation of a cryptic splice site and consequently a stop-loss mutation. This variant is predicted to be disease-causing by MutationTaster. The heterozygous variant c.842+1G>C (rs199516208) in *CATSPER2* affects a donor splice site. This may cause the activation of a cryptic splice site and the introduction of a premature stop codon and is considered disease-causing by MutationTaster. The heterozygous truncating variant c.90T>A; p.Cys30* in *ADCY10*. This variant is considered pathogenic for the autosomal dominant inherited condition of susceptibility to absorptive hypercalciuria (OMIM #143870).

The genetic variants identified in the 12 infertile patients enrolled in this study using an NGS pre-diagnostic genes panel are reported in **Table 3**. Almost half of the variants identified by NGS in the 12 patients included in this study belong to the cytoplasmic dynein genes. The distribution of pre-diagnostic genes variants is shown in **Figure 2**.

**TABLE 2** | Clinical features of the patients positive for pre-diagnostic genes.

| | Gene(s) | Clinical suspect | Sperm parameters[1] | FSH serum levels (IU/ml) | Testicular volume (right and left)[2] | Testicular histology | FSH responsiveness[3] |
|---|---|---|---|---|---|---|---|
| **Subject 1** | *DNAH11, DNAI1, GALNTL5* | Primary defects of spermatogenesis | Mild OAT | 6.6 | 9.6 ml and 14.9 ml | — | No |
| **Subject 2** | *DNAH5, AMELY* | Primary defects of spermatogenesis | Azoospermia | 8.0 | 19.5 ml and 19.9 ml | NA | NA |
| **Subject 3** | *CCDC40* | Primary defects of spermatogenesis | OAT | 5.4 | 9.8 ml and 11.2 ml | — | NA |
| **Subject 4** | *DNAH10* | Primary defects of spermatogenesis | ? | ? | ? | ? | ? |
| **Subject 5** | *KLK4* | Primary defects of spermatogenesis | Mild OAT | 3.6 | 15.1 ml and 11.7 ml | — | NA |
| **Subject 6** | *DNAH10* | Primary defects of spermatogenesis | Mild OAT | 7.3 | 7.5 ml and 12.6 ml | — | No |
| **Subject 7*** | *DNAH11* | Primary defects of spermatogenesis | Normozoospermia | 5.7 | 10.9 ml and 10.7 ml | — | Yes |
| **Subject 8** | *CFTR* | Primary defects of spermatogenesis | OAT | 16.3 | 6.3 ml and 9.8 ml | — | — |
| **Subject 9** | *CATSPER2, KLK14* | Primary defects of spermatogenesis | Azoospermia | 32.7 | 6.7 ml and 8.7 ml | Sertoli cell only syndrome | — |
| **Subject 10** | *ADCY10* | Primary defects of spermatogenesis | OAT | 3.6 | 10.1 ml and 12.5 ml | — | No |

[1]*Assessed using WHO 2010 guidelines.*
[2]*Evaluated by ultrasound (ml).*
[3]*FSH responsiveness was defined by the doubling of sperm concentration or total sperm count vs. pre-treatment values.*
**The patient was diagnosed for reversal central hypogonadism. The values shown have been measured following 5 months from treatment withdrawal.*
*Severe oligozoospermia was defined for total sperm count <1.0 million; mild oligozoospermia was defined for total sperm count enclosed between 1.0 and 5.0 million; oligozoospermia for total sperm count enclosed between 5.0 and 39.0 million (21).*
*FSH, follicle-stimulating hormone; NA, not available; OAT, oligo-astheno-teratozoospermia.*

## DISCUSSION

Male infertility is a condition with highly heterogeneous phenotypic representation and a complex multifactorial etiology including environmental and genetic factors. The elevated number of candidate genes makes it hard to find a genetic cause of infertility in the majority of the cases (22–24). Anyway, a multi-disease gene panel can improve the identification of the etiology of male infertility (3, 25, 26). In several cases, idiopathic infertility has a genetic origin, therefore a correct phenotyping and medical history of the infertile patient may represent an initial basis for the genetic interpretation of the disorder (27), especially for the genetic variants of uncertain

**TABLE 3** | Genetic variants of the pre-diagnostic genes identified in infertile patients negative to an NGS diagnostic test consisting of 110 genes.

| | Gene | HGVS[1] cDNA | HGVS[1] protein | Reference ID according to NCBI | Consequence | Clinic relevance[2] | *In silico* prediction | ClinVar accession |
|---|---|---|---|---|---|---|---|---|
| **Subject 1** | *DNAH11* | NM_001277115.1:c.5805G>C | NP_001264044.1:p.Leu1935Phe | – | missense variant | – | deleterious | SCV001432675 |
| | *DNAI1* | NM_001281428.1:c.1960C>T | NP_001268357.1:p.Arg654Cys | rs140820295 | missense variant | uncertain significance | deleterious | SCV001432676 |
| | *GALNTL5* | NM_145292.3:c.1256G>C | NP_660335.2:p.Arg419Pro | – | missense variant | – | deleterious | SCV001432677 |
| **Subject 2** | *DNAH5* | NM_001369.2:c.5557A>T | NP_001360.1:p.Lys1853Ter | rs748618094 | stop gained | pathogenic | – | SCV001432678 |
| | *AMELY* | NM_001143.1:c.574-1G>A | – | rs760519968 | splice acceptor variant | – | – | SCV001432679 |
| **Subject 3** | *CCDC40* | NM_001243342.1:c.1945T>C | NP_001230271.1:p.Phe649Leu | – | missense variant | – | deleterious | SCV001432680 |
| | *CCDC40* | NM_001243342.1:c.850G>C | NP_001230271.1:p.Asp284His | rs201042940 | missense variant | uncertain significance | deleterious | SCV001432681 |
| **Subject 4** | *DNAH10* | NM_207437.3:c.10174C>G | NP_997320.2:p.Pro3392Ala | rs143987578 | missense variant | – | deleterious | SCV001432682 |
| **Subject 5** | *KLK4* | NM_001302961.1:c.395C>T | NP_001289890.1:p.Pro132Leu | rs144350395 | missense variant | – | deleterious | SCV001432683 |
| **Subject 6** | *DNAH10* | NM_207437.3:c.10954G>A | NP_997320.2:p.Ala3652Thr | – | missense variant | – | deleterious | SCV001432684 |
| | *DNAH10* | NM_207437.3:c.3514C>T | NP_997320.2:p.Leu1172Phe | rs778218750 | missense variant | – | deleterious | SCV001432685 |
| | *DNAH10* | NM_207437.3:c.3221A>G | NP_997320.2:p.Asn1074Ser | rs771006247 | missense variant | – | benign | SCV001432686 |
| **Subject 7** | *DNAH11* | NM_001277115.1:c.11804C>T | NP_001264044.1:p.Pro3935Leu | rs72658814 | missense variant | uncertain significance | deleterious | SCV001432687 |
| **Subject 8** | *CFTR* | NM_000492.3:c.3454G>C | NP_000483.3:p.Asp1152His | rs75541969 | missense variant | pathogenic & drug response | deleterious | SCV001432688 |
| **Subject 9** | *CATSPER2* | NM_001282309.2:c.842+1G>C | – | rs199516208 | splice donor variant | – | – | SCV001432689 |
| | *KLK14* | NM_001311182.1:c.700G>A | NP_001298111.1:p.Val234Met | rs201317571 | missense variant | – | deleterious | SCV001432690 |
| **Subject 10** | *ADCY10* | NM_001297772.1:c.90T>A | NP_001284701.1:p.Cys30Ter | – | stop gained | – | – | SCV001432691 |

*[1]All identified variants are indicated both by cDNA base sequence (third column) and by protein sequence (fourth column) according to the HGVS (Human Genome Variation Society) nomenclature guidelines.*

*[2]Information reported in NCBI (National Centre for Biotechnology Information) database.*

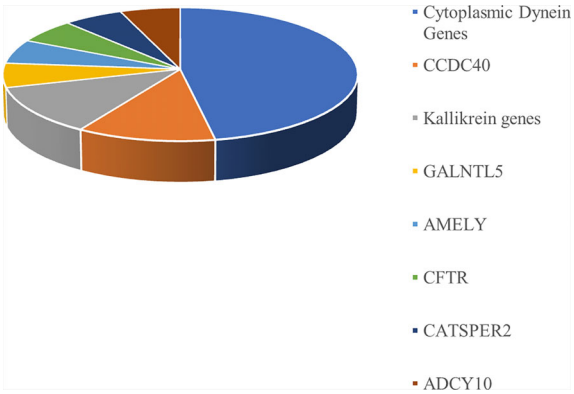


**FIGURE 2** | Pre-diagnostic gene variants distribution.

significance (VUS). To classify genetic variants, a prior likelihood of pathogenicity, based on *in silico* analysis, can be associated with the available genetic and epidemiological data to calculate the probability that a variant is pathogenic, in a multifactorial likelihood model.

Based on references of the American College of Medical Genetics and Genomics, genetic variants can be distinguished into five classes: pathogenic, likely pathogenic, variant of uncertain significance, likely benign, or benign (28). A VUS is a genetic change with unclear implications for gene function. Interpretation of VUS represents a difficult challenge for genetic counseling and clinical management of infertile male patients. It is fundamental to identify VUS and to evaluate them since, at moment, they are not clearly associated with a phenotype but may be classified as pathogenic in the future (29–31).

We have successfully developed a genetic test based on NGS that covers the main male infertility indications (9, 32, 33). We developed a custom-made panel of 65 additional pre-diagnostic genes that we tested in 12 infertile patients who were negative to a diagnostic panel consisting of 110 genes. Eleven patients had a primary spermatogenic failure and one patient had central hypogonadism.

In our analysis, 17 filtered variants were found in the following 12 out of the 65 genes analyzed (18%): *DNAH11*, *DNAH10*, *DNAH5*, DNAI1, *CCDC40*, *CFTR*, *GALNTL5*, *AMELY*, *KLK4*, *KLK14*, *CATSPER2*, and *ADCY10*. Some reports have described the involvement of the mutations of these genes in the pathogenesis of male infertility. As an example, *DNAH11*, *DNAH5*, *DNAI1*, and *CCDC40* genes have been linked to primary ciliary dyskinesia (34, 35). Similarly, the *GALNTL5* and the *KLK* genes may be involved in the pathogenesis of asthenozoospermia (36, 37).

Almost half of the variants identified by NGS belong to the cytoplasmic dynein genes (**Figure 2**). Dynein genes are known to be involved in the syndromic forms of asthenozoospermia, including primary ciliary dyskinesia/Kartagener syndrome (38–40). A possible association between variants of dynein genes and isolated non-syndromic asthenozoospermia has also been reported (41).

Two pathogenic variants in two patients with primary spermatogenic failure were identified: p.Lys1853*, rs748618094 in *DNAH5*, and p.Asp1152His, rs75541969 in *CFTR* (42). *DNAH5 (*Dynein Axonemal Heavy Chain 5), mapping on the chromosome 5p15.2, encodes an axonemal heavy chain dynein protein. Variations in this gene mainly cause primary ciliary dyskinesia type 3 and Kartagener syndrome, which are diseases due to ciliary defects. Truncating variants in *DNAH5* results in the absence of the outer dynein arm of the cilia, leading to abnormal ciliary structure and motor function (43, 44). In this specific case, Subject 2 has azoospermia and carries this variant in a heterozygous state, a trait that may be associated with mutations in *DNAH5*. However, pathologic phenotype associated with mutations in *DNAH5* is inherited in a recessive manner. We cannot exclude the presence of a large deletion/insertion in the other allele or the contribution of other genes. *CFTR* (CF Transmembrane Conductance Regulator), mapping on chromosome 7q31.2, encodes a membrane protein and chloride channel. Notoriously, mutations in this gene cause cystic fibrosis (45). *CFTR* is important for spermatogenesis (46). Genetic variants of the *CFTR* gene are a relatively frequent cause of male infertility, due to obstructive azoospermia, or in atypical forms of CF such as the congenital absence of the vas deferens, bilateral ejaculatory duct obstruction, or bilateral obstructions (47, 48). However, the patient studied here (Subject 8) has oligo-astheno-teratozoospermia, a trait never associated with this gene. We cannot exclude the presence of a large deletion/insertion in the other allele or the contribution of other genes.

Moreover, in our analysis three VUS were found: p.Arg654Cys, rs140820295 in *DNAI1*, p.Pro3935Leu, rs72658814 in *DNAH11*, and p.Asp284His, rs201042940 in *CCDC40*.

*DNAI1* (Dynein Axonemal Intermediate Chain 1), mapping on the chromosome 9p13.3, and *DNAH11* (Dynein Axonemal Heavy Chain 11), mapping on the chromosome 7p15.3, are other genes of the dynein family related to primary ciliary dyskinesia and involved in male infertility (48), especially in isolated non-syndromic asthenozoospermia (32). The variant in *DNAI1* is heterozygous; however primary ciliary dyskinesia caused by mutations in *DNAI1* is inherited in an autosomal recessive manner. We cannot exclude that heterozygous variants in *DNAI1* may cause a milder phenotype characterized only by infertility. In this specific case, Subject 1 showed oligo-astheno-teratozoospermia. Variants of *DNAH11* are found also in primary ciliary dyskinesia patients with normal ciliary ultrastructure. Interestingly, we found a patient (Subject 7) that carries the p.Pro3935Leu variant in a homozygous state. In gnomAD this variant is always reported in a heterozygous state. *CCDC40* (Coiled-Coil Domain Containing 40) mapping on the chromosome 17q25.3, is another gene associated with ciliary dyskinesia. The coiled-coil domain-containing protein CCDC40 is essential for motile cilia function and left-right axis formation (49). The variant p.Asp284His was found in compound heterozygosity with p.Phe649Leu, therefore we may speculate that both variants cannot cause major developmental defects like primary ciliary dyskinesia but they can cause oligo-astheno-teratozoospermia as observed in Subject 3. Interestingly, other variants with high impact requiring further functional and family segregation studies were identified. For instance, the splice variants rs760519968 in *AMELY* and rs199516208 in *CATSPER2*, and the stop gained variant p.Cys30* in *ADCY10*. To date, no loss-of-function mutations have been reported in the *AMELY* (Amelogenin Y-linked) gene in association with infertility. Structural rearrangements involving *AMELY*, mapping on the chromosome Yp11.2, have been found in patients with hypogonadism (50), although a direct link between the phenotype and the rearrangement has not been proven. *CATSPER2* (Cation Channel Sperm Associated 2) mapping on the chromosome 15q15.3 is the main $Ca^{2+}$ channel mediating extracellular $Ca^{2+}$ influx into spermatozoa. *CATSPER*-related infertility is associated with azoospermia. This is consistent with the phenotype reported in Subject 9 (51). *ADCY10* (Adenylate Cyclase 10) mapping on the chromosome 1q24.2, encodes for soluble adenylyl cyclase, which is the predominant adenylate cyclase in sperm crucial to sperm motility regulation, and it is associated with severe recessive asthenozoospermia (52). Subject 10 shows oligo-astheno-teratozoospermia, therefore his phenotype is partially overlapping with asthenozoospermia. Although truncating variants in *ADCY10* are recessively inherited when associated with infertility, we cannot exclude the presence of a large insertion/deletion in the other allele that was not detected with NGS.

Therefore, an NGS custom-made panel test including pre-diagnostic genes can give an improvement to genetic diagnostic testing and can influence male infertility clinical management. The precise prevalence of male infertility is not known and, at present, there are not complete systematic reviews or meta-analyses on the epidemiology of male infertility (53, 54). Making the diagnosis of genetic infertility is of relevance, also because the available epidemiological observations indicate lower life expectancy and higher morbidity in infertile patients (55, 56).

In conclusion, we showed the efficacy of NGS-based approaches also employing pre-diagnostic genes. This panel of genes may help to identify the etiology underlying the disorder and guide clinical management.

## DATA AVAILABILITY STATEMENT

The dataset presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found in the article/supplementary material.

## ETHICS STATEMENT

The experimental protocol was performed in the Division of Andrology and Endocrinology of the Teaching hospital "G. Rodolico," University of Catania, Catania, Italy. The internal Institutional Review Board approved the study protocol. An exhaustive explanation of the study purpose was given to each participant and informed written consent was obtained in compliance with Helsinki's declaration. The patients/

participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

VP wrote the article. RC collected clinical data and critically revised the article. SP, GMB, TB, LS, GT, and AZ analyzed the data and critically revised the article. GM performed the bioinformatic analysis and critically revised the article. AEC conceived the study, collected clinical data, supervised the work, and critically revised the article. MB conceived the study, supervised the work, and critically revised the article. All authors contributed to the article and approved the submitted version.

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## REFERENCES

1. Hotaling J, Carrell DT. Clinical genetic testing for male factor infertility: current applications and future directions. *Andrology* (2014) 2:339–50. doi: 10.1111/j.2047-2927.2014.00200.x
2. Cariati F, D'Argenio V, Tomaiuolo R. The evolving role of genetic tests in reproductive medicine. *J Transl Med* (2019) 17:267. doi: 10.1186/s12967-019-2019-8
3. Robay A, Abbasi S, Akil A, El-Bardisi H, Arafa M, Crystal RG, et al. A systematic review on the genetics of male infertility in the era of next-generation sequencing. *Arab J Urol* (2018) 16:53–64. doi: 10.1016/j.aju.2017.12.003
4. World Health Organization. *WHO Laboratory manual for the examination and processing of human semen*. Geneva: World Health Organization (2010).
5. Güney A, Javadova D, Kırac D, Ulucan K, Arafa G, Ergec D, et al. Detection of Y chromosome microdeletions and mitochondrial DNA mutations in male infertility patients. *Genet Mol Res* (2012) 11(2):1039–48. doi: 10.4238/2012.April.27.2
6. Stouffs K, Seneca S, Lissens W. Genetic causes of male infertility. *Ann d'endocrinol* (2014) 75(2):109–11. doi: 10.1016/j.ando.2014.03.004
7. https://omim.org/.
8. Bracke A, Peeters K, Punjabi U, Hoogewijs D, Dewilde S. A search for molecular mechanisms underlying male idiopathic infertility. *Reprod BioMed Online* (2018) 36:327–39. doi: 10.1016/j.rbmo.2017.12.005
9. Krausz C, Riera-Escamilla A. Genetics of male infertility. *Nat Rev Urol* (2018) 15:369–84. doi: 10.1038/s41585-018-0003-3
10. Pereira R, Sousa M. NGS and Male Infertility: Biomarkers Wanted. *Annu Res Rev Biol* (2015) 8:1–4. doi: 10.9734/ARRB/2015/20263
11. Cheung S, Parrella A, Rosenwaks Z, Palermo GD. Genetic and epigenetic profiling of the infertile male. *PLoS One* (2019) 14:e0214275. doi: 10.1371/journal.pone.0214275
12. Babakhanzadeh E, Nazari M, Ghasemifar S, Khodadadian A. Some of the Factors Involved in Male Infertility: A Prospective Review. *Int J Gen Med* (2020) 13:29–41. doi: 10.2147/IJGM.S241099
13. Tüttelmann F, Ruckert C, Röpke A. Disorders of spermatogenesis: Perspectives for novel genetic diagnostics after 20 years of unchanged routine. *Med Genet* (2018) 30:2–20. doi: 10.1007/s11825-018-0181-7
14. Punab M, Poolamets O, Paju P, Vihljajev V, Pomm K, Ladva R, et al. Causes of male infertility: a 9-year prospective monocentre study on 1737 patients with reduced total sperm counts. *Hum Reprod* (2017) 32:18–31. doi: 10.1093/humrep/dew284
15. Cannarella R, Condorelli RA, Duca Y, La Vignera S, Calogero AE. New insights into the genetics of spermatogenic failure: a review of the literature. *Hum Genet* (2019) 138:125–40. doi: 10.1007/s00439-019-01974-1
16. Cannarella R, Condorelli RA, Paolacci S, Barbagallo F, Guerri G, Bertelli M, et al. Next-generation sequencing: toward an increase in the diagnostic yield in patients with apparently idiopathic spermatogenic failure. *Asian J Androl* (2020) 138:125–40. doi: 10.4103/aja.aja_25_20
17. https://www.ncbi.nlm.nih.gov/books/NBK1116/.
18. Mattassi R, Manara E, Colombo PG, Manara S, Porcella A, Bruno G, et al. Variant discovery in patients with Mendelian vascular anomalies by next-generation sequencing and their use in patient clinical management. *J Vasc Surg* (2018) 67:922–32.e11. doi: 10.1016/j.jvs.2017.02.034
19. Marceddu G, Dallavilla T, Guerri G, Zulian A, Marinelli C, Bertelli M. Analysis of machine learning algorithms as integrative tools for validation of next generation sequencing data. *Eur Rev Med Pharmacol Sci* (2019) 23:8139–47. doi: 10.26355/eurrev_201909_19034
20. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* (2015) 17:405–24. doi: 10.1038/gim.2015.30
21. Tournaye H, Krausz C, Oates RD. Novel concepts in the aetiology of male reproductive impairment. *Lancet Diabetes Endocrinol* (2017) 5:544–53. doi: 10.1016/S2213-8587(16)30040-7
22. Zorrilla M, Yatsenko AN. The Genetics of Infertility: Current Status of the Field. *Curr Genet Med Rep* (2013) 1(4). doi: 10.1007/s40142-013-0027-1
23. Mallepaly R, Butler PR, Herati AS, Lamb DJ. Genetic basis of male and female infertility. *Monogr Hum Genet* (2017) 21:1–16. doi: 10.1159/000477275
24. Oud MS, Volozonoka L, Smits RM, Vissers LELM, Ramos L, Veltman JA. A systematic review and standardized clinical validity assessment of male infertility genes. *Hum Reprod* (2019) 5:932–41. doi: 10.1093/humrep/dez022
25. Patel B, Parets S, Akana M, Kellogg G, Jansen M, Chang C, et al. Comprehensive genetic testing for female and male infertility using next-generation sequencing. *J Assist Reprod Genet* (2018) 35:1489–96. doi: 10.1007/s10815-018-1204-7
26. Lorenzi D, Fernández C, Bilinski M, Fabbro M, Galain M, Menazzi S, et al. First custom next-generation sequencing infertility panel in Latin America: design and first results. *JBRA Assist Reprod* (2020) 24:104–14. doi: 10.5935/1518-0557.20190065
27. Normand EA, Alaimo JT, Van den Veyver IB. Exome and genome sequencing in reproductive medicine. *Fertil Steril* (2018) 109:213–20. doi: 10.1016/j.fertnstert.2017.12.010
28. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* (2015) 17:405. doi: 10.1038/gim.2015.30
29. Lazaridis KN, Schahl KA, Cousin MA, Babovic-Vuksanovic D, Riegert-Johnson DL, Gavrilova RH, et al. Outcome of Whole Exome Sequencing for Diagnostic Odyssey Cases of an Individualized Medicine Clinic: The Mayo Clinic Experience. *Mayo Clin Proc* (2016) 91:297–307. doi: 10.1016/j.mayocp.2015.12.018
30. Reuter C, Chun N, Pariani M, Hanson-Kahn A. Understanding variants of uncertain significance in the era of multigene panels: Through the eyes of the patient. *J Genet Couns* (2019) 28:878–86. doi: 10.1002/jgc4.1130
31. Clift K, Macklin S, Halverson C, McCormick JB, Abu Dabrh AM, Hines S. Patients' views on variants of uncertain significance across indications. *J Community Genet* (2020) 11:139–45. doi: 10.1007/s12687-019-00434-7
32. Neto FT, Bach PV, Najari BB, Li PS, Goldstein M. Genetics of Male Infertility. *Curr Urol Rep* (2016) 17:70. doi: 10.1007/s11934-016-0627-x
33. Miyamoto T, Minase G, Shin T, Ueda H, Okada H, Sengoku K. Human male infertility and its genetic causes. *Reprod Med Biol* (2017) 16:81–8. doi: 10.1002/rmb2.12017

34. Storm van's Gravesande K, Omran H. Primary ciliary dyskinesia: clinical presentation, diagnosis and genetics. *Ann Med* (2005) 37(6):439–49. doi: 10.1080/07853890510011985
35. Sui W, Hou X, Che W, Ou M, Sun G, Huang S, et al. CCDC40 mutation as a cause of primary ciliary dyskinesia: a case report and review of literature. *Clin Respir J* (2016) 10(5):614–21. doi: 10.1111/crj.12268
36. Emami N, Scorilas A, Soosaipillai A, Earle T, Mullen B, Diamandis EP. Association between kallikrein-related peptidases (KLKs) and macroscopic indicators of semen analysis: their relation to sperm motility. *Biol Chem* (2009) 390(9):921–9. doi: 10.1515/BC.2009.094
37. Takasaki N, Tachibana K, Ogasawara S, Matsuzaki H, Hagiuda J, Ishikawa H, et al. A heterozygous mutation of GALNTL5 affects male infertility with impairment of sperm motility. *Proc Natl Acad Sci U S A* (2014) 111(3):1120–5. doi: 10.1073/pnas.1310777111
38. Zariwala MA, Knowles MR, Leigh MW. Primary Ciliary Dyskinesia. In: MP Adam, HH Ardinger, RA Pagon, editors. *GeneReviews®*. Seattle (WA: University of Washington, Seattle (1993-2020). Available at: https://www.ncbi.nlm.nih.gov/books/NBK1122/.
39. Ji ZY, Sha YW, Ding L, Li P. Genetic factors contributing to human primary ciliary dyskinesia and male infertility. *Asian J Androl* (2017) 19:515–20. doi: 10.4103/1008-682X.181227
40. Leigh MW, Pittman JE, Carson JL, Ferkol TW, Dell SD, Davis SD, et al. Clinical and genetic aspects of primary ciliary dyskinesia/Kartagener syndrome. *Genet Med* (2009) 11:473–87. doi: 10.1097/GIM.0b013e3181a53562
41. Zuccarello D, Ferlin A, Cazzadore C, Pepe A, Garolla A, Moretti A, et al. Mutations in dynein genes in patients affected by isolated non-syndromic asthenozoospermia. *Hum Reprod* (2008) 23:1957–62. doi: 10.1093/humrep/den193
42. Pereira SV, Ribeiro JD, Ribeiro AF, Bertuzzo CS, Marson FAL. Novel, rare and common pathogenic variants in the CFTR gene screened by high-throughput sequencing technology and predicted by in silico tools. *Sci Rep* (2019) 9:6234. doi: 10.1038/s41598-019-42404-6
43. Hornef N, Olbrich H, Horvath J, Zariwala MA, Fliegauf M, Loges NT, et al. DNAH5 mutations are a common cause of primary ciliary dyskinesia with outer dynein arm defects. *Am J Respir Crit Care Med* (2006) 174:120–6. doi: 10.1164/rccm.200601-084OC
44. Wang K, Chen X, Guo CY, Liu FQ, Wang JR, Sun LF. Cilia ultrastructural and gene variation of primary ciliary dyskinesia: report of three cases and literatures review. *Zhonghua Er Ke Za Zhi* (2018) 56:134–7. doi: 10.3760/cma.j.issn.0578-1310.2018.02.012
45. Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, et al. Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* (1989) 245:1059–65. doi: 10.1126/science.2772657
46. Chen H, Ruan YC, Xu WM, Chen J, Chan HC. Regulation of male fertility by CFTR and implications in male infertility. *Hum Reprod Update* (2012) 18:703–13. doi: 10.1093/humupd/dms027
47. Stuhrmann M, Dörk T. CFTR gene mutations and male infertility. *Andrologia* (2000) 32:71–83. doi: 10.1046/j.1439-0272.2000.00327.x
48. Knowles MR, Leigh MW, Carson JL, Davis SD, Dell SD, Ferkol TW, et al. Mutations of DNAH11 in patients with primary ciliary dyskinesia with normal ciliary ultrastructure. *Thorax* (2012) 67:433–41. doi: 10.1136/thoraxjnl-2011-200301
49. Pathak D, Yadav SK, Rawal L, Ali S. Mutational landscape of the human Y chromosome-linked genes and loci in patients with hypogonadism. *J Genet* (2015) 94(4):677–87. doi: 10.1007/s12041-015-0582-1
50. Becker-Heck A, Zohn IE, Okabe N, Pollock A, Lenhart KB, Sullivan-Brown J, et al. The coiled-coil domain containing protein CCDC40 is essential for motile cilia function and left-right axis formation. *Nat Genet* (2011) 43:79–84. doi: 10.1038/ng.727
51. Hildebrand MS, Avenarius MR, Smith RJH, et al. CATSPER-Related Male Infertility. In: MP Adam, HH Ardinger, RA Pagon, editors. *GeneReviews*. Seattle, WA: University of Washington, Seattle (2009).
52. Akbari A, Pipitone GB, Anvar Z, Jaafarinia M, Ferrari M, Carrera P, et al. ADCY10 frameshift variant leading to severe recessive asthenozoospermia and segregating with absorptive hypercalciuria. *Hum Reprod* (2019) 34:1155–64. doi: 10.1093/humrep/dez048
53. Pastuszak AW, Sigalos JT, Lipshultz LI. The role of the urologist in the era of in vitro fertilization-intracytoplasmic sperm injection. *Urology* (2017) 103:19–26. doi: 10.1016/j.urology.2016.12.025
54. Mehta A, Nangia AK, Dupree JM, Smith JF. Limitations and barriers in access to care for male factor infertility. *Fertil Steril* (2016) 105:1128–37. doi: 10.1016/j.fertnstert.2016.03.023
55. Salonia A, Matloob R, Gallina A, Abdollah F, Saccà A, Briganti A, et al. Are infertile men less healthy than fertile men? Results of a prospective case-control survey. *Eur Urol* (2009) 56:1025–31. doi: 10.1016/j.eururo.2009.03.001
56. Eisenberg ML, Li S, Behr B, Cullen MR, Galusha D, Lamb DJ, et al. Semen quality, infertility and mortality in the USA. *Hum Reprod* (2014) 2:91567–74. doi: 10.1093/humrep/deu106


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frontiers in Endocrinology

REVIEW
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# Is HPV the Novel Target in Male Idiopathic Infertility? A Systematic Review of the Literature

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Infertility is an important health problem that affects up to 16% of couples worldwide. Male infertility is responsible for about 50% of the cases, and the various causes of male infertility may be classified in pre-testicular (for example hypothalamic diseases), testicular, and post-testicular (for example obstructive pathologies of seminal ducts) causes. Sexually transmitted infections (STI) are increasingly widely accepted by researchers and clinicians as etiological factors of male infertility. In particular, several recent reports have documented the presence of HPV in seminal fluid and observed that sperm infection can also be present in sexually active asymptomatic male and infertile patients. In this review, we aimed to perform a systematic review of the whole body of literature exploring the impact of HPV infection in natural and assisted fertility outcomes, from both an experimental and a clinical point of view. Starting from *in-vitro* studies in animals up to *in-vivo* studies in humans, we aimed to study and evaluate the weight of this infection as a possible cause of idiopathic infertility in males with any known cause of conception failure.

**Keywords: human papillomavirus, HPV seminal infection, male infertility, anti-sperm antibodies, assisted reproductive technique, HPV treatment**

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## INTRODUCTION

Human Papillomavirus (HPV) is the etiological agent of the most common sexually transmitted infection worldwide, with an estimated 6.2 million new cases annually (1). HPV comprises a group of small non-enveloped epitheliotropic viruses with a double-stranded circular DNA genome made-up of 8000 bp. Its virion has an icosaedral shape, of 55 nm diameter, constructed of 52 capsomeres, each containing five molecules of the major capsid protein L1 and a smaller number of the minor capsid protein L2 (2). HPV consists of more than 200 genotypes, adapted to particular epithelial tissues, such as anogenital skin and mucosa (3). According to the basis of oncogenic potential, HPV can be divided in two different groups: high-risk (HR-HPV) and low-risk (LR-HPV). The former ones, that include the well-known 16 and 18 types, have been classified as oncogenic to humans according the International Agency for Research on Cancer (4), and may cause neoplastic transformations in the following epithelial areas: cervix, vagina, vulva, anus, penis and oropharynx (5). The latter ones, such us 6 and 11 types, are responsible of benign diseases such as genital warts (6). HPV infections are primarily contracted by direct contact of the skin or

mucoses with an infected lesion. Genital HPV infection is largely transmitted through sexual intercourse, mostly insertive intercourses, although non-penetrative types of contact, such us genital-genital, oral-genital and manual-genital are also possible routes of transmission (7). Concerning the epidemiology of HPV infection, an evident difference occurs in the prevalence of the infection between females and males: while in the formers the prevalence is high in the first years after the sex debut and thereafter it decreases, in males the prevalence remains high during the whole life (8). Despite HPV-related diseases have been historically almost exclusively studied in females, recently, a growing interest is developing towards HPV infection in males. Recently it has been definitely demonstrated that, in addition to the well-known external genital areas, HPV virions may also be detected inside the male reproductive tract. In particular, it has been detected in male accessory glands were it can represent a possible cause of MAGI (male accessory gland infection), a condition which may play an important role in the impairment of seminal fluid and thus of fertility (9, 10). Finally, it was found in semen, both in exfoliated cells and even bound to spermatozoa (1, 11). Since the first studies which focused about this topic, it was clear that HPV does not enter the sperm cells, differently from the infection of epithelial cells and the exact localization of HPV in sperm was clarified by some important *in vitro* studies. Through immunofluorescence techniques, some authors clarified both the mechanism of sperm-HPV binding and its exact localization (12, 13). They reported that HPV-L1 capsid protein is able to bind the glycosamino-glycan Syndecan-I on the sperm surface and located in the equatorial region of the head. On this basis, different authors investigated the possible role of HPV semen infection in male infertility and recent metanalyses showed that this condition can impair couples fertility through different mechanisms (11, 14–16).

## AIMS OF THIS REVIEW

The first aim of this review was to summarize the implications of HPV semen infection on the following topics: i) effect on sperm parameters, ii) development of anti-sperm antibodies, iii) impact on both natural and assisted reproductive outcomes, such us pregnancy rate and miscarriage rate. We also discussed *in-vitro* studies reporting the possible role of HPV infected spermatozoa on blastocyst development and trophoblastic invasiveness. Moreover, we summarized the diagnostic and therapeutic strategies available in infertile couples aimed to improve the reproductive outcome.

## DATA SOURCES AND METHODS

Literature analysis was performed on the electronic databases Medline, Embase, ScienceDirect and the Cochrane Library, considering the time interval from January 1995 to October 2020. Key terms included: "HPV semen infection," "HPV male infection," "HPV and male infertility," "HPV and anti-sperm antibodies," "HPV and sperm parameters," "HPV and sperm DNA fragmentation," "anti-sperm antibodies and fertility," "HPV-infected spermatozoa and fertilization," "HPV and fertility outcome," "HPV and blastocyst," and "HPV and trophoblast." We considered randomized trials, observational and retrospective studies, original articles having as topic the relationship between HPV sperm infection and the following items: altered sperm parameters, anti-sperm antibodies (ASA), sperm apoptosis, sperm DNA alteration and infertility. In the included studies, all known causes of male infertility had been ruled out. We also included experimental *in vitro* studies focused on the effects of HPV infection on oocyte fertilization, blastocyst development, and trophoblastic cell invasiveness. Also, studies describing the adjuvant administration of the HPV quadrivalent vaccine Gardasil (Merck Serono S.p.A., Milan, Italy) as a possible strategy to promote HPV clearance from semen in infected males, were included. An accurate analysis of the references of the main works was successively performed. We considered data from eligible studies separately, according to different topics: "HPV and impairment of sperm parameters," "HPV and development of anti-sperm antibodies," "HPV and impairment of natural and assisted fertility outcome." Manuscripts were selected according to the use of the following methods: semen parameters and the related alterations were defined according to WHO laboratory manual for examination and processing of human semen (17) and Hamilton Thorn motility analyzer (18); HPV-DNA detection in spermatozoa, whole semen and fertilized oocyte, was performed by the use of polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) techniques. The evaluation of apoptotic events, related to HPV, in spermatozoa and embryo was detected through a terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling assay (TUNEL) test, DNA-Comet assay, DNA Disk chip assay, and Cell Death Detection ELISA assays or SCSA (flow cytometric technique). Sperm washing procedures relied on two-layer isolate colloid wash, test-yolk buffer procedures, swim-up procedure, modified swim-up with enzymatic treatment (Hyaluronidase) and discontinuous Ficoll gradients. Foresta et al. (13) used the hamster egg–human sperm penetration test (HEPT) to assess the ability of HPV-infected spermatozoa to fertilize and transfer viral genome into oocytes.

## RESULTS

The literature search, based on previously mentioned key terms, identified a total of 24 papers meeting all the eligibility criteria for this review. **Figure 1** reports the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram which shows the process of study selection. The reported evidences ranged between 1997 and 2020. Some papers evaluated more than one topic. Among these manuscripts, 14 papers focused on the clinical impact of HPV detection in semen related to the alteration of sperm parameters (11, 14, 19–30); three papers (already included in the previous group) focused on

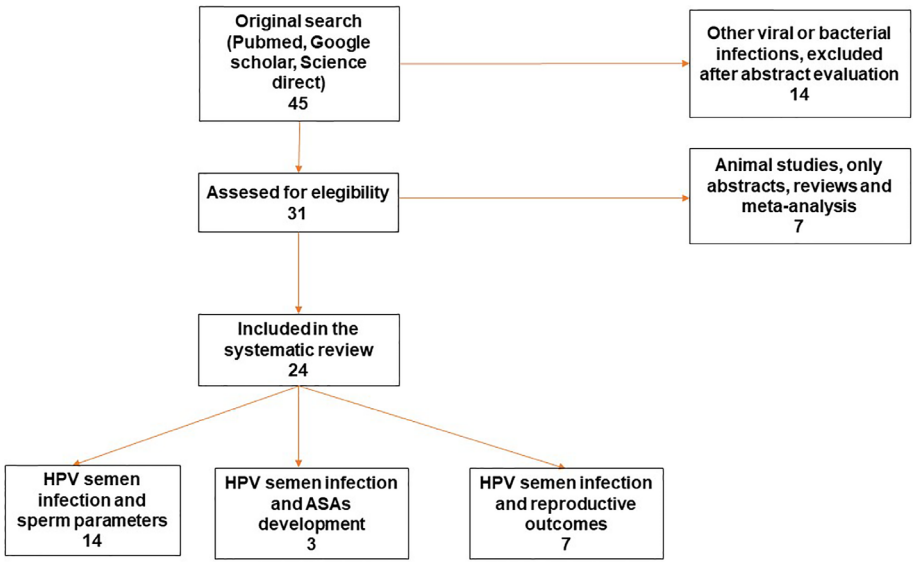


**FIGURE 1** | Preffered Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) flow diagram of the review study on literature analysis for HPV semen infection and sperm parameters, ASAs development and reproductive outcome. Some of the studies evaluated more than a topic. HPV, Human Papillomavirus; ASAs, antisperm antibodies.

the correlation between HPV semen infection in males, development of anti-sperm antibodies and their impact on male fertility (22, 25, 27) and seven papers (of which one already included in the first group) focused on the HPV semen infection in males and impairment of natural and assisted fertility outcomes (29, 31–36).

## HPV Semen Infection and Sperm Parameters

Because HPV infection is usually considered to be transient in males and without detrimental clinical consequences, its presence in semen has not been adequately investigated in the past. However, recently, an increasing number of studies have suggested the possible role of HPV in male infertility. In fact, several authors have confirmed the presence of the virus in the seminal fluid of men suffering from idiopathic infertility (15, 28, 37, 38). This data, combined with the higher prevalence of sperm HPV-infection in infertile subjects compared to general population (10%–35.7% *vs.* 2%–31%) (39) suggested that HPV may an important role in the impairment of sperm quality and, consequently, of male fertility.

**Table 1** reports the main studies which investigated the consequences of HPV seminal infections towards sperm quality seminal parameters and, consequently sperm quality. Among the 14 included studies reported, five reported a relation between HPV seminal infection and a reduced sperm motility (11, 14, 22, 24, 27). In addition, Piroozmand showed also a significant reduction of the sperm count. Boeri et al., documented an increased Sperm DNA fragmentation index when semen infections involved high-risk HPV genotypes (19) while Yang et al., reported that HPV seminal infection may cause an alteration of sperm normal morphology (30). In 2017, Damke et al. observed a correlation between HPV seminal infection and prostatic function, in terms reduced volume and increased pH and viscosity (20), while Rintala et al. demonstrated a seminal pH alteration in male patients with high-risk HPV seminal infection (28). Ultimately, Moghimi et al., observed a significantly higher prevalence of high-risk HPV in infertile men, compared to fertiles, associated with an impairment of sperm morphology and motility (26). Only four out of the 14 studies did not report any significant relationship between HPV seminal infection and sperm parameters (21, 23, 25, 29).

## HPV Semen Infection and Anti-Sperm Antibodies

In addition to the possible impairment of sperm parameters, different kind of semen infections have been associated with the development of anti-sperm antibodies (ASAs). Also HPV semen infection, has been recognized as a risk factor for the ASAs (40, 41). In fact, the prevalence of ASAs seems to be higher in infected infertile patients compared to non-infected infertiles and general population. Moreover, in infected infertile subjects, presence of antibodies is associated with a further reduction of sperm motility (42). Although the role of ASAs is controversial in reproduction, various mechanisms have been proposed as to how they affect male fertility, including sperm agglutination, impaired cervical mucus penetration, complement-mediated sperm injury through the female genital tract and interference with sperm-egg interaction (43). Different authors evaluated the role of HPV as an antigenic stimulus for the development of ASAs.

**Table 2** reports the main studies which investigated the relationship between HPV seminal infection, the development of ASAs and their implication in the worsening of fertility. In the study by Garolla et al., the authors studied the association between HPV infection and ASAs and their clearance time

**TABLE 1** | Association between Human Papillomavirus (HPV) seminal infection and impairment of sperm parameters.

| First Author | Country | Study setting | Kind of infertility | Years of infertility | Outcomes | Conclusions |
|---|---|---|---|---|---|---|
| **Boeri et al.** (19) | Italy | Cross-sectional s. | I. I. | N.A. | Volume, concentration, morphology, motility, leukocytes | HR-HPV genotypes induce reduced progressive sperm motility and increased sperm DNA fragmentation. |
| **Damke et al. (**20) | Brazil | Prospective cohort s. | I. I. | N.A. | Volume, concentration, morphology, motility, leukocytes | Altered prostate function with abnormal seminal volume, pH and viscosity. |
| **Fedder et al. (**21) | Denmark | Prospective cohort s. | N.O.A. | N.A. | Volume, concentration, sperm count | No impact on sperm parameters. |
| **Foresta et al. (**11) | Italy | Cross-sectional clinical s. | I. I. | N.A. | Volume, sperm count, concentration, motility, morphology | Reduced progressive sperm motility |
| **Foresta et al. (**14) | Italy | Cross-sectional clinical s. | N.A. | N.A. | Motility | Detrimental effect on sperm motility when HPV is bound to sperm |
| **Garolla et al. (**22) | Italy | Cross-sectional clinical s. | I. I. | 2 years | HPV sperm infection, sperm aneuploidies, and sperm ASAs. | Reduced sperm motility and presence of ASAs, that may further reduce male fertility. |
| **Kim et al. (**23) | Korea | Cross-sectional s. | I. I. | N.A. | Volume, concentration, spem-count, motility, morphology | No impact on sperm parameters. |
| **Lai et al. (**24) | China | Cross-sectional clinical s. | I. I. | N.A. | Morphology, motility | Higher incidence of asthenozoospermia. |
| **Luttmer et al. (**25) | Netherlands | Cross-sectional s. | N. A. | N.A. | Volume, concentration, sperm-count, motility | No impact on sperm parameters |
| **Moghimi et al. (**26) | Iran | Case-control s. | I. I. | N.A. | Concentration, morphology, motility | HR-HPV genotypes induce reduced progressive sperm motility |
| **Piroozmand et al. (**27) | Iran | Cross-sectional s. | N. A. | N.A. | HPV sperm infection and presence of sperm ASAs | Reduced sperm count and motility. |
| **Rintala et al. (**28) | Finland | Cross-sectional s. | N. A. | N.A. | Volume, Sperm count, concentration, motility | Impairment of seminal pH. |
| **Tanaka et al. (**29) | Japan | Case-control s. | N. A. | N.A. | Concentration, motility | No impact on semen parameters. |
| **Yang et al. (**30) | China | Case-control s. | I. I. | N.A. | Volume, concentration, motility, morphology | Impaired sperm motility and morphology |

*HPV, Human papillomavirus; HR-HPV, High-risk Papilloma viruses; ASAs, Anti-sperm antibodies; s, study; I. I., Idiopathic infertility; N.O.A., Non-obstructive azoospermia; N.A., not applicable.*

semen samples from infected and non-infected infertile subjects. They pointed out that more than 40% of HPV infected infertile patients had ASAs on the sperm surface. In contrast, this condition was significantly lower in non-infected infertile men and in fertile control subjects. Moreover, infected patients had a higher mean percentage of ASAs compared with non-infected ones. These findings suggested that sperm autoimmunity could probably be HPV-dependent. In order to confirm this finding, they documented the presence of both viral proteins and immunoglobulins in the same sperm cells of samples with positive sperm-mixed antiglobulin reaction (Mar) test results. Notably, when immunofluorescence for HPV 16-L1 was present on the sperm surface, they observed co-staining for IgA and IgG. This observation, according to the authors, suggested that semen infection could represent a new clinical condition associated with the presence of ASAs. Finally, they also reported that, in infected males, a significant viral clearance (approximately 85.3%) was obtained after 24 months of follow-up. Interestingly, the reduction in sperm infection paralleled the disappearance of ASAs and was significantly related to a progressive improvement of sperm motility (22). Likewise, Piroozmand et al. demonstrated that, compared with non-infected ones, patients with HPV semen infection had a higher rate of ASAs and a worse sperm quality, suggesting that young infertile couples should be tested for HPV and ASAs along with other causes of infertility (27). On the contrary, Luttmer et al. stated that the presence of HPV in semen was not associated with impaired semen parameters and presence of sperm ASAs (25).

## HPV Semen Infection and Reproductive Outcomes

Due to its ability to affect seminal parameters and to induce ASAs development, HPV semen infection seems to be a significant risk factor for male infertility. In this regard, many original studies, reviews and meta-analyses reported that HPV semen infection is related to reduced fertility both in natural and assisted conceptions. However, the exact mechanisms responsible for this condition have been never definitely assessed (16, 19, 31, 33, 39, 44, 45).

**Table 3** reports the main studies which investigated how HPV seminal infection in males may impair reproductive outcome in natural and/or assisted fertility. Depuydt et al., studying the HPV infection in semen samples (samples of sperm donors used for assisted reproductive techniques) coming from three different cryo-banks, observed that no pregnancies were obtained when using HPV-infected semen samples coming from sperm donors (31). The following year, in the context of the intra-uterine insemination technique (IUI), the detection of HPV virions in sperm from partners was associated with a negative IUI outcome (32). On this basis, they suggested to introduce in the counselling of infertile couples the examination of HPV in semen. In Italy, Garolla et al., reported that the presence of HPV in seminal fluid was associated with a reduction of both natural and assisted cumulative pregnancy rate and an increase in miscarriage rate (33). In another study, the same group reported that the administration of HPV quadrivalent vaccine Gardasil

**TABLE 2 |** Association between Human Papillomavirus (HPV) seminal infection and presence of anti-sperm antibodies.

| First Author | Country | Study setting | Kind of infertility | Years of infertility | Outcomes | Conclusions |
|---|---|---|---|---|---|---|
| **Garolla et al. (22)** | Italy | Cross-sectional clinical s. | I. I. | 2 years | HPV sperm infection, sperm aneuploidies, and sperm ASAs. | Association with ASAs and long-lasting persistence |
| **Luttmer et al. (25)** | Netherlands | Cross-sectional s. | N. A. | N. A. | HPV sperm infection and presence of sperm ASAs | No association with ASAs. |
| **Piroozmand et al. (27)** | Iran | Cross-sectional s. | N.A. | N. A. | HPV sperm infection and presence of sperm ASAs | Association with ASAs. |

*HPV, Human papillomavirus; ASAs, Anti-sperm antibodies; s, study; I. I., Idiopathic infertility; N.A., not applicable.*

(Merck Serono S.p.A., Milan, Italy) as an adjuvant tool to counteract seminal infection was associated with enhanced clearance of HPV from semen and, in parallel, with an increased rate of natural pregnancies and live births in idiopathic infertile couples (34). Even Perino et al. observed a significant correlation between pregnancy rate and seminal HPV DNA in males of infertile couples compared to HPV- negative (66.7% *vs.* 15%) (35). On the contrary, neither Tanaka et al., nor Tangal et al. reported any significant correlation between HPV semen infection and adverse natural or assisted fertility outcomes (29, 36). However, their results can be explained as follows: the first study evaluated only HPV 16 infection, while all HPV types seems to have a role in infertility; the second, did not test the presence of HPV in the day of failed Intra-cytoplasmic sperm injection (ICSI) procedure, but only in a following moment.

## Effect of HPV Semen Infection on Embryo-Development (*In-Vitro* Studies)

Many *in-vitro* studies have shown a negative influence of HPV infection upon several aspects of human fertility. For example, Gomez et al., in 2008, reported that HPV transfected blastocyst and trophoblastic cells were affected by a reduction in decidua invasion capacity, potentially responsible for a failure of maternal uterine wall invasion by trophoblastic cell, subsequent placental dysfunction and adverse pregnancy outcome (46). Furthermore, several experimental studies have demonstrated the role of HPV in causing pregnancy loss by transmission of viral genes to oocytes and determining DNA fragmentation and apoptosis of embryonic cells (47, 48). However, there is little evidence regarding the possibility that HPV infected sperm are able to interfere with embryo development when injected into the oocyte cytoplasm (such as during the procedure of *in-vitro* fertilization). To better understand this process, Foresta et al. performed an *in-vitro* study evaluating the ability of the virus- infected sperm to transfer HPV DNA and capsid proteins to oocyte during fertilization. After transfecting a human sperm with a plasmidic episome containing HPV E6 and E7 proteins, they used the hamster egg-human sperm penetration test to show the ability of infected sperm to transfer the capsid protein L1 to oocyte and the expression of E6 and E7 viral protein in the fertilized oocyte (13). Their findings demonstrated that both spermatozoa transfected with E6 and E7 genes and exposed to HPV L1 capsid protein were able to penetrate the oocyte. These laboratory data, combined with the observation that HPV DNA is found in a larger proportion of abortions rather than voluntary termination of pregnancy (35), may suggest an active role for HPV (which is carried to the egg by the spermatozoa) in the etiology of premature term gestation. This phenomenon could lead to an increase in the fragmentation of embryonic DNA resulting in alteration and apoptosis of the embryo (13). It should be remembered, however, that the situations described above refer to *in vitro* models that may not reflect the *in vivo* situation, where the entry of viral DNA into the egg has never been proven (22). In fact, the actual state of literature is insufficient to draw definite conclusion regarding the effect of HPV infection on the most important reproductive outcomes following natural and assisted fertility in women.

# POSSIBLE CLINICAL MANAGEMENT OF INFERTILE INFECTED PATIENTS

It is well known that HPV infections frequently affect individuals for a long time and it has been suggested that certain anatomic sites could act as viral reservoirs able to sustain the persistence of the infection (39, 49). Nowadays, there is a lack of an effective and resolutive treatment for HPV infection and related problems, including the consequences of HPV infection on fertility. Therefore, it is mandatory, while waiting for the development of effective prevention and therapy solutions, to educate and counsel infected couples through the suggestion of strategies able to counteract the infection, especially when they are seeking fertility, both naturally and by assisted reproductive technologies (ARTs). Firstly, it is important to educate and provide careful counselling in couples where at least one of the members is infected with HPV. A 2014 controlled study showed the effectiveness of this strategy. Couples in which both partners had HPV infection at genital site, were carefully counselled to follow some strict advices aimed to clear the virus (like: hygiene of both of their reproductive tract and their hand; using personal underwear and personal towels only; avoiding oral and anal sex) and monitored at 6, 12, 18, and 24 months. Counselled couples had a significantly higher clearance rate and shorter time of viral persistence, compared to non-counselled infected controls (50). Furthermore, recent evidence has suggested that HPV vaccination is a valid tool even in patients who have already contracted the infection. In fact, it has been highlighted that patients with HPV semen infection receiving vaccination, had a faster rate of seroconversion and greater viral clearance,

**TABLE 3** | Association between Human Papillomavirus (HPV) seminal infection and natural and/or assisted fertility outcomes.

| First Author | Country | Study setting | Kind of infertility | Years of infertility | Outcomes | Conclusions |
|---|---|---|---|---|---|---|
| **Depuydt et al.** (31) | Belgium | Prospective non interventional multicenter s. | N. A. | N. A. | Pregnancy rate in IUI from infected and non-infected sperm donors | No clinical pregnancy using infected sperm from donors |
| **Depuydt et al.** (32) | Belgium | Prospective non interventional multicenter s. | I. I. | N. A. | Pregnancy rate in IUI with infected and non-infected semen | Four times fewer clinical pregnancy using infected semen |
| **Garolla et al.** (33) | Italy | Cross-sectional clinical s. | I. I. | N. A. | Spontaneous and assisted reproductive outcomes (pregnancy rate, live births, and miscarriages) in infected and non-infected infertile couples | Reduction in natural and assisted cumulative pregnancy rate and increased miscarriage rate |
| **Garolla et al.** (34) | Italy | Case-control s. | I. I. | N. A. | Reproductive outcomes after HPV vaccination in infected infertile subjects | Better reproductive outcomes following HPV adjuvant vaccination |
| **Perino et al.** (35) | Italy | Prospective clinical s. | Oligo-astheno-teratozoospermia (58.6%) Idiopathic infertility (10.5%) | N. A. | Assisted reproductive outcomes in infected couples | Increased pregnancy loss using infected sperm |
| **Tanaka et al.** (29) | Japan | Case-control s. | N. A. | N. A. | In-vitro fertilization outcome in type 16 semen infection | No association with adverse fertility outcome |
| **Tangal et al.** (36) | Turkey | Prospective cohort s. | I. I. | N. A. | Prevalence of infection in failed ICSI cycles | No higher prevalence of HPV semen infection in patients with previous ICSI failures |

*HPV, Human papillomavirus; IUI, intra-uterine insemination; ICSI, intra-cytoplasmic sperm injection; s, study; I. I., Idiopathic infertility; N.A., not applicable.*

identified as the percentage of HPV DNA detectable in seminal fluid compared to infected patients who did not undergo vaccination (51). In the specific context of HPV and fertility outcome, a more recent study of 2018 analyzed 151 infertile couples in which the male partner had HPV semen infection. Among them, 79 males received the quadrivalent vaccination, while 71 refused it and served as controls. In 1 year of follow-up, an improvement of sperm parameters and natural fertility outcome was recorded in vaccinated patients compared to controls. In particular, vaccinated patients showed a higher viral clearance that paralleled an improvement of sperm motility and a reduction in the percentage of anti-sperm antibodies. Furthermore, couples where the male partner received vaccination recorded higher pregnancy and delivery rates and a lower miscarriage rate during the follow-up (34). In addition to counselling and adjuvant vaccination, different techniques of sperm selection (centrifugation, discontinuous density gradient and direct Swim-up) have been tested aimed to remove HPV from the sperm surface. However, all techniques had very poor or even absent effect in the complete removal of the virus. Very recently, our group tested a modified swim-up technique with the addiction of hyaluronidase enzyme obtaining the complete elimination of HPV from infected samples (52). The rationale of this treatment was to cleave the binding of HPV to its putative ligand, Syndecan-I located on the sperm surface (13). Compared to normal swim-up technique, the modified swim-up with hyaluronidase was able to abolish the binding between HPV and sperm in 100% cases of infected sperm, confirmed by negative fluorescent in-situ hybridization (FISH) for HPV, without any significant impairment of either motility or DNA fragmentation in the spermatozoa.

On the basis of these evidences, we recommend testing for HPV in the male partner of the infertile couples in the following cases: male affected by unexplained couple infertility (not related to known male or female factors), asthenozoospermia, presence of ASA, positive medical history for HPV infection or evidence of ongoing HPV-related diseases. **Figure 2** describes the management suggested for infertile men candidate to HPV detection in semen. As a first line diagnosis, we suggest performing HPV detection and genotyping on semen by Polymerase-chain reaction (PCR) or by Inno-LiPA Genotyping Extra assay as previously described (22, 53). In negative cases, the role of HPV in male infertility can be excluded. In patients tested positive, a FISH analysis for HPV should be performed to detect the presence of HPV-DNA bound to sperm. In patients with a negative FISH analysis, it is possible to search for fertility both naturally or by ART. In patients showing the presence of HPV on the spermatozoa, the management is different in cases of younger or older couples since it should consider other clinical conditions and include a recommendation not to delay the initiation of infertility treatment. In younger couples, we suggest the previously described counselling, adjuvant vaccination and a new FISH analysis after 6 months of follow-up. If negative, couples may undergo through natural or assisted fertility seeking. If still positive, couples have to be redirected towards the same route or counselled to change strategy. In the latter case and in older couples we suggest performing ARTs through a

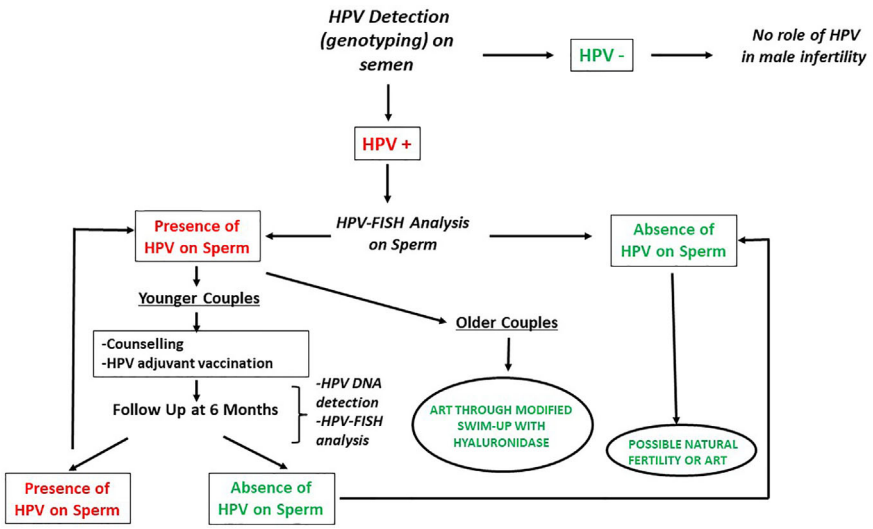


**FIGURE 2** | Management of infertile men candidate to HPV detection in semen (modified by Foresta et al. (39). Andrology). HPV, Human Papillomavirus; FISH, Fluorescent *in-situ* hybridization; ART, Assisted reproductive techniques.

modified technique of sperm selection by a modified swim-up with hyaluronidase to blunt virions attached to sperm.

## CONCLUSIONS

From the above reported literature, we can conclude that HPV semen infection can impair sperm quality and induce ASAs development, thus reducing couple fertility, particularly when viral DNA is present on the sperm surface. The impact of HPV genital tract infection in females is less clear, however the presence of the virus in embryos has been related to reduced pregnancy rate and increased abortion rate.

In the present systematic review, we summarize the state of the art on the link between HPV infection and couple infertility and suggest a flow-chart for the management and counselling of infertile couples in whom the male partner has HPV semen infection. The aim of this clinical management is to ensure the best reproductive outcome, both natural and through ARTs, particularly in those men without any other factor explaining infertility other than the presence of HPV. In the light of the large body of literature showing that HPV semen infection has a negative impact on reproductive outcome, we are strongly convinced that the time has come to follow this path. Further studies are required in order to demonstrate that the described strategy and, in particular, the clearance of HPV from semen is able to improve the delivery rate of infertile couples unable to conceive either naturally or *via* assisted reproduction.

## STRENGTH AND LIMITATIONS

The present review comprehensively summarizes, in a systematic way, the results of studies analyzing the correlation between HPV semen infection and male idiopathic infertility and provides the state of the art on the diagnostic and therapeutic strategies available in infertile couples aimed to improve the reproductive outcome. The main limitation of this analysis is that many of the studies, included in this review, did not consider important factors such as infertility duration and age of the female partner. Moreover, not all the studies clearly ruled out all known causes of infertility.

## AUTHOR CONTRIBUTIONS

AG, FM, and CF designed the study. LT, FM, GG, and AC collected the data. LT and FM interpreted the results and drafted the report. All authors contributed to the article and approved the submitted version.

## REFERENCES

1. Dunne EF, Nielson CM, Stone KM, Markowitz LE, Giuliano AR. Prevalence of HPV infection among men: A systematic review of the literature. *J Infect Dis* (2006) 194:1044–57. doi: 10.1086/507432
2. Doorbar J, Quint A, Banks L, Bravo IG, Stoler M, Broker TR, et al. The biology and life-cycle of human papillomaviruses. *Vaccine* (2012) 30:F55–70. doi: 10.1016/j.vaccine.2012.06.083
3. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, et al. WHO International Agency for Research on Cancer Monograph Working Group. A review of human carcinogens–Part B: biological agents. *Lancet Oncol* (2009) 10:321–2. doi: 10.1016/S1470-2045(09)70096-8
4. International Agency for Research on Cancer. Human papillomaviruses. *IARC Monogr Eval Carcinog Risks to Hum* (2007) 90:1–636.
5. Cubie HA. Diseases associated with human papillomavirus infection. *Virology* (2013) 445:21–34. doi: 10.1016/j.virol.2013.06.007

6. de Sanjosé S, Brotons M, Pavón MA. The natural history of human papillomavirus infection *Best Pract Res Clin Obstet Gynaecol* (2018) 48:2–13. doi: 10.1016/j.bpobgyn.2017.08.015
7. Dunne EF, Park IU. HPV and HPV-associated diseases. *Infect Dis Clin North Am* (2013) 27(4):765–78. doi: 10.1016/j.idc.2013.09.001
8. De Vuyst H, Clifford G, Li N, Franceschi S. HPV infection in Europe. *Eur J Cancer* (2019) 45:2632–9. doi: 10.1016/j.ejca.2009.07.019
9. La Vignera S, Vicari E, Condorelli RA, Franchina C, Scalia G, Morgia G, et al. Prevalence of human papilloma virus infection in patients with male accessory gland infection. *Reprod BioMed Online* (2015) 30:385–91. doi: 10.1016/j.rbmo.2014.12.016
10. La Vignera S, Condorelli RA, Cannarella R, Giacone F, Mongioi' L, Scalia G, et al. High rate of detection of ultrasound signs of prostatitis in patients with HPV-DNA persistence on semen: role of ultrasound in HPV-related male accessory gland infection. *J Endocrinol Invest* (2019) 42:1459–65. doi: 10.1007/s40618-019-01069-8
11. Foresta C, Pizzol D, Moretti A, Barzon L, Palù G, Garolla A. Clinical and prognostic significance of human papillomavirus DNA in the sperm or exfoliated cells of infertile patients and subjects with risk factors. *Fertil Steril* (2010) 94:1723–7. doi: 10.1016/j.fertnstert.2009.11.012
12. Pérez-Andino J, Buck CB, Ribbeck K. Adsorption of human papillomavirus 16 to live human sperm. *PloS One* (2009) 4:e5847. doi: 10.1371/journal.pone.0005847
13. Foresta C, Patassini C, Bertoldo A, Menegazzo M, Francavilla F, Barzon L, et al. Mechanism of Human Papillomavirus Binding to Human Spermatozoa and Fertilizing Ability of Infected Spermatozoa. *PloS One* (2011) 6:e15036. doi: 10.1371/journal.pone.0015036
14. Foresta C, Garolla A, Zuccarello D, Pizzol D, Moretti A, Barzon L, et al. Human papillomavirus found in sperm head of young adult males affects the progressive motility. *Fertil Steril* (2010) 93:802–6. doi: 10.1016/j.fertnstert.2008.10.050
15. Laprise C, Trottier H, Monnier P, Coutlée F, Mayrand MH. Prevalence of human papillomaviruses in semen: a systematic review and meta-analysis. *Hum Reprod* (2014) 29:640–51. doi: 10.1093/humrep/det453
16. Lyu Z, Feng X, Li N, Zhao W, Wei L, Chen Y, et al. Human papillomavirus in semen and the risk for male infertility: a systematic review and meta-analysis. *BMC Infect Dis* (2017) 17:714. doi: 10.1186/s12879-017-2812-z
17. World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*. World Health Organization, Cambridge, UK: Cambridge University Press (1999). Viral cancers.
18. Burkman LJ. Discrimination between non hyperactivated and classical hyperactivated motility patterns in human sperm using computerized analysis. *Fertil Steril* (1991) 55:363–71. doi: 10.1016/S0015-0282(16)54131-4
19. Boeri L, Capogrosso P, Ventimiglia E, Pederzoli F, Cazzaniga W, Chierigo F, et al. High-risk human papillomavirus in semen is associated with poor sperm progressive motility and a high sperm DNA fragmentation index in infertile men. *Hum Reprod* (2019) 34:209–17. doi: 10.1093/humrep/dey348
20. Damke E, Kurscheidt FA, Balani VA, Takeda KI, Irie MMT, Gimenes F, et al. Male Partners of Infertile Couples with seminal infections of human papillomavirus have impaired fertility parameters. *BioMed Res Int* (2017) 2017:4684629. doi: 10.1155/2017/4684629
21. Fedder J, Ørnskov D, Engvad B, Kristensen TK, Lomholt M, Marcussen N, et al. Seminal human papillomavirus originates from the body surface and is not a frequent aetiological factor in azoospermia. *Andrologia* (2019) 51:e13202. doi: 10.1111/and.13202
22. Garolla A, Pizzol D, Bertoldo A, De Toni L, Barzon L, Foresta C. Association, prevalence, and clearance of human papillomavirus and antisperm antibodies in infected semen samples from infertile patients. *Fertil Steril* (2013) 99:125–31. doi: 10.1016/j.fertnstert.2012.09.006
23. Kim SJ, Paik DJ, Lee JS, Lee HS, Seo JT, Jeong MS, et al. Effects of infections with five sexually transmitted pathogens on sperm quality. *Clin Exp Reprod Med* (2017) 44:207–2013. doi: 10.5653/cerm.2017.44.4.207
24. Lai YM, Lee JF, Huang HY, Soong YK, Yang FB. The effect of human papillomavirus infection on sperm cell motility. *J Infect Dis* (1997) 67:1152–5. doi: 10.1016/S0015-0282(97)81454-9
25. Luttmer R, Dijkstra MG Snijders PJF, Hompes PGA, Pronk DTM, Hubeek I, Johannes Berkhof J, et al. Presence of human papillomavirus in semen in relation to semen quality. *Hum Reprod* (2016) 31:380–6. doi: 10.1093/humrep/dev317
26. Moghimi M, Zabihi-Mahmoodabadi S, Kheirkhah-Vakilabad A, Kargar Z. Significant Correlation between High-Risk HPV DNA in Semen and Impairment of Sperm Quality in Infertile Men. *Int J Fertil Steril* (2019) 12:306–9. doi: 10.22074/ijfs.2019.5421
27. Piroozmand A, Mousavi Nasab SD, Erami M, Hashemi SMA, Khodabakhsh E, Ahmadi N, et al. Distribution of Human Papillomavirus and Antisperm Antibody in Semen and Its Association with Semen Parameters Among Infertile Men. *J Reprod Infertil* (2020) 21:183–8.
28. Rintala MA, Grénman SE, Pöllänen PP, Suominen JJ, Syrjänen SM. Detection of high-risk HPV DNA in semen and its association with the quality of semen. *Int J STD AIDS* (2004) 15:740–3. doi: 10.1258/0956462042395122
29. Tanaka H, Karube A, Kodama H, Fukuda J, Tanaka T. Mass screening for human papillomavirus type 16 infection in infertile couples. *J Reprod Med* (2000) 45:907–11.
30. Yang Y, Jia CW, Ma YM, Zhou LY, Wang SY. Correlation between HPV sperm infection and male infertility. *Asian J Androl* (2013) 15:529–32. doi: 10.1038/aja.2013.36
31. Depuydt CE, Donders GGG, Verstraete L, Vanden Broeck D, Beert JFA, Salembier G, et al. Time has come to include Human Papillomavirus (HPV) testing in sperm donor banks. *Facts Views Vis Obgyn* (2018) 10:201–5.
32. Depuydt CE, Donders GGG, Verstraete L, Vanden Broeck D, Beert JFA, Salembier G, et al. Infectious human papillomavirus virions in semen reduce clinical pregnancy rates in women undergoing intrauterine insemination. *Fertil Steril* (2019) 111:1135–44. doi: 10.1016/j.fertnstert.2019.02.002
33. Garolla A, Engl B, Pizzol D, Ghezzi M, Bertoldo A, Bottacin A, et al. Spontaneous fertility and in vitro fertilization outcome: New evidence of human papillomavirus sperm infection. *Fertil Steril* (2016) 105:65–72.e1. doi: 10.1016/j.fertnstert.2015.09.018
34. Garolla A, De Toni L, Bottacin A, Valente U, De Rocco Ponce M, Di Nisio A, et al. Human Papillomavirus Prophylactic Vaccination improves reproductive outcome in infertile patients with HPV semen infection: a retrospective study. *Sci Rep* (2018) 17:912. doi: 10.1038/s41598-018-19369-z
35. Perino A, Giovannelli L, Schillaci R, Ruvolo G, Fiorentino FP, Alimondi P, et al. Human papillomavirus infection in couples undergoing in vitro fertilization procedures: impact on reproductive outcomes. *Fertil Steril* (2011) 95:1845–8. doi: 10.1016/j.fertnstert.2010.11.047
36. Tangal S, Taşçı Y, Göksan Pabuçcu E, Çağlar SG, Haliloğlu AH, Yararbaş K. DNA fragmentation index and human papilloma virus in males with previous assisted reproductive technology failures. *Turk J Urol* (2018) 45(1):12–6. doi: 10.5152/tud.2018.96393
37. Chan PJ, Su BC, Kalugdan T, Seraj IM, Tredway DR, King A. Human papillomavirus gene sequences in washed human sperm deoxyribonucleic acid. *Fertil Steril* (1994) 61:982–5. doi: 10.1016/s0015-0282(16)56719-3
38. Nielson CM, Flores R, Harris RB, Abrahamsen M, Papenfuss MR, Dunne EF, et al. Human papillomavirus prevalence and type distribution in male anogenital sites and semen. *Cancer Epidemiol Biomarkers Prev* (2007) 16:1107–14. doi: 10.1158/1055-9965.EPI-06-0997
39. Foresta C, Noventa M, De Toni L, Gizzo S, Garolla A. HPV-DNA sperm infection and infertility: From a systematic literature review to a possible clinical management proposal. *Andrology* (2015a) 3:163–73. doi: 10.1111/andr.284
40. Ochsendorf FR. Sexually transmitted infections: impact on male fertility. *Andrology* (2008) 40:72–5. doi: 10.1111/j.1439-0272.2007.00825.x
41. Rusz A, Pilatz A, Wagenlehner F, Linn T, Diemer T, Schuppe HC, et al. Influence of urogenital infections and inflammation on semen quality and male fertility. *World J Urol* (2012) 30:23–30. doi: 10.1007/s00345-011-0726-8
42. Heidereich A, Bonfig R, Wilbert DM, Strohmaier WL, Engelmann UH. Risk factors for antisperm antibodies in infertile men. *Am J Reprod Immunol* (1994) 31:69–76. doi: 10.1111/j.1600-0897.1994.tb00849.x
43. Francavilla F, Santucci R, Barbonetti A, Francavilla S. Naturally-occurring antisperm antibodies in men: interference with fertility and clinical implications. An update. *Front Biosci* (2007) 12:2890–911. doi: 10.2741/2280
44. Siristatidis C, Vaidakis D, Sertedaki E, Martins WP. Effect of human papilloma virus infection on in-vitro fertilization outcome: systematic review and meta-analysis. *Ultrasound Obstet Gynecol* (2018) 51:87–93. doi: 10.1002/uog.17550
45. Xiong YQ, Chen YX, Cheng MJ, He WQ, Chen Q. The risk of human papillomavirus infection for male fertility abnormality: a meta-analysis. *Asian J Androl* (2018) 20:493–7. doi: 10.4103/aja.aja_77_17

46. Gomez LM, Ma Y, Ho C, McGrath CM, Nelson DB, Parry S. "Placental infection with human papillomavirus is associated with spontaneous preterm delivery". *Hum Reprod* (2008) 23(3):709–15. doi: 10.1093/humrep/dem404
47. Henneberg AA, Patton WC, Jacobson JD, Chan PJ. Human papilloma virus DNA exposure and embryo survival is stage-specific. *J Assist Reprod Genet* (2006) 23:255–9. doi: 10.1007/s10815-006-9030-8
48. Noventa M, Andrisani A, Gizzo S, Nardelli GB, Ambrosini G. Is it time to shift the attention on early stages embryo development to avoid inconclusive evidence on HPV-related infertility: debate and proposal. *Reprod Biol Endocrinol* (2014) 31:48. doi: 10.1186/1477-7827-12-48
49. Giuliano AR, Anic G, Nyitray AG. Epidemiology and patholohy pf HPV disease in males. *Gynecol Oncol* (2010) 117:15–9. doi: 10.1016/j.ygyno.2010.01.026
50. Garolla A, Pizzol D, Vasoin F, Barzon L, Bertoldo A, Foresta C. Counseling reduces HPV persistence in coinfected couples. *J Sex Med* (2014) 11:127–35. doi: 10.1111/jsm.12358
51. Foresta C, Garolla A, Parisi S, Ghezzi M, Bertoldo A, Di Nisio A, et al. HPV Prophylactic Vaccination in Males Improves the Clearance of Semen Infection. *EBioMedicine* (2015) 2:1487–93. doi: 10.1016/j.ebiom.2015.09.005
52. De Toni L, Cosci I, Carosso A, Barzon L, Engl B, Foresta C, et al. Hyaluronidase-based swim-up for semen selection in patients with human papillomavirus semen infection. *Biol Reprod* (2020) 10:ioaa173. doi: 10.1093/biolre/ioaa173
53. Foresta C, Ferlin A, Bertoldo A, Patassini C, Zuccarello D, Garolla A. Human papilloma virus in the sperm cryobank: An emerging problem? *Int J Androl* (2010) 34:242–6. doi: 10.1111/j.1365-2605.2010.01075.x

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# Sperm DNA Methylation at Metabolism-Related Genes in Vegan Subjects

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**Objective:** To investigate if epigenome of sperm cells could be dynamically affected by nutrition.

**Design and Methods:** We assessed 40 healthy volunteers with different dietary habits and collected their demographic characteristics, as well as clinical and anthropometric parameters. We compared methylation profiles in sperm quantified by bisulfite pyrosequencing, at promoter-associated CpG sites of genes involved in metabolism including fat mass and obesity-associated (*FTO*) and melanocortin-4 receptor (*MC4R*) from six vegans and 34 omnivores. In addition, the *FTO* rs9939609 (T>A) was genotyped.

**Results:** Higher DNA methylation levels were detected in the sperm of vegan at *FTO* gene CpG1 (p=0.02), CpG2 (p=0.001), CpG3 (p=0.004), and CpG4 (p=0.003) sites and at *MC4R*-CpG2 site [p=0.016] as compared to sperm of omnivores. This association was not related to *FTO* genotype.

**Conclusions:** Although limited by the small number of investigated cases, our data provide insight into the role of diet on sperm DNA methylation in genes involved in metabolism.

**Keywords: sperm, vegan, epigenetic, reproduction, nutrition**

## INTRODUCTION

Epigenetic modifications, including DNA methylation, histone marks and small non-coding RNAs, are stable and mitotically heritable changes that modulate normal and disease-related phenotypic differences. Among environmental factors able to induce epigenetic modifications, a key role is played by diet. Dietary habits can result in epigenetic modifications by acting directly on metabolism genes to up- or downregulate pathways involved in the bioavailability of nutrients (1). In addition, it has been also suggested that nutrition induced epigenetic modifications of gene expression can influence metabolism and susceptibility to non-communicable diseases (NCDs) (2–4).

To date, growing evidence suggests that the sperm epigenome can be dynamically affected by lifestyle (nutrient supply, physical exercise, alcohol and tobacco consumption) influencing not only male individual health status, but also his reproductive fitness and even the future offspring's health (5–9). Several studies have demonstrated that the spermatozoa from obese humans as well as from rats exposed to different models of diet, such as high-fat or low-protein diet, show an altered epigenetic signature, raising the question of a possible epigenetic inheritance of metabolic dysfunction (8, 10). Therefore, the possible role of diet in improving the quality of human sperm is becoming widely explored in the context of male infertility focusing on sperm epigenetic modifications (11).

Unlike omnivore diet, defined as a diet consuming all types of foods, the vegan diet is characterized by total exclusion of any animal derived substance, being thus very rich in fibres, but poor in proteins and fats (12). To date, this kind of diet is increasingly widespread in western societies, but its prevalence remains low (13). Although few studies described vegan diet as healthy, no conclusive data have been obtained yet (14–16), and vegan diet often requires supplementation of additional nutrients, at least during pregnancy (17).

To the best of our knowledge, the association between vegan diet and sperm DNA methylation patterns in human has not been investigated yet. In order to fill this gap, therefore, in the present study we investigated the effects of vegan diet as compared to omnivore diet on DNA methylation profiles in sperm at promoter-associated CpG sites of genes involved in metabolism, namely fat mass and obesity-associated (*FTO*) and melanocortin-4 receptor (*MC4R*).

*FTO* gene encodes for an AlkB-like 2-oxoglutarate-dependent nucleic acid demethylase, a potential regulator of RNA modification. It is highly expressed in the hypothalamus, visceral fat and liver but its function remains undefined. Recent studies reported that *FTO* seems to influence the Iroquois homeobox 3 (IRX3) expression with effects on body weight (18).

MC4R protein is a membrane-bound G-protein-coupled receptor found in brain regions, including the paraventricular nucleus in the hypothalamus (19) and common genetic variations near *MC4R* are involved in food intake by participating in appetite control and energy balance regulation (20–26).

Polymorphisms in these genes have been associated with body weight and composition, obesity, Type 2 Diabetes Mellitus (T2DM) and eating behavior (27–37).

The aim of this study is to evaluate the effect of vegan diet on sperm DNA methylation as a model to understanding the epigenetic effect of nutrition on male gametes and provide information for possible diet-based therapeutic strategies for improve male reproductive fitness.

# MATERIALS AND METHODS

## Study Participants

This is a multicentric study involving the Unit of Andrology and Reproductive Medicine of the Department of Medicine of the University of Padua (Italy), the Laboratory of Molecular Genetics, School of Medicine and Health Sciences, "G. D'Annunzio" University of Chieti (Italy), the Center for Diagnosis and Cure of Couple infertility, University of Siena (Italy) and the Spatocco Clinic, Chieti (Italy). Forty healthy subjects with different dietary habits were recruited. In particular, six cases have been following a vegan diet for at least 2 years, and the remaining 34 an omnivore diet. Exclusion criteria were obstructive azoospermia and known causes of infertility (previous or concomitant testicular cancer, orchitis, testicular torsion and trauma, use of gonadotoxic drugs, oncological diseases, karyotype anomalies, Y chromosome long arm microdeletions, mutations in the androgen receptor and varicocele).

The study of sperm DNA methylation was conducted at the Laboratory of Molecular Genetics, School of Medicine and Health Sciences, "G. D'Annunzio" University of Chieti, Italy.

## Patients

During the visit, data on demographic characteristics, anthropometric, and clinical parameters were collected. In addition, smoking habits and supplement intake were reported. Finally, physical activity (PA) was assessed registering the different levels of intensity (low, moderate, and high PA). All subjects underwent semen donation by masturbation into sterile containers after 2–5 days of sexual abstinence. Samples were allowed to liquefy for 30 min and were examined for sperm count, viability, motility and morphology according to the WHO criteria (38).

Sperm were isolated by using Percoll gradient centrifugation. After decondensation with proteinase K for total of 4 h DNA was extracted using the QIAamp® DNA Mini Kit according to manufacturer's recommendations.

The study was approved by the Ethics Committee of the University of Padua, Italy (protocol number #2208). In accordance with the Declaration of Helsinki, all participants gave their written informed consent prior to their inclusion in the study.

## DNA Methylation Analysis

The promoter associated CpG sites were tested in *FTO* gene (16q12.2, 4 CpGs sites), *MC4R* gene (18q21.32, 2 CpGs sites) and the imprinted gene *H19* (11p15.5, 5 CpGs sites). *FTO* and *MC4R* genes were selected due to their correlation with obesity, metabolism and appetite control. *H19* gene was included as a control, being maternally expressed only and thus expected to show a full methylation in male gametes. After bisulfite treatment, DNA (~20 ng) was amplified by PCR using the Kapa Hifi Hotstart Uracil+ HotStart Ready mix (Roche Diagnostics), according to manufacturer's recommendations.

Once NaBis-DNA amplified, the pyrosequencing was carried out using the PyroMark Q96 MD pyrosequencing instrument (Qiagen) with PCR and sequencing primers for the region of interest in the *FTO* and *MC4R* genes selected according to previous studies (39). In addition, PCR and sequencing primers for analysis of the *H19* CpGs were designed with PyroMark Assay Design (version 2.0.1.15; Qiagen). Primers information for these genes can be found in **Table 1**.

**TABLE 1** | Primers for bisulfite PCR and pyrosequencing.

| Gene | Primers | Product size (pb) | Number of CpGs analyzed |
|---|---|---|---|
| ***FTO*** | F: TTTGGAGTTATTTTTTTTTTGAGTAGAAAR: [Btn]ATTCTCCTTAAACTCTAACCTATTTACTS: TTTTAGGTTAGATAGTTGGAAGA | 168 | 4 |
| ***MC4R*** | F: AGGGTGATATAGATTTAGATGTAGAAGTR: [Btn]AAACAATATACTTTCCATTTCATTTTACACS: GTAGAAGTTTTTGAAGTTTG | 220 | 2 |
| ***H19*** | F: GGTTTTGGAGGTTAGTGTTTTR: [Btn]CTCAACCCCTAAAACTAACTTAACAS: TTGTATTATTTTTTTTTTTGAGAGT | 322 | 5 |

## Genotyping

The rs9939609 (T>A) SNP in *FTO* was genotyped in twenty-five subjects by PCR amplification (95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min) and direct sequencing procedure using BigDye Term v3.1 CycleSeq Kit (Life Technologies, Monza, Italy) followed by automatic sequencing analysis.

## Statistical Analysis

The subjects were stratified according to dietary habits and differences between the two groups were tested by the Mann-Whitney U for continuous variables or the Chi-square test for categorical variables. Shapiro-Wilk test showed a non-normal distribution of methylation levels. Thus, non-parametric analysis was conducted.

Moreover, to test the effect of genotypes on body mass index (BMI) as well as the effect of genotypes on methylation levels, a Nonparametric Kruskal-Wallis test was performed.

The quantitative variables were summarized as means and standard deviation (SD). Qualitative variables were summarized as percentage. All tests were 2-sided, and a level of statistical significance was set at $p < 0.05$. All analyses were performed with SPSS version 20.

# RESULTS

The demographic and clinical characteristics of study participants are summarised in **Table 2**. No difference was present in mean age and in BMI between vegan and omnivorous men.

The vegans reported no assumption of any type of dietary supplement. In addition, no significant difference was reported in physical activity and smoking between the subjects in the two groups. Sperm count parameters showed no significant difference in the two groups. All subjects had a normal sperm count, except one subject in the omnivore group and one in the vegan group who had mild oligozoospermia (<15 million/ml) and severe oligozoospermia (<5 million/ml), respectively.

Methylation profiles of sperm DNA from all men recruited are reported in **Table 3**. Vegan subjects showed higher sperm DNA methylation levels as compared to omnivorous men at *FTO* gene CpG1 (p=0.02), CpG2 (p=0.001), CpG3 (p=0.004), and CpG4 (p=0.003) sites. In addition, the mean DNA methylation percentages for the overall CpGs at *FTO* gene in sperm of vegan men were higher than in sperm of omnivorous men (p=0.001).

DNA methylation levels at *MC4R*-CpG1 site were not significantly different between vegan and omnivorous men, whereas DNA methylation levels at *MC4R*-CpG2 site were significantly different between the two groups (p=0.016). On the other hand, all the analyzed CpG sites of the *H19* gene were close to 100% of methylation in all the samples, consistently with their maternal imprinting. However, DNA methylation levels at *H19* gene CpG sites were slightly lower in sperm DNA of omnivores than in vegan men, although only CpG3 value did reach significance (p=0.007).

**TABLE 2** | Demographic and clinical characteristics of healthy vegans and omnivore.

| Characteristics | omnivorous men (n=34) | Vegan (n=6) | *p-value** |
|---|---|---|---|
| Age (yr)[a] | 35.5 ± 7.07 | 36.3 ± 7.58 | *0.86* |
| BMI (kg/m$^2$)[a] | 24.5 ± 2.47 | 25.7 ± 2.8 | *0.19* |
| Smoking[b] | | | *0.34* |
| No | 63.0 | 83.3 | |
| Yes | 37.0 | 16.7 | |
| Physical activity[b] | | | *0.63* |
| Low | 51.9 | 66.7 | |
| Moderate | 37.0 | 16.7 | |
| High | 11.1 | 16.7 | |
| Sperm concentration[a] ($10^6$/ml) | 79.7 ± 56.9 | 44.3 ± 21.2 | *0.14* |
| Progressive Sperm motility[b] | 57.2 ± 11.6 | 46.4 ± 22.4 | *0.29* |
| Total Sperm motility[b] | 59.2 ± 11.8 | 59 ± 23.7 | *0.60* |
| Sperm morphology (normal forms)[b] | 9 ± 3.3 | 10.2 ± 4.9 | *0.64* |

**Mann–Whitney U Test or Chi-square test.*
[a]*Data are presented as means ± SD.*
[b]*Data are presented as percent (%).*

**TABLE 3 |** Methylation profiles in sperm from vegans and omnivore.

| CpG Sites | omnivorous men (n=34) | Vegan (n=6) | *p-value** |
|---|---|---|---|
| ***FTO* gene** | | | |
| CpG1% | 1.44 ± 1.11 | 2.83 ± 1.33 | ***0.024*** |
| CpG2% | 2.53 ± 2.11 | 7.0 ± 2.28 | ***0.001*** |
| CpG3% | 3.65 ± 2.45 | 7.83 ± 4.75 | ***0.004*** |
| CpG4% | 0.85 ± 1.02 | 2.33 ± 0.82 | ***0.003*** |
| Mean all CpG sites % | 2.06 ± 1.48 | 5.0 ± 2.19 | ***0.001*** |
| ***MC4R* gene** | | | |
| CPG1% | 4.12 ± 1.53 | 4.17 ± 1.17 | *0.80* |
| CpG2% | 7.0 ± 2.26 | 10.17 ± 3.31 | ***0.016*** |
| Mean all CpG sites % | 5.65 ± 1.76 | 7.0 ± 2.28 | *0.17* |
| ***H19* gene** | | | |
| CpG1% | 91.5 ± 2.63 | 90.5 ± 1.38 | *0.23* |
| CpG2% | 94.41 ± 2.66 | 96.33 ± 3.14 | *0.17* |
| CpG3% | 92.26 ± 2.38 | 95.17 ± 1.60 | ***0.007*** |
| CpG4% | 92.38 ± 3.95 | 95.33 ± 4.08 | *0.11* |
| CpG5% | 98.32 ± 2.52 | 100 ± 0 | *0.05* |
| Mean all CpG sites % | 93.74 ± 2.06 | 95.33 ± 1.86 | *0.12* |

**Mann–Whitney U Test.*
*Data are presented as means ± SD.*
*Statistically significant values are in bold.*

No significant association was found for *FTO* genotype neither with BMI (**Supplementary Table 1**) nor with DNA methylation levels at *FTO* gene (**Supplementary Table 2**).

## DISCUSSION

The epigenetics of human sperm currently represents a very promising field of investigation in relation to the growing evidence of the role played by epigenetic alterations in male infertility. Very recently, the presence of sperm epigenomic alterations has been used as a biomarker of the success rate of ART protocols, since patients with specific pattern of sperm DNA methylation are characterized by a poor outcome of these protocols (40). Given the reversible nature of epigenetic modifications, males with aberrant sperm DNA methylation can be considered as excellent candidates to investigate treatments aimed to induce a reversibility of epigenetic alterations. It has been suggested that personalized nutrition could prevent or reverse the detrimental epigenetic modifications induced by unhealthy lifestyles. Since the spermatogenesis process is completed in a few weeks, it could be suggested that a few months period of personal lifestyle care could restore a physiological epigenetic mark of the sperm (10, 41). In this view, a crucial point is represented by the knowledge of the specific effect of different diets on the epigenetic pattern of human sperm.

Despite the exact relationship between diet and the epigenome remains unclear, several studies reported an association between epigenetic modifications and dietary depletion or supplementation (1, 42–48).

Recent study suggests substantial differences in methylation of CpG sites and genes, particularly in regulatory regions, between vegans and non-vegetarians, laying the foundation for the identification of transcriptional alterations associated with diet-influenced methylation patterns (49). In addition, genome-wide methylation analysis in the DNA of blood cells showed epigenetic differences in methylation in vegans versus pescatarians and in vegans versus nonvegetarians. In particular, the authors found that vegans had a higher methylation in the majority of the differentially methylated sites, with DNA hypomethylation occurring in only 4% of all DM probes in the nonvegetarian comparison; this value was 33% for the pescatarian comparison (50).

Moreover, it has been demonstrated that the sperm epigenome may be responsive to dietary factors (41, 51). In this view, we investigated for the first time, to the best of our knowledge, DNA methylation profiles of metabolism-related genes and their associations with vegan diet in sperm of healthy subjects. Our results showed that a vegan diet was associated with higher sperm DNA methylation at the *FTO* and *MC4R* genes.

These findings seem to confirm that a specific diet can induce epigenetic modifications in human sperm. The mechanism underlying this process and the possible consequences must be analyzed in detail. A possible explanation is that hypermethylation of *FTO* and *MC4R* genes is the result of the increased number of dietary methyl donors available in vegan diet. In fact, nutrients include molecules that constitute DNA and histone methylation, such as methylfolate, choline, betaine, methionine, vitamins B12, B6, and B2 (52). Several studies showed the critical nutrients occurrence for each dietary regime (53–56). In particular, on the micronutrient level, one study carried out on the largest sample of vegan dieters worldwide reported that the men following a vegan diet have lower intake of saturated fatty acids (SFA), retinol, vitamin B12 and D, calcium, zinc than omnivorous diet. On the other hand, higher levels of magnesium, iron, folic acid, vitamin B1, C, and a higher intake of dietary fiber have been shown in vegan compared to omnivore dieters (57). The risk of nutrient deficiencies for specific micronutrients is the major criticism of plant-based diets. In fact, 4% of vegans are more likely to need supplements and food fortified compared to omnivores (13). Noteworthy, in the present study the vegans reported no kind of dietary supplement. Our findings suggest that the possible imbalances in the metabolism of the methyl nutrients such as vitamins (folate, riboflavin, vitamin B6, choline) and amino acids (methionine, cysteine, serine, glycine) could potentially modulate hypo- or hyper- methylation of DNA. An interesting point is the comparison between our results and those reported in the literature on obese subjects. In fact, it has been described that *FTO* and *MC4R* genes show different levels on DNA methylation in obese as compared to lean subjects (41). However, these authors analyzed different CpG islands in these genes and results of the different islands were not consistent. Nevertheless, these results confirm that *FTO* and *MC4R* methylation in human sperm can be affected by individual lifestyle. In our study, surprisingly, the vegans had a higher BMI than omnivores, although not statistically significant. This is in contrast with previous studies reporting lower BMI as compared to omnivores whose diet included more proteins and less fibres (58, 59).

Given the potential role of genotype of *FTO* on gene expression (6, 60), we analyzed the effect of genotypes on methylation levels. No significant association was found between genetic variant and DNA methylation levels at *FTO* gene. To note, rs9939609 was not evaluated in the studies of Doaei et al. (60, 61), which pointed to another *FTO* SNP.

This study has some limitations. First of all, the cross-sectional study design. Secondly, the main limit of the study is the small sample size. In the last years, the number of subjects who began to adopt a vegan dietary pattern has increased, but still limited to a minority. To confirm these data, an increase in the number of subjects enrolled and the representation of different dietary groups are needed. The literature on the effects of diet on epigenetic alterations in sperm is limited as studies in this field are hardly available, largely due to difficulties implied by the collection of samples. The possible role of a diet in improving the quality of human sperm is underexplored in the context of sperm epigenetic modifications. As matter of fact, the connection between a vegan diet and sperm DNA methylation patterns in human is widely unknown and needs further insight.

Thirdly, the assessment of diet was occurred by self-report. Fourthly, the examined genes do not play a role in sperm development and function.

A strength of this study is that, to our knowledge, this is the first attempt to evaluate the effects of vegan diet on DNA methylation level at genes involved in metabolism in sperm.

Interestingly, Soubry introduced the Paternal Origins of Health and Disease (POHaD) paradigm emphasizing the paternal transgenerational epigenetic inheritance of metabolic disorders (62). Therefore, the epigenetic modifications of spermatozoa can be transmitted to the offspring and subsequent generations, thus influencing their lifetime health. In this regard, it has been demonstrated that obesity is a factor able to induce reversible sperm epigenetic modifications (8), providing insight into a possible role of specific diets in improving the human sperm quality. In accordance with these observations, we suggest that genes related to metabolism could be susceptible to germ cell epigenetic modulation in response to nutritional status and diet. A greater understanding of epigenetic pattern as modifiable component in the periconceptional period may provide new findings and propose a novel conceptualization of susceptibility to metabolic disturbances. This domain of research is solid, but the knowledge of the underlying mechanism is currently still lacking.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Padua, Italy protocol number #2208. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

The study was designed by CF and LS. CF, IS, PP, AL, and MS contributed to clinical evaluation and support to the recruitment of patients. IS, SF, and MF conducted the experiments. MF, IS, and LS contributed to data acquisition. MF, LS, and EV contributed to interpretation of results. MF performed the statistical analysis. The manuscript was drafted by MF and LS. All authors contributed to the article and approved the submitted version. LS and CF are the guarantors of this work.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.633943/full#supplementary-material

## REFERENCES


1. Ideraabdullah FY, Zeisel SH. Dietary Modulation of the Epigenome. *Physiol Rev* (2018) 98(2):667–95. doi: 10.1152/physrev.00010.2017
2. Parrillo L, Spinelli R, Nicolò A, Longo M, Mirra P, Raciti GA, et al. Nutritional Factors, DNA Methylation,and Risk of Type 2 Diabetes and Obesity: Perspectives and Challenges. *Int J Mol Sci* (2019) 20(12):2983. doi: 10.3390/ijms20122983
3. Franzago M, Fraticelli F, Stuppia L, Vitacolonna E. Nutrigenetics, epigenetics and gestational diabetes: consequences in mother and child. *Epigenetics* (2019) 14(3):215–35. doi: 10.1080/15592294.2019.1582277
4. Franzago M, Santurbano D, Vitacolonna E. Stuppia L.Genes and Diet in the Prevention of Chronic Diseases in Future Generations. *Int J Mol Sci* (2020) 21 (7):2633. doi: 10.3390/ijms21072633
5. Stuppia L, Franzago M, Ballerini P, Gatta V, Antonucci I. Epigenetics and male reproduction: the consequences of paternal lifestyle on fertility, embryo development, and children lifetime health. *Clin Epigenet* (2015) 7:120. doi: 10.1186/s13148-015-0155-4
6. Denham J, O'Brien BJ, Harvey JT, Charchar FJ. Genome-wide sperm DNA methylation changes after 3 months of exercise training in humans. *Epigenomics* (2015) 7(5):717–31. doi: 10.2217/epi.15.29
7. Soubry A, Guo L, Huang Z, Hoyo C, Romanus S, Price T, et al. Obesity-related DNA methylation at imprinted genes in human sperm: Results from the TIEGER study. *Clin Epigenet* (2016) 8:51. doi: 10.1186/s13148-016-0217-2
8. Donkin I, Versteyhe S, Ingerslev LR, Qian K, Mechta M, Nordkap L, et al. Obesity and bariatric surgery drive epigenetic variation of spermatozoa in humans. *Cell Metab* (2016) 23(2):369–78. doi: 10.1016/j.cmet.2015.11.004
9. Ingerslev LR, Donkin I, Fabre O, Versteyhe S, Mechta M, Pattamaprapanont P, et al. Endurance training remodels sperm-borne small RNA expression and methylation at neurological gene hotspots. *Clin Epigenet* (2018) 25(10):12. doi: 10.1186/s13148-018-0446-7
10. de Castro Barbosa T, Ingerslev LR, Alm PS, Versteyhe S, Massart J, Rasmussen M, et al. High fat diet reprograms the epigenome of rat spermatozoa and transgenerationally affects metabolism of the offspring. *Mol Metab* (2015) 5 (3):184–97. doi: 10.1016/j.molmet.2015.12.002

11. Franzago M, Rovere M, Franchi PG, Vitacolonna E, Stuppia L. Epigenetics and human reproduction: the primary prevention of the noncommunicable diseases. *Epigenomics* (2019) 11(12):1441–60. doi: 10.2217/epi-2019-0163
12. Dinu M, Abbate R, Gensini GF, Casini A, Sofi F. Vegetarian, vegan diets and multiple health outcomes: A systematic review with meta-analysis of observational studies. *Crit Rev Food Sci Nutr* (2017) 57(17):3640–9. doi: 10.1080/10408398.2016.1138447
13. Allès B, Baudry J, Méjean C, Touvier M, Péneau S, Hercberg S, et al. Comparison of sociodemographic and nutritional characteristics between self-reported vegetarians, vegans, and meat-eaters from the Nutrinet-Sante study. *Nutrients* (2017) 9(9):1023. doi: 10.3390/nu9091023
14. Craig WJ. Health effects of vegan diets. *Am J Clin Nutr* (2009) 89:1627S–33S. doi: 10.3945/ajcn.2009.26736N
15. Le LT, Sabaté J. Beyond meatless, the health effects of vegan diets: Findings from the Adventist cohorts. *Nutrients* (2014) 6:2131–47. doi: 10.3390/nu6062131
16. Medawar E, Huhn S, Villringer A, Veronica Witte A. The effects of plant-based diets on the body and the brain: a systematic review. *Transl Psychiatry* (2019) 9(1):226. doi: 10.1038/s41398-019-0552-0
17. Cetin I, Bühling K, Demir C, Kortam A, Prescott SL, Yamashiro Y, et al. Impact of Micronutrient Status during Pregnancy on Early Nutrition Programming. *Ann Nutr Metab* (2019) 74(4):269–78. doi: 10.1159/000499698
18. Gholamalizadeh M, Jarrahi AM, Akbari ME, Rezaei S, Doaei S, Mokhtari Z, et al. The possible mechanisms of the effects of IRX3 gene on body weight: an overview. *Arch Med Sci Atheroscler Dis* (2019) 4:e225–30. doi: 10.5114/amsad.2019.87545
19. Hinney A, Volckmar A-L, Knoll N. Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis. *In:Prog Mol Biol Transl Sci* (2013) 114:147–91. doi: 10.1016/B978-0-12-386933-3.00005-4
20. Dina C, Meyre D, Gallina S, Durand E, Körner A, Jacobson P, et al. Variation in FTO contributes to childhood obesity and severe adult obesity. *Nat Genet* (2007) 39(6):724–6. doi: 10.1038/ng2048
21. Fredriksson R, Hagglund M, Olszewski PK, Stephansson O, Jacobsson JA, Olszewska AM, et al. The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain. *Endocrinology* (2008) 149(5):2062–71. doi: 10.1210/en.2007-1457
22. Cecil JE, Tavendale R, Watt P, Hetherington MM, Palmer CN. An obesity-associated FTO gene variant and increased energy intake in children. *N Engl J Med* (2008) 359(24):2558–66. doi: 10.1056/NEJMoa0803839
23. Peng S, Zhu Y, Xu F, Ren X, Li X, Lai M. FTO gene polymorphisms and obesity risk: a meta-analysis. *BMC Med* (2011) 8(9):71. doi: 10.1186/1741-7015-9-71
24. Loos RJ. Genetic determinants of adiposity. In: ME Symonds, editor. *Adipose Tissue Biology*. New York: Springer Science (2012). p. 317–3.
25. Brunkwall L, Ericson U, Hellstrand S, Gullberg B, Orho-Melander M, Sonestedt E. Genetic variation in the fat mass and obesity-associated gene (FTO) in association with food preferences in healthy adults. *Food Nutr Res* (2013) 12:57. doi: 10.3402/fnr.v57i0.20028
26. Qi Q, Downer MK, Kilpeläinen TO, Taal HR, Barton SJ, Ntalla I, et al. Dietary intake, FTO genetic variants and adiposity: a combined analysis of over 16,000 children and adolescents. *Diabetes* (2015) 64:2467–76. doi: 10.2337/db14-1629
27. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* (2007) 316:889–94. doi: 10.1126/science.1141634
28. Stutzmann F, Cauchi S, Durand E, Calvacanti-Proença C, Pigeyre M, Hartikainen AL, et al. Common genetic variation near MC4R is associated with eating behaviour patterns in European populations. *Int J Obes (Lond)* (2009) 33:373–8. doi: 10.1038/ijo.2008.279
29. Bauer F, Elbers CC, Adan RA, Loos RJ, Onland-Moret NC, Grobbee DE, et al. Obesity genes identified in genome-wide association studies are associated with adiposity measures and potentially with nutrient-specific food preference. *Am J Clin Nutr* (2009) 90:951–9. doi: 10.3945/ajcn.2009.27781
30. Hardy R. Life course variations in the associations between FTO and MC4R gene variants and body size. *Hum Mol Genet* (2009) 120:2345–59. doi: 10.1093/hmg/ddp504
31. Church C, Moir L, McMurray F, Girard C, Banks GT, Teboul L, et al. Overexpression of Fto leads to increased food intake and results in obesity. *Nat Genet* (2010) 42:1086–92. doi: 10.1038/ng.713
32. Ahmad T, Chasman DI, Mora S, Paré G, Cook NR, Buring JE, et al. The fat-mass and obesity-associated (FTO) gene, physical activity, and risk of incident cardiovascular events in white women. *Am Heart J* (2010) 160:1163–9. doi: 10.1016/j.ahj.2010.08.002
33. Cecil J, Dalton M, Finlayson G, Blundell J, Hetherington M, Palmer C. Obesity and eating behaviour in children and adolescents: contribution of common gene polymorphisms. *Int Rev Psychiatry* (2012) 24:200–10. doi: 10.3109/09540261.2012.685056
34. Castellini G, Franzago M, Bagnoli S, Lelli L, Balsamo M, Mancini M, et al. Fat mass and obesity-associated gene (FTO) is associated to eating disorders susceptibility and moderates the expression of psychopathological traits. *PloS One* (2017) 12(3):10. doi: 10.1371/journal.pone.0173560
35. Fairbrother U, Kidd E, Malagamuwa T, Walley A. Genetics of Severe Obesity. *Curr Diabetes Rep* (2018) 18(10):85. doi: 10.1007/s11892-018-1053-x
36. Franzago M, Fraticelli F, Marchetti D, Celentano C, Liberati M, Stuppia L, et al. Nutrigenetic variants and cardio-metabolic risk in women with or without gestational diabetes. *Diabetes Res Clin Pract* (2018) 137:64–71. doi: 10.1016/j.diabres.2018.01.001
37. Crovesy L, Rosado EL. Interaction between genes involved in energy intake regulation and diet in obesity. *Nutrition* (2019) 67-68:110547. doi: 10.1016/j.nut.2019.06.027
38. WHO. *World Health Organization laboratory manual for the examination and processing of human semen. 5th ed.* Geneva: WHO Press (2010).
39. Mendoza-Pérez J, Gu J, Herrera LA, Tannir NM, Zhang S, Matin S, et al. Prognostic significance of promoter CpG island methylation of obesity-related genes in patients with nonmetastatic renal cell carcinoma. *Cancer* (2017) 123(18):3617–27. doi: 10.1002/cncr.30707
40. Jenkins TG, Aston KI, James ER, Carrell DT. Sperm epigenetics in the study of male fertility, offspring health, and potential clinical applications. *Syst Biol Reprod Med* (2017) 63(2):69–76. doi: 10.1080/19396368.2016.1274791
41. Donkin I, Barrès R. Sperm epigenetics and influence of environmental factors. *Mol Metab* (2018) 14:1–11. doi: 10.1016/j.molmet.2018.02.006
42. Hoek J, Steegers-Theunissen RP, Willemsen SP, Schoenmakers S. Paternal Folate Status and Sperm Quality, Pregnancy Outcomes, and Epigenetics: A Systematic Review and Meta-Analysis. *Mol Nutr Food Res* (2020) 64(9):1900696. doi: 10.1002/mnfr.201900696
43. Curtis EM, Krstic N, Cook E, D'angelo S, Crozier SR, Moon RJ, et al. Gestational vitamin D supplementation leads to reduced perinatal RXRA DNA methylation: results from the MAVIDOS trial. *J Bone Miner Res* (2019) 34(2):231–40. doi: 10.1002/jbmr.3603
44. Nur SM, Rath S, Ahmad V, Ahmad A, Ateeq B, Khan MI. Nutritive vitamins as epidrugs. *Crit Rev Food Sci Nutr* (2021) 61(1):1–13. doi: 10.1080/10408398.2020.1712674
45. Ong LT, Booth DR, Parnell GP. Vitamin D and its Effects on DNA Methylation in Development, Aging, and Disease. *Mol Nutr Food Res* (2020) 64(23):2000437. doi: 10.1002/mnfr.202000437
46. Keleher MR, Zaidi R, Hicks L, Shah S, Xing X, Li D, et al. A high-fat diet alters genome-wide DNA methylation and gene expression in SM/J mice. *BMC Genom* (2018) 19:888. doi: 10.1186/s12864-018-5327-0
47. Keleher MR, Zaidi R, Shah S, Oakley ME, Pavlatos C, El Idrissi S, et al. Maternal high-fat diet associated with altered gene expression, DNA methylation, and obesity risk in mouse offspring. *PloS One* (2018) 13:e0192606. doi: 10.1371/journal.pone.0192606
48. ElGendy K, Malcomson FC, Lara JG, Bradburn DM, Mathers JC. Effects of dietary interventions on DNA methylation in adult humans: Systematic review and meta-analysis. *Br J Nutr* (2018) 120:961–76. doi: 10.1017/S000711451800243X
49. Miles FL, Mashchak A, Filippov V, Orlich MJ, Duerksen-Hughes P, Chen X, et al. DNA Methylation Profiles of Vegans and Non-Vegetarians in the Adventist Health Study-2 Cohort. *Nutrients* (2020) 12(12):3697. doi: 10.3390/nu12123697
50. Filippov V, Jaceldo-Siegl K, Eroshkin A, Loskutov V, Chen X, Wang C, et al. Methylomes in Vegans versus Pescatarians and Nonvegetarians. *Epigenomes* (2020) 4:28. doi: 10.3390/epigenomes4040028

51. Bodden C, Hannan AJ. Reichelt AC Diet-Induced Modification of the Sperm Epigenome Programs Metabolism and Behavior. *Trends Endocrinol Metab* (2020) 31(2):131–49. doi: 10.1016/j.tem.2019.10.005
52. Obeid R. The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway. *Nutrients* (2013) 5 (9):3481–95. doi: 10.3390/nu5093481
53. Clarys P, Deliens T, Huybrechts I, Deriemaeker P, Vanaelst B, De Keyzer W, et al. Comparison of nutritional quality of the vegan, vegetarian, semi-vegetarian, pesco-vegetarian and omnivorous diet. *Nutrients* (2014) 6:1318–32. doi: 10.3390/nu6031318
54. Schüpbach R, Wegmüller R, Berguerand C, Bui M, Herter-Aeberli I. Micronutrient status and intake in omnivores, vegetarians and vegans in Switzerland. *Eur J Nutr* (2017) 56:283–93. doi: 10.1007/s00394-015-1079-7
55. Weikert C, Trefflich I, Menzel J, Obeid R, Longree A, Dierkes J, et al. Vitamin and Mineral Status in a Vegan Diet. *Deutsches Aerzteblatt Int* (2020) 117(35–36):575–82. doi: 10.3238/arztebl.2020.0575
56. Bakaloudi DR, Halloran A, Rippin HL, Oikonomidou AC, Dardavesis TI, Williams J, et al. Intake and adequacy of the vegan diet. A systematic review of the evidence. *Clin Nutr* (2020) 2020:S0261-5614(20)30656-7. doi: 10.3238/arztebl.2020.0575
57. Davey GK, Spencer EA, Appleby PN, Allen NE, Knox KH, Key TJ. EPIC–Oxford: lifestyle characteristics and nutrient intakes in a cohort of 33 883 meat-eaters and 31 546 non meat-eaters in the UK. *Public Health Nutr* (2003) 6:259–68. doi: 10.1079/PHN2002430
58. Spencer EA, Appleby PN, Davey GK, Key TJ. Diet and body mass index in 38000 EPIC-Oxford meat-eaters, fish-eaters, vegetarians and vegans. *Int J Obes Relat Metab Disord* (2003) 27(6):728–34. doi: 10.1038/sj.ijo.0802300
59. Tonstad S, Butler T, Yan R, Fraser GE. Type of vegetarian diet, body weight, and prevalence of type 2 diabetes. *Diabetes Care* (2009) 32(5):791–6. doi: 10.2337/dc08-1886
60. Doaei S, Kalantari N, Izadi P, Salonurmi T, Jarrahi AM, Rafieifar S, et al. Interactions between macro-nutrients' intake, FTO and IRX3 gene expression,and FTO genotype in obese and overweight male adolescents. *Adipocyte* (2019) 8(1):386–91. doi: 10.1080/21623945.2019.1693745
61. Doaei S, Kalantari N, Izadi P, Salonurmi T, Mosavi Jarrahi A, Rafieifar S, et al. Changes in FTO and IRX3 gene expression in obese and overweight male adolescents undergoing an intensive lifestyle intervention and the role of FTO genotype in this interaction. *J Transl Med* (2019) 17(1):176. doi: 10.1186/s12967-019-1921-4
62. Soubry A. POHaD: why we should study future fathers. *Environ Epigenet* (2018) 4(2):dvy007. doi: 10.1093/eep/dvy007


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# Testicular Growth and Pubertal Onset in GH-Deficient Children Treated With Growth Hormone: A Retrospective Study

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The prevalence of idiopathic oligozoospermia has been esteemed as high as 75%. An Italian survey has reported bilateral testicular hypotrophy in 14% of final-year high school students. The search for determinants of testicular growth in childhood is important for the primary prevention of spermatogenic failure. Therefore, this retrospective study aimed to evaluate the testicular growth and pubertal onset in deficient children treated recombinant human growth hormone (rhGH). To accomplish this, the clinical charts of 93 patients with GH deficiency (GHD) were carefully reviewed. Their mean age at the time of diagnosis was 11.2 ± 2.4 years. rhGH was administered for 44.0 ± 22.4 months, and the onset of puberty was recorded after a mean of 25.8 ± 22.4 months from the first rhGH administration. As expected, serum insulin-like growth factor 1 (IGF1) levels increased significantly after treatment. Before rhGH therapy, the Tanner stage was I in 59 out of 70 boys (84.3%), II in 8/70 (11.4%), III in 3/70 (4.3%). No one was on stage IV or V. The mean Tanner stage was 1.19 ± 0.51. At the last visit, the Tanner stage was I in 8/72 boys (11.1%), II in 6/72 (8.3%), III in 6/72 (8.3%), IV in 16/72 (22.2%), and V in 36/72 (50.0%). After a mean of 44.0 ± 22.4 months of rhGH treatment, the mean Tanner stage was 4.05 ± 1.30. Patients treated with rhGH showed a significant testicular volume (TV) growth over time, whereas no growth was observed in age-matched but not yet treated patients, even when the age was compatible with a spontaneous start of puberty. The multivariate regression analysis showed that the duration of treatment and the mean rhGH dose significantly predicted the percentage of TV increase. In contrast, age, serum FSH, and IGF1 levels, and final rhGH dose did not impact TV growth over time. In conclusion, these findings suggest that GH may play a role in testicular growth and pubertal onset, despite the descriptive nature of this study. Further properly designed studies are needed to confirm these findings. This knowledge may be useful to implement the diagnostic-therapeutic algorithm in case of a lack of testicular growth in childhood.

**Keywords: oligozoospermia, GH deficiency, GHD, IGF1, testicular volume, testicular growth, infertility**

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## INTRODUCTION

Male infertility represents an increasingly emergent issue in Western countries, since it affects ~7% of the male population (1). However, despite a through diagnostic work-up (including genetic testing), its etiology remains elusive in the vast majority of the cases (2). A longitudinal single center study carried out in 1737 patients with oligozoospermia esteemed the rate of idiopathy as high as 75% (3). Meta-regression data on secular trends of sperm parameters worryingly show that sperm concentration and total sperm count halved in the last forty years. The amount of the annual decrease of total sperm count was 1.6%, overall corresponding to a decline of 59.3% (4). These evidences push toward the urgent need of searching for the causes of apparently idiopathic male infertility.

In recent years, the knowledge on testicular physiology has increased (see 5–7 for review). The Sertoli cells (SCs), the main components of the prepubertal testis, are "nurse cells", as they release factors enhancing spermatogenesis in the adulthood. Also, SCs constitute the blood-testicular-barrier with the tight-junctions between them, which makes the testicular tubules immunologically silent, so that germ cells are protected by the attack of the immune system. SCs secrete androgen binding protein and anti-Müllerian hormone (AMH), needed for Müllerian ducts regression. Before puberty, SCs are in an immature state during which they actively proliferate and secrete AMH. When puberty starts, SCs switch from an immature to a mature state and lose the ability to mitotically divide. In this phase, they start to release inhibin B and the secretion of AMH declines (5–7).

Every SC provides the niche for the male germline and is able to support the proliferation and differentiation of a definite number of spermatogonia (8, 9). Thus, factors that impact on SC proliferation can likely influence fertility and sperm output in the adulthood. Therefore, at least some of the cases of apparently idiopathic oligozoospermia may be addressed to an abnormal proliferation of SCs in childhood, which entails low testicular volume (TV) in the adulthood. Accordingly, a low TV is associated with low sperm concentration and total sperm count (10). An Italian survey carried out in 3816 final-year high school students found bilateral testicular hypotrophy in up to 14% of cases (11). Hence, to find the factors involved in pre- and peri-pubertal testicular growth and SC proliferation may reasonably be of relevance for the fertility of the tomorrow's fathers.

Our *in vitro* experience on a model of porcine neonatal SCs suggests that insulin-like growth factor 1 (IGF1) promotes SC proliferation (12). In particular, incubation with IGF1 but not with growth hormone (GH), nor (surprisingly) with follicle-stimulating hormone (FSH), stimulates the proliferation of cultured SCs (12). Despite other data on the experimental animal support the relevance of IGF1 on testicular development and growth (13), the possible implication(s) of this knowledge in the clinical practice is unknown. In particular, the impact of IGF 1 serum levels on testicular growth in childhood has not been fully acknowledged yet. This information could be useful to implement the diagnostic-therapeutic algorithm in case of evidence of poor testicular volumetric growth in childhood.

GH deficiency (GHD) represent a useful clinical model which provides information on the *in-vivo* consequences of the lack of IGF1 and recombinant human GH (rhGH) replacement therapy on testicular growth and pubertal onset in a predefined window of life. Thus, this retrospective study aimed to evaluate the role of GH treatment on testicular growth and pubertal onset in a cohort of GHD children.

## PATIENTS AND METHODS

### Study Population

This is a retrospective study performed on male GHD children who were diagnosed and followed-up at the Unit of Pediatric Endocrinology, University of Catania (Catania, Italy), from May 2002 to December 2019. Specifically, all children referred for short stature [-2 SDS according to the Italian population-based reference (14)] underwent a complete medical evaluation, including anamnesis, physical examination, blood testing for measurements of complete blood count, fasting glucose, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), thyroid hormones, and insulin-like growth factor 1 (IGF1). Screening for celiac disease was also accomplished, by the measurement of serum total IgA, transglutaminase IgA, gliadin IgA, and deamidated gliadin IgA. In the case of negative values and no IgA deficiency, the algorithm stopped. In case of IgA deficiency, transglutaminase IgG, gliadin IgG, and deamidated gliadin IgG were measured. Patients with positive values underwent duodenal biopsy for diagnosis confirmation. In those with the suspicion of GHD, plasma GH response to both arginine infusion and glucagon was evaluated and the diagnosis was made when the $GH_{max}$ value at the two GH stimulation tests was <10 μg/l, as for the GH Research Society consensus guidelines (15). All GHD patients received rhGH treatment at a starting dose of 25 μg/kg/day. During follow-ups, dose adjustment was performed within a range of 25–50 μg/kg/day, according to guidelines (15) and in conformity with Note 39 of the Italian Drug Agency (AIFA) (http://www.agenziafarmaco.gov.it/content/nota-39).

Small for gestational age (SGA) patients, those with a head injury, endocrine disorders (hypogonadism, hyperprolactinemia, Cushing syndrome, and hypopituitarism), any form of tumor, exposure to radio- and/or chemotherapy, abnormal FSH levels, systemic diseases (kidney and/or liver diseases), and genetic disorders were excluded.

### Follow-Ups

Patients were followed-up every six months, as the guidelines suggest (15), for auxological measurements and puberty staging. IGF1 and testicular volume were evaluated at each follow-up time. Luteinizing hormone (LH), FSH, total testosterone (TT),

and bone age were assessed less frequently when needed (e.g., for monitoring pubertal onset or adverse effects).

Children were followed-up until they reached the near-adult height, or until the end of the study. The treatment was administered until the target height was achieved, independently of the pubertal stage. Pubertal onset was defined for a TV >3 ml by Prader orchidometer. The assessment of puberty was accomplished using the Tanner and Whitehouse staging method (16). The duration of puberty was calculated as the time elapsing between the first measurement of TV >3 ml and the age when the maximum TV was achieved.

## Hormonal Measurements

Hormone evaluation was performed in the central laboratory of the University-Teaching Hospital Policlinico "G. Rodolico-San Marco", by electrochemiluminescence (ECLIA) (Hitachi-Roche equipment, Cobas 6000, Roche Diagnostics, Indianapolis, IN, USA). Blood was collected at the Pediatrics Endocrinology outpatient clinic, in the morning.

## Statistical Analysis

Results are reported as mean ± SD throughout the study. Outcomes were classified according to rhGH administration. The normality of data distribution was evaluated using the Shapiro-Wilk test. Significant differences between mean TV, gonadotropin, TT, and IGF1 in age-matched treated and still untreated GHD patients were analyzed using the Student *t*-test for independent samples or the Mann-Whitney U test, as appropriate. Multivariate regression analysis was performed for the TV change over time, that is the percentage of TV increase during treatment. It was calculated as the ratio between the TV value at the end of rhGH therapy and TV value before rhGH therapy was started. The variables included in the model were: the length of treatment (number of months during which patients received rhGH therapy), mean IGF1, mean FSH serum levels, calculated from the beginning of puberty to the time when the full testicular development was reached, mean rhGH dosage, final rhGH dosage, age at the end of treatment. SPSS 22.0 for Windows (SPSS Inc., Chicago, USA) and RealStatistics add-on for Excel were used for statistical analysis. The results were considered statistically significant when the *p*-value was lower than 0.05.

**TABLE 1** | Anthropometric and auxological characteristics of the 93 patients with growth hormone (GH) deficiency included in this study at baseline [before the start of recombinant human GH (rhGH) therapy] and at the end of the study.

| Parameters | Baseline | End of the study |
|---|---|---|
| Age (years) | 11.2 ± 2.4 | 15.4 ± 3.1 |
| Dose of rhGH (mg/kg/daily) | 0.024 ± 0.004 | 0.03 ± 0.01 |
| Height (cm) | 127.01 ± 14.88 | 158.36 ± 11.0 |
| SDS of height | -2.54 ± 0.78 | -1.62 ± 0.79 |
| Body mass index (BMI) (Kg/m$^2$) | 17.93 ± 5.32 | 20.15 ± 4.60 |
| BMI standard deviation-score (SDS) | -0.40 ± 1.50 | -0.77 ± 1.68 |
| Growth velocity (cm/year) | 4.18 ± 1.20 | 5.03 ± 2.55 |
| Growth velocity SDS | -1.72 ± 1.66 | 2.13 ± 3.47 |
| Tanner stage | 1.19 ± 0.51 | 4.05 ± 1.30 |

*Tanner stage was available for 70 patients at baseline and 72 patients at the end of the study.*

## Ethical Approval

This study was conducted at the Division of Endocrinology, Metabolic Diseases and Nutrition of the University-Teaching hospital "G. Rodolico-San Marco", University of Catania (Catania, Italy). The protocol was approved by the internal Institutional Review Board, and informed written consent was obtained from the parents of each participant after full explanation of the purpose and nature of all procedures used. The study has been conducted in accordance with the principles expressed in the Declaration of Helsinki.

# RESULTS

Medical records of 115 patients were initially evaluated and 93 GHD patients were ultimately included in this study. The remaining 22 were excluded because they had craniofaringioma (n = 3), neuropsychiatric disorder under treatment with risperidone (n = 1), leukemia (n = 1), thalassemia major (n = 3), *short-stature homeobox* (*SHOX*) gene mutations (n = 2), Nijhegen syndrome (n = 1), 1q32.2p43 chromosome deletion (n = 1), Noonan syndrome (n = 1), renal failure (n = 1), medulloblastoma (n = 2), multiple exostoses (n = 1), and abnormalities at brain MRI (n = 2). Three patients with incomplete medical records were also excluded.

At the time of GHD diagnosis, the patients' mean age was 11.2 ± 2.4 years. The mean rhGH dose initially prescribed was 0.025 ± 0.003 mg/Kg daily; it was modulated at each follow-up according to its efficacy and IGF1 levels. The mean rhGH dose during therapy was 0.026 ± 0.003 mg/Kg daily. rhGH was administered for a mean of 44.0 ± 22.4 months. At the end of the study, patients showed an increase in height and BMI, compared to baseline (**Table 1**). Similarly, the Tanner stage was expectably higher at the end of the study (4.1 ± 1.3) compared to baseline (1.2 ± 0.5), and the onset of puberty was recorded after 25.8 ± 22.4 months from the first rhGH administration.

The majority of the patients were pre-pubertal before rhGH was prescribed. In particular, before rhGH therapy, the Tanner stage was I in 59 out of 70 boys, II in 8/70, and III in 3/70. None was on Tanner stage IV or V. At the last visit, the Tanner stage was I in 8 out of 72 boys, II in 6/72, III in 6/72, IV in 16/72, and V in 36/72 (**Figure 1**). Overall, at the end of the study, the treatment was ongoing in 17 patients since they had not already reached the target height. Among them, 10 had a Tanner stage V, 2 a Tanner stage IV, 1 a Tanner stage III, 3 a Tanner stage II, and 1 a Tanner stage I.

To appraise the effects of rhGH on the onset of puberty and testicular growth, we evaluated mean IGF1 and TV values at each follow-up visit during GH therapy, and these results were compared with those of the age-matched patients who were not on treatment yet. As showed in **Figure 2**, TV was significantly higher in the treated GHD patients, compared to

**TABLE 2** | Stepwise multivariate regression analysis.

**Model with all the variables entered.**

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 6 | 3613744.43 | 602290.74 | 9.9742 | 0.0000 |
| Regression | 6 | 3613744.43 | 602290.74 | 9.9742 | 0.0000 |
| Residual | 29 | 1751160.33 | 60384.84 | | |
| Total | 35 | 5364904.76 | | | |

**Parameters of multivariate regression analysis.**

| | *β coeff* | *std err* | *t stat* | *p-value* | *Lower 95%* | *Upper 95%* | VIF |
|---|---|---|---|---|---|---|---|
| Intercept | -1317.0624 | 816.2324 | -1.6136 | 0.1174 | -2986.4452 | 352.3202 | – |
| Mean IGF1 | 0.0059 | 0.3976 | 0.0148 | 0.9883 | -0.8073 | 0.8191 | 1.242 |
| Mean FSH | -19.8334 | 23.2197 | -0.8542 | 0.4000 | -67.3230 | 27.6562 | 1.61 |
| Age | 28.3438 | 38.5955 | 0.7344 | 0,4686 | -50.5928 | 107.2805 | 1.475 |
| Duration of treatment (months) | 12.4452 | 1.9576 | 6.3573 | 0.0000 | 8.4415 | 16.4490 | 1.226 |
| Mean rhGH dose | 31595.3224 | 22148.8406 | 1.4265 | 0.1644 | -13704.1430 | 76894.7878 | 4.76 |
| Final rhGH dose | 226.7791 | 17306.4988 | 0.0131 | 0.9896 | -35168.9852 | 35622.5434 | 4.051 |

**Model after removal of variables with p>0.05.**

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 2 | 3536761,7 | 1768381 | 31,92123 | 0.0000 |
| Residual | 33 | 1828143,1 | 55398,27 | | |
| Total | 35 | 5364904,8 | | | |

**Parameters after Stepwise procedure.**

| | *β coeff* | *std err* | *t stat* | *p-value* | *Lower 95%* | *Upper 95%* | VIF |
|---|---|---|---|---|---|---|---|
| Intercept | -861.5613 | 363.1699 | -2.3723 | 0.0237 | -1600.4360 | -122.6865 | – |
| Duration of treatment (months) | 12.3217 | 1.7792 | 6.9253 | 0.0000 | 8.7018 | 15.9416 | 1.000 |
| Mean rhGH dose | 30447.4389 | 13889.9927 | 2.1920 | 0.0355 | 2188.0362 | 58706.8416 | 1.000 |

*IGF1, insulin-like growth factor 1; FSH, follicle-stimulating hormone; rhGH, recombinant human growth hormone.*

the age-matched GHD patients who were not on treatment yet. In the patients not already treated, who served as controls, the TV volume remained pre-pubertal, even in those with an age compatible with a spontaneous start of puberty (13-15 years). Only an untreated 15.5 years old patient had a TV of 11 ml (**Figure 2** and **Supplementary Table 1**).

However, baseline TV values were available in 59 patients. Among these, only 5 had a TV >3 ml, which corresponded to 11.4% (5/44) of the patients aged ≥10 years. All the other patients had a TV ≤3 ml (**Figure 3A**). TV at the end of rhGH administration was available in 60 patients, and a full testicular development was reached in 70% of cases (**Figure 3B**). Among patients with TV <12 ml at the last visit, 53.3% (8/15) withdrew from therapy before the age of 16 years.

Expectably, treated patients showed significantly higher IGF1 levels compared to the age-matched not yet treated ones (**Figure 4**). No difference was found in serum LH, FSH, and TT (**Supplementary Table 2**). These findings suggest the influence of the GH-IGF1 system on TV growth over-time and the onset of puberty.

To evaluate whether the increase in TV was influenced by the duration of rhGH therapy, we built a multivariate regression model, including the length of treatment (number of months during which patients received rhGH therapy), mean IGF1, mean FSH serum levels, calculated from the beginning of puberty to the time when the full testicular development was reached, mean rhGH dosage, final rhGH dosage, and age at the end of treatment. The stepwise procedure showed that the duration of treatment and the mean dose were the only two variables that significantly influenced the percentage of TV increase. By contrast, the other variables were shown not to influence TV growth over time (**Table 2**).

## DISCUSSION

This retrospective study was undertaken to evaluate whether treatment with rhGH can influence testicular growth and the onset of puberty in a cohort of patients with GHD, the vast majority of whom were pre-pubertal. We found that only the patients receiving rhGH had a progressive increase of the TV over time, whereas still untreated patients did not show any significant TV increase. However, the final TV of the patients treated with rhGH remained lower than the normative values

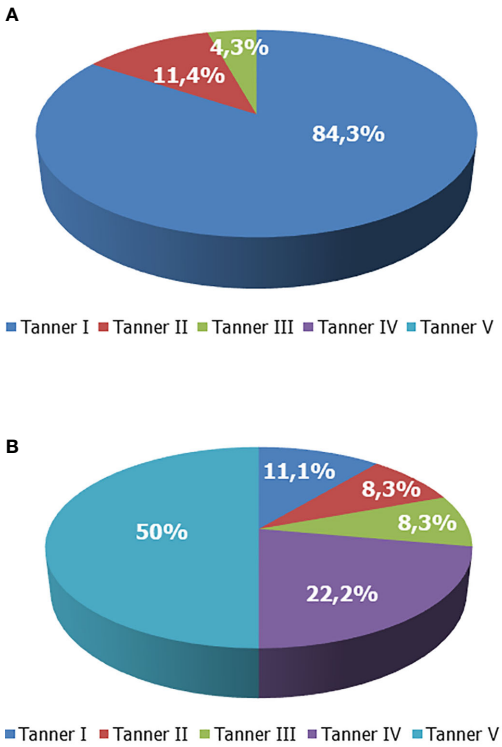


**FIGURE 1** | Tanner stage of boys with growth hormone (GH) deficiency. **(A)** Tanner stage at enrollment. **(B)** Tanner stage at the end of the study.

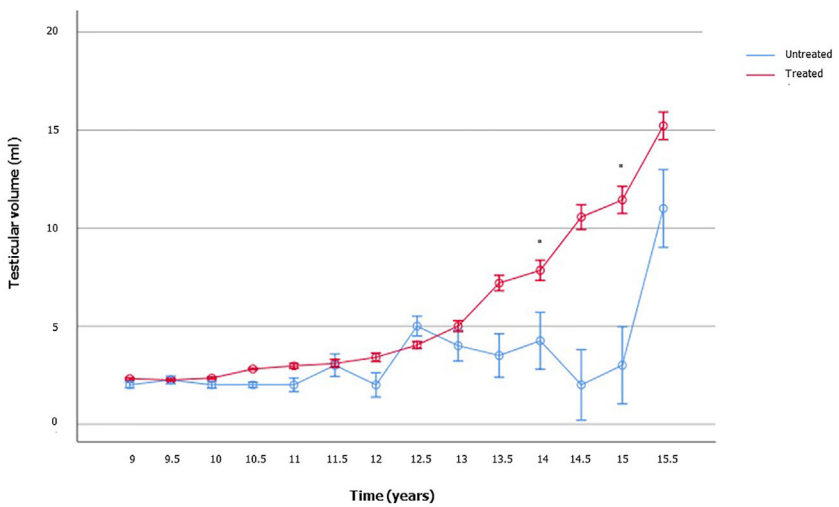


**FIGURE 2** | Testicular volume changes over time in boys with growth hormone (GH) deficiency who received recombinant human GH (rhGH) and in aged-matched not yet treated GHD boys. The number of patients available for each time point is reported in the **Supplementary Table 1**.

reported in healthy children and adolescents (17). Moreover, TV growth was influenced by the duration of rhGH treatment. Limitedly to the retrospective design of this study, these results suggest a role of GH (and of IGF1) in testicular growth and pubertal onset. On note, the treatment was ongoing in 17 patients at the end of the study, since they had not yet

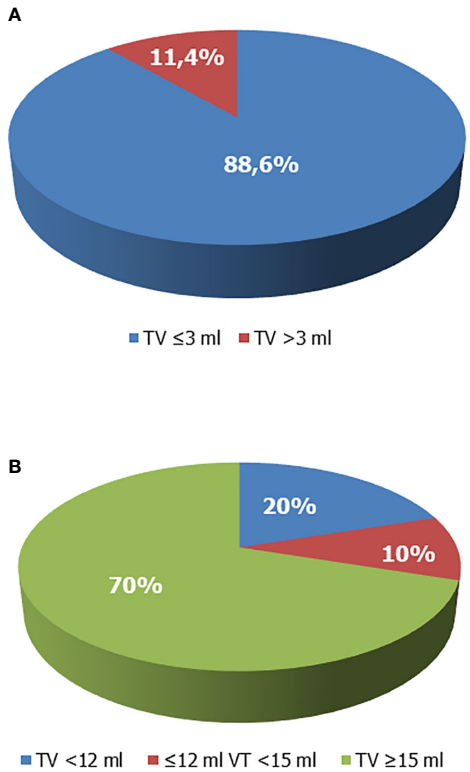


**FIGURE 3** | Testicular volume (TV) of boys with growth hormone (GH) deficiency. **(A)** TV at enrollment. Overall, 88.6% of patients whose TV values were available prior to recombinant human GH (rhGH) administration and aged ≥10 years showed TV values ≤3 ml and had a Tanner stage 1. **(B)** TV at the end of rhGH administration. The 70% of patients on rhGH therapy had TV ≥15 ml at the end of therapy, 20% had values <12 ml, compatible with testicular hypotrophy. Ten percent had borderline values (12 ml ≤ TV < 15 ml).

reached the target height. However, the majority of them (10/17) had a Tanner stage V and only 1 was prepubertal (Tanner stage I). Hence, the results were not influenced by the ongoing treatment.

These findings are in agreement with those of the previous literature. However, few studies have assessed the effects of rhGH administration on the onset of puberty and testicular health in the past because this aspect has poorly been explored in GHD. Nevertheless, pubertal delay is a common finding in GHD patients, which has led to treatment with oxandrolone or T in the past (18). The onset of puberty has been reported to occur after 19.0 ± 3.5 months after rhGH therapy initiation and no other specific treatments, in a cohort of eight GHD children. Interestingly, no abnormality of conventional sperm parameters nor TV was found in these patients (19). Subsequently, Bertelloni and colleagues reported low TV and hypergonadotropic hypogonadism in four male patients with non-GHD-dependent short stature treated with rhGH (20). These discrepant data prompted other research groups to assess whether rhGH treatment could be deleterious for the testicular function. Leschek and colleagues designed a randomized, double-blind, placebo-controlled trial to evaluate the effect of rhGH administration on pre-pubertal or early pubertal male patients with non-GH-deficient short stature. The results of this study showed that GH treatment did not have a detrimental effect on testicular function and pubertal onset or pace, as no difference of pubertal onset age or final TV was found compared with placebo (21). Similar findings were reported in a cohort of 84 GHD patients and 27 boys with idiopathic short stature (22). Also, in a cohort of 107 patients (79 with GHD and 28 with idiopathic short stature) treated with rhGH, no difference was found in final TV when compared with that of the reference population, thus confirming that rhGH administration does not impact negatively on testicular volume progression (23). A subsequent study carried out in eight patients with non-GH-deficient short stature (constitutional delay of puberty or idiopathic short stature) showed no adverse effect of rhGH therapy on final TV, gonadotropins, and sperm conventional parameters (24). Finally, in the last prospective, randomized, controlled study on 124 non-GHD-related short children (91 male) randomized in rhGH treatment or placebo, rhGH treatment reported increasing TV (25).

Altogether, these data suggest 1) no detrimental effect of rhGH administration on testicular function in childhood; 2) that

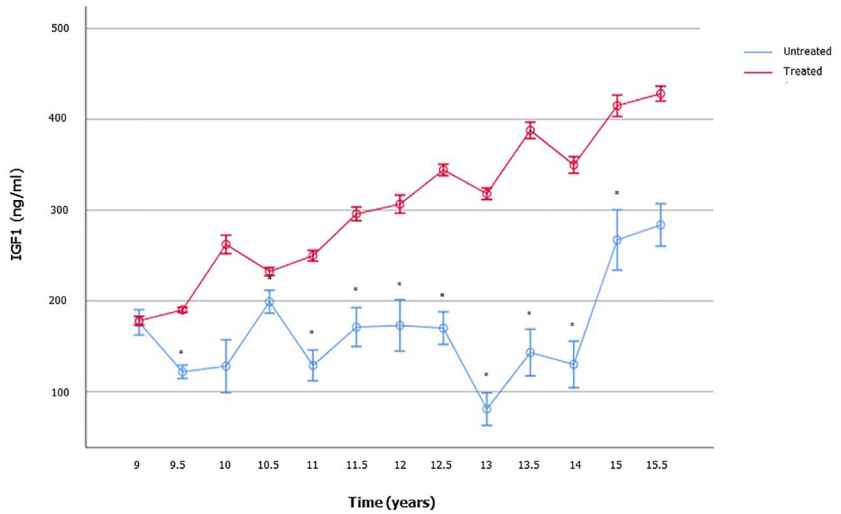


**FIGURE 4** | Insulin-like growth factor 1 (IGF1) serum levels in boys with growth hormone (GH) deficiency who received recombinant human GH (rhGH) and in aged-matched not yet treated GHD boys. The number of patients available for each time point is reported in the **Supplementary Table 2**.

rhGH administration may enhance testicular growth in GHD patients that likely occurs *via* an IGF1-mediated mechanism. This hypothesis has been recently suggested also by other authors (26). Indeed, by observing the physiological elevated (acromegalic) levels of IGF1 during puberty in both sexes, IGF1 has been supposed to promote the development of sexual organs and gonads (26). Moreover, IGF1 receptor (IGF1R) has been identified in gonadotropin hormone-releasing hormone (GnRH) neurons of mice, and mice with a selective knock-out of the *IGF1R* gene in the GnRH neurons show marked delay of puberty (26). Hence, IGF1 may act with both a peripheral mechanism – by enhancing SC proliferation, and with a central one – by inducing the firing of the GnRH neurons.

On this account, IGF1 may be suggested as a diagnostic target (with potential therapeutic implications) for cases with poor testicular volumetric growth in childhood. Its serum levels are known to augment with the increase of TV in healthy children (27). Several previous studies have tried to understand if rhGH treatment may somehow improve sperm parameters in adulthood (28–30) or whether a relationship between IGF1 serum levels in adulthood and sperm parameters do exist (31). The results of these studies showed no effect, and, therefore, the idea that the GH-IGF1 may play a role in the reproductive system was abandoned. However, those studies focused on the adult testis, which is made of mature SCs that are unable to proliferate. The physiology of the childhood testis is somewhat different as SCs actively proliferate with an IGF1-dependent mechanism (12). Testicular growth and function in childhood are not frequently investigated by pediatricians. The evaluation of testicular function in prepubertal children may be really important to early recognize markers of testicular tubulopathy and to prevent the onset of apparently idiopathic (and irreversible) oligozoospermia in adulthood (32). In fact, a poor SC proliferation in childhood could lead to a reduced SC number in adulthood, which will support the proliferation and differentiation of a lower number of germ cell, in turn leading to irreversible (since SCs can no more divide) oligozoospermia (8, 9). In this scenario, the measurement of IGF1 may be suggested in children with poor testicular volumetric growth.

We are aware of some of the limitations of the present study. The reasons for cautiously interpreting our results include the retrospective design, which limits the strength of the findings, as well as the lack of an appropriate control group made of untreated GHD patients. However, ethical issues do not allow overcoming this last aspect, since it is not possible to deny rhGH prescription to GHD children. Moreover, the study lacks entirely or in part of some parameters. For example, IGF1 SDS or $GH_{AUC}$ were not available in all children included in the study; also, information on the Tanner stage was available in 70 boys before they were prescribed rhGH and in 72 at the last visit, whereas data on TV were found in 59 boys before therapy and 60 afterward. Finally, not all patients had completed the rhGH therapy at the last visit.

In conclusion, according to the scanty previous literature, a role for GH treatment in testicular growth and pubertal onset in GHD children cannot be excluded. Due to the high prevalence of apparently idiopathic oligozoospermia, the assessment of testicular growth and function in childhood is of importance to prevent the onset of male infertility in adulthood. Longitudinal studies are needed to understand whether a poor testicular volumetric growth may underlie borderline-low IGF1 serum levels in otherwise healthy children. This knowledge may implement the diagnostic-therapeutic algorithm in case of evidence of poor testicular volumetric growth in childhood and could be used for the primary prevention of infertility.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

RC conceived the study, analyzed the data, and wrote the paper. MC conceived the study and revised the paper. ACr, MB, and SP collected the data. RAC and SV revised the paper. ACa conceived the study and revised the paper. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.619895/full#supplementary-material

## REFERENCES


1. Cannarella R, Condorelli RA, Mongioì LM, Barbagallo F, Calogero AE, La Vignera S. Effects of the selective estrogen receptor modulators for the treatment of male infertility: a systematic review and meta-analysis. *Expert Opin Pharmacother* (2019) 20(12):1517–25. doi: 10.1080/14656566.2019.1615057
2. Tüttelmann F, Ruckert C, Röpke A. Disorders of spermatogenesis: Perspectives for novel genetic diagnostics after 20 years of unchanged routine. *Med Genet* (2018) 30(1):2–20. doi: 10.1007/s11825-018-0181-7
3. Punab M, Poolamets O, Paju P, Vihljajev V, Pomm K, Ladva R, et al. Causes of male infertility: a 9-year prospective monocentre study on 1737 patients with reduced total sperm counts. *Hum Reprod* (2017) 32(1):18–31. doi: 10.1093/humrep/dew284
4. Levine H, Jørgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. *Hum Reprod Update* (2017) 23(6):646–59. doi: 10.1093/humupd/dmx022
5. Edelsztein NY, Grinspon RP, Schteingart HF, Rey RA. Anti-Müllerian hormone as a marker of steroid and gonadotropin action in the testis of children and adolescents with disorders of the gonadal axis. *Int J Pediatr Endocrinol* (2016) 2016:20. doi: 10.1186/s13633-016-0038-2
6. Condorelli RA, Cannarella R, Calogero AE, La Vignera S. Evaluation of testicular function in prepubertal children. *Endocrin* (2018) 62(2):274–80. doi: 10.1007/s12020-018-1670-9
7. La Vignera S, Cannarella R, Condorelli RA, Calogero AE. Disorders of Puberty: Endocrinology of the Pre-Pubertal Testis. *J Clin Med* (2020) 9 (3):780. doi: 10.3390/jcm9030780
8. Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. *Endocrinology* (1988) 122(3):787–94. doi: 10.1210/endo-122-3-787
9. Orth JM. Cell biology of testicular development in the fetus and neonate. In: C Desjardins, LL Ewing, editors. *Cell and molecular biology of the testis*. Oxford: Oxford University Press (1993). p. 3–42.
10. Condorelli R, Calogero AE, La Vignera S. Relationship between Testicular Volume and Conventional or Nonconventional Sperm Parameters. *Int J Endocrinol* (2013) 2013:145792. doi: 10.1155/2013/145792
11. Gianfrilli D, Ferlin A, Isidori AM, Garolla A, Maggi M, Pivonello R, et al. 'Amico-Andrologo' Study Group. Risk behaviours and alcohol in adolescence are negatively associated with testicular volume: results from the Amico-Andrologo survey. *Andrology* (2019) 7(6):769–77. doi: 10.1111/andr.12659
12. Cannarella R, Mancuso F, Condorelli RA, Arato I, Mongioì LM, Giacone F, et al. Effects of GH and IGF1 on Basal and FSH-Modulated Porcine Sertoli Cells In-Vitro. *J Clin Med* (2019) 8(6):811. doi: 10.3390/jcm8060811
13. Cannarella R, Condorelli RA, La Vignera S, Calogero AE. Effects of the insulin-like growth factor system on testicular differentiation and function: a review of the literature. *Andrology* (2018) 6(1):3–9. doi: 10.1111/andr.12444
14. Cacciari E, Milani S, Balsamo A, Spada E, Bona G, Cavallo L, et al. Italian cross-sectional growth charts for height, weight and BMI (2 to 20 yr). *J Endocrinol Invest* (2006) 29(7):581–93. doi: 10.1007/BF03344156
15. Growth Hormone Research Society. Consensus guidelines for the diagnosis and treatment of growth hormone (GH) deficiency in childhood and adolescence: summary statement of the GH Research Society. GH Research Society. *J Clin Endocrinol Metab* (2000) 85(11):3990–3. doi: 10.1210/jcem.85.11.6984
16. Tanner JM, Whitehouse RH. Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. *Arch Dis Child* (1976) 51(3):170–9. doi: 10.1136/adc.51.3.170
17. Goede J, Hack WW, Sijstermans K, van der Voort-Doedens LM, Van der Ploeg T, Meij-de Vries A, et al. Normative values for testicular volume measured by ultrasonography in a normal population from infancy to adolescence. *Horm Res Paediatr* (2011) 76(1):56–64. doi: 10.1159/000326057
18. Albanese A, Stanhope R. Treatment of growth delay in boys with isolated growth hormone deficiency. *Eur J Endocrinol* (1994) 130(1):65–9. doi: 10.1530/eje.0.1300065
19. Tatò L, Zamboni G, Antoniazzi F, Piubello G. Gonadal function and response to growth hormone (GH) in boys with isolated GH deficiency and to GH and gonadotropins in boys with multiple pituitary hormone deficiencies. *FertilSteril* (1996) 65(4):830–4. doi: 10.1016/S0015-0282(16)58222-3
20. Bertelloni S, Baroncelli GI, Viacava P, Massimetti M, Simi P, Saggese G. Can growth hormone treatment in boys without growth hormone deficiency impair testicular function? *J Pediatr* (1999) 135(3):367–70. doi: 10.1016/S0022-3476(99)70136-8
21. Leschek EW, Troendle JF, Yanovski JA, Rose SR, Bernstein DB, Cutler GBJr, et al. Effect of growth hormone treatment on testicular function, puberty, and adrenarche in boys with non-growth hormone-deficient short stature: a randomized, double-blind, placebo-controlled trial. *J Pediatr* (2001) 138 (3):406–10. doi: 10.1067/mpd.2001.111332
22. Ankarberg-Lindgren C, Norjavaara E, AlbertssonWikland K. Short boys treated with growth hormone show normal progression of testicular size and achieve normal serum testosterone concentrations. *Eur J Endocrinol* (2002) 146(5):681–5. doi: 10.1530/eje.0.1460681
23. Lindgren AC, Chatelain P, Lindberg A, Price DA, Ranke MB, Reiter EO, et al. Normal progression of testicular size in boys with idiopathic short stature and isolated growth hormone deficiency treated with growth hormone: experience from the KIGS. *Horm Res* (2002) 58(2):83–7. doi: 10.1159/000064658
24. Radicioni AF, Paris E, De Marco E, Anzuini A, Gandini L, Lenzi A. Testicular function in boys previously treated with recombinant-human growth hormone for non-growth hormone-deficient short stature. *J Endocrinol Invest* (2007) 30(11):931–6. doi: 10.1007/BF03349240
25. Albin AK, Ankarberg-Lindgren C, Tuvemo T, Jonsson B, Albertsson-Wikland K, Ritzén EM, et al . Does growth hormone treatment influence pubertal development in short children? *Horm Res Paediatr* (2011) 76(4):262–72. doi: 10.1159/000329743
26. Juul A, Skakkebæk NE. Why Do Normal Children Have Acromegalic Levels of IGF-I During Puberty? *J Clin Endocrinol Metab* (2019) 104(7):2770–6. doi: 10.1210/jc.2018-02099
27. Juul A, Bang P, Hertel NT, Main K, Dalgaard P, Jørgensen K, et al. Serum insulin-like growth factor-I in 1030 healthy children, adolescents, and adults: relation to age, sex, stage of puberty, testicular size, and body mass index. *J Clin Endocrinol Metab* (1994) 78(3):744–52. doi: 10.1210/jc.78.3.744
28. Radicioni A, Paris E, Dondero F, Bonifacio V, Isidori A. Recombinant-growth hormone (rec-hGH) therapy in infertile men with idiopathic oligozoospermia. *Acta Eur Fertil* (1994) 25(5):311–7.
29. Lee KO, Ng SC, Lee PS, Bongso AT, Taylor EA, Lin TK, et al. Effect of growth hormone therapy in men with severe idiopathic oligozoospermia. *Eur J Endocrinol* (1995) 132(2):159–62. doi: 10.1530/eje.0.1320159

30. Carani C, Granata AR, De Rosa M, Garau C, Zarrilli S, Paesano L, et al. The effect of chronic treatment with GH on gonadal function in men with isolated GH deficiency. *Eur J Endocrinol* (1999) 140(3):224–30. doi: 10.1530/eje.0.1400224
31. Andreassen M, Jensen RB, Jørgensen N, Juul A. Association between GH receptor polymorphism (exon 3 deletion), serum IGF1, semen quality, and reproductive hormone levels in 838 healthy young men. *Eur J Endocrinol* (2014) 170(4):555–63. doi: 10.1530/EJE-13-0729
32. La Vignera S, Condorelli RA, Cimino L, Cannarella R, Giacone F, Calogero AE. Early Identification of Isolated Sertoli Cell Dysfunction in Prepubertal and Transition Age: Is It Time? *J Clin Med* (2019) 8(5):636. doi: 10.3390/jcm8050636

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REVIEW
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# Investigating the Role of the microRNA-34/449 Family in Male Infertility: A Critical Analysis and Review of the Literature

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There is a great body of evidence suggesting that in both humans and animal models the microRNA-34/449 (miR-34/449) family plays a crucial role for normal testicular functionality as well as for successful spermatogenesis, regulating spermatozoa maturation and functionality. This review and critical analysis aims to summarize the potential mechanisms *via* which miR-34/449 dysregulation could lead to male infertility. Existing data indicate that miR-34/449 family members regulate ciliogenesis in the efferent ductules epithelium. Upon miR-34/449 dysregulation, ciliogenesis in the efferent ductules is significantly impaired, leading to sperm aggregation and agglutination as well as to defective reabsorption of the seminiferous tubular fluids. These events in turn cause obstruction of the efferent ductules and thus accumulation of the tubular fluids resulting to high hydrostatic pressure into the testis. High hydrostatic pressure progressively leads to testicular dysfunction as well as to spermatogenic failure and finally to male infertility, which could range from severe oligoasthenozoospermia to azoospermia. In addition, miR-34/449 family members act as significant regulators of spermatogenesis with an essential role in controlling expression patterns of several spermatogenesis-related proteins. It is demonstrated that these microRNAs are meiotic specific microRNAs as their expression is relatively higher at the initiation of meiotic divisions during spermatogenesis. Moreover, data indicate that these molecules are essential for proper formation as well as for proper function of spermatozoa per se. MicroRNA-34/449 family seems to exert significant anti-oxidant and anti-apoptotic properties and thus contribute to testicular homeostatic regulation. Considering the clinical significance of these microRNAs, data indicate that the altered expression of the miR-34/449 family members is strongly associated with several aspects of male infertility. Most importantly, miR-34/449 levels in spermatozoa, in testicular tissues as well as in seminal plasma seem to be directly associated with severity of male infertility, indicating

that these microRNAs could serve as potential sensitive biomarkers for an accurate individualized differential diagnosis, as well as for the assessment of the severity of male factor infertility. In conclusion, dysregulation of miR-34/449 family detrimentally affects male reproductive potential, impairing both testicular functionality as well as spermatogenesis. Future studies are needed to verify these conclusions.

**Keywords: male infertility, idiopathic male infertility, micro-ribonucleic acids (microRNAs), miR-34/449, spermatogenesis, ciliogenesis, biomarkers, personalized medicine**

## INTRODUCTION

According to the World Health Organization (WHO) infertility is defined as the inability to achieve a pregnancy following at least twelve months of unprotected sexual intercourse (1, 2). Recent data demonstrate that the prevalence of infertility is very high, affecting 8-12% of couples of reproductive age worldwide (1–3). Infertility could be attributed to both female and male factors, detrimentally affecting reproductive potential. Male factor infertility represents the sole infertility aetiology for 20-30% of infertile couples. Additionally, and in combination with female etiology, male infertility presents as a contributory factor for 50% of infertility cases (3, 4). Male infertility is a multifactorial condition caused by a wide variety of congenital, acquired, and idiopathic factors (2, 3, 5). Moreover, several environmental stressors and life-style parameters, including nutrition, alcohol consumption and smoking, could detrimentally affect male reproductive system functionality and dynamic (6). However, and despite advances in the field of human reproduction, the exact infertility aetiology remains unknown regarding 30% of infertile men, highlighting the need for a more accurate and a more precise understanding of the molecular mechanisms involved in the pathogenesis of male infertility (7).

Aiming to instigate, present, discuss and critically analyze novel possible molecular targets of male infertility, authors of the present review focused their attention on the role of micro-ribonucleic acids (microRNAs). Micro-ribonucleic acids are a class of small non-coding ribonucleic acids (RNAs), constructed by an average sequence of 22 ribonucleotides (8). Their loci is commonly found in transcription units and are produced following the transcription of these gene regions. Moreover, microRNAs are commonly located in clusters throughout the genome, except for the Y chromosome (9, 10). Generally, microRNAs act as epigenetic modifiers regulating gene expression in the post-translation level, causing translation repression and/or degradation of their mRNA targets (11). Thus, microRNAs are described as master regulators of several biological processes both in the cellular and the organism level (8). Micro-ribonucleic acids are involved in cell cycle regulation, in cell programming as well as in cell differentiation and tissue formation (12). Moreover, microRNAs exert a pivotal role on embryo development, on organ formation as well as on growth (13–15). Due to their pivotal role on regulation of cellular processes, microRNA dysregulation is considered to be directly associated with the pathogenesis of several diseases and pathophysiological conditions, including developmental diseases, cancer and infertility (16).

This narrative review is focused on identifying and underlying the possible role of a specific microRNA family, namely microRNA-34/449 (miR-34/449) on male infertility pathophysiology. Briefly, the miR-34/449 family is comprised by six highly conserved microRNAs, namely miR-34a/b/c and miR-449a/b/c, respectively (17). The miR-34a gene is located on chromosome 1p36.22 (18). The other five family members are classified into two functional clusters, namely miR-34b/c cluster and miR-449a/b/c cluster, respectively (17). Members of both of these clusters are co-expressed in the testis and present with common characteristics (19). The miR-34b/c cluster is located on chromosome 11q23.1 and the miR-449a/b/c cluster is originated from polycistronic transcripts encoded on chromosome 5q11.2 and more specifically in the second intron of the cell division cycle 20b (cdc20b) gene (20). Despite the fact that the members of the miR-34/449 family are transcribed from different gene loci, these present with similar mature sequences and share an identical "seed sequence" (21, 22). As a result, these microRNAs are sharing common features and properties. In both animals and humans, the members of miR-34/449 family exert several significant functions, including cell cycle regulation as well as regulation of cell differentiation and functionality. These microRNAs are expressed in several tissues and organs such as in airway epithelium, in ovaries, in fallopian tubes, in epithelial cells located on the surface of the ventricular system of the brain and finally and most importantly in testis (19, 23–25). All these tissues are sharing a common dominator: among their different cell types, these tissues contain ciliated cells and/or flagellate cells, such as spermatozoa. Considering the topology of their expression, it becomes clear that miR-34/449 members constitute master regulators of the ciliated cells' differentiation and function (22, 26). Most importantly, considering the topic of the present review, there is increasing evidence suggesting that miR-34/449 family members play crucial roles in testicular functionality as well as in regulating spermatogenesis and mature spermatozoa formation (19, 25, 27). The essential role that miR-34/449 family members appears to play in the male reproductive system served as the incentive for authors to proceed to this critical analysis, while the rational for this study was fueled by the need to provide further in depth analysis on male infertility.

In summary, data presented from several studies both in humans and animal models indicate that miR-34/449 family members play crucial roles during spermatogenesis by regulating

spermatozoa maturation and testicular functionality. It is suggested that miR-34/449 dysregulation is related with several aspects of idiopathic male infertility ranging from oligozoospermia to non-obstructive azoospermia (NOA). This critical analysis aims to highlight the potential mechanisms *via* which miR-34/449 dysregulation could lead to male infertility, highlighting the role of these molecules as potential biomarkers and possible therapeutic targets for improving management of male infertility in the era of personalized and precision medicine.

## MULTICILIATED CELLS AND NORMAL TESTICULAR FUNCTIONALITY

Prior to elaborating on the role of miR-34/449 family members on male infertility it is significant to understand their contribution in proper development and function of testicular multiciliated cells. Cilia are dynamic microtubule-based cell organelles that extend outside the cell body (28). These organelles are highly conserved among eukaryotes and consist of the basal body, which resides in the apical surface inside the cell and the extracellular axoneme structure (29). Cilia can be motile or immotile exerting different cell functions. Generally, almost all human cells possess a single-non motile cilium, commonly characterized as primary cilium, which plays crucial roles mainly with regard to cell signaling serving like a sensor (30, 31). However, there is a class of hostile specialized differentiated epithelial cells, which possess multiple motile cilia. These cells, which project multiple cilia on their apical surface, are commonly referred to as multiciliated cells (32–36). The multiple cilia of these cells are generally moving in a synchronous wave-like manner to facilitate movement of luminal contents in several tissues and organs, including the airway epithelium, the ventricular system of the brain as well as the spinal column (19, 23–25, 37). Interestingly, multiciliated cells are observed in both the female and male reproductive system (38).

Considering the female reproductive system, multiciliated cells are mainly located in the epithelium of the fallopian tubes and *via* the synchronous wave-like motion of their multiple cilia these oviduct epithelial cells assist the oocyte and embryo passage towards the uterus (39). With regards to the male reproductive system, data indicate that multiciliated cells are present in the epithelium of the efferent ductules (27, 40). Similarly to the fallopian tubes, efferent ductules are small tubes *via* which the immature immotile spermatozoa are transported from the rete testes to the caput epididymis, where the final maturation of the spermatozoa is taking place (27, 40). In the efferent ductules immature spermatozoa are suspended in an abundance of seminiferous tubular fluid, the great amount of which is reabsorbed prior to reaching the caput epididymis (41). Seminiferous tubular fluid reabsorption is of paramount importance since immature spermatozoa should reach the caput epididymis in high concentrations in order for the final spermatozoa maturation to be achieved (27). The multiciliated cells of the efferent ductules seem to play crucial roles in both immature spermatozoa transportation into the epididymis, as well as in the successful reabsorption of the seminiferous tubular fluids (27). Recently, published data demonstrate that cilia in the efferent ductules are not moving in a synchronous wave-like manner, but in contrast are performing whip-like beatings and continually change the direction of the fluids' flow, generating turbulence, which is turn keeps spermatozoa suspended in the seminiferous tubular fluids (27, 42). Therefore, it becomes evident that the multiciliated cells located in the efferent ductules are significant contributors for normal testicular functionality.

## MIR-34/449 FAMILY: A REQUIREMENT FOR MULTICILIATED CELL DIFFERENTIATION

Despite the fact that the multiciliated cells are present in several different organs and tissues, data demonstrate that cilia formation, being a differentiation process called ciliogenesis, is taking place *via* shared molecular mechanisms. During the first stage of ciliogenesis, progenitor cells should exit the cell cycle to acquire the multiciliated phenotype. Several endocrine, paracrine, mechanical and chemical stimuli could induce ciliogenesis (43). In progenitor undifferentiated basal cells ciliogenesis is initiated by the amplification of centrioles, representing the second stage of the whole process. This event is regulated by two independent pathways, the canonical parental centriole dependent pathway, and the deuterostome dependent pathway (44). Centriole abundance is proportional to the surface area and is not associated with cell volume (45). Following the amplification stage, centrioles move to the apical membrane of the cell, where they dock and maturation is achieved giving rise to basal bodies (28). The axoneme is the scaffold of the cilia and is a dynamic structure, which originates from the basal bodies. The axoneme is comprised of several proteins, including A and B tubulins and dynein (46).

Considering the role of miR-34/449 family in ciliogenesis, there is a great amount of evidence suggesting that these microRNAs share common developmental roles, serving as master regulators of the ciliogenesis process in several tissues and organs, including the testis (19, 23–25). This becomes evident considering that the miR-34/449 family members are overexpressed during ciliogenesis in vertebrates and constitutes the most highly expressed microRNA family in the ciliated epithelia (20). Moreover, data indicate that genetic ablation of both miR-34b/c and miR-449a/b/c clusters could detrimentally affect ciliogenesis *via* dysregulation of several genes involved in cell cycle regulation. In the absence of miR-34/449 several genes encoding cell cycle regulatory proteins, namely cyclin-dependent kinases (CDKs), are upregulated resulting to impaired ciliogenesis. These proteins keep progenitor cells to a proliferative stage, rendering them incapable of exiting the cell cycle in order to adopt the multiciliated phenotype (12). It is well established that the members of the miR-34/449 family present with pro-apoptotic and anti-proliferative properties, which are exerted by their ability to silence the expression of several

proteins promoting cell proliferation, including CDKs as well as cyclins (47–49). It seems that during ciliogenesis, these properties are also important for the successful differentiation of the multicillia epithelium in several tissues, including the testis.

In order to better understand the significant role of the miR-34/449 family during ciliogenesis it is of high significance to present the molecular pathways mediating respective actions. Data originating from both animal models and humans indicate that the members of the miR-34/449 family work synergistically during ciliogenesis. Firstly, during early ciliogenesis these microRNAs suppress both Notch and bone morphogenetic protein (BMP) signaling pathways, enabling progenitor cells to exit the cell cycle. Moreover, Notch and BMP suppression mediates centriole amplification and migration of the amplified centrioles to the apical surface of the progenitor cells (20, 50, 51). Several studies indicate that Notch suppression stands as a prerequisite condition in order for the progenitor cells to be successfully differentiated into multiciliated cells. Experiments performed, employing both Xenopus epidermis and cultured human aortic endothelial cells (HAEC), demonstrate that miR-449a/b/c cluster's members can directly suppress the Nocth1 receptor as well as its ligand delta like ligand 1 (DLL1), promoting ciliogenesis. In contrast, when Notch1 and DLL1 messenger RNAs (mRNAs) were protected against the miR-449a/b/c, an impaired ciliogenesis was observed (20). These data indicate that miR-34/449 family is required for successful determination of the multiciliated cell fate by silencing significant pathways, including Notch and BMP signaling pathways.

Members of the miR-34/449 family are not only required at the early stages of the ciliogenesis, but also act as significant regulators of the following steps. There is evidence suggesting that miR-34/449 family members work together for the formation of the apical actin meshwork as well as for the formation and the maturation of the basal bodies. Data indicate that miR-34/449 family members can directly or indirectly suppress the mRNA of the RAS related protein (R-Ras), controlling the redistribution of Filamin A protein on the apical actin meshwork of the multiciliated cells. It seems that these specific microRNAs serve as regulators of the apical actin meshwork's architecture, which is a crucial structure necessary for basal body anchoring (50, 51). In addition, miR-34/449 members also control cilia biogenesis *via* the regulation of the cp110 mRNA. The cp110 protein is a regulatory molecule necessary for both cilia formation and function. During ciliogenesis, cp110 levels should be accurately controlled to enable proper formation and function of apical actin meshwork as well as of basal bodies. Strong correlation of the cp110 protein with the miR-34/449 family members is highlighted considering that both cp110 and miR-34/449 can be regulated by the same transcription factors. However, the miR-34/449 members can post-transcriptionally control expression of the cp110 protein, and thus they can regulate optimal cp110 proteins' levels for the apical actin meshwork formation as well as for the basal bodies anchoring (22, 26, 52). Moreover, miR-34b can regulate levels of c-Myb protein. The c-Myb protein is a well-known transcription factor, which controls several genes involved in cell differentiation and cell survival (53–55). The c-Myb is also one of the master regulators of ciliogenesis, controlling expression of several ciliary-related genes, including Polo like kinase-4 (PLK4) and Stil (Scl/Tal1 interrupting locus) genes. Experiments performed in zebrafish kidney's multiciliated cells indicate that the miR-34b/c-Myb signaling serves as an important regulator of centriole migration and docking. Genetic ablation of miR-34 or c-Myb overexpression can equally impair the normal centriole migration and docking. In the middle kidney ducts of c-Myb mutants, a fewer number of centrioles lining the apical membrane of the multiciliated cells was observed, indicating that optimal levels of the c-Myb transcription factor are required for proper centriole migration. Although the c-Myb transcript presents binding sites for the miR-34b, its regulation seems to be indirect and more date is required to elucidate whether miR-34b controls c-Myb levels (56). These data demonstrate that miR-34/449 members represent essential elements of the molecular mechanisms regulating apical actin meshwork formation as well as basal body anchoring.

## IMPAIRED TESTICULAR MULTICILIOGENESIS AND MIR-34/449 DYSREGULATION

Several studies have been so far conducted in order to elucidate whether miR-34/449 dysregulation could impair ciliogenesis. Experiments performed in Xenopus epidermis as well as in ex vivo cultured cells demonstrate that the miR-449 cluster inhibition could impair multiciliated cell functionality by reducing the number of basal bodies (20). In contrast to the aforementioned observation, no significant change in the number of basal bodies was reported in the multiciliated cells of the respiratory tract of miR-34/449 knock out mice. However, defects in basal body migration and docking were observed as the majority of them were localized into the cytoplasm and did not migrate to the apical surface of the cells (22). Triple knock out (TKO) mice regarding all the miR-34/449 family genome loci presented with respiratory deficiency as well as with respiratory infections and were characterized by a phenotype resembling a condition called Primary Cilia Disorder (PCD). Further analysis revealed a dysfunction in generation of the multiciliated cells of the respiratory tracks, which was caused by defective basal body formation and docking (39). Same outcomes were observed regarding the ciliated epithelium of the reproductive tracks regarding both female and male mice, where ciliogenesis was also significantly impaired, leading to infertility (39). Similar findings were also reported by Otto et al., in mice in which both miR-34b/c and miR-449a/b/c clusters were genetically ablated. Impaired multiciliogenesis was observed in the respiratory and reproductive epithelia of both sexes. Histological examination of both embryos and adult mice confirmed that in the respiratory system, in the fallopian tubes as well as in the efferent ductules, ciliogenesis was defective. A microarray analysis of these tissues revealed that several cell cycle genes were significantly

upregulated in both mRNA and protein levels, indicating the incapability of the progenitor cells to obtain the multiciliated phenotype. These finding were confirmed following proliferation assays, indicating that the miR-34b/c and miR-449a/b/c deficient mice displayed a higher fraction of proliferating cells compared to the wild-type controls (12). These data suggest that miR-34/449 family members are required for proper ciliogenesis, especially regarding respiratory and reproductive tracks epithelia. Their role is of paramount importance not only for the progenitor cells to acquire the ciliated phenotype, but also for the maturation, as well as for ensuring proper functionality of the differentiated multiciliated cells. Thus, alterations in their expression leads to both respiratory defects and infertility in both sexes.

In order to elaborate on the role of miR-34/449 family members on the female reproductive system's physiology, it is of significance to note that genetic ablation of these microRNAs leads to impaired ciliogenesis in the epithelium of the fallopian tubes and subsequently to female infertility. These observations were confirmed following histological examination performed on TKO female mice which were treated with gonadotrophins. In these female mice, a significant reduction regarding the number of oocytes identified in the fallopian tubes was observed, a phenomenon which is likely attributed to the impaired multiciliogenesis of the fallopian epithelium (12, 22, 57, 58).

Regarding the male reproductive system, several studies demonstrated that genetic ablation of both miR-34b/c and miR-449a/b/c clusters results in spermatogenic impairment and subsequently to male infertility (27, 59, 60). Data originating from these studies indicate that male infertility is probably attributed to the impaired ciliogenesis of the efferent ductules. As previously highlighted, the motile cilia of the internal lumen of the efferent ductules present with unique properties and are required for the proper functionality of the testis (59). In 2019, Yuan et al. published high-quality evidence highlighting the significance of these unique properties of the multiciliated cells located in the efferent ductules. In this study, authors generated spermatogenic cell-specific and multiciliated cell-specific double miR-34 and miR-449 cluster knockout mice. Data demonstrated that in the absence of both miR-34 and miR-449 clusters, spermatogenic cells remained unaffected and these mice were totally fertile. In contrast, the mice of the multiciliated cell-specific double miR-34 and miR-449 cluster were infertile and presented with testicular atrophy. In addition, these mice exhibited impaired ciliogenesis of the efferent ducts due to the dysregulation of several genes contributing to ciliogenesis, namely Ccdc113 and Dnah6. In concordance with previous data, image analysis *via* electron microscopy revealed that the epithelium of the efferent ductules had fewer number of cilia, presenting with less motility compared with the wild-type mice (27). More specifically, data originating from this study indicate that genetic ablation of both the miR-34 and miR-449 clusters led to abnormal multicillia cell differentiation in the efferent ductules, which in turn led to sperm aggregation and agglutination, testicular tubular obstruction, impaired spermatogenesis, testicular atrophy and subsequently to male infertility (27).

In conclusion, altered miR-34/449 expression motifs can cause defective ciliogenesis in the efferent ductules epithelium, leading to progressive spermatogenic failure. In this case, spermatogenic failure is attributed to testicular dysfunction, mainly originating from impaired reabsorption of the seminiferous tubular fluids, which in turn leads to high hydrostatic pressure into the testis. High hydrostatic pressure progressively leads to testicular atrophy and subsequently to male factor infertility, which could range from OAT to azoospermia. These data demonstrate that clinicians should consider multicillia cell dysfunction and efferent ductules obstruction in cases where abnormal semen parameters and/or azoospermia co-exist with sperm aggregation and agglutination as well as with luminal enlargement (27).

## THE ROLE OF MIR-34/449 FAMILY IN SPERMATOGENESIS

Taking into account the spermatozoa architecture, it may be hypothesized that the spermatozoon may in fact represent a highly differentiated type of ciliated cells. It is this exact hypothesis that drove the authors' extrapolation that miR-34/449, ciliogenesis and spermatozoa are governed by a relationship that may hold the key to explaining how disorders of the ciliogenesis process, defined by miR-34/449, ultimately lead to infertility. Spermatozoa are equipped with a single-motile cilia which is called flagellum. Considering the role of miR-34/449 family members during ciliogenesis, and taking into account that spermatozoa represent a special type of ciliated cells, it is of value to analyze the role of miR-34/449 family members on the physiological events entailed during spermatogenesis. The role of miR-34/449 family members on the physiological events entailed during spermatogenesis is summarized in **Figure 1**.

The miR-34/449 family members are expressed in reproductive organs (60–62). Transcriptome analysis in primates and rodents revealed that the miR-34/449 family members are highly expressed in the mature testis, indicating their possible role during sperm maturation (36, 63, 64). In adult male mice, the expression of both miR-34b/c and miR-449a/b/c clusters is progressively increased in the postnatal testis. In contrast, the miR-34a expression seems to be stable during spermatogenesis. This finding could be attributed to the fact that, in the mouse testis, miR-34a is predominantly expressed in spermatogonia rather than in spermatocytes or spermatids (19, 62). In addition, both miR-34b/c and miR-449a/b/c clusters present similar expression patterns in male germ cells. They are considered to be meiotic specific microRNAs as their expression is relatively higher at the initiation of meiotic divisions during spermatogenesis (19, 65, 66).

Considering their expression partners, researchers attempted to investigate the molecular mechanism *via* which the miR-34/449 family members contribute to generation of the mature

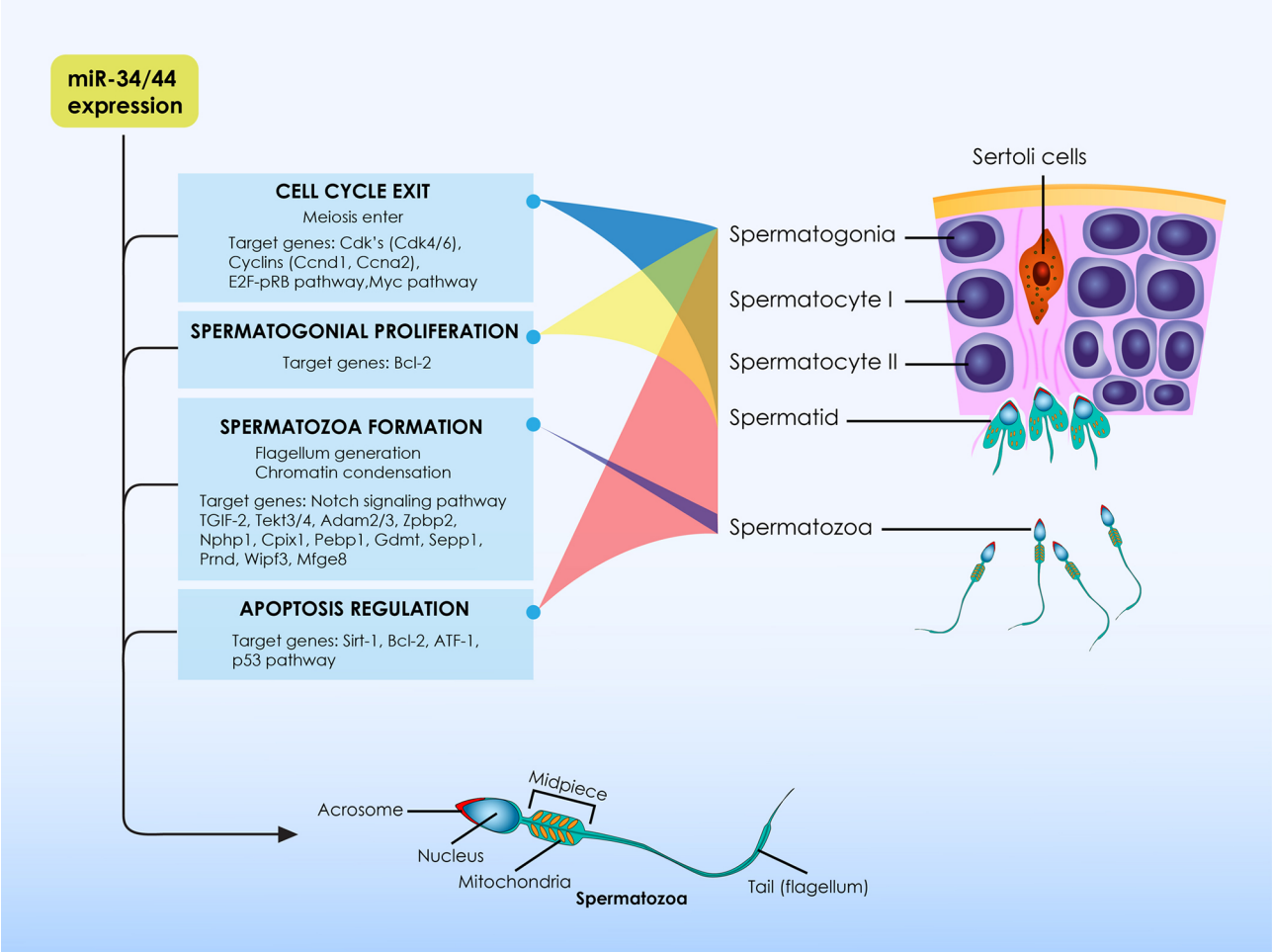


**FIGURE 1** | The role of miR-34/449 family members on the physiological events entailed during spermatogenesis. At the initial stages of spermatogenesis miR-34/449 family members target several transcripts of cell-cycle regulatory proteins, leading germ cells to exit the cell cycle in order for spermatogenesis to be initiated. They are considered to be meiotic specific microRNAs as their expression is relatively higher at the initiation of meiotic divisions. During spermatogenesis these microRNAs are essential molecules promoting normal spermatozoa formation *via the* differential regulation of expression levels of several spermatogenesis-related proteins depending on spermatogenesis stages. In addition, miR-34/449 family members do not only exert significant actions for the regulation of spermatogenesis, but also these molecules are essential for proper formation as well as for proper function of spermatozoa per se. Except from spermatogenesis, evidence also suggest that members of the miR-34/449 family exert significant anti-oxidant and anti-apoptotic properties and thus stand as significant elements of the testicular homeostatic mechanisms.

spermatozoa. Using bioinformatic analysis, several gene-targets were identified, including CDKs, Notch1, Bcl-2 and Casp3. These molecules are known to play essential roles during spermatogenesis (36, 64, 67–69). For example, in the mouse testis Notch1 downregulation is required for optimal spermatozoa maturation. In contrast, the activation of the Notch signaling pathway in mutant mice resulted in impaired spermatogenesis. These mutant mice experienced an age-related sperm count reduction and were also presenting with sperm morphological defects. This defective spermatogenesis, noted in the mutant mice overexpressing Notch, was principally attributed to the inability of the germ cells to properly differentiate to mature spermatozoa. Moreover, Notch overexpression increased apoptosis levels in spermatogonia of the mutant mice (70). To elaborate on that, miR-34b/c and miR-449a/b/c clusters' members have been characterized as principal post-transcriptional controllers for the transition of germ cells from the pro-meiotic to the meiotic stage (66). These microRNAs target several transcripts of cell-cycle regulatory proteins, leading germ cells to exit the cell cycle, in order for spermatogenesis to be initiated. Interestingly, experiments performing luciferase report assays demonstrated that, in germ cells, the miR-34b/c and miR-449a/b/c clusters' members are sharing common targets. Some of them include different types of cyclins and CDKs as well as elements of the RB/E2F pathway. The significant role of miR-34b/c and miR-449a/b/c clusters' members during spermatogenesis is highlighted in experiments performed on animal models presenting with chemotherapy-induced

azoospermia. A significant reduction in the expression levels of miR-34b/c and miR-449a/b/c was observed in rats following treatment with busulfan, leading to azoospermia. Following chemotherapy some of the affected rats were subjected to mesenchymal stem cell (MSC) transplantation in the testis. Notably, MSC transplantation was able not only to restore fertility but also to upregulate the previously altered expression of the miR-34b/c and miR-449a/b/c clusters' members. Following regulation of these microRNAs expression, expression patterns of several other genes, including Cdk1, E2F1, c-Myc and PLCXD3 was normalized (71). This data suggests that miR-34b/c and miR-449a/b/c regulate sperm maturation by controlling expression levels of several proteins involved in spermatogenesis. For instance, and as previously mentioned, the RB/E2F pathway is controlled by the miR-34b/c and miR-449a/b/c clusters' members during cell cycle control. During spermatogenesis, the E2F1 protein, being a transcription factor, is essential for the maintenance of spermatogonia in a proliferative status, promoting mitotic divisions of these germ cells. However, in later stages of spermatogenesis, the transition of germ cells from the pro-meiotic to the meiotic stage requires silence of the RB/E2F pathway. Upon miR-34b/c and miR-449a/b/c dysregulation, the E2F1 activity during the later stages of spermatogenesis results in Sertoli cell dysfunction, as well as in a progressive reduction of the number of mature spermatocytes and spermatids (72, 73). Moreover, studies indicate that miR-34b stands as a significant controller of spermatogenesis, as it targets the mRNA of the cyclin dependent kinase 6 (Cdk6). It is well established that Cdk6 protein plays crucial roles during spermatogenesis, enabling germ cells to exit the cell cycle in order to differentiate to mature spermatozoa. Indeed, overexpression of the miR-34b in cultured cells significantly downregulated expression levels of the Cdk6 protein (74). Similar studies indicate that also miR-449 mimics could lead to proliferation inhibition (19). MicroRNA-34/449 family members are essential molecules promoting normal spermatogenesis *via* the differential regulation of expression levels of several spermatogenesis-related proteins depending on spermatogenesis stages.

The crucial contribution of miR-34/449 family members during spermatogenesis is demonstrated in several studies investigating spermatogenesis outcomes upon the absence of these specific microRNAs. Knockdown male mice for either miR-34b/c or miR-449a/b/c clusters, presented with no significant spermatogenic defects and were phenotypically normal. In both cases, the knockdown male mice had normal fertility and normal sperm parameters and were also presenting with normal testicular morphology. The normal phenotype of the single knockdown mice could be attributed to the fact that miR-34b/c or miR-449a/b/c clusters are sharing common targets and roles. Interestingly, upon miR-449a/b/c cluster's genetic ablation, an upregulation of miR-34b/c expression was observed, confirming the synergetic action of these microRNAs (19, 60). However, simultaneous inactivation of both clusters resulted to male infertility while mice presented with a phenotype resembling OAT. Sperm analysis revealed impaired spermatogenesis and both the number, and the motility of the spermatozoa were detrimentally affected. Spermatozoa found in the epididymis were headless to an extent of 80%. Image analysis employing transmission electron microscopy revealed major defects in the formation of the flagellum architecture. The defects included a disorganization of the mitochondrial sheath and the lack of the typical "9+2" microtubular organization. However, and despite the major structural defects of the mature spermatozoa, mutant round spermatids showed normal fertility potential and the lack of the miR-34b/c and miR-449a/b/c clusters did not affect development of the generated embryos (25, 60). In light of the above, miR-34/449 family members do not only exert significant actions for the regulation of spermatogenesis, but are also essential for proper formation as well as for proper function of spermatozoa per se.

Current data also demonstrate that miR-34/449 family members represent significant regulators of the molecular mechanisms involved in maintaining testicular homeostasis. During spermatogenesis, miR-34/449 family members induce apoptosis, eliminating defective cells, including abnormal germ cells and immature spermatozoa. *In vitro* experiments employing GC-2 lines highlight that miR-34c possibly regulates apoptosis through repression of the ATF1 mRNA (75). In addition, the miR-34a seems to play crucial roles for oxidative stress mediated apoptosis, inhibiting the Sirt1 protein in the testis. The induction of oxidative stress in the mouse testis results in an upregulation of miR-34a and downregulation of SIRT1. Upon oxidative stress, the testis displays higher expression of apoptotic markers and higher apoptotic index in male germ cells, indicating that oxidative stress is a major cause of male infertility (76, 77). The members of the miR-34/449 family seems to exert significant anti-oxidant and anti-apoptotic properties and are vital for the testicular homeostatic mechanisms. Upregulation of their expression observed upon oxidative stress indicates that members of the miR-34/449 family ensure-via their unique actions-maintenance of spermatogenesis when the microenvironment of the testis is jeopardized. Thus, alterations with regard to miR-34/449 expression have been correlated with defective anti-oxidant capacity, ultimately leading to male infertility (78).

## MALE INFERTILITY IN HUMANS AND MIR-34/449 ASSOCIATIONS

Similarly to animal models, several studies suggest that in humans the dysregulated expression of miR-34/449 family members is strongly associated with failed spermatogenesis, with impaired testicular functionality and subsequently with male infertility (79–82). Considering the expression motifs of the miR-34/449 family members in the male reproductive system, data indicate that these microRNAs are expressed in both spermatozoa and testicular tissues but not in the accessory glands, highlighting the possible implication of miR-34/449 family in male infertility originating from testicular dysfunction (83).

More specifically, studies investigating microRNAs' expression patterns in patients with male infertility, revealed that members of the miR-34b/c were significantly downregulated

in both spermatozoa and in seminal plasma of men presenting with oligozoospermia and azoospermia (79–82). Between the two members of the miR-34b/c cluster, miR-34b was positively correlated with spermatozoa vitality and concentration (81, 82). Furthermore, it is demonstrated that miR-34b levels are significantly reduced in spermatozoa as well as in testicular samples of men presenting with different types of NOA, in comparison to men with normal spermatogenesis. Analysis employing receiver operating characteristic curve (AUC) indicated that miR-34b could successfully separate different types of NOA and control fertile men, as it presented with a high AUC value reaching 0.944 (95% CI 0.9131–0.9713) (80). In concordance to the aforementioned studies, Salas Huetos et al. indicated that miR-34b levels were significantly reduced in semen samples of oligozoospermic and asthenozoospermic men. This is a significant observation considering that semen could be used as a material for non-invasive differential diagnosis of male infertility compared with tissue analysis, which requires invasive testicular biopsies. In the same study, bioinformatic analysis revealed altered expression patterns of several genes involved in spermatogenesis, chromatin modification and cell cycle regulation, highlighting that several of these genes are targets of the miR-34b/c. These data are of paramount importance for better understanding the connection between the miR-34/449 alterations with male infertility. As anticipated, considering the shared mechanisms of action between miR-34b/c and miR-449a/b/c clusters, the miR-449a/b/c cluster also displays similar expression patterns as the miR-34b/c cluster's members. These entail decreased levels in semen samples of infertile patients presenting with abnormal semen parameters (84). When critically analyzing data, it appears that there is a great body of evidence suggesting that the altered expression of the miR-34/449 family members is strongly associated with several aspects of male infertility ranging from oligozoospermia to NOA. Most importantly, miR-34/449 levels in spermatozoa, in testicular tissues as well as in seminal plasma seem to be directly associated with severity of male infertility. Thus, miR-34/449 family members could serve as potential sensitive biomarkers for an accurate individualized differential diagnosis, as well as for assessment of male infertility severity.

It has been noted that upon miR-34/449 dysregulation both testicular functionality and spermatogenesis are severely compromised, commonly leading to idiopathic male infertility. However, the exact mechanisms involved in miR-34/449 dysregulation in infertile men presenting with impaired spermatogenesis as well as with diminished testicular functionality are poorly understood. Current evidence suggests that epigenetic alterations, including abnormal methylation of the miR-34/449 loci, probably stands as one of the main pathological mechanisms inducing abnormal miR-34/449 expression patterns. Indeed, two recently published studies reported a higher methylation status in the promoters of miR-34b/c and miR-449a/b/c clusters in men presenting with idiopathic male infertility and abnormal semen parameters (84, 85). In both studies, infertile men presented with a higher percentage of methylation in the miR-34/449 promoters and subsequently with a significant reduction of the expression levels of miR-34/449 compared with fertile men featuring normal semen parameters. Interestingly, these alterations in miR-34/449 methylation were documented as directly associated with the severity of semen abnormalities, with OAT patients presenting with the highest methylation percentage, as well as the lowest miR-34/449 levels (84, 85). Moreover, defective genomic imprinting of miR-34/449 loci was also observed in patients presenting with male infertility (86–88). The underlying causes of altered imprinting as well as of defective methylation of the miR-34/449 loci are yet poorly understood. In the study of Najafipour and colleagues it was suggested that smoking can increase the methylation status of the miR-449 cluster's promoter (84). Other researchers have similarly associated smoking with aberrant methylation patterns in several genes as well as with abnormal semen parameters, indicating the detrimental epigenetic effect of smoking on male fertility (89–91). Evidence suggests that several other lifestyle parameters, physical activity levels, stress as well as aging are described as factors that could affect DNA methylome in spermatozoa, leading to abnormal methylation patterns in specific genome loci, including the miR-34/449 loci, eventually leading to structural and functional abnormalities of spermatozoa (86). For example, there are studies reporting an age-related reduction of miR-34b levels, indicating that aging could negatively affect male reproductive potential (Salas-Huetos et al., 2019). It has also been voiced that early life stress is associated with reduction of miR-34/449 levels in semen samples. Men with high scores of early life stress showed a reduced expression of miR-34/449 in semen, coupled by poorer semen parameters in comparison to individuals experiencing low early life stress (92). These data indicate that stress could impair miR-34/449 expression, reducing male reproductive capacity, however the underlying mechanisms remain unknown (93). Except from the epigenetic alterations, several genetic and chromosomal abnormalities have been also associated with impaired miR-34/449 expression. For instance, interesting results emerge from a recently published study, where the expression profile of several microRNAs in the testes of patients with Klinefelter (46, XXY) syndrome were investigated (94). It is well-known that individuals with Klinefelter syndrome are commonly presenting with NOA. Results of this study revealed a significant reduction of miR-34b/c in testicular samples of Klinefelter patients compared with individuals diagnosed with obstructive azoospermia. Notably, miR-449 cluster's members were undetectable in the testicular samples obtained from Klinefelter patients (94). These interesting findings suggest, that despite the fact that miR-34/449 are encoded from transcriptional units located in autosomal chromosomes, their expression is possibly regulated by factors encoded from loci on the sex chromosomes. Alterations in sex chromosomes could detrimentally affect miR-34/449 expression patterns, highlighting the need for further research to better understand the complex network regulating the miR-34/449 family. Apparently, more data are required in order to unveil the pathophysiological mechanisms leading to miR-34/449

alterations, which in turn cause male factor infertility. Several inherited or/and acquired genetic and epigenetic conditions, such as genome imprinting and DNA methylation, have been so far correlated with impaired miR-34/449 expression patterns. It is of high clinical significance to note that life-style parameters, environmental stressors as well as aging, are at the top of the list of the cascade of events leading to miR-34/449 dysregulation and to male infertility.

Considering the significant role of miR-34/449 in idiopathic male infertility, it is of added value to further study the possible implication of these microRNAs on pathologies that represent known causes of male infertility, such as varicocele. It is well-established that varicocele is associated with increased testicular temperature, leading to germ cell damage and finally to temperature-depending spermatogenic failure (95). Several molecular mechanisms are involved in heat-induced germ cell damage, including apoptosis, oxidative stress, DNA damage and autophagy (95). Indeed, a significant reduction of miR-34c levels has been reported in patients presenting with varicocele and impaired semen parameters in comparison to fertile men with varicocele and normal testicular functionality. In the same study, miR-34c levels were positively correlated with semen parameters, namely spermatozoa concentration, motility and morphology. In contrast, miR-34c levels were negatively correlated with markers indicating oxidative stress and apoptosis (96). Lower levels of miR-34a were also reported in varicocele patients, according to the results provided by a recently published study, which similarly suggests that miR-34/449 family dysregulation constitutes part of varicocele's pathophysiology. More specifically, varicocele patients presented with reduced expression of miR-34a as well as with increased levels of oxidative stress markers in their semen samples, compared with healthy fertile controls (93). In order to connect the molecular events entailed in varicocele pathophysiology with the miR-34/449 family, we should consider that these microRNAs exert anti-apoptotic properties (97). Moreover, there are reports suggesting that miR-34/449 family members also serve as oxidative stress-responsive elements (98). In light of that it becomes evident that miR-34/449 dysregulation could be associated with increased germ cell apoptosis as well as with increased oxidative stress in the testis of varicocele patients. Notably, data also demonstrate that the levels of miR-34/449 family members are strongly associated with the severity of impairment observed in semen parameters of varicocele patients, highlighting their possible role as biomarkers for properly categorizing these patients. In addition, miR-34/449 members may stand as a possible non-invasive and reliable tool. This in the future could be employed to assist andrologists in successfully assessing the reproductive potential of varicocele patients subjected to surgical treatment, in a personalized, patient-friendly and precise manner. However, future studies are needed to verify the value of this hypothesis.

As discussed in previous sections of this manuscript, the miR-34/449 family is implicated in several aspects of male infertility. At this point, authors focused on presenting the current evidence regarding the role of miR-34/449 family members on the pathophysiological mechanisms involved in azoospermia, which represents by far the most severe expression of male infertility. Azoospermia is characterized by the absence of spermatozoa in the ejaculate and is a well-studied condition (99). Data indicate that azoospermia is a multifactorial condition caused by a wide variety of congenital, acquired, and idiopathic factors, including genetic, anatomical, endocrine, as well as environmental factors (100–102). However, for a large proportion of azoospermic patients the exact causes of azoospermia cannot be identified, a condition called idiopathic azoospermia (103). Management of these patients is highly challenging, especially with regards to NOA, where histological examination of testicular tissues can reveal different types of spermatogenic failure, ranging from a mild hypo-spermatogenesis to complete loss of spermatozoa, a syndrome called Sertoli Cell Only (SCO) syndrome (104, 105). Considering the pathophysiology of azoospermia, data originating from several studies indicate the possible role of several microRNAs on the molecular basis of testicular impairment observed in this condition (82, 106–108). Numerous studies indicate abnormal expression patterns of several microRNAs in patients with azoospermia (107, 109–111). Notably, miR-34/449 family members seem to represent the most affected class of microRNAs. In 2009 Lian and colleagues were the first to examine the microRNAs' profile in NOA patients and discovered multiple alterations in the expression patterns of several microRNAs in the testicular tissues obtained from these patients. Results also highlighted that both miR-34b and miR-449a were significantly under-expressed in NOA patients (109). Following this evidence, a series of similar studies was conducted, where more data on the distinct microRNA expression patterns of azoospermic patients was sourced. In studies, where microarray analysis was employed on testicular biopsies of patients presenting with different subtypes of NOA diagnosis, including SCO syndrome, mixed atrophy and germ cell arrest, it was revealed that, in all of the aforementioned groups, both miR-34b/c and miR-449a/b/c clusters were the most downregulated class out of all the examined microRNAs (112). Interestingly, miR-34b/c and miR-449a/b/c clusters seem to exert different expression profiles according to the type of NOA. This is indicated by the study of Munoz et al., where the reduction observed in miR-34/449 levels was higher in patients with SCO syndrome compared to patients that presented with maturation arrest. These interesting findings suggest that miR-34/449 family members exert significant regulating actions, which are a prerequisite for germ cell differentiation, especially following the beginning of meiosis during testicular development as well as in adult spermatogenesis (19, 94). Other studies also highlight these observations (82). At this point it is interesting to note that despite the differences, NOA subgroups are sharing common downregulated microRNAs, which underlies that NOA conditions may possibly arise from identical molecular alterations (107). To conclude, evidence suggest that miR-34/449 family members play crucial roles on azoospermia pathogenesis. Alterations of miR-34/449 expression patterns are associated with impaired spermatogenesis, especially

following the stage, where meiotic divisions are initiated. Expression patterns of the miR-34/449 family merit further investigation to be showcased as an effective tool for better categorizing NOA patients.

Regarding NOA caused by spermatogenic failure, the gold standard management is sperm retrieval employing micro-testicular sperm extraction (TESE). However, TESE fails to ascertain a high percentage of success resulting in emotional distress for couples (113). It is imperative to develop reliable and non-invasive markers that could reflect not only testicular histopathology, but that could accurately predict success of a sperm retrieval. The miR-34 family members could be a promising predictive molecule for the clinician alone or in combination with other miRNAs. It is interesting to note that when miR-34b and miR-10b were combined as predictive biomarkers, the ROC curve analysis indicated a high predictive diagnostic accuracy in distinguishing NOA from fertile individuals (114). Additionally, Fang et al. investigated and compared the microRNA profiles of NOA patients with successful and unsuccessful sperm retrieval. They found that the two groups had 180 differentially expressed microRNAs, with miR-34 and miR-449 clusters being the most downregulated in testicular biopsies and seminal plasma of the unsuccessful sperm retrieval group (115). These significant data demonstrate that these microRNAs could not only be used for the accurate diagnosis of NOA, but also for prognosis of TESE effectiveness.

## DISCUSSION

This review is all-inclusively presenting current data on the implication of the miR-34/449 family members in male infertility. Evidence provided is summarized in **Figure 2** and highlights that alterations in expression patterns as well as in functionality of the miR-34/449 family members detrimentally affect male reproductive potential, impairing both testicular functionality as well as spermatogenesis *per se*.

In the present review authors highlight that altered miR-34/449 expression patterns can cause defective ciliogenesis in the efferent ductules epithelium, leading to progressive spermatogenic failure, which could range from OAT to azoospermia. In this case, spermatogenic failure is attributed to testicular dysfunction, resulting from impaired reabsorption of the seminiferous tubular fluids, which in turn leads to high hydrostatic testicular pressure. These cases, where multicillia cell dysfunction and efferent ductules obstruction occur, are characterized by a unique phenotype, where abnormal semen parameters and/or azoospermia co-exist with sperm aggregation and agglutination, as well as with luminal enlargement. The clinical significance of these findings is of paramount importance, considering that these abnormalities resemble the condition of NOA as well as of the SCO syndrome. Considering the small size of the efferent ductules as well as the topographic anatomy of this organ, we can assume that efferent ductules' obstructions could-in some cases-be overseen, bypassed and misdiagnosed. Indeed, there are reports indicating that patients presenting with immotile cilia syndrome also present with azoospermia (116, 117). This could be attributed to the efferent ductules' obstruction in these patients, resulting to fluid accumulation and subsequently generation of testicular hydrostatic pressure. Proper diagnosis of these cases is of value since surgical interventions may provide an effective way for relieving hydrostatic pressure, aiming to improve testicular functionality in order to recover sperm production and restore-to some extent-male fertility (27).

This review further highlights data demonstrating that miR-34/449 family members also constitute significant regulators of spermatogenesis. These microRNAs control expression of several regulatory proteins and are essential for proper transition of the germ cells from the pro-meiotic to meiotic stages. Most importantly, these microRNAs play crucial roles during the final sperm maturation, and upon their dysregulation both spermatozoa formation, as well as functionality are jeopardized. Their added value becomes evident considering that their expression is significantly correlated with semen parameters, including spermatozoa concentration, morphology and motility. In the future, these microRNAs may be employed as specific biomarkers assisting clinicians to in diagnose, and design an efficient management protocol. This may be of value especially in cases of idiopathic male infertility. We herein also demonstrate that miR-34/449 could not only be used to distinguish fertile from infertile men, but also to perform a non-invasive accurate diagnosis defining male infertility etiology, especially regarding NOA patients. Today, male factor infertility diagnosis and evaluation principally includes medical history assessment, physical examination, hormonal assessment and semen analysis. Under specific circumstances, further assessment may be required, including genetic testing, imaging analysis such as scrotal ultrasonography and specific semen testing such as sperm DNA fragmentation testing (3). However, and despite the great advances observed in the field considering development of a variety of diagnostic tools, their interpretation is often imprecise, subjective, vague and inaccurate, highlighting the need for more precise diagnostic tools (118). Data suggest that miR-34/449 members may serve as such biomarkers.

Data summarized herein indicate that miR-34/449 family members are implicated in male reproductive system physiology and pathology. The question is "which physiological mechanisms and factors regulate their expression patterns?" In a recently published review, Ioannis Loukas and colleagues summarize the current evidence with regard to the molecular mechanisms and factors regulating miR-34/449 expression levels during ciliogenesis (119). Authors highlight that both tumour suppressor protein P53 and E2 promoter binding factor 1 (E2F1) stimulate miR-34/449 expression, however their role during multiciliogenesis remains unclear (119–121). The transcriptional domain-containing active p73 (TAp73) has been recently introduced as a master regulator of multiciliogenesis process in several tissues and organs (119, 122, 123). This factor directly binds to miR-34b/c loci and transactivates the expression of this specific microRNA cluster.

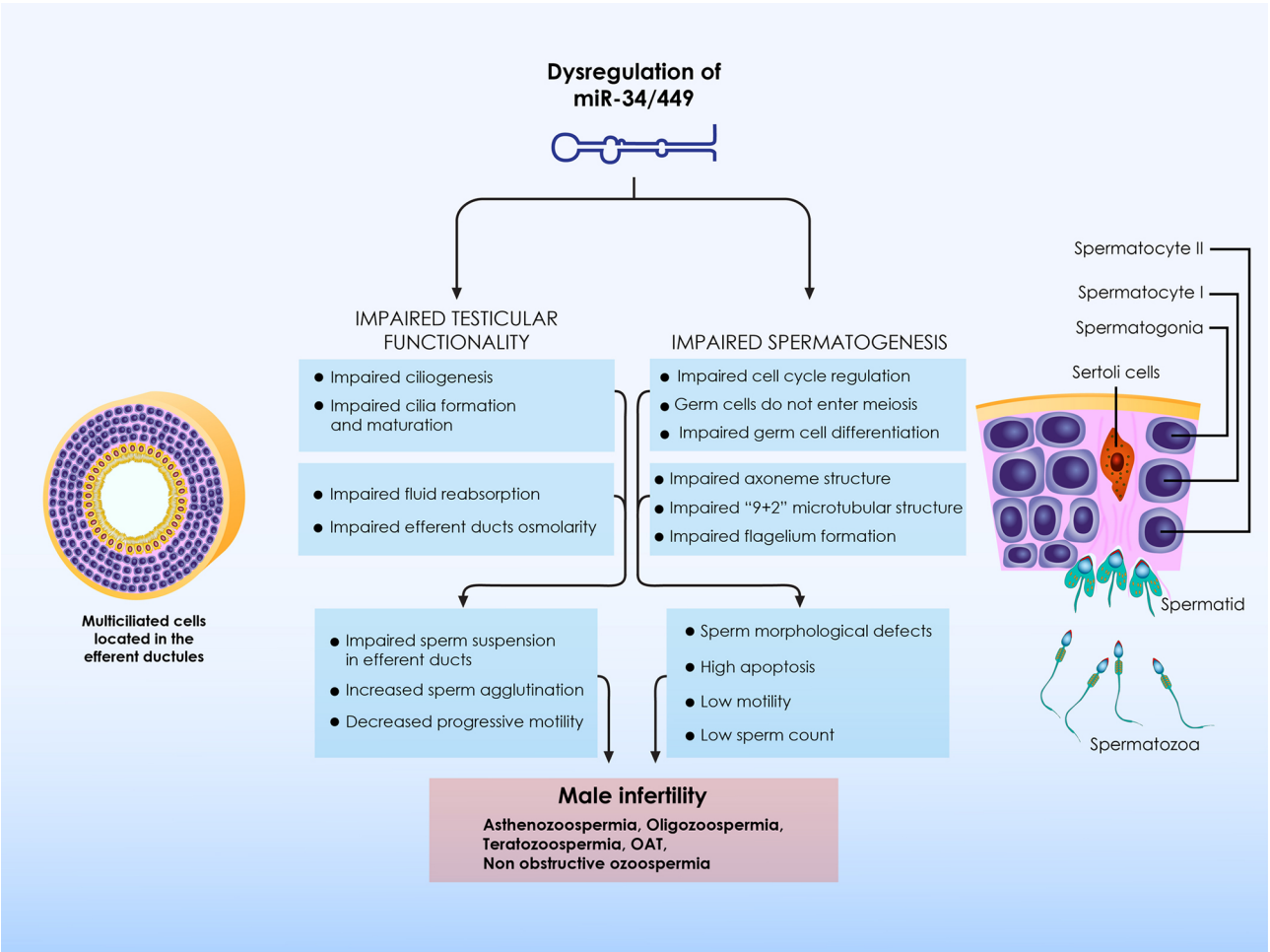


**FIGURE 2** | Summary of the role of miR-34/449 on male infertility. Alterations in the expression patterns as well as in functionality of miR-34/449 family members detrimentally affect male reproductive potential, impairing both testicular functionality as well as spermatogenesis per se. On the one hand, impaired miR-34/449 expression patterns result to defective ciliogenesis in the efferent ductules epithelium, leading to impaired reabsorption of the seminiferous tubular fluids. Failed reabsorption of the fluids in turns leads to epididymal spermatozoa agglutination and aggregation. In these conditions the number of spermatozoa reaching to caput epididymis is significantly reduced and as a result the number of mature motile spermatozoa is significantly decreased causing OAT or even azoospermia. Impaired ciliogenesis also leads to fluid accumulation into the rete testis, which in turns lead to high hydrostatic pressure into the testis. Hydrostatic pressure progressively leads to spermatogenic failure, finally resulting to male infertility. On the other hand, alterations regarding miR-34/449 expression patterns also directly affect spermatogenesis. Upon miR-34/449 dysregulation, the expression patterns of several spermatogenesis-related proteins are disrupted, preventing germ cells to properly enter meiosis and thus to differentiate to mature spermatozoa. Moreover, miR-34/449 dysregulation also directly impairs spermatozoa formation and maturation, mainly considering flagellum construction and function. Thus, alterations with regard to miR-34/449 expression patterns are directly associated with impaired semen parameters, including abnormal spermatozoa concentration, morphology and motility. *OAT, oligoasthenoteratozoospermia*.

However, it seems that p73 is differentially expressed among the different multiciliated tissues, as its expression is not a prerequisite for multicillia cell differentiation in either the respiratory or the reproductive track epithelia (124). These data support the hypothesis that alternative pathways have been developed in diverse tissues to support multicillia cell differentiation (119). Geminin superfamily and more specifically GemC1 and McIdas have been recently introduced as key-regulators of multiciliogenesis in the airway epithelium as well as in the brain (37, 44, 125, 126). Interestingly, GemC1 constitutes a predicted miR-34/449 target, however their complex interactions during ciliogenesis remain unclear (119). Considering the aforementioned, the possible role of Geminin family during multicillia cell differentiation in other tissues and organs, including the testis and the fallopian tubes, merits further investigation. Focusing on testicular functionality, limited data exist with regard to the upstream elements controlling miR-34/449 expression patterns. It seems that two transcription factors, namely cAMP-responsive element modulator τ (CREM) and SRY-box transcription factor 5 (SOX5), serve as upstream regulators and are able to transactivate miR-449 cluster in the mouse testes (19). Interestingly, both of these factors also regulate male germ cell gene expression patterns, and their dysregulation is strongly associated with severe male factor

infertility, such as NOA (127). In summary, investigation of the upstream pathways controlling miR-34/449 expression patterns in the testis may open a new line of investigation, unveiling potential biomarkers as well as novel therapeutic targets, towards better understanding and efficiently managing male infertility.

Considering the clinical perspective in addressing male infertility, significant information could be retrieved by analyzing miR-34/449 levels in both testicular tissues and semen, namely predicting a successful sperm retrieval following biopsy. The prognostic value of these microRNAs could ascertain improvement of *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) outcomes, reducing the cost as well as well as psychological discomfort associated with male infertility (128). The significance of these findings is highlighted, considering that infertility is not strictly a medical issue but rather extends to social, psychological, and even bioethical aspects. An infertility diagnosis impacts both partners on various levels, entailing psychological distress and financial strain as treatment may be costly. National Health systems-where involved-may equally be financially burdened (129, 130). The need to provide solutions through research in addressing infertility, from diagnosis to treatment has been thoroughly documented (129). Thus, identifying biomarkers that allow proper patient categorization is a powerful tool in the era of personalized and precision medicine.

Taking a closer look into the advanced insemination techniques employed today to address male infertility, as part of our clinical routine tools we identify ICSI and intracytoplasmic morphologically selected sperm injection (IMSI), and development of accurate surgical interventions for sperm collection direct from the testis, such as testicular sperm aspiration (TESA) and TESE. These options have led clinicians and clinical embryologists to successfully overcome several types of male infertility, including the most severe namely azoospermia. Coupled by high efficiency, these techniques were quickly adopted by clinicians and fertility centers all over the word (131). Despite several couples achieving their reproductive goals employing these state-of-the-art techniques, it should be highlighted that these techniques and methods have been designed to merely bypass the barrier of male factor infertility, albeit the exact infertility aetiology remains untreated. Several reports highlight that perhaps availability and-to some extent-overuse of these advanced techniques lessens the urgency for investigating in depth treatment options, and improving male fertility status at its core (132). Even though advanced assisted reproductive technology (ART) techniques can circumvent male infertility and provide solutions for fertilizing an oocyte, nonetheless, the solution to properly address and treat male infertility still eludes us. It appears that research is more clinically driven and focused on addressing the "symptom", rather than investigating means to understand and treat the actual condition. Providing clinically driven solutions fails to effectively address male infertility in depth. This should be taken into consideration as there is a great body of evidence suggesting that sperm quality as well as paternal health at the time of conception could significantly affect outcomes (133, 134). These outcomes range from the IVF treatment's efficiency, to embryo development, pregnancy, and finally to the offspring's' health, and even the children's future reproductive potential (133, 134). Data also indicate that harmful epigenetic modifications could be inherited from damaged spermatozoa to the next generation (135). To add to that, several studies suggest that embryos resulting from spermatozoa of impaired quality are characterized by reduced developmental and implantation potential, increasing the incidence of pregnancy failure (136, 137).

Taking into account this critical analysis, it is of paramount importance to focus not only on developing advanced technologies aiming to override the barriers of male infertility, but most importantly to focus on performing research to understand male infertility pathophysiology in depth. Better understanding the pathological basis of male infertility, in a personalized and precise manner, and following on the concept of individualization and precision could significantly assist the reproductive scientist. Orienting research to develop higher performance diagnostic and evaluating tools, will enable design of accurate, targeted and efficient therapies, in order to personalize patients' management and finally to ensure the health of future generations. Only by aspiring to understand and treat the condition of male infertility itself, and not merely bypass it, we may advance towards ascertaining health of the next generations. It is this realization that fuels and justifies research on identifying areas focusing on novel molecular aspects of male infertility. This review uniquely presents the case of miR-34/449 family members, all-inclusively describing their involvement while showcasing their value in investigating and addressing male infertility.

## AUTHOR CONTRIBUTIONS

KP, SG, KS, and MS conceived and designed the project. SG, PT, and IL performed the literature review. KP, SG, PT, IL, EM, AP, NN, TV, and GK contributed to drafting and editing the manuscript. KP, SG, GK, AA, KS, and MS revised the manuscript. GK, AA, KS, and MS contributed to the critical discussion and provided intellectual content. KP, KS, and MS supervised the study. All authors contributed to the article and approved the submitted version.

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## REFERENCES


1. Vander Borght M, Wyns C. Fertility and Infertility: Definition and Epidemiology. *Clin Biochem* (2018) 62:2–10. doi: 10.1016/j.clinbiochem.2018.03.012
2. Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, et al. The International Glossary on Infertility and Fertility Care, 2017. *Fertil Steril* (2017) 108(3):393–406. doi: 10.1016/j.fertnstert.2017.06.005
3. Agarwal A, Baskaran S, Parekh N, Cho C-L, Henkel R, Vij S, et al. Male Infertility. *Lancet* (2021) 397(10271):319–33. doi: 10.1016/S0140-6736(20)32667-2
4. Anderson JE, Farr SL, Jamieson DJ, Warner L, Macaluso M. Infertility Services Reported by Men in the United States: National Survey Data. *Fertil Steril* (2009) 91(6):2466–70. doi: 10.1016/j.fertnstert.2008.03.022
5. Krausz C. Male Infertility: Pathogenesis and Clinical Diagnosis. *Best Pract Res Clin Endocrinol Metab* (2011) 25(2):271–85. doi: 10.1016/j.beem.2010.08.006
6. Ilacqua A, Izzo G, Emerenziani GP, Baldari C, Aversa A. Lifestyle and Fertility: The Influence of Stress and Quality of Life on Male Fertility. *Reprod Biol Endocrinol* (2018) 16(1):115. doi: 10.1186/s12958-018-0436-9
7. Duca Y, Calogero AE, Cannarella R, Condorelli RA, La Vignera S. Current and Emerging Medical Therapeutic Agents for Idiopathic Male Infertility. *Expert Opin Pharmacother* (2019) 20(1):55–67. doi: 10.1080/14656566.2018.1543405
8. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulatio. *Front Endocrinol (Lausanne)* (2018) 9:402. doi: 10.3389/fendo.2018.00402
9. Ghorai A, Ghosh U. miRNA Gene Counts in Chromosomes Vary Widely in a Species and Biogenesis of miRNA Largely Depends on Transcription or Post-Transcriptional Processing of Coding Genes. *Front Genet* (2014) 5:100. doi: 10.3389/fgene.2014.00100
10. Wang Y, Luo J, Zhang H, Lu J. microRNAs in the Same Clusters Evolve to Coordinately Regulate Functionally Related Gene. *Mol Biol Evol* (2016) 33(9):2232–47. doi: 10.1093/molbev/msw089
11. Lin Q, Ma L, Liu Z, Yang Z, Wang J, Liu J, et al. Targeting microRNAs: A New Action Mechanism of Natural Compounds. *Oncotarget* (2016) 8(9):15961–70. doi: 10.18632/oncotarget.14392
12. Otto T, Candido SV, Pilarz MS, Sicinska E, Bronson RT, Bowden M, et al. Cell Cycle-Targeting microRNAs Promote Differentiation by Enforcing Cell-Cycle Exit. *Proc Natl Acad Sci USA* (2017) 114(40):10660–5. doi: 10.1073/pnas.1702914114
13. Cirillo F, Catellani C, Lazzeroni P, Sartori C, Street ME. The Role of MicroRNAs in Influencing Body Growth and Developmen. *Horm Res Paediatr* (2020) 93(1):7–15. doi: 10.1159/000504669
14. Gross N, Kropp J, Khatib H. MicroRNA Signaling in Embryo Development. *Biol (Basel)* (2017) 6(3):34. doi: 10.3390/biology6030034
15. Ivey KN, Srivastava D. microRNAs as Developmental Regulators. *Cold Spring Harb Perspect Biol* (2015) 7(7):a008144. doi: 10.1101/cshperspect.a008144
16. Dwivedi S, Purohit P, Sharma P. MicroRNAs and Diseases: Promising Biomarkers for Diagnosis and Therapeutic. *Indian J Clin Biochem* (2019) 34(3):243–5. doi: 10.1007/s12291-019-00844-x
17. Lv J, Zhang Z, Pan L, Zhang Y. MicroRNA-34/449 Family and Viral Infections. *Virus Res* (2019) 260:1–6. doi: 10.1016/j.virusres.2018.11.001
18. Li XJ, Ren ZJ, Tang JH. MicroRNA-34a: A Potential Therapeutic Target in Human Cancer. *Cell Death Dis* (2014) 5(7):e1327. doi: 10.1038/cddis.2014.270
19. Bao J, Li D, Wang L, Wu J, Hu Y, Wang Z, et al. MicroRNA-449 and MicroRNA-34b/C Function Redundantly in Murine Testes by Targeting E2F Transcription Factor-Retinoblastoma Protein (E2F-pRb) Pathwa. *J Biol Chem* (2012) 287(26):21686–98. doi: 10.1074/jbc.M111.328054
20. Marcet B, Chevalier B, Luxardi G, Coraux C, Zaragosi L-E, Cibois M, et al. Control of Vertebrate Multiciliogenesis by miR-449 Through Direct Repression of the Delta/Notch Pathway. *Nat Cell Biol* (2011) 13(6):693–9. doi: 10.1038/ncb2241
21. Comazzetto S, Di Giacomo M, Rasmussen KD, Much C, Azzi C, Perlas E, et al. Oligoasthenoteratozoospermia and Infertility in Mice Deficient for miR-34b/C and miR-449 Loci. *PloS Genet* (2014) 10(10):e1004597. doi: 10.1371/journal.pgen.1004597
22. Song R, Walentek P, Sponer N, Klimke A, Lee JS, Dixon G, et al. miR-34/449 miRNAs are Required for Motile Ciliogenesis by Repressing Cp110. *Nature* (2014) 510(7503):115–20. doi: 10.1038/nature13413
23. Fededa JP, Esk C, Mierzwa B, Stanyte R, Yuan S, Zheng H, et al. MicroRNA-34/449 Controls Mitotic Spindle Orientation During Mammalian Cortex Development. *EMBO J* (2016) 35(22):2386–98. doi: 10.15252/embj.201694056
24. Solberg OD, Ostrin EJ, Love MI, Peng JC, Bhakta NR, Hou L, et al. Airway Epithelial miRNA Expression Is Altered in Asthm. *Am J Respir Crit Care Med* (2012) 186(10):965–74. doi: 10.1164/rccm.201201-0027OC
25. Wu J, Bao J, Kim M, Yuan S, Tang C, Zheng H, et al. Two miRNA Clusters, miR-34b/C and miR-449, are Essential for Normal Brain Development, Motile Ciliogenesis, and Spermatogenesis. *Proc Natl Acad Sci USA* (2014) 111(28):E2851–2857. doi: 10.1073/pnas.1407777111
26. Walentek P, Song R, He L. microRNAs and Cilia. An Ancient Connection. *Cell Cycle* (2014) 13(15):2315–6. doi: 10.4161/cc.29827
27. Yuan S, Liu Y, Peng H, Tang C, Hennig GW, Wang Z, et al. Motile Cilia of the Male Reproductive System Require miR-34/miR-449 for Development and Function to Generate Luminal Turbulence. *Proc Natl Acad Sci USA* (2019) 116(9):3584–93. doi: 10.1073/pnas.1817018116
28. Avasthi P, Marshall WF. Stages of Ciliogenesis and Regulation of Ciliary Length. *Differentiation* (2012) 83(2):S30–42. doi: 10.1016/j.diff.2011.11.015
29. Ishikawa T. Axoneme Structure From Motile Cilia. *Cold Spring Harb Perspect Biol* (2017) 9(1):a028076. doi: 10.1101/cshperspect.a028076
30. Goetz SC, Anderson KV. The Primary Cilium: A Signalling Centre During Vertebrate Development. *Nat Rev Genet* (2010) 11(5):331–44. doi: 10.1038/nrg2774
31. Wheway G, Nazlamova L, Hancock JT. Signaling Through the Primary Ciliu. *Front Cell Dev Biol* (2018) 6:8. doi: 10.3389/fcell.2018.00008
32. Brooks ER, Wallingford JB. Multiciliated Cells: A Review. *Curr Biol* (2014) 24(19):R973–82. doi: 10.1016/j.cub.2014.08.047
33. Fliegauf M, Benzing T, Omran H. When Cilia Go Bad: Cilia Defects and Ciliopathies. *Nat Rev Mol Cell Biol* (2007) 8(11):880–93. doi: 10.1038/nrm2278
34. Kirchhoff C, Osterhoff C, Samalecos A. HE6/GPR64 Adhesion Receptor Co-Localizes With Apical and Subapical F-Actin Scaffold in Male Excurrent Duct Epithelia. *Reproduction* (2008) 136(2):235–45. doi: 10.1530/REP-08-0078
35. Takeda S, Narita K. Structure and Function of Vertebrate Cilia, Towards a New Taxonomy. *Differentiation* (2012) 83(2):S4–11. doi: 10.1016/j.diff.2011.11.002
36. Yan N, Lu Y, Sun H, Qiu W, Tao D, Liu Y, et al. Microarray Profiling of microRNAs Expressed in Testis Tissues of Developing Primates. *J Assist Reprod Genet* (2009) 26(4):179–86. doi: 10.1007/s10815-009-9305-y
37. Arbi M, Pefani D-E, Kyrousi C, Lalioti M-E, Kalogeropoulou A, Papanastasiou AD, et al. GemC1 Controls Multiciliogenesis in the Airway Epithelium. *EMBO Rep* (2016) 17(3):400–13. doi: 10.15252/embr.201540882
38. Spassky N, Meunier A. The Development and Functions of Multiciliated Epithelia. *Nat Rev Mol Cell Biol* (2017) 18(7):423–36. doi: 10.1038/nrm.2017.21
39. Li S, O'Neill SRS, Zhang Y, Holtzman MJ, Takemaru K-I, Korach KS, et al. Estrogen Receptor α is Required for Oviductal Transport of Embryos. *FASEB J* (2017) 31(4):1595–607. doi: 10.1096/fj.201601128R
40. Francavilla S, Moscardelli S, Bruno B, Barcellona PS, De Martino C. The Postnatal Maturation of Efferent Tubules in the Rat: A Light and Electron Microscopy Study. *J Embryol Exp Morphol* (1986) 96:51–63. doi: 10.1242/dev.96.1.51
41. Clulow J, Jones RC, Hansen LA, Man SY. Fluid and Electrolyte Reabsorption in the Ductuli Efferentes Testis. *J Reprod Fertil Suppl* (1998) 53:1–14.
42. Rosenfeld CS. Male Reproductive Tract Cilia Beat to a Different Drummer. *Proc Natl Acad Sci USA* (2019) 116(9):3361–3. doi: 10.1073/pnas.1900112116
43. Avidor-Reiss T, Gopalakrishnan J. Cell Cycle Regulation of the Centrosome and Cilium. *Drug Discov Today Dis Mech* (2013) 10(3–4):e119–24. doi: 10.1016/j.ddmec.2013.03.002

44. Arbi M, Pefani D-E, Taraviras S, Lygerou Z. Controlling Centriole Numbers: Geminin Family Members as Master Regulators of Centriole Amplification and Multiciliogenesis. *Chromosoma* (2018) 127(2):151–74. doi: 10.1007/s00412-017-0652-7
45. Nanjundappa R, Kong D, Shim K, Stearns T, Brody SL, Loncarek J, et al. Regulation of Cilia Abundance in Multiciliated Cells. Reiter JF, Akhmanova A, Tsou M-FB, Editors. *eLife* (2019) 8:e44039. doi: 10.7554/eLife.44039
46. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Cilia and Flagella: Structure and Movemen, in: *Mol Cell Biolog* (2000). Available at: https://www.ncbi.nlm.nih.gov/books/NBK21698/ (Accessed cited 2021 May 12).
47. Achari C, Winslow S, Ceder Y, Larsson C. Expression of miR-34c Induces G2/M Cell Cycle Arrest in Breast Cancer Cells. *BMC Cancer* (2014) 14:538. doi: 10.1186/1471-2407-14-538
48. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA Component of the P53 Tumour Suppressor Network. *Nature* (2007) 447 (7148):1130–4. doi: 10.1038/nature05939
49. Lee Y-M, Lee J-Y, Ho C-C, Hong Q-S, Yu S-L, Tzeng C-R, et al. miRNA-34b as a Tumor Suppressor in Estrogen-Dependent Growth of Breast Cancer Cells. *Breast Cancer Res* (2011) 13(6):R116. doi: 10.1186/bcr3059
50. Chevalier B, Adamiok A, Mercey O, Revinski DR, Zaragosi L-E, Pasini A, et al. miR-34/449 Control Apical Actin Network Formation During Multiciliogenesis Through Small GTPase Pathways. *Nat Commun* (2015) 6(1):8386. doi: 10.1038/ncomms9386
51. Mercey O, Kodjabachian L, Barbry P, Marcet B. MicroRNAs as Key Regulators of GTPase-Mediated Apical Actin Reorganization in Multiciliated Epithelia. *Small GTPases* (2016) 7(2):54–8. doi: 10.1080/21541248.2016.1151099
52. Cao J, Shen Y, Zhu L, Xu Y, Zhou Y, Wu Z, et al. miR-129-3p Controls Cilia Assembly by Regulating CP110 and Actin Dynamics. *Nat Cell Biol* (2012) 14 (7):697–706. doi: 10.1038/ncb2512
53. Fahl SP, Daamen AR, Crittenden RB, Bender TP. C-Myb Coordinates Survival and the Expression of Genes That Are Critical for the Pre-BCR Checkpoin. *J Immunol* (2018) 200(10):3450–63. doi: 10.4049/jimmunol.1302303
54. Mitra P. Transcription Regulation of MYB: A Potential and Novel Therapeutic Target in Cancer. *Ann Transl Med* (2018) 6(22):443. doi: 10.21037/atm.2018.09.62
55. Oh I-H, Reddy EP. The Myb Gene Family in Cell Growth, Differentiation and Apoptosis. *Oncogene* (1999) 18(19):3017–33. doi: 10.1038/sj.onc.1202839
56. Wang L, Fu C, Fan H, Du T, Dong M, Chen Y, et al. miR-34b Regulates Multiciliogenesis During Organ Formation in Zebrafish. *Development* (2013) 140(13):2755–64. doi: 10.1242/dev.092825
57. Spektor A, Tsang WY, Khoo D, Dynlacht BD. Cep97 and CP110 Suppress a Cilia Assembly Progra. *Cell* (2007) 130(4):678–90. doi: 10.1016/j.cell.2007.06.027
58. Tsang WY, Dynlacht BD. CP110 and its Network of Partners Coordinately Regulate Cilia Assembly. *Cilia* (2013) 2(1):9. doi: 10.1186/2046-2530-2-9
59. Joseph A, Shur BD, Hess RA. Estrogen, Efferent Ductules: Structure and the Epididymis. *Biol Reprod* (2011) 84(2):207–17. doi: 10.1095/biolreprod.110.087353
60. Yuan S, Tang C, Zhang Y, Wu J, Bao J, Zheng H, et al. Mir-34b/C and Mir-449a/B/C are Required for Spermatogenesis, But Not for the First Cleavage Division in Mice. *Biol Open* (2015) 4(2):212–23. doi: 10.1242/bio.201410959
61. Liu W-M, Pang RTK, Chiu PCN, Wong BPC, Lao K, Lee K-F, et al. Sperm-Borne microRNA-34c is Required for the First Cleavage Division in Mouse. *PNAS* (2012) 109(2):490–4. doi: 10.1073/pnas.1110368109
62. Zhang S, Yu M, Liu C, Wang L, Hu Y, Bai Y, et al. MIR-34c Regulates Mouse Embryonic Stem Cells Differentiation Into Male Germ-Like Cells Through RARg: MIR-34c Regulates Mescs Differentiation. *Cell Biochem Funct* (2012) 30(8):623–32. doi: 10.1002/cbf.2922
63. Buchold GM, Coarfa C, Kim J, Milosavljevic A, Gunaratne PH, Matzuk MM. Analysis of MicroRNA Expression in the Prepubertal Testis. Stanton J-AL, Editor. *PloS One* (2010) 5(12):e15317. doi: 10.1371/journal.pone.0015317
64. Yan N, Lu Y, Sun H, Tao D, Zhang S, Liu W, et al. A Microarray for microRNA Profiling in Mouse Testis Tissues. *Reproduction* (2007) 134 (1):73–9. doi: 10.1530/REP-07-0056
65. Bouhallier F, Allioli N, Lavial F, Chalmel F, Perrard M-H, Durand P, et al. Role of miR-34c microRNA in the Late Steps of Spermatogenesis. *RNA* (2010) 16(4):720–31. doi: 10.1261/rna.1963810
66. Hilz S, Fogarty EA, Modzelewski AJ, Cohen PE, Grimson A. Transcriptome Profiling of the Developing Male Germ Line Identifies the miR-29 Family as a Global Regulator During Meiosis. *RNA Biol* (2017) 14(2):219–35. doi: 10.1080/15476286.2016.1270002
67. Hayashi T, Kageyama Y, Ishizaka K, Xia G, Kihara K, Oshima H. Requirement of Notch 1 and its Ligand Jagged 2 Expressions for Spermatogenesis in Rat and Human Testes. *J Androl* (2001) 22(6):999–1011. doi: 10.1002/j.1939-4640.2001.tb03441.x
68. Yamamoto CM, Hikim APS, Lue Y, Portugal AM, Guo TB, Hsu SY, et al. Impairment of Spermatogenesis in Transgenic Mice With Selective Overexpression of Bcl-2 in the Somatic Cells of the Testi. *J Androl* (2001) 22(6):981–91. doi: 10.1002/j.1939-4640.2001.tb03439.x
69. Zheng S, Turner TT, Lysiak JJ. Caspase 2 Activity Contributes to the Initial Wave of Germ Cell Apoptosis During the First Round of Spermatogenesis1. *Biol Reproduction* (2006) 74(6):1026–33. doi: 10.1095/biolreprod.105.044610
70. Huang Z, Rivas B, Agoulnik AI. NOTCH1 Gain of Function in Germ Cells Causes Failure of Spermatogenesis in Male Mice. Lobaccaro J-MA, Editor. *PloS One* (2013) 8(7):e71213. doi: 10.1371/journal.pone.0071213
71. Badawy AA, El-Magd MA, AlSadrah SA, Alruwaili MM. Altered Expression of Some miRNAs and Their Target Genes Following Mesenchymal Stem Cell Treatment in Busulfan-Induced Azoospermic Rats. *Gene* (2020) 737:144481. doi: 10.1016/j.gene.2020.144481
72. Hoja M-R, Liu J-G, Mohammadieh M, Kvist U, Yuan L. E2F1 Deficiency Impairs Murine Spermatogenesis and Augments Testicular Degeneration in SCP3-Nullizygous Mice. *Cell Death Dif* (2004) 11(3):354–6. doi: 10.1038/sj.cdd.4401362
73. Nalam RL, Andreu-Vieyra C, Braun RE, Akiyama H, Matzuk MM. Retinoblastoma Protein Plays Multiple Essential Roles in the Terminal Differentiation of Sertoli Cell. *Mol Endocrinol* (2009) 23(11):1900–13. doi: 10.1210/me.2009-0184
74. Smorag L, Zheng Y, Nolte J, Zechner U, Engel W, Pantakani DVK. MicroRNA Signature in Various Cell Types of Mouse Spermatogenesis: Evidence for Stage-Specifically Expressed miRNA-221, -203 and -34b-5p Mediated Spermatogenesis Regulation. *Biol Cell* (2012) 104(11):677–92. doi: 10.1111/boc.201200014
75. Liang X, Zhou D, Wei C, Luo H, Liu J, Fu R, et al. MicroRNA-34c Enhances Murine Male Germ Cell Apoptosis Through Targeting ATF1. Rota R, Editor. *PloS One* (2012) 7(3):e33861. doi: 10.1371/journal.pone.0033861
76. Ghazipour AM, Shirpoor A, Ghiasi R, Pourheydar B, Khalaji N, Naderi R. Cyclosporine A Induces Testicular Injury via Mitochondrial Apoptotic Pathway by Regulation of Mir-34a and Sirt-1 in Male Rats: The Rescue Effect of Curcumin. *Chem-Biol Interact* (2020) 327:109180. doi: 10.1016/j.cbi.2020.109180
77. Jiao D, Zhang H, Jiang Z, Huang W, Liu Z, Wang Z, et al. MicroRNA-34a Targets Sirtuin 1 and Leads to Diabetes-Induced Testicular Apoptotic Cell Death. *J Mol Med (Berl)* (2018) 96(9):939–49. doi: 10.1007/s00109-018-1667-0
78. Fatemi N, Sanati MH, Shamsara M, Moayer F, Zavarehei MJ, Pouya A, et al. TBHP-Induced Oxidative Stress Alters microRNAs Expression in Mouse Testis. *J Assist Reprod Genet* (2014) 31(10):1287–93. doi: 10.1007/s10815-014-0302-4
79. Abu-Halima M, Hammadeh M, Schmitt J, Leidinger P, Keller A, Meese E, et al. Altered microRNA Expression Profiles of Human Spermatozoa in Patients With Different Spermatogenic Impairments. *Fertil Steril* (2013) 99 (5):1249–55.e16. doi: 10.1016/j.fertnstert.2012.11.054
80. Abu-Halima M, Hammadeh M, Backes C, Fischer U, Leidinger P, Lubbad AM, et al. Panel of Five microRNAs as Potential Biomarkers for the Diagnosis and Assessment of Male Infertility. *Fertil Steril* (2014) 102 (4):989–97. e1. doi: 10.1016/j.fertnstert.2014.07.001
81. Eikmans M DH, Anholts J, Blijleven L, Meuleman T, van Beelen E, van der Hoorn M-LP, et al. Optimization of microRNA Acquirement From Seminal Plasma and Identification of Diminished Seminal microRNA-34b as Indicator of Low Semen Concentratio. *IJMS* (2020) 21(11):4089. doi: 10.3390/ijms21114089
82. Muñoz X, Mata A, Bassas L, Larriba S. Altered miRNA Signature of Developing Germ-Cells in Infertile Patients Relates to the Severity of Spermatogenic Failure and Persists in Spermatozo. *Sci Rep* (2015) 5 (1):17991. doi: 10.1038/srep17991

83. Abu-Halima M, Galata V, Backes C, Keller A, Hammadeh M, Meese E. MicroRNA Signature in Spermatozoa and Seminal Plasma of Proven Fertile Men and in Testicular Tissue of Men With Obstructive Azoospermia. *Andrologia* (2020) 52(2):e13503. doi: 10.1111/and.13503
84. Najafipour R, Momeni A, Yousefipour F, Mousavi S, Moghbelinejad S. Underexpression of hsa-miR-449 Family and Their Promoter Hypermethylation in Infertile Men: A Case-Control Study. *Int J Reprod Biomed* (2021) 19(1):23–34. doi: 10.18502/ijrm.v19i1.8177
85. Momeni A, Najafipour R, Hamta A, Jahani S, Moghbelinejad S. Expression and Methylation Pattern of hsa-miR-34 Family in Sperm Samples of Infertile Me. *Reprod Sci* (2020) 27(1):301–8. doi: 10.1007/s43032-019-00025-4
86. Åsenius F, Danson AF, Marzi SJ. DNA Methylation in Human Sperm: A Systematic Review. *Hum Reprod Update* (2020) 26(6):841–73. doi: 10.1093/humupd/dmaa025
87. Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT. Alterations in Sperm DNA Methylation Patterns at Imprinted Loci in Two Classes of Infertility. *Fertil Steril* (2010) 94(5):1728–33. doi: 10.1016/j.fertnstert.2009.09.010
88. Kobayashi N, Miyauchi N, Tatsuta N, Kitamura A, Okae H, Hiura H, et al. Factors Associated With Aberrant Imprint Methylation and Oligozoospermia. *Sci Rep* (2017) 7(1):42336. doi: 10.1038/srep42336
89. Dai J-B, Wang Z-X, Qiao Z-D. The Hazardous Effects of Tobacco Smoking on Male Fertility. *Asian J Androl* (2015) 17(6):954–60. doi: 10.4103/1008-682X.150847
90. Gunes S, Metin Mahmutoglu A, Arslan MA, Henkel R. Smoking-Induced Genetic and Epigenetic Alterations in Infertile Men. *Andrologia* (2018) 50 (9):e13124. doi: 10.1111/and.13124
91. Ranganathan P, Rao KA, Thalaivarasai Balasundaram S. Deterioration of Semen Quality and Sperm-DNA Integrity as Influenced by Cigarette Smoking in Fertile and Infertile Human Male Smokers-A Prospective Study. *J Cell Biochem* (2019). doi: 10.1002/jcb.28458
92. Dickson DA, Paulus JK, Mensah V, Lem J, Saavedra-Rodriguez L, Gentry A, et al. Reduced Levels of miRNAs 449 and 34 in Sperm of Mice and Men Exposed to Early Life Stress. *Transl Psychiatry* (2018) 8(1):101. doi: 10.1038/s41398-018-0146-2
93. Ashrafzade AM, Sadighi Gilani MA, Topraggaleh TR, Khojasteh M, Sepidarkish M, Borjian Boroujeni P, et al. Oxidative Stress-Related miRNAs in Spermatozoa may Reveal the Severity of Damage in Grade III Varicocele. *Andrologia* (2020) 52(9):e13598. doi: 10.1111/and.13598
94. Ibarra-Ramírez M, Calvo-Anguiano G, Lugo-Trampe J de J, Martínez-de-Villarreal LE, Rodríguez-Torres D, Nistal M, et al. Expression Profile of microRNAs in the Testes of Patients With Klinefelter Syndrome. *Sci Rep* (2020) 10(1):11470. doi: 10.1038/s41598-020-68294-7
95. Durairajanayagam D, Agarwal A, Ong C. Causes, Effects and Molecular Mechanisms of Testicular Heat Stress. *Reprod BioMed Online* (2015) 30 (1):14–27. doi: 10.1016/j.rbmo.2014.09.018
96. Mostafa T, Rashed LA, Nabil NI, Osman I, Mostafa R, Farag M. Seminal miRNA Relationship With Apoptotic Markers and Oxidative Stress in Infertile Men With Varicocel. *BioMed Res Int* (2016) 2016:1–9. doi: 10.1155/2016/4302754
97. Adams BD, Parsons C, Slack FJ. The Tumor-Suppressive and Potential Therapeutic Functions of miR-34a in Epithelial Carcinoma. *Expert Opin Ther Targ* (2016) 20(6):737–53. doi: 10.1517/14728222.2016.1114102
98. Wan Y, Cui R, Gu J, Zhang X, Xiang X, Liu C, et al. Identification of Four Oxidative Stress-Responsive MicroRNAs, miR-34a-5p, miR-1915-3p, miR-638, and miR-150-3p, in Hepatocellular Carcinoma. *Oxid Med Cell Longev* (2017) 2017:5189138. doi: 10.1155/2017/5189138
99. Practice Committee of the American Society for Reproductive Medicine in Collaboration With the Society for Male Reproduction and Urology. Evaluation of the Azoospermic Male: A Committee Opinion. *Fertil Steril* (2018) 109(5):777–82. doi: 10.1016/j.fertnstert.2018.01.043
100. Cocuzza M, Alvarenga C, Pagani R. The Epidemiology and Etiology of Azoospermia. *Clinics (Sao Paulo)* (2013) 68(Suppl 1):15–26. doi: 10.6061/clinics/2013(Sup01)03
101. Kasak L, Laan M. Monogenic Causes of Non-Obstructive Azoospermia: Challenges, Established Knowledge, Limitations and Perspectives. *Hum Genet* (2021) 140(1):135–54. doi: 10.1007/s00439-020-02112-y
102. Wosnitzer M, Goldstein M, Hardy MP. Review of Azoospermia. *Spermatogenesis* (2014) 4:e28218. doi: 10.4161/spmg.28218
103. Kim SY, Lee BY, Oh AR, Park SY, Lee HS, Seo JT. Clinical, Hormonal, and Genetic Evaluation of Idiopathic Nonobstructive Azoospermia and Klinefelter Syndrome Patient. *Cytogenet Genome Res* (2017) 153(4):190–7. doi: 10.1159/000487039
104. Jarow JP, Espeland MA, Lipshultz LI. Evaluation of the Azoospermic Patien. *J Urol* (1989) 142(1):62–5. doi: 10.1016/S0022-5347(17)38662-7
105. McLachlan RI, Rajpert-De Meyts E, Hoei-Hansen CE, de Kretser DM, Skakkebaek NE. Histological Evaluation of the Human Testis–Approaches to Optimizing the Clinical Value of the Assessment: Mini Review. *Hum Reprod* (2007) 22(1):2–16. doi: 10.1093/humrep/del279
106. Dabaja AA, Mielnik A, Robinson BD, Wosnitzer MS, Schlegel PN, Paduch DA. Possible Germ Cell-Sertoli Cell Interactions Are Critical for Establishing Appropriate Expression Levels for the Sertoli Cell-Specific MicroRNA, miR-202-5p, in Human Testis. *Basic Clin Androl* (2015) 25(1):2. doi: 10.1186/s12610-015-0018-z
107. Noveski P, Popovska-Jankovic K, Kubelka-Sabit K, Filipovski V, Lazarevski S, Plaseski T, et al. MicroRNA Expression Profiles in Testicular Biopsies of Patients With Impaired Spermatogenesis. *Andrology* (2016) 4(6):1020–7. doi: 10.1111/andr.12246
108. Zhang H-T, Zhang Z, Hong K, Tang W-H, Liu D-F, Mao J-M, et al. Altered microRNA Profiles of Testicular Biopsies From Patients With Nonobstructive Azoospermia. *Asian J Androl* (2020) 22(1):100–5. doi: 10.4103/aja.aja_35_19
109. Lian J, Zhang X, Tian H, Liang N, Wang Y, Liang C, et al. Altered microRNA Expression in Patients With Non-Obstructive Azoospermia. *Reprod Biol Endocrinol* (2009) 7(1):13. doi: 10.1186/1477-7827-7-13
110. Rahbar S, Pashaiasl M, Ezzati M, AsrBadr YA, Mohammadi-Dehcheshmeh M, Mohammadi SA, et al. MicroRNA-Based Regulatory Circuit Involved in Sperm Infertility. *Andrologia* (2020) 52(1):e13453. doi: 10.1111/and.13453
111. Wu W, Hu Z, Qin Y, Dong J, Dai J, Lu C, et al. Seminal Plasma microRNAs: Potential Biomarkers for Spermatogenesis Status. *Mol Hum Reprod* (2012) 18(10):489–97. doi: 10.1093/molehr/gas022
112. Abu-Halima M, Backes C, Leidinger P, Keller A, Lubbad AM, Hammadeh M, et al. MicroRNA Expression Profiles in Human Testicular Tissues of Infertile Men With Different Histopathologic Patterns. *Fertil Steril* (2014) 101(1):78–86. doi: 10.1016/j.fertnstert.2013.09.009
113. Schlegel PN. Testicular Sperm Extraction: Microdissection Improves Sperm Yield With Minimal Tissue Excision. *Hum Reprod* (1999) 14(1):131–5. doi: 10.1093/humrep/14.1.131
114. Zhang H-T, Zhang Z, Hong K, Tang W-H, Liu D-F, Mao J-M, et al. Altered microRNA Profiles of Testicular Biopsies From Patients With Nonobstructive Azoospermia. *Asian J Androl* (2020) 22(1):100–5. doi: 10.4103/aja.aja_35_19
115. Fang N, Cao C, Wen Y, Wang X, Yuan S, Huang X. MicroRNA Profile Comparison of Testicular Tissues Derived From Successful and Unsuccessful Microdissection Testicular Sperm Extraction Retrieval in non-Obstructive Azoospermia Patients. *Reprod Fertil Dev* (2019) 31(4):671–82. doi: 10.1071/RD17423
116. Bashi S, Khan MA, Guirjis A, Joharjy IA, Abid MA. Immotile-Cilia Syndrome With Azoospermia: A Case Report and Review of the Literature. *Br J Dis Chest* (1988) 82(2):194–6. doi: 10.1016/0007-0971(88)90043-5
117. Dixit R, Dixit K, Jindal S, Shah KV. An Unusual Presentation of Immotile-Cilia Syndrome With Azoospermia: Case Report and Literature Review. *Lung India* (2009) 26(4):142–5. doi: 10.4103/0970-2113.56352
118. Esteves SC. Are Specialized Sperm Function Tests Clinically Useful in Planning Assisted Reproductive Technology? *Int Braz J Urol* (2020) 46 (1):116–23. doi: 10.1590/s1677-5538.ibju.2020.01.03
119. Loukas I, Skamnelou M, Tsaridou S, Bournaka S, Grigoriadis S, Taraviras S, et al. Fine-Tuning Multiciliated Cell Differentiation at the Post-Transcriptional Level: Contribution of miR-34/449 Family Members. *Biol Rev* (2021). doi: 10.1111/brv.12755
120. Lizé M, Pilarski S, Dobbelstein M. E2F1-Inducible microRNA 449a/B Suppresses Cell Proliferation and Promotes Apoptosis. *Cell Death Differ* (2010) 17(3):452–8. doi: 10.1038/cdd.2009.188

121. Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, et al. P53-Mediated Activation of Mirna34 Candidate Tumor-Suppressor Genes. *Curr Biol* (2007) 17(15):1298–307. doi: 10.1016/j.cub.2007.06.068
122. Lalioti M-E, Arbi M, Loukas I, Kaplani K, Kalogeropoulou A, Lokka G, et al. GemC1 Governs Multiciliogenesis Through Direct Interaction With and Transcriptional Regulation of P73. *J Cell Sci* (2019) 132(11):jcs228684 doi: 10.1242/jcs.228684
123. Marshall CB, Mays DJ, Beeler JS, Rosenbluth JM, Boyd KL, Santos Guasch GL, et al. P73 Is Required for Multiciliogenesis and Regulates the Foxj1-Associated Gene Networ. *Cell Rep* (2016) 14(10):2289–300. doi: 10.1016/j.celrep.2016.02.035
124. Wildung M, Esser TU, Grausam KB, Wiedwald C, Volceanov-Hahn L, Riedel D, et al. Transcription Factor TAp73 and microRNA-449 Complement Each Other to Support Multiciliogenesis. *Cell Death Differ* (2019) 26(12):2740–57. doi: 10.1038/s41418-019-0332-7
125. Kyrousi C, Arbi M, Pilz G-A, Pefani D-E, Lalioti M-E, Ninkovic J, et al. Mcidas and GemC1 are Key Regulators for the Generation of Multiciliated Ependymal Cells in the Adult Neurogenic Niche. *Development* (2015) 142 (21):3661–74. doi: 10.1242/dev.126342
126. Ortiz-Álvarez G, Daclin M, Shihavuddin A, Lansade P, Fortoul A, Faucourt M, et al. Adult Neural Stem Cells and Multiciliated Ependymal Cells Share a Common Lineage Regulated by the Geminin Family Member. *Neuron* (2019) 102(1):159–72.e7. doi: 10.1016/j.neuron.2019.01.051
127. Gu X, Li H, Chen X, Zhang X, Mei F, Jia M, et al. PEX10, SIRPA-SIRPG, and SOX5 Gene Polymorphisms are Strongly Associated With Nonobstructive Azoospermia Susceptibility. *J Assist Reprod Genet* (2019) 36(4):759–68. doi: 10.1007/s10815-019-01417-w
128. Cao H-M, Wan Z, Gao Y, Zhang J-L, Zhang Y, Xiao H-P, et al. Psychological Burden Prediction Based on Demographic Variables Among Infertile Men With Sexual Dysfunction. *Asian J Androl* (2019) 21(2):156–62. doi: 10.4103/aja.aja_86_18
129. ESHRE Capri Workshop Group. Economic Aspects of Infertility Care: A Challenge for Researchers and Clinicians. *Hum Reprod* (2015) 30(10):2243–8. doi: 10.1093/humrep/dev163
130. Patel M. The Socioeconomic Impact of Infertility on Women in Developing Countries. *Facts Views Vis Obgyn* (2016) 8(1):59–61.
131. O'Neill CL, Chow S, Rosenwaks Z, Palermo GD. Development of ICSI. *Reproduction* (2018) 156(1):F51–8. doi: 10.1530/REP-18-0011
132. McLernon DJ, Steyerberg EW, Te Velde ER, Lee AJ, Bhattacharya S. Predicting the Chances of a Live Birth After One or More Complete Cycles of *In Vitro* Fertilisation: Population Based Study of Linked Cycle Data From 113 873 Women. *BMJ* (2016) 355:i5735. doi: 10.1136/bmj.i5735
133. Colaco S, Sakkas D. Paternal Factors Contributing to Embryo Quality. *J Assist Reprod Genet* (2018) 35(11):1953–68. doi: 10.1007/s10815-018-1304-4
134. Oseguera-López I, Ruiz-Díaz S, Ramos-Ibeas P, Pérez-Cerezales S. Novel Techniques of Sperm Selection for Improving IVF and ICSI Outcomes. *Front Cell Dev Biol* (2019) 7:298. doi: 10.3389/fcell.2019.00298
135. Craig JR, Jenkins TG, Carrell DT, Hotaling JM. Obesity, Male Infertility, and the Sperm Epigenome. *Fertil Steril* (2017) 107(4):848–59. doi: 10.1016/j.fertnstert.2017.02.115
136. Chapuis A, Gala A, Ferrières-Hoa A, Mullet T, Bringer-Deutsch S, Vintejoux E, et al. Sperm Quality and Paternal Age: Effect on Blastocyst Formation and Pregnancy Rates. *Basic Clin Androl* (2017) 27:2. doi: 10.1186/s12610-016-0045-4
137. Ménézo YJR, Sakkas D, Janny L. Co-Culture of the Early Human Embryo: Factors Affecting Human Blastocyst Formation *In Vitro*: CO-CULTURE OF THE EARLY HUMAN EMBRYO. *Microsc Res Tech* (1995) 32(1):50–6. doi: 10.1002/jemt.1070320105

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# Decreased piRNAs in Infertile Semen Are Related to Downregulation of Sperm MitoPLD Expression

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Currently, the molecular mechanisms underlining male infertility are still poorly understood. Our previous study has demonstrated that PIWI-interacting RNAs (piRNAs) are downregulated in seminal plasma of infertile patients and can serve as molecular biomarkers for male infertility. However, the source and mechanism for the dysregulation of piRNAs remain obscure. In this study, we found that exosomes are present in high concentrations in human seminal plasma and confirmed that piRNAs are predominantly present in the exosomal fraction of seminal plasma. Moreover, we showed that piRNAs were significantly decreased in exosomes of asthenozoospermia patients compared with normozoospermic men. By systematically screening piRNA profiles in sperms of normozoospermic men and asthenozoospermia patients, we found that piRNAs were parallelly reduced during infertility. At last, we investigated the expression of some proteins that are essential for piRNAs biogenesis in sperms and therefore identified a tight correlation between the levels of spermatozoa piRNA and MitoPLD protein, suggesting that the loss-of-function of MitoPLD could cause a severe defect of piRNA accumulation in sperms. In summary, this study identified a parallel reduction of piRNAs and MitoPLD protein in sperms of asthenozoospermia patients, which may provide pathophysiological clues about sperm motility.

**Keywords: infertility, piRNAs, exosome, sperm, MitoPLD**

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## INTRODUCTION

Infertility is a prevalent health problem and affects nearly 15% of couples all over the world (1, 2). Male factors contribute to about 50% of childless couples. As a complex disease, male infertility is caused by a series of multifactorial genetic and environment factors, but the underlying molecular mechanisms have not yet been elucidated (3–5).

piRNAs are approximately 26-31 nucleotides in length and expressed mainly in pachytene spermatocytes and round spermatids in the testis of mammals (6–9). They are named PIWI-interacting RNAs because of their close relationship with the PIWI subfamily members. Two pathways for piRNA biogenesis have been identified: namely, primary and secondary pathways

(10–12). The primary pathway is thought to produce piRNAs (primary piRNAs) from long single-strand piRNA precursors, which are derived from genomic regions called piRNA clusters. The pathway about primary piRNAs generation is not well studied, but a mitochondrial protein, MitoPLD (also known as Zucchini or PLD6), a member of the nuclease/phospholipase D family, has been proposed to function as an endonuclease to generate the 5' ends of piRNAs (13–15). In the secondary pathway, piRNAs (secondary piRNAs) are produced by the ping-pong amplification cycles from the 5' portions of RNA fragments cleaved by PIWI-piRNA complexes. The piRNAs from primary and secondary pathway guide each other's production in the ping-pong cycle to accelerate production of piRNAs (12, 16, 17). Currently, the main proposed function of piRNAs is to protect the germline and gonadal somatic cells and to avoid transposable elements related harmful expression and thus maintains the genomic integrity of germ cells. Increasing evidence has also shown that piRNAs may be involved in post-transcriptional regulation of protein-coding genes (18–20). Because of the diverse and pivotal roles of piRNAs in the male reproductive system, the dysregulation and dysfunction of piRNA often cause male infertility.

Since piRNAs are specifically expressed in germ-cell and essential for spermatogenesis, it is not surprising to find that the levels of spermatozoa piRNAs are directly correlated to semen quality and male fertility. We have previously shown that the concentration of seminal plasma piRNAs were significantly decreased in infertile patients compared with the normozoospermic men. Several specific piRNAs in seminal plasma were even identified as molecular biomarkers for male infertility (3). However, the source of seminal plasma piRNAs remains elusive, and the cause of massive reduction of piRNAs in seminal plasma of infertile patients has not been definitively identified. Extracellular RNA profile in human semen was comprehensively characterized in a recent study and a great number of small RNAs were found within seminal exosomes (21). Thus, it is rational to speculate that piRNAs in seminal plasma are mostly derived from the secretion of exosomes from the male germ cells. In this study, we validated that a majority of piRNAs were present within the exosomal fraction of seminal plasma. Moreover, we showed the piRNAs' types and levels were significantly decreased in the sperms of asthenozoospermia patients compared with those in normozoospermic men. Finally, we identified a tight correlation between the levels of spermatozoa piRNA and MitoPLD protein, suggesting that the loss-of-function of MitoPLD could cause severe defects in the piRNA accumulation in sperms.

# MATERIALS AND METHODS

## Semen Samples

Semen samples were provided from Nanjing Drum Tower Hospital, and all protocols in this study were approved by the Medical Ethics Committee of Hangzhou medical college and Nanjing Drum Tower Hospital. Informed consents were signed by both normozoospermic men volunteers and asthenozoospermia patients before sample collection for the further study. The study recruited 42 asthenozoospermia patients with infertility more than 2 years and 41 normozoospermic men volunteers who conceived naturally within 1-2 years. The demographic characteristics of all tested persons were listed in **Table 1**. The volunteers in this study did not receive any treatment, semen samples were analyzed in the Reproductive Laboratory of Nanjing Drum Tower Hospital.

**TABLE 1** | Demographic and clinical features of the Asthenospermia patients and normozoospermic men[a].

| Variables (sequencing set) | Normozoospermic men (n=10) | Asthenospermia patients (n=10) | *P*-Value[b] |
|---|---|---|---|
| Average age,years | 28.4 (2.41) | 29.9 (3.57) | 0.2789 |
| Sexual abstinence time,days | 4.1 (0.74) | 3.9 (0.88) | 0.5554 |
| Semen parameters | | | |
| pH | 7.53 (0.28) | 7.44 (0.16) | 0.2869 |
| Total volume,mL | 4.62 (6.71) | 4.22 (0.77) | 0.2003 |
| Sperm parameters | | | |
| Sperm density,×$10^6$/mL | 47.84 (14.23) | 43.37 (10.34) | 0.4916 |
| Sperm viability,% | 70.39 (4.94) | 12.75 (7.03) | $3.18\times10^{-9}$ |
| Progressive motility (PR)[e] | 51.9 (7.09) | 5.99 (4.46) | $6.36\times10^{-8}$ |
| **Variables (validation set)** | **(n=31)** | **(n=32)** | |
| Average age,years | 31.29 (5.72) | 29.44 (4.84) | 0.1711 |
| Sexual abstinence time,days | 4.03 (0.66) | 3.88 (0.66) | 0.3472 |
| Semen parameters | | | |
| pH | 7.34 (0.19) | 7.44 (0.19) | 0.0655 |
| Total volume,mL | 4.68 (1.02) | 4.16 (1.70) | 0.1038 |
| Sperm parameters | | | |
| Sperm density,×$10^6$/mL | 49.13 (8.86) | 44.11 (8.85) | 0.0916 |
| Sperm viability,% | 67.98 (8.3) | 15.46 (6.94) | $7.14\times10^{-35}$ |
| Progressive motility (PR)[e] | 50.2 (8.21) | 10.88 (5.34) | $3.34\times10^{-28}$ |

[a]*Data are presented as mean (SD).*
[b]*Normozoospermic men vs Asthenozoospermia patients.*
[e]*a,rapid progressive motility.*

## Sample Preparation

Semen samples obtained through by masturbation after 3-5 days of abstinence and then were transferred into a 15 mL centrifuge tube (Corning) and liquefied for 30 min at 37°C. Sperm concentration and viability were assessed by Sperm analysis system (SAS medical). Routine semen analysis was based on the World Health Organization (WHO) criteria (22). The sperms isolated from semen samples by centrifuging at 3000 rpm for 5 min at room temperature were resuspended in PBS and stored at -80°C for further protein analysis.

## Isolation of Exosomes From Seminal Plasma

Differential centrifugation was employed to isolate exosomes from seminal plasma that was obtained by centrifuging semen samples (850 g, 5min at room temperature). In brief, cell debris was removed by spinning at low speed (3,000 g, for 30 min) at the first step. Then, the shedding vesicles and the other larger vesicles were removed by centrifugation at 10,000 g for 30 min. Finally, the exosome pellets were collected by centrifugation at 110,000 g for 70 min, and then re-suspended in PBS buffer, and the supernatant was kept as exosome-free seminal plasma. All procedures steps carried out at 4°C.

## Transmission Electron Microscopy Assay (TEM)

The morphology of exosome was imaged by TEM. Briefly, the exosome pellet was fixed in 2.5% glutaraldehyde overnight at 4°C, and then was rinsed with PBS and post-fixed with 1% osmium tetroxide for 1 h at room temperature. Then the exosome pellet was embedded in 10% gelatin and fixed with glutaraldehyde at 4°C and carved into blocks. Subsequently, the exosome pellet was dehydrated by incubation for 10 min with graded alcohol series (30%, 50%, 70%, 90%, 95%, and 100%, 3 times), then the samples were incubated with propylene oxide, and infiltrated with increasing concentrations of Quetol-812 epoxy resin mixed with propylene oxide (25%, 50%, 75%, and 100%). At last, exosome samples embedded in pure, fresh Quetol-812 epoxy resin and polymerized increasing temperature for 12-24 h (35°C for 12 h, 45°C for 12 h, and 60°C for 24 h), were cut into ultrathin sections using Leica UC6 ultramicrotome, and stained with uranyl acetate (10 min) and lead citrate (5 min) at room temperature. The samples were imaged with TEM (FEI Tecnai T20) at a voltage of 120 kV.

## Illumina High-Throughput Sequencing

Total RNA from pooled sperm samples of normozoospermic men and asthenozoospermia patients (each pooled from 10 individuals) was prepared using TRIzol Reagent (Takara, Dalian, China). About 1~2 μg of quantified total RNA was performed high-throughput sequencing on Illumina NextSeq500 system according to the manufacturer's instructions. The sequences corresponding to known piRNAs after data analysis were determined by perfect sequence matching to the piRNA database piRNABank (http://pirnabank.ibab.ac.in/). All data have been uploaded to the GEO database (Accession number: GSE172486).

## RNA Isolation and qRT-PCR Assays

TRIzol Reagent (TaKaRa, Dalian, China) was used to isolate total RNA from sperm, exosome and exosome-free seminal plasma. Briefly, sperms derived from 1 ml of semen, exosome from 100 μl of seminal plasma and 100 μl of exosome-free seminal plasma were mixed with 1 ml TRIzol, after being vortexed vigorously for 10 s, the mixture was incubated with 200 μl of chloroform for 10 min on ice, The RNA containing phase was then transferred to a fresh RNase-free tube after centrifugation at 16,000 g for 20 min at 4°C, Then the supernatant was incubated with equal volume of isopropanol at -20°C for 1.5 h to precipitate RNA. The isolated RNA was collected by centrifugation (16,000 g, 4°C, 20 min), was then washed rinsed once with 75% ethanol and dried for 20 min at room temperature. Finally, the RNA was dissolved in 20 μl RNase-free $H_2O$ and stored at -80°C for further analysis.

Total RNA (2 μl) was reverse transcribed to cDNA using AMV reverse transcriptase based on the manufacturer's instruction. Then, 1 μl of cDNA was used for subsequent qRT-PCR analysis on a Roche LightCycler 480 PCR system. Taqman piRNA probes (GenePharma, Shanghai, China) was used to measure the piRNA level in this study. All reactions were performed in triplicate. RNU6-6P was used as an internal control.

## Protein Extraction and Western Blotting

Sperms were lysed in RIPA lysis buffer with freshly added PMSF for 30 min on ice, and sonication (Sonics&materials Inc.VCX 130 PB) was used to facilitate sperm cell disruption, Insoluble debris were removed by centrifugation at 16,000 g, 4°C for 10 min. The protein concentration was quantified using a BCA proteinassay kit (Thermo Scientific, Rockford, IL, USA). The expression of indicated proteins were detected using their specific antibodies, including anti-MitoPLD antibody (ab170183) and anti-PIWIL1 (ab12337) purchased from Abcam (Cambridge, MA, USA), β-actin antibody (sc-69879) was served as the reference, which purchased from Santa Cruz (Dallas, TX, USA). Western blot image acquisition was performed using the Bio-Rad ChemiDoc imaging system, and Image J was used for densitometric analysis.

## Statistical Analysis

All images are representative of at least three different experiments. The data shown are the mean ± SE of at least three independent experiments. Student's t-test was used for statistical analysis and $p$ value < 0.05 (indicated by *), < 0.01 (indicated by **) or < 0.001 (indicated by ***) were considered statistically significant.

# RESULTS

## Characterization of Exosomes From Seminal Plasma

We purified exosomes from seminal plasma of normozoospermic men and asthenozoospermia patients by ultracentrifugation and examined the particle size and morphology by transmission

electron microscopy. Under electron microscopy, the exosomes isolated from both normozoospermic men and asthenozoospermia patients appeared as lipid bilayer-bound single and small clumps of particles of 30-150 nm in diameter, consistent with the expected size range of exosomes (**Figure 1A**). The exosomes isolated from normozoospermic men and asthenozoospermia patients were further identified by the presence of equal amounts of universal exosomal markers (CD63 and TSG101) based on immunoblotting (**Figure 1B**). Moreover, the particle size and concentration of exosomes were determined by nanoparticle tracking analysis. Exosomes from normozoospermic men had an average diameter of 123 nm, most exosomes (> 85%) range from 80 to 150 nm, and the concentration of exosomes was $2.89\times10^9$ particles/mL (**Figure 1C**). For exosomes from asthenozoospermia patients, the mean diameter was 121 nm, and the concentration was $3.0\times10^9$ particles/mL (**Figure 1D**). The results confirmed that exosomes were present in high concentrations in human seminal plasma, but the size range and concentration of exosomes were not differentially present between normozoospermic men and asthenozoospermia patients.

## Differentially Expressed piRNAs in Seminal Plasma Exosomes

A previous study has identified a great amount of small RNA in the exosomal fraction of seminal plasma (21). In this study, we determined to compared the ratio of piRNA levels in exosomes to that in exosome-free seminal plasma. We selected *piR-1207*, *piR-2107*, *piR-5937* and *piR-5939* as the representative piRNAs and measured their levels in exosomes and exosome-free seminal plasma by quantitative RT-PCR assay because our previous study have identified these piRNAs as the significantly downregulated piRNAs in seminal plasma of asthenozoospermia patients compared with normozoospermic men (3). *piR-1207*, *piR-2107*, *piR-5937* and *piR-5939* were mainly stored in exosomes (**Figure 2A**), suggesting that the majority of piRNAs were present in exosomal fraction of seminal plasma. Meanwhile, we also compared the levels of these piRNAs in exosomes between

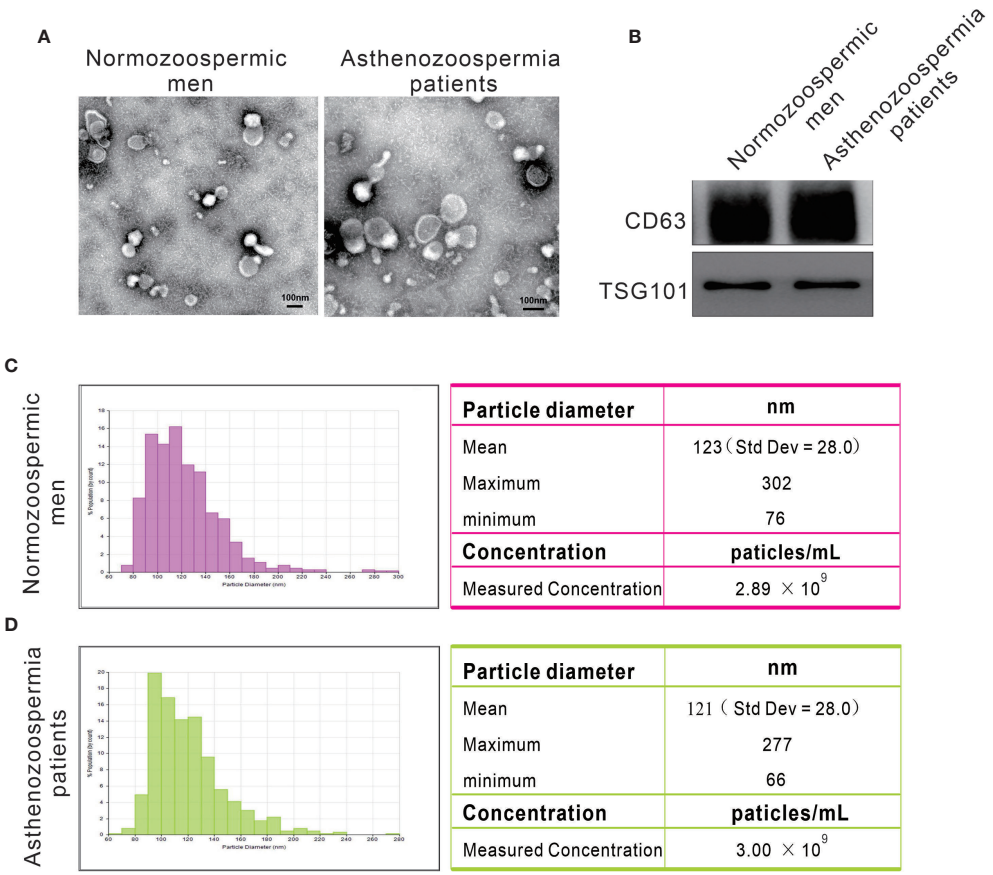


**FIGURE 1** | Particles isolated from seminal plasma exhibit the characteristic features of exosomes. **(A)** Representative transmission electron micrographs of exosomes. **(B)** Western blots on exosomes using antibodies against the common exosomal markers CD63 and TSG101. **(C, D)** Representative particle size distribution profiles from seminal plasma of normozoospermic men and asthenozoospermia patients.

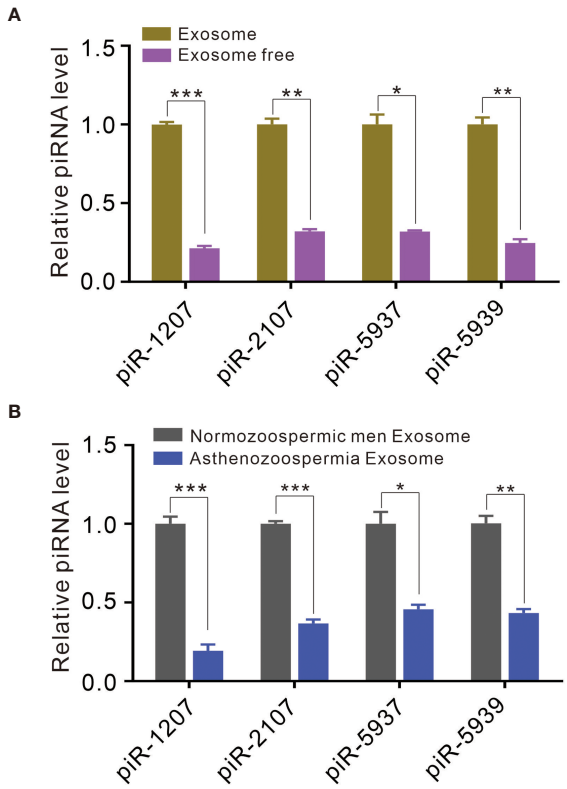


**FIGURE 2** | Exosomes contain piRNAs and differential presence of piRNAs between asthenozoospermia patients and normozoospermic men. **(A)** Quantitative RT-PCR analysis of *piR-1207, piR-2107, piR-5937 and piR-5939 in* exosomes and exosome-free seminal plasma. **(B)** Quantitative RT-PCR analysis of *piR-1207*, *piR-2107*, *piR-5937* and *piR-5939* in exosomes from asthenozoospermia patients and normozoospermic men. *p < 0.05; **p < 0.01; ***p < 0.001

normozoospermic men and asthenozoospermia patients. The result proved that *piR-1207*, *piR-2107*, *piR-5937* and *piR-5939* were significantly reduced in exosomes from asthenozoospermia patients compare with the normozoospermic men (**Figure 2B**). These results indicated that the reduction of piRNAs in exosomes contributed to the reduction of piRNAs in seminal plasma of asthenozoospermia patients.

## Profiling Sperm piRNAs by High-Throughput Sequencing

Although piRNAs were significantly reduced in exosomes of asthenozoospermia patients, the source and mechanism for the dysregulation of piRNAs remain obscure. piRNAs are expressed abundantly in pachytene spermatocytes and round spermatids (23). Given that exosomes are likely secreted from multiple cellular sources in the male genital tract, we speculate that piRNAs may be actively secreted from spermatocytes during the spermatogenesis process. However, it is very difficult to get spermatocytes from asthenozoospermia patients. Alternatively, we systematically characterized the piRNA profiles in mature sperms. Total RNA was extracted from pooled sperm samples of normozoospermic men and asthenozoospermia patients (each pooled from 10 individuals), and was qualified by agarose gel electrophoresis and quantified using Nanodrop. Subsequently, commercial kit was used for piRNA-seq library preparation, which includes 3'-adapter and 5'-adapter ligation, cDNA synthesis and library PCR amplification. The prepared piRNA-seq libraries were finally quantified using Agilent BioAnalyzer 2100, then sequenced by using Illumina NextSeq 500. After removing 5'and 3' adaptor sequences and aligned to the piRBase, a total of 8,245,354 and 4,220,714 reads of piRNAs were obtained in the sperm of normozoospermic men group and asthenozoospermia patient group, respectively (**Figure 3A**). Moreover, the types of piRNAs were decreased from sperm of normozoospermic men group (17657) to asthenozoospermia patient group (15742) (**Figure 3B**). Analysis of the length distribution revealed that sperm of normozoospermic men group and asthenozoospermia patient group contained a number of small RNAs with size that was consistent with common the size of piRNAs (25–32 nucleotides) (**Figures 3C, D**). Next, we narrowed the list of piRNAs. Firstly, we narrowed down and selected 33 known highly expressed piRNAs under the condition of sequencing reads are larger than 20000 in the group of normozoospermic men, Heatmap analysis was performed based on these criteria, the result showed that 17 piRNAs have at least 2-fold higher reads in normozoospermic men group than in the asthenozoospermia patient group (**Figure 3E**, **Supplementary Table 1**). Subsequently, we compared asthenozoospermia patient group with the normozoospermic men group by scatter plots with the following parameters: sequencing reads are larger than 1000 in the group of normozoospermic men. The result was shown in **Figure 3F** and **Supplementary Table 2**, indicating a remarkable different expression level of piRNAs between asthenozoospermia patient group and normozoospermic men group. Generally, the comparison among the sperm piRNA profiles revealed a considerable reduction of sperm piRNAs in asthenozoospermia patients relative to normozoospermic men (from 8,245,354 to 4,220,714 reads and from 17,657 to 15,742 types of piRNAs).

## Individual Quantification of Sperm piRNAs by Quantitative RT-PCR

Next, a TaqMan probe-based quantitative RT-PCR assay was performed to measure the presence of piRNAs in individual samples. The representative *piR-1207* and *piR-2107* were assessed in sperm samples of 20 normozoospermic men and 20 asthenozoospermia patients. Consistent with the results from deep sequencing, *piR-1207* and *piR-2107* levels were markedly downregulated in sperm of asthenozoospermia patients (**Figures 4A, B**). We further performed receiver-operating characteristic (ROC) curve analysis to assess the usefulness of *piR-1207* and *piR-2107* in discriminating asthenozoospermia patients from normozoospermic men. ROC curve analysis for *piR-1207* revealed an AUC of 0.845 and an optimal cut-off value of 1717.93 with a corresponding sensitivity of 70.0% and specificity of 95.2% (**Figure 4C**), and for *piR-2107* revealed an

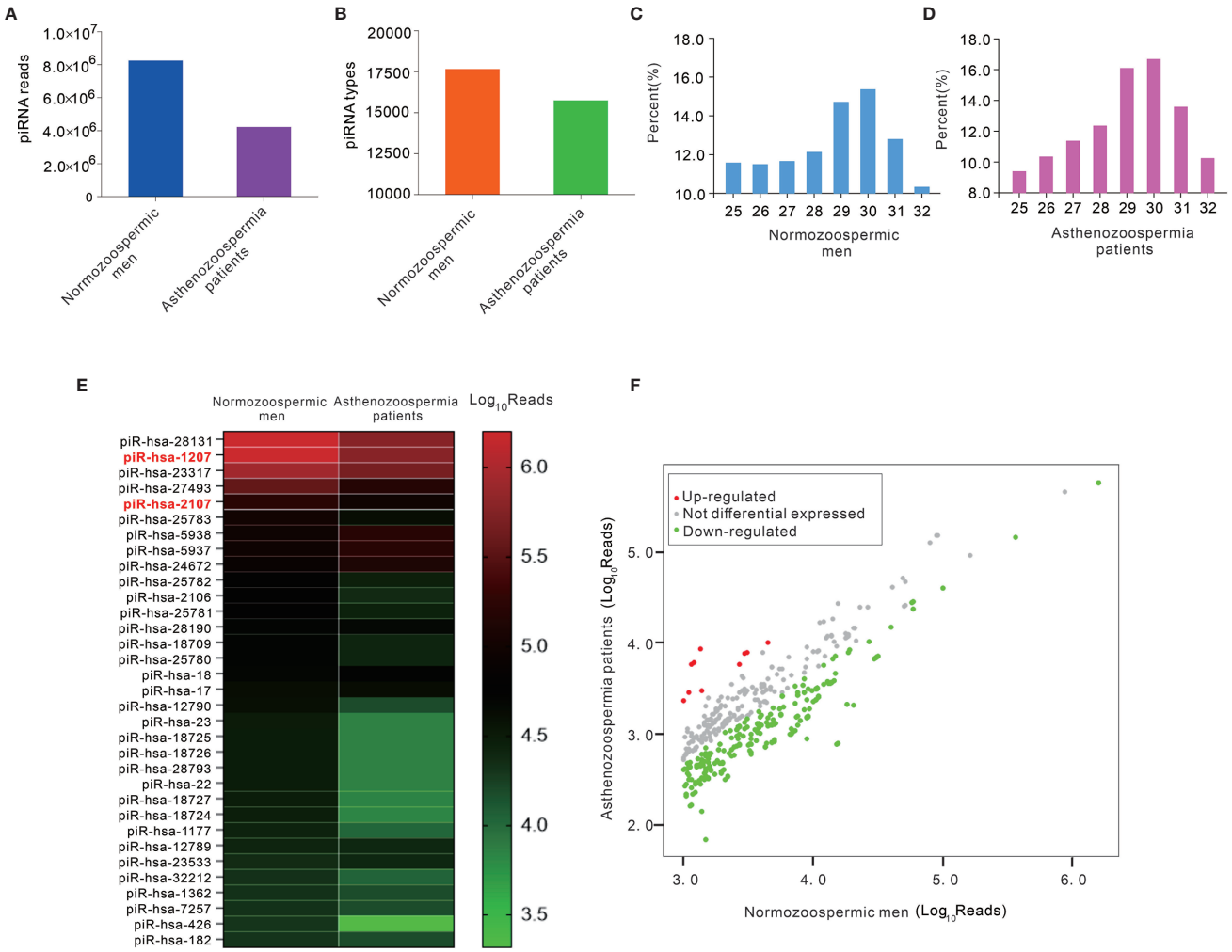


**FIGURE 3** | The analysis of high-throughput sequencing of piRNAs in sperms from asthenozoospermia patients and normozoospermic men. **(A, B)** Total sequencing reads **(A)** and types **(B)** of piRNAs were decreased in sperms of asthenozoospermia patients compared with those in normozoospermic men. **(C, D)** The distribution of small RNAs of various lengths (25-32 bp) measured by high-throughput sequencing in sperms. **(E, F)** The heatmap **(E)** and scatter plots **(F)** analysis of sequencing reads of piRNAs between asthenozoospermia patients and normozoospermic men.

AUC of 0.93 and an optimal cut-off value of 7476.52 with a corresponding sensitivity of 85% and specificity of 90% (**Figure 4D**). These results showed that *piR-1207* and *piR-2107* could serve as valuable indicators for distinguishing asthenozoospermia patients from normozoospermic men.

## Expression Level of MitoPLD Is Decreased in Asthenozoospermia Patient Sperms

Since the biogenesis and function of piRNA is tightly associated with that of the PIWI protein subfamily, and MitoPLD is essential for the creation of the 5' ends of primary piRNAs, the marked reduction of mature piRNAs in sperm of asthenozoospermia patients suggested the dysfunction or loss of expression of these essential enzymes in sperms. Therefore, we measured the expression level of MitoPLD and PIWIL1 in the sperms of normozoospermic men and asthenozoospermia patients. The results revealed that the expression of MitoPLD protein was significantly reduced in asthenozoospermia patient sperms (**Figures 5A–C**). In contrast, the alteration of PIWIL1 in asthenozoospermia patient sperms was irregular, PIWIL1 expression level were increased in the sperms of some asthenozoospermia patients (6 out of 11 patients), but were significantly decreased in 5 patients (**Figures 5D–F**).

# DISCUSSION

Although piRNAs have been found to have obvious expression in germ cells across various animal species, specifically in male germ cells of mammals, researches related to their detailed function and mechanism remain obscure. Up to now, what is becoming clear is that piRNAs are participate in posttranscriptional regulation of protein-coding genes as well

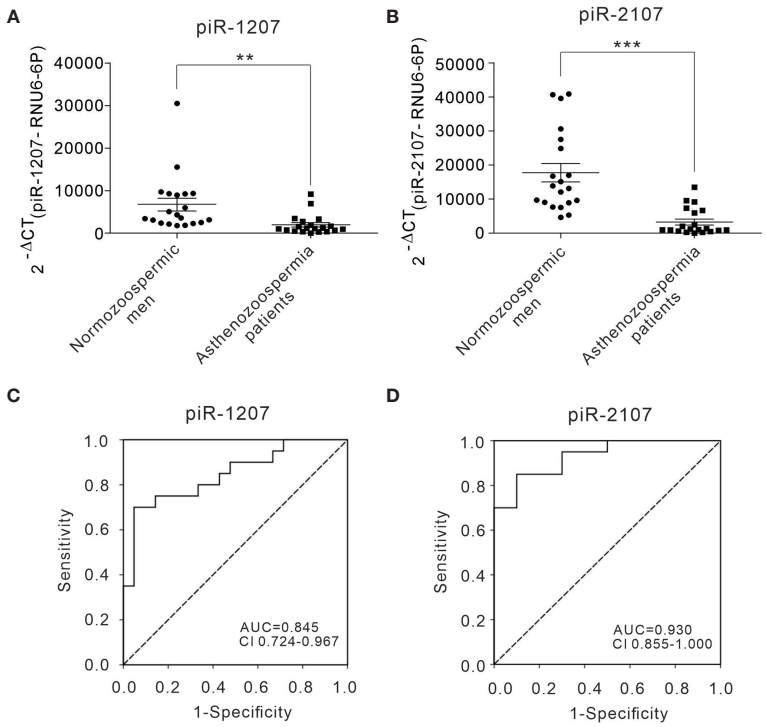


**FIGURE 4** | Differential presence of piRNAs from sperms between asthenozoospermia patients and normozoospermic men and ROC curve analysis. **(A, B)** The relative level of *piR-1207* and *piR-2107* were determined by quantitative RT-PCR in the sperms of asthenozoospermia patients (n=20) compared with those in the sperms of normozoospermic men (n=20). **(C, D)** ROC curve for the *piR-1207* **(C)** and *piR-2107* **(D)** to separate asthenozoospermia patients from normozoospermic men. $^{**}p < 0.01$; $^{***}p < 0.001$

as in the repression of retrotransposons and are indispensable for male fertility, and a recent study showed that PIWI/piRNA pathway genes repression by hypermethylation is probably contributed to unsuccessful spermatogenesis (24). These results suggest that the critical role and function of piRNAs during spermatogenesis process has been well documented, whether piRNAs in sperm may regulate sperm motility remain largely unknown. Increasing studies have shown that miRNAs expressed in mature sperm could regulate sperm motility (25, 26). In contrast to miRNAs, piRNAs were just discovered in 2006, and the biogenesis and functions remain largely unexplored. However, piRNAs are known to be much more abundant and germ cell-specific than miRNAs, suggesting that piRNAs may potentially play a more fundamental role in regulating the sperm motility. In this study, we found that piRNAs were enriched in sperms and observed that a massive amount of piRNAs were lost in sperms of asthenozoospermia patients. Furthermore, ROC curve analysis revealed a strong relationship between the low presence of sperm piRNAs and asthenozoospermia patients, suggesting that sperm piRNAs may be essential for sperm motility. The biological roles of these piRNAs in sperm motility with male infertility, which may provide some pathophysiological clues for the molecular mechanisms of this disease, call for further investigations.

Based on their biogenesis models, they are typically classified into two groups: the primary processing pathway and amplification loop (secondary processing pathway). MitoPLD, a mitochondria-anchored endonuclease belonging to the member of the phospholipase D superfamily proteins, is conserved among diverse species and is implicated in the primary processing pathway of piRNAs. MitoPLD is previously known to be a phospholipase that hydrolyzes cardiolipin to generate phosphatidic acid and is involved in the regulation of mitochondrial morphology (27, 28). MitoPLD is also implicated in the formation of nuage (also known as inter-mitochondrial cement or chromatoid body), which works as a pivotal cytoplasmic structure comprising most piRNA-related proteins (15, 27). Surprisingly, MitoPLD was found to be essential to piRNA biogenesis. MitoPLD acts as an endonuclease and conducts the first cleavage of piRNAs precursors to generate the 5' ends of secondary piRNAs, and then the cleaved piRNAs are transferred to PIWI proteins to trigger the secondary piRNA processing pathway (29, 30). Knockout of MitoPLD abolishes the majority of piRNA in male germ cells and resulted in transposon activation and arrest of spermatogenesis, characteristic phenotypes of piRNA pathway mutants (12).In this study, we measured the expression levels of MitoPLD in sperms of normozoospermic men and asthenozoospermia patients and found that MitoPLD protein

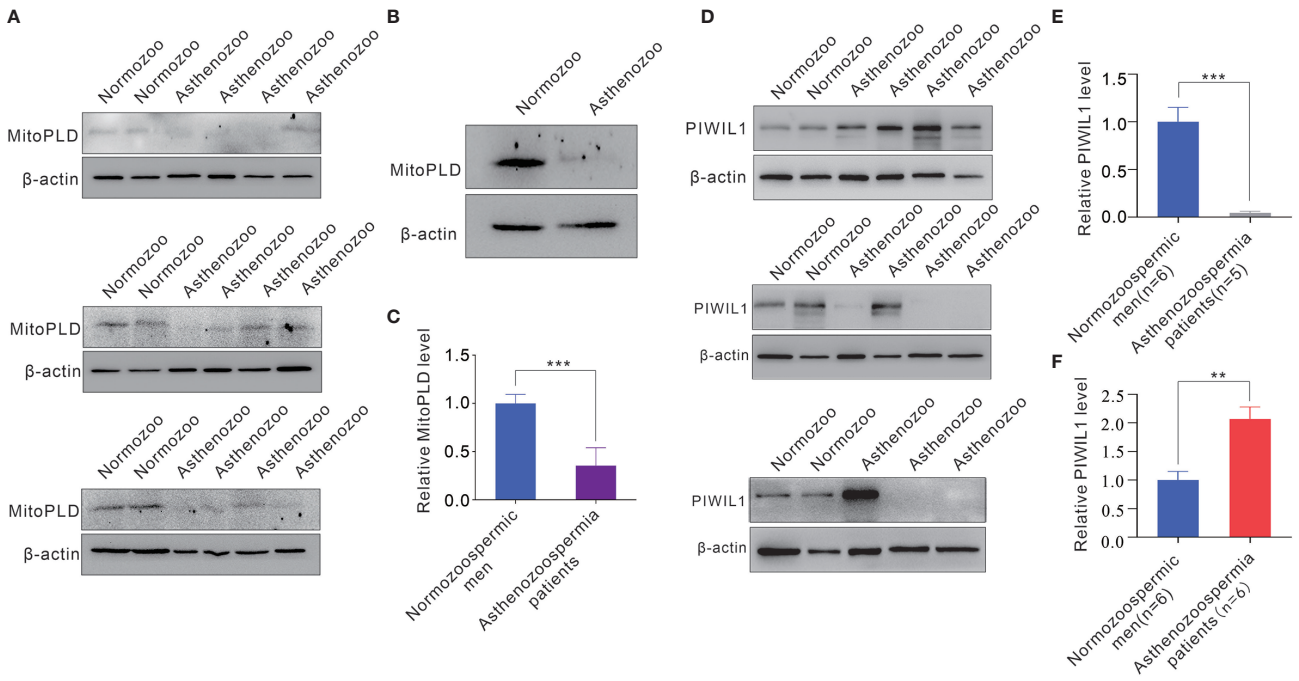


**FIGURE 5** | The expression level of MitoPLD and PIWIL1 protein from sperms between asthenozoospermia patients and normozoospermic men. **(A)** Western blotting analysis of the expression levels of MitoPLD in individual sperm samples between asthenozoospermia patients and normozoospermic men. **(B)** Western blotting analysis of the expression levels of MitoPLD in pooled sperm samples between asthenozoospermia patients and normozoospermic men (each pooled from 10 individuals). **(C)** Quantitative analysis of panel **(A, B)**. **(D)** Western blotting analysis of the expression levels of PIWIL1 in individual sperm samples between asthenozoospermia patients and normozoospermic men. **(E, F)** Quantitative analysis of expression levels of PIWIL1 were decreased **(E)** or increased **(F)** in sperms of asthenozoospermia patients. $^{**}p < 0.01$; $^{***}p < 0.001$.

was significantly downregulated in sperms of asthenozoospermia patients. Massive reduction of piRNAs in the sperms of asthenozoospermia patients may be caused, at least in part, by the parallel reduction of MitoPLD protein in sperms. Further studies are required to investigate whether dysregulation or dysfunction of MitoPLD is involved in the pathology of infertility.

Exosomes are nano-sized vesicles with a diameter ranging between 30 and 150 nm. Released by multiple cell types, exosomes are present in a variety of body fluids and can transfer bioactive molecules (e.g., proteins, lipids and nucleic acids) between neighboring and distant cells (31). Scientists have reached a consensus that exosomes play a key role in intercellular communication *via* the horizontal transfer of miRNAs. However, the presence of piRNAs in exosomes has only currently been noted. A recent study has shown that a large number of small RNAs (including miRNAs and piRNAs) were contained and protected within seminal exosomes (21). In this study, we also identified a majority of piRNAs in the seminal exosomes. In addition, we found that piRNAs in seminal exosomes of asthenozoospermia patients were significantly decreased compared with normozoospermic men. However, it has not been established yet if the piRNA in seminal exosomes can have regulatory functions in the recipient cells and act as a new role in the intercellular communication system. Future studies are needed to characterize the functions of piRNAs in seminal exosomes and to investigate the role of communicators of exosomal piRNAs in the microenvironment of genital tract.

In conclusion, we systematically characterized the piRNA profiles in sperms of normozoospermic men and asthenozoospermia patients and found that the amounts of piRNAs were significantly decreased in the sperms of asthenozoospermia patients. We also investigated the mechanism for the dysregulation of piRNAs in sperms and revealed that the parallel reduction of MitoPLD may be the cause and consequence of male infertility.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GEO repository, GSE172486.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hangzhou Medical College Ethics Committee, Nanjing Drum Tower Hospital Ethics Committee. The

patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Study conceptualization: YH and JZ. Data acquisition, analysis and interpretation: YH, YW, CY, and LS. Clinical samples and data collection: XZ. Technical or material support: LC, HC, and FG. Manuscript writing and editing: YH, CY, LS, and JZ. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.696121/full#supplementary-material

## REFERENCES


1. Abu-Halima M, Ludwig N, Hart M, Leidinger P, Backes C, Keller A, et al. Altered Micro-Ribonucleic Acid Expression Profiles of Extracellular Microvesicles in the Seminal Plasma of Patients With Oligoasthenozoospermia. *Fertil Steril* (2016) 106(5):1061–9.e3. doi: 10.1016/j.fertnstert.2016.06.030
2. Cardona Barberan A, Boel A, Vanden Meerschaut F, Stoop D, Heindryckx B. Diagnosis and Treatment of Male Infertility-Related Fertilization Failure. *J Clin Med* (2020) 9(12):3899. doi: 10.3390/jcm9123899
3. Hong Y, Wang C, Fu Z, Liang H, Zhang S, Lu M, et al. Systematic Characterization of Seminal Plasma Pirnas as Molecular Biomarkers for Male Infertility. *Sci Rep* (2016) 6:24229. doi: 10.1038/srep24229
4. Wang C, Yang C, Chen X, Yao B, Yang C, Zhu C, et al. Altered Profile of Seminal Plasma Microrrnas in the Molecular Diagnosis of Male Infertility. *Clin Chem* (2011) 57(12):1722–31. doi: 10.1373/clinchem.2011.169714
5. Okada H, Tajima A, Shichiri K, Tanaka A, Tanaka K, Inoue I. Genome-Wide Expression of Azoospermia Testes Demonstrates a Specific Profile and Implicates ART3 in Genetic Susceptibility. *PLoS Genet* (2008) 4(2):e26. doi: 10.1371/journal.pgen.0040026
6. Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, et al. Characterization of the Pirna Complex From Rat Testes. *Science* (2006) 313 (5785):363–7. doi: 10.1126/science.1130164
7. Grivna ST, Beyret E, Wang Z, Lin H. A Novel Class of Small Rnas in Mouse Spermatogenic Cells. *Genes Dev* (2006) 20(13):1709–14. doi: 10.1101/gad.1434406
8. Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P, Iovino N, et al. A Novel Class of Small Rnas Bind to MILI Protein in Mouse Testes. *Nature* (2006) 442(7099):203–7. doi: 10.1038/nature04916
9. Girard A, Sachidanandam R, Hannon GJ, Carmell MA. A Germline-Specific Class of Small Rnas Binds Mammalian Piwi Proteins. *Nature* (2006) 442 (7099):199–202. doi: 10.1038/nature04917
10. Aravin AA, Hannon GJ, Brennecke J. The Piwi-Pirna Pathway Provides an Adaptive Defense in the Transposon Arms Race. *Science* (2007) 318 (5851):761–4. doi: 10.1126/science.1146484
11. Kim VN, Han J, Siomi MC. Biogenesis of Small Rnas in Animals. *Nat Rev Mol Cell Biol* (2009) 10(2):126–39. doi: 10.1038/nrm2632
12. Watanabe T, Chuma S, Yamamoto Y, Kuramochi-Miyagawa S, Totoki Y, Toyoda A, et al. MITOPLD is a Mitochondrial Protein Essential for Nuage Formation and Pirna Biogenesis in the Mouse Germline. *Dev Cell* (2011) 20 (3):364–75. doi: 10.1016/j.devcel.2011.01.005
13. Nishimasu H, Ishizu H, Saito K, Fukuhara S, Kamatani MK, Bonnefond L, et al. Structure and Function of Zucchini Endoribonuclease in Pirna Biogenesis. *Nature* (2012) 491(7423):284–7. doi: 10.1038/nature11509
14. Voigt F, Reuter M, Kasaruho A, Schulz EC, Pillai RS, Barabas O. Crystal Structure of the Primary Pirna Biogenesis Factor Zucchini Reveals Similarity to the Bacterial PLD Endonuclease Nuc. *RNA* (2012) 18(12):2128–34. doi: 10.1261/rna.034967.112
15. Kabayama Y, Toh H, Katanaya A, Sakurai T, Chuma S, Kuramochi-Miyagawa S, et al. Roles of MIWI, MILI and PLD6 in Small RNA Regulation in Mouse Growing Oocytes. *Nucleic Acids Res* (2017) 45(9):5387–98. doi: 10.1093/nar/gkx027
16. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, et al. Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila. *Cell* (2007) 128(6):1089–103. doi: 10.1016/j.cell.2007.01.043
17. Gunawardane LS, Saito K, Nishida KM, Miyoshi K, Kawamura Y, Nagami T, et al. A Slicer-Mediated Mechanism for Repeat-Associated Sirna 5' End Formation in Drosophila. *Science* (2007) 315(5818):1587–90. doi: 10.1126/science.1140494
18. Gou LT, Dai P, Yang JH, Xue Y, Hu YP, Zhou Y, et al. Pachytene Pirnas Instruct Massive Mrna Elimination During Late Spermiogenesis. *Cell Res* (2014) 24(6):680–700. doi: 10.1038/cr.2014.41
19. Zhang P, Kang JY, Gou LT, Wang J, Xue Y, Skogerboe G, et al. MIWI and Pirna-Mediated Cleavage of Messenger Rnas in Mouse Testes. *Cell Res* (2015) 25(2):193–207. doi: 10.1038/cr.2015.4
20. Dai P, Wang X, Gou LT, Li ZT, Wen Z, Chen ZG, et al. A Translation-Activating Function of MIWI/Pirna During Mouse Spermiogenesis. *Cell* (2019) 179(7):1566–81.e16. doi: 10.1016/j.cell.2019.11.022
21. Vojtech L, Woo S, Hughes S, Levy C, Ballweber L, Sauteraud RP, et al. Exosomes in Human Semen Carry a Distinctive Repertoire of Small Non-Coding Rnas With Potential Regulatory Functions. *Nucleic Acids Res* (2014) 42(11):7290–304. doi: 10.1093/nar/gku347
22. World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed* Vol. xiv. Geneva: World Health Organization (2010). 271 p.p.
23. He Z, Kokkinaki M, Pant D, Gallicano GI, Dym M. Small RNA Molecules in the Regulation of Spermatogenesis. *Reproduction* (2009) 137(6):901–11. doi: 10.1530/REP-08-0494
24. Zhang GW, Wang L, Chen H, Guan J, Wu Y, Zhao J, et al. Promoter Hypermethylation of PIWI/Pirna Pathway Genes Associated With Diminished Pachytene Pirna Production in Bovine Hybrid Male Sterility. *Epigenetics* (2020) 15(9):914–31. doi: 10.1080/15592294.2020.1738026
25. Curry E, Safranski TJ, Pratt SL. Differential Expression of Porcine Sperm Microrrnas and Their Association With Sperm Morphology and Motility. *Theriogenology* (2011) 76(8):1532–9. doi: 10.1016/j.theriogenology.2011.06.025
26. Wang W, Liang K, Chang Y, Ran M, Zhang Y, Ali MA, et al. Mir-26a is Involved in Glycometabolism and Affects Boar Sperm Viability by Targeting PDHX. *Cells* (2020) 9(1):146. doi: 10.3390/cells9010146
27. Huang H, Gao Q, Peng X, Choi SY, Sarma K, Ren H, et al. Pirna-Associated Germline Nuage Formation and Spermatogenesis Require Mitopld Profusogenic Mitochondrial-Surface Lipid Signaling. *Dev Cell* (2011) 20 (3):376–87. doi: 10.1016/j.devcel.2011.01.004
28. Choi SY, Huang P, Jenkins GM, Chan DC, Schiller J, Frohman MA. A Common Lipid Links Mfn-Mediated Mitochondrial Fusion and SNARE-Regulated Exocytosis. *Nat Cell Biol* (2006) 8(11):1255–62. doi: 10.1038/ncb1487
29. Brennecke J, Malone CD, Aravin AA, Sachidanandam R, Stark A, Hannon GJ. An Epigenetic Role for Maternally Inherited Pirnas in Transposon Silencing. *Science* (2008) 322(5906):1387–92. doi: 10.1126/science.1165171
30. Ishizu H, Siomi H, Siomi MC. Biology of PIWI-Interacting Rnas: New Insights Into Biogenesis and Function Inside and Outside of Germlines. *Genes Dev* (2012) 26(21):2361–73. doi: 10.1101/gad.203786.112

31. Zhou Y, Zhou G, Tian C, Jiang W, Jin L, Zhang C, et al. Exosome-Mediated Small RNA Delivery for Gene Therapy. *Wiley Interdiscip Rev RNA* (2016) 7 (6):758–71. doi: 10.1002/wrna.1363

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# Seminal Plasma Lipidomics Profiling to Identify Signatures of Kallmann Syndrome

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**Background:** Kallmann syndrome (KS) is a rare developmental disorder. Our previous metabolomics work showed substantial changes in linoleic acid and glycerophospholipid metabolism in KS. Here, we performed targeted lipidomics to further identify the differential lipid species in KS.

**Methods:** Twenty-one patients with KS (treatment group) and twenty-two age-matched healthy controls (HC, control group) were enrolled. Seminal plasma samples and medical records were collected. Targeted lipidomics analysis of these samples was performed using ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS).

**Results:** Lipidomics profiling of patients with KS and the HCs showed clear separation in the orthogonal projections to latent structures-discriminant analysis (OPLS-DA). There were many differential lipids identified, with the main differential lipid species being triacylglycerols (TAGs), phosphatidylcholines (PCs) and phosphatidylethanolamine (PE).

**Conclusions:** The lipidomics profile of patients with KS changed. It was also determined that TAGs, PCs and PE are promising biomarkers for KS diagnosis. To our knowledge, this is the first report to analyze lipidomics in men with Kallmann syndrome.

**Keywords: targeted lipidomics, Kallmann syndrome, biomarker, rare disease, UPLC-MS/MS**

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## INTRODUCTION

Kallmann syndrome (KS) is a genetic disorder characterized by hypogonadotropic hypogonadism accompanied by anosmia or hyposmia, which is caused by congenital gonadotropin-releasing hormone (GnRH) deficiency and olfactory bulb hypoplasia (1). KS is a rare disease that can be familial or sporadic. KS has genetic heterogeneity, including three methods of inheritance:

autosomal recessive inheritance, autosomal dominant inheritance, and X-linked recessive inheritance (2, 3). Currently, it is challenging for doctors to match genetic mutations to phenotypes, leading to the difficult diagnosis of KS (4–6).

Metabolomics is an emerging "omics" method that quantitatively analyzes metabolites with a molecular mass of less than 1,000 and finds the relative relationship between pathological changes and metabolites (7). It is an integral part of systems biology and has been used to develop biomarkers of seminal fluid in men with male factor infertility (8–11). The reported potential biomarkers in seminal plasma in male infertility include fatty acids, oxidative stress (OS)-related metabolites and amino acids. Of note, omega-3 supplements might improve semen quality parameters in men with infertility and men from couples seeking fertility treatment (9). Our previous metabolomics study also showed that linoleic acid and glycerophospholipid metabolism are the main affected pathways in patients with KS (12). To further clarify the lipid species, we performed targeted lipidomics analysis of seminal plasma.

# MATERIALS AND METHODS

## Participants

All subjects participating in this study were volunteers recruited from the Peking Union Medical College Hospital (PUMCH) from January 2020 to October 2020. The recruited series included 21 KS and 22 HC. The clinical evaluations and hospital records of all participants were collected (**Table 1**).

KS is a developmental disorder characterized by hypogonadotropic hypogonadism accompanied by anosmia (21/21). Diagnosis is made by an experienced doctor according to examination results. The diagnostic criteria of KS included the following: (a) patients with absent/incomplete puberty by the age of 18; (b) serum T ≤100 ng/dl with low or normal serum gonadotropin levels; (c) normal hypothalamic–pituitary region magnetic resonance imaging (MRI); (d) normal pituitary–adrenal/pituitary–thyroid/pituitary–IGF-1 axis function; and (e) anosmia or hyposmia (6). An age-matched control group (healthy volunteers) with normal semen parameters was recruited from PUMCH. Semen samples were produced by masturbation after ≥3 days of abstinence.

## Materials and Instrument

The SPLASH LIPIDOMIX Mass Spec Standard was from AVANTI. Chromatographic grade methanol and methyl tert-butyl ether (MTBE) were purchased from the CNW Technologies, Shanghai, China. The Lipidyzer TM was from the AB Sciex Pte. Ltd., Massachusetts, United States. Chromatographic grade ammonium acetate, dichloromethane and isopropanol were obtained from the Merck Company, Darmstadt, Germany. The SCIEX ExionLC system was coupled to a SCIEX QTrap 6500+ (AB Sciex Pte. Ltd., Massachusetts, USA). ACQUITY UPLC HSS T3 (2.1 ∗ 100 mm, 1.8 μm, Waters Corporation, Milford, MA, USA) was used for lipidomics analysis.

## Sample Collection

The preparation before sampling, blood sample collection and specimen processing were conducted according to IFCC/C-RIDL protocols. Fasting blood samples were taken *via* venipuncture into Vacuette tubes containing procoagulant, and within 15–30 min after sample collection, the samples were centrifuged at 1,200×*g* for 10 min.

A semen sample from each participant was obtained by means of masturbation and ejaculation directly into noncytotoxic sterile containers. Freshly collected semen was liquefied for 30–60 min at room temperature and processed within 1 h of ejaculation for analysis of the sperm characteristics according to the criteria published by the WHO. The samples

**TABLE 1** | Clinical characteristics of all participants.

| Parameter | Patients with KS (n = 20) | Healthy controls (n = 23) | Normal reference ranges |
|---|---|---|---|
| Age at diagnosis (years), mean ± SD | 22.2 ± 5.1 | 24.3 ± 8.4 | – |
| Olfactory (anosmia) | 21/21 | Negative | Negative |
| Deafness | 2/21 | Negative | Negative |
| Cryptorchidism | 2/21 | Negative | Negative |
| Gynecomastia | 5/21 | Negative | Negative |
| Agenesis of kidney | 1/21 | Negative | Negative |
| Semen non-liquefaction | 13/21 | 0/23 | liquefaction |
| Semen condensed | 12/21 | 0/23 | Negative |
| Sperm count (million) | 13.9 ± 21.5 | 367.4 ± 206.1* | ≥39 |
| Sperm concentration (million/ml) | 5.7 ± 9.8 | 102.8 ± 57.2* | ≥15 |
| Sperm motility (%) | 7.4 ± 9.8 | 67.1 ± 11.1* | ≥40 (PR + NP) |
| Seminal fructose (Positive) | 17/17 | 17/17 | Positive |
| FSH (IU/L) | 1.27 (0.62–2.64) | 5.61 (1.95–16.32)* | 1.27–19.26 |
| LH (IU/L) | 0.33 (0.16–1.66) | 3.93 (1.67–7.42)* | 1.24–8.62 |
| T (ng/ml) | 0.55 (0.31–2.79) | 4.22 (2.24–7.30)* | 1.75–7.81 |
| PRL (ng/ml) | 7.49 (6.75–11.25) | 6.98 (4.71–10.24) | 2.6–13.1 |
| E2 (pg/ml) | 18 (8–31) | 20 (9–34) | <39 |

*FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone; E2, estradiol; PRL, prolactin.*
**Indicates p <0.05.*

were centrifuged at 1,200×*g* for 10 min and frozen at −80°C to obtain 200 µl of seminal plasma.

## Laboratory Assays

All sex hormones were measured using an automated chemiluminescence immunoassay analyzer (Beckman Coulter UniCel DXI 800, Beckman Coulter; Brea, CA, USA) using the corresponding reagents, calibration materials and quality control materials. Sperm motility and concentration assessments were performed using computer-assisted sperm analysis systems (Suiplus SSA-II, Suiplus Software Co. Ltd.; Beijing, China). The microscope used with SSA-II was a Nikon 80i with a 20-phase objective.

## Sample Preparation for Metabolomics Profiling

Ten microliters of the seminal plasma was mixed with 190 µl of water in a sterile 1.5 ml Eppendorf tube, and 480 µl of extract solution (MTBE/MeOH = 5:1) with internal standard was added. The samples were vortexed for 60 s and sonicated for 10 min in an ice water bath. Then, the samples were centrifuged at 3,000 rpm for 15 min at 4°C, and 250 µl of supernatant was transferred to a fresh tube. Another 250 µl of MTBE was added to the rest of the sample, and the extraction step was repeated twice. The combined supernatants were freeze-dried and resuspended in 100 µl of DCM/MeOH/$H_2O$ (60/30/4.5) by sonication on ice for 10 min. The samples were centrifuged at 12,000 rpm for 15 min at 4°C, and 30 µl of the supernatant was transferred to a fresh glass vial for liquid chromatography-mass spectrometry (LC/MS) analysis.

## Quality Control

The quality control (QC) sample was prepared by mixing 15 µl of the supernatants from all of the seminal plasma samples. The QC sample was stored at −80°C and used according to the sequence.

## LC-MS/MS Analysis

A SCIEX ExionLC series UHPLC System was used to perform ultrahigh-performance liquid chromatography (UHPLC) separation. Mobile phase A consisted of water/acetonitrile (40%/60%) with 10 mmol/L ammonium acetate. Mobile phase B consisted of acetonitrile/isopropanol (10%/90%) with 10 mmol/L ammonium acetate. A gradient elution procedure was used (0.0–16.0 min, 80–2% mobile phase A; 16.01–18.0 min, 80% mobile phase A). The autosampler temperature was set as 6°C, and the injection volume was set as 2 µl. The column temperature was 40°C. The flow rate was set as 0.3 ml/min.

Assay development was performed with an AB Sciex QTrap 6500+ mass spectrometer. The ion spray voltage was +5,500/−4,500 V, ion source gas 1 was 50 psi, ion source gas 2 was 50 psi, the DP was ±80 V, the curtain gas was 40 psi, and the temperature was 350°C.

## Data Processing and Multivariate Data Analysis

Quantification of the target compounds was performed with Skyline 20.1 software. The absolute content of the individual lipids was calculated based on the peak area corresponding to the internal standard (IS) of the identical lipid class.

## Statistical Analysis

Experimental values are expressed as the mean ± SD. SIMCA software was used to perform statistical analysis. Variable importance in the projection (VIP) >1 and $p < 0.05$ (Student's t test) were used to screen the significance of metabolite levels. A Euclidean distance matrix was calculated to cluster the differential lipids with a complete linkage method.

# RESULTS

## Baseline Clinical Characteristics of Patients With KS

All of the patients were ethnically Han Chinese (100%). Their average age was 22.2 ± 5.1 years old. All of the patients showed anosmia (21/21), and many patients had semen non-liquefaction (13/21) and condensed semen (12/21). Patients with KS showed lower sperm counts ($13.9 \pm 21.5 * 10^6$), sperm concentrations ($5.7 \pm 9.8 * 10^6$/ml) and sperm motility (7.4 ± 9.8%). The serum follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) levels were significantly lower in the patients with KS than those in the control group (**Table 1**).

## Seminal Plasma Lipidomics

We analyzed the lipidomics data to discover lipid species related to KS. The effects of high variance of the variables and noise were minimized using scaled and logarithmic transformations. SIMCA software (V16.0.2, Sartorius Stedim Data Analytics AB, Umea, Sweden) was used to generate orthogonal projections to latent structures-discriminant analysis (OPLS-DA) plots (**Figure 1**). The OPLS-DA chart shows that the separation of the control group (HC, red) and the treatment group (patients with KS, blue) had a small overlap. Furthermore, a volcano plot was used to screen the statistically significant lipids (**Figure 2**), and blue dots indicate the presence of significantly downregulated metabolites in patients with KS. The value of the first principal component from the OPLS-DA analysis was obtained. The criterion of significantly changed metabolites was set as variable importance in the projection (VIP) >1 and $p < 0.05$ (Student's t test).

As shown in **Figure 2**, many lipid species such as triacylglycerol (TAG), sphingomyelin (SM), phosphatidylethanolamine (PE), lyso phosphatidylethanolamine (LPE), free fatty acid (FFA) and hexosylceramides (HexCer) changed in the patients with KS, TAG(53:2)_FA 17:0, TAG(56:5)_FA20:4, TAG(56:4)_FA18:1, TAG(54:4)_FA20:3, TAG(54:4)_FA16:0, PC (16:0/20:4), PC (16:0/16:1), PC (18:1/20:3), PC (14:0/18:1), and PC (16:0/14:0) decreased in patients with KS. Then, we calculated the Euclidean distance matrix, clustered the differential lipids with a complete linkage method, and displayed them in a heat map (**Figure 3**). The Euclidean distance matrix can be regarded as a weighted form of the adjacency matrix, which includes two levels of information: (1) whether the elements (points) are connected to each other and

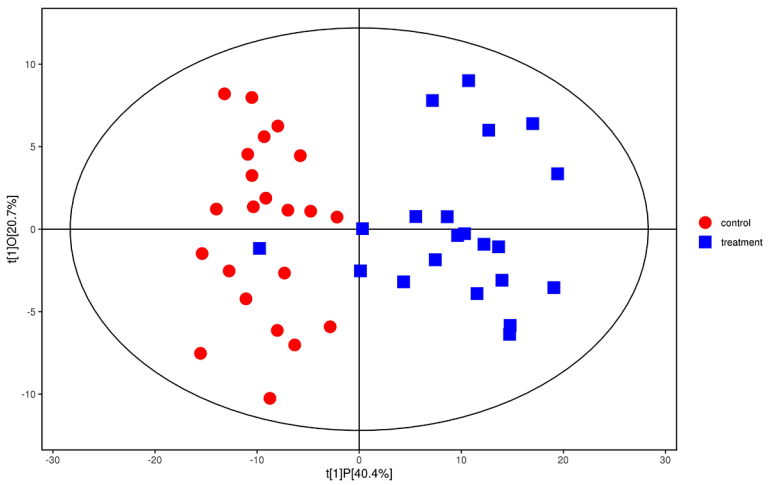


**FIGURE 1** | Orthogonal partial least squares-discriminant analysis (OPLS-DA) model for group treatment (KS, blue) *vs* control (HC, red). Ions marked in blue show a significant difference in intensity between the KS and HC groups.

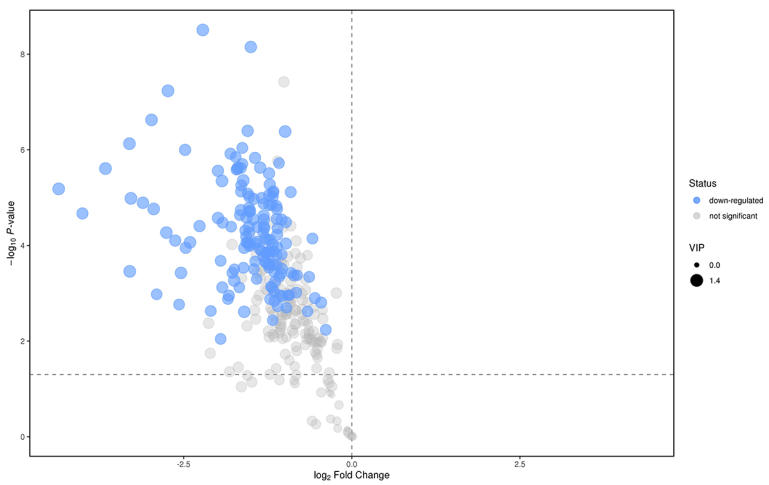


**FIGURE 2** | Volcano plot for the treatment (KS) *vs* control (HC) groups.

(2) the cost or distance of the connectivity between the elements (points). Therefore, the Euclidean distance matrix can be used to perform hierarchical cluster analysis. The ratio of the quantitative value of the different lipids was calculated using logarithmic conversion, and the corresponding content trend changes are displayed in the radar chart (**Figure 4**). For each group of comparisons, we calculated the corresponding ratio of the quantitative value of the different metabolites and took the logarithmic conversion with base 2 as the logarithmic conversion. The figure is represented with red font, each grid line represents a difference multiple, and the purple shading is represented by the connection of the lines of the difference multiples of each substance. The corresponding content trend changes are displayed in the radar chart. As shown in **Figure 4**, free fatty acids (FFAs) and diacylglycerols (DAGs) were downregulated in patients with KS. We also used a lipid group bubble plot to display the metabolite content change degree, difference significance and classification information (**Figure 5**). Each point in the lipid group bubble chart represents a metabolite. The size of the point represents the P-value of Student's t test. The larger the point, the smaller the P-value. Gray dots represent nonsignificant differences with a P-value of not less than 0.05, and colored dots represent significant differences with a P-value of less than 0.05 (different colors are marked according to lipid classification). The abscissa of the lipid group bubble chart represents the percent relative change of the content of each substance in the group. The ordinate of the lipid group bubble chart represents the lipid classification information. The black line at the bottom shows the distribution density of the

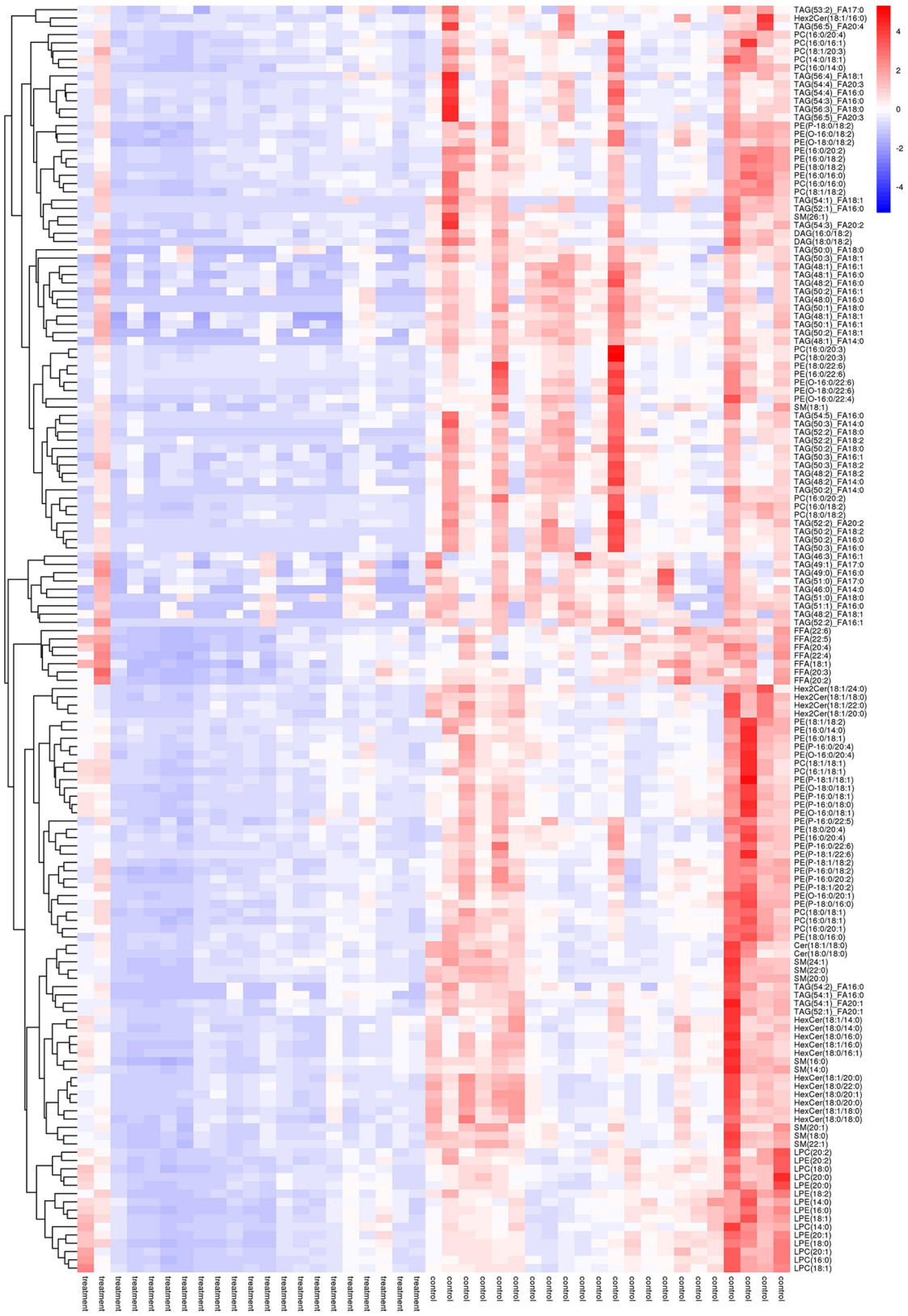


**FIGURE 3** | Heatmap of hierarchical clustering analysis for the treatment (KS) *vs* control (HC) groups.

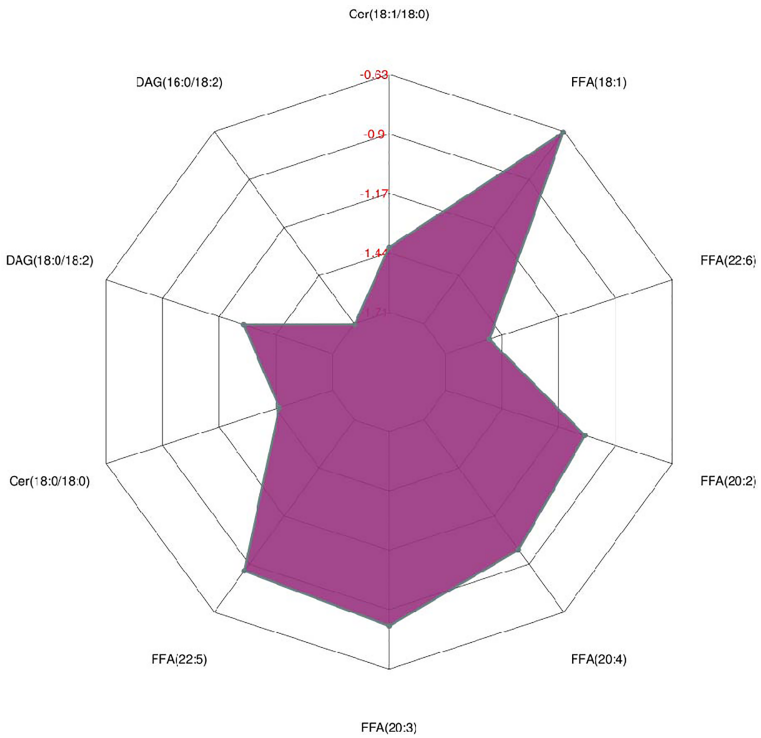


**FIGURE 4** | Radar chart analysis for the treatment (KS) *vs* control (HC) groups.

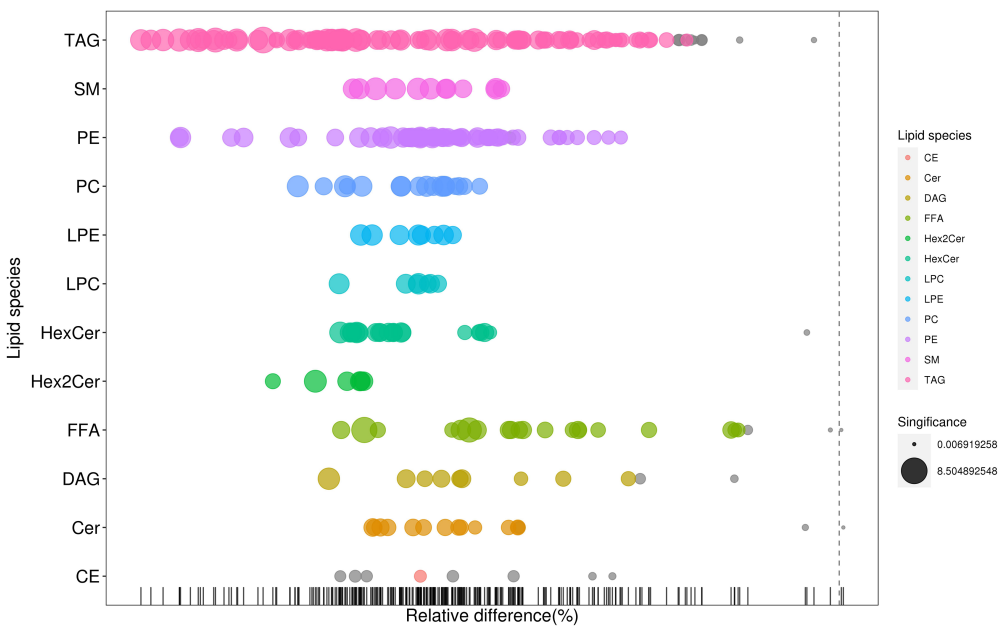


**FIGURE 5** | Bubble plot for the treatment (KS) *vs* control (HC) groups.

metabolites (one line represents one metabolite). As shown in **Figure 5**, TAGs, PCs and PE were the main differential species.

## DISCUSSION

KS is a rare disease lacking specific markers for early diagnosis. In our previous work, we used an untargeted metabolomics approach and found that linoleic acid metabolism and glycerophospholipid metabolism changed substantially in KS (12). In this project, we used lipidomics analysis for further study. Many lipid species, such as triacylglycerols (TAGs), sphingomyelin (SM), phosphatidylethanolamine (PE), lyso-phosphatidylethanolamine (LPE), free fatty acids (FFAs) and hexosylceramides (HexCers), changed in patients with KS, and TAGs, PCs and PE were the main differential species.

Triacylglycerols (TAGs) are important lipid components that have various fatty acyl groups with different chain lengths, degrees of unsaturation and fatty acid isomers. In the cell, triacylglycerols can be hydrolyzed to form free fatty acids (FFAs) and 2-monoacylglycerol, and free fatty acids can be metabolized to biologically active compounds, oxidized to provide energy, or resynthesized into triacylglycerols for storage. A previous study on complete fatty acid profiling revealed potential candidate markers of semen quality (13). Our data showed that TAG(50:2)_FA18:2,TAG(52:1)_FA16:0, TAG(50:2)_FA16:0,TAG(52:2)_FA18:0, TAG(52:2)_FA18:2, TAG(48:0)_FA16:0, TAG(50:3)_FA16:0, and TAG(54:1)_FA18:1 decreased in patients with KS, which is in accordance with previous findings (**Table S1**) (13–15). In addition, the different FFAs also decreased in patients with KS (**Figure 4**). We think that a lower level of TAGs, to a certain extent, reveals a microenvironment without adequate nutrition. In the study, the patients with KS had lower sperm counts ($13.9 \pm 21.5 \times 10^6$/ml *vs* HC of $39 \times 10^6$/ml), sperm concentrations ($5.7 \pm 9.8 \times 10^6$/ml *vs* HC of $15 \times 10^6$/ml) and sperm motility (7.4 ± 9.8% *vs* HC of 38 to 42%), which may be associated with the microenvironment.

Phosphatidylcholines (PCs) are a class of glycerophospholipids that, along with other phospholipids, account for more than half of the lipids in most membranes. For example, lysophospholipids are PCs that affect lysophospholipid receptors as lipid signaling regulators (16, 17). In our work, PC (16:0/20:4), PC (16:0/16:1), PC (18:1/20:3), PC (14:0/18:1), and PC (16:0/14:0) decreased in patients with KS, indicating diagnostic value. The change in phosphatidylcholine composition and the saturation of their fatty acids may cause the deterioration of sperm membranes. This is consistent with a previous report that showed that glycerylphosphorylcholine (GPC) had potential diagnostic value for infertile men (18–22).

Phosphatidylethanolamine (PE) is a major component of membranes of many species. PE (16:0/22:6) and PE (O-16:0/22:6) were the significantly different metabolites (**Table S1**). For example, 50% of the total PE lipids are PE plasmalogens, which represent a major source of arachidonic acid and play an important role in the inflammatory response and also cause semen non-liquefaction (13/21) and semen condensation (12/21) (23, 24).

## CONCLUSIONS

In conclusion, we presented lipidomics profiling of semen plasma in patients with KS, and the main differential metabolic pathways focused on TAGs, PCs and PE. The results from this new report are consistent with our previous work, suggesting the promising value of these biomarkers. However, the exact mechanism remains to be further elucidated. In addition, since this is a single-center study with a relatively small sample size, the performance of these biomarkers needs to be further evaluated.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by institutional committee that protects human subjects from PUMCH. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

(I) Conception and design: XL and YG. (II) Administrative support: YL. (III) Provision of study materials or patients: YG. (IV) Collection and assembly of data: XL and YG. (V) Data analysis and interpretation: XL. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.692690/full#supplementary-material

## REFERENCES


1. Schwanzel-Fukuda M, Bick D, Pfaff DW. Luteinizing Hormone-Releasing Hormone (LHRH)-Expressing Cells Do Not Migrate Normally in an Inherited Hypogonadal (Kallmann) Syndrome. *Brain Res Mol Brain Res* (1989) 6:311–26. doi: 10.1016/0169-328X(89)90076-4
2. Pitteloud N, Meysing A, Quinton R, Acierno JS, Crowley WF. Mutations in Fibroblast Growth Factor Receptor 1 Cause Kallmann Syndrome With a Wide Spectrum of Reproductive Phenotypes. *Mol Cell Endocrinol* (2006) 254-255:60–9. doi: 10.1016/j.mce.2006.04.021
3. Maione L, Dwyer A, Francou B, Guiochon-Mantel A, Binart N, Bouligand J, et al. Genetic Counseling for Congenital Hypogonadotropic Hypogonadism and Kallmann Syndrome: New Challenges in the Era of Oligogenism and Nextgeneration Sequencing. *Eur J Endocrinol* (2018) 178:R55–80. doi: 10.1530/EJE-17-0749
4. Caronia LM, Martin C, Welt CK, Sykiotis GP, Quinton R, Thambundit A, et al. A Genetic Basis for Functional Hypothalamic Amenorrhea. *N Engl J Med* (2011) 64:215–25. doi: 10.1056/NEJMoa0911064
5. Kaplan JD, Bernstein JA, Kwan A, Hudgins L. Clues to An Early Diagnosis of Kallmann Syndrome. *Am J Med Genet A* (2010) 152A:2796–801. doi: 10.1002/ajmg.a.33442
6. Nie M, Yu B, Chen R, Sun B, Mao J, Wang X, et al. Novel Rare Variants in FGFR1 and Clinical Characteristics Analysis in a Series of Congenital Hypogonadotropic Hypogonadism Patients. *Clin Endocrinol (Oxf)* (2021) 00:1–10. doi: 10.1111/cen.14436
7. Nicholson JK, Lindon JC. Systems Biology: Metabonomics. *Nature* (2008) 455:1054–6. doi: 10.1038/4551054a
8. Wu H, Xue R, Tang Z, Deng C, Liu T, Zeng H, et al. Metabolomic Investigation of Gastric Cancer Tissue Using Gas Chromatography/Mass Spectrometry. *Anal Bioanal Chem* (2010) 396:1385–95. doi: 10.1007/s00216-009-3317-4
9. Falsi g AML, Gleerup CS, Knudsen UB. The Influence of Omega-3 Fatty Acids on Semen Quality Markers: A Systematic PRISMA Review. *Andrology* (2019) 7:6. doi: 10.1111/andr.12649
10. Zanetti SR, Monclus MdLÁ, Rensetti DE, Fornés MW, Aveldaño MI. Differential Involvement of Rat Sperm Choline Glycerophospholipids and Sphingomyelin in Capacitation and the Acrosomal Reaction. *Biochimie* (2010) 92(12):1886–94. doi: 10.1016/j.biochi.2010.08.015
11. Boguenet M, Bocca C, Bouet P-E, Serri O, Chupin S, Tessier L, et al. Metabolomic Signature of the Seminal Plasma in Men With Severe Oligoasthenospermia. *Andrology* (2020) 8:1859–66. doi: 10.1111/andr.12882
12. Guo Y, Li X, Yan S, Li Y. Metabolomic Alterations Associated With Kallmann Syndrome. *Ann Transl Med* (2020) 8(8):529. doi: 10.21037/atm.2020.04.03
13. Zerbinati C, Caponecchia L, Rago R, Leoncini E, Bottaccioli AG, Ciacciarelli M, et al. Fatty Acids Profiling Reveals Potential Candidate Markers of Semen Quality. *Andrology* (2016) 4(6):1094–101. doi: 10.1111/andr.12236
14. Mu H, Porsgaard T. The Metabolism of Structured Triacylglycerols. *Prog Lipid Res* (2005) 44:430–48. doi: 10.1016/j.plipres.2005.09.002
15. Masaki H, Kim N, Nakamura H, Kumasawa K, Kamata E, Hirano KI, et al. Long-Chain Fatty Acid Triglyceride (TG) Metabolism Disorder Impairs Male Fertility: A Study Using Adipose Triglyceride Lipase Deficient Mice. *Mol Hum Reprod* (2017) 23(7):452–60. doi: 10.1093/molehr/gax031
16. Pietiläinen KH, Róg T, Seppänen-Laakso T, Virtue S, Gopalacharyulu P, Tang J, et al. Association of Lipidome Remodeling in the Adipocyte Membrane With Acquired Obesity in Humans. *PloS Biol* (2011) 9:e1000623. doi: 10.1371/journal.pbio.1000623
17. Wymann MP, Schneiter R. Lipid Signalling in Disease. *Nat Rev Mol Cell Biol* (2008) 9:162–76. doi: 10.1038/nrm2335
18. Vorkas PA, Isaac G, Holmgren A, Want EJ, Shockcor JP, Holmes E, et al. Perturbations in Fatty Acid Metabolism and Apoptosis Are Manifested in Calcific Coronary Artery Disease: An Exploratory Lipidomic Study. *Int J Cardiol* (2015) 197:192–9. doi: 10.1016/j.ijcard.2015.06.048
19. Vouk K, Ribič-Pucelj M, Adamski J, Rižner TL. Altered Levels of Acylcarnitines, Phosphatidylcholines, and Sphingomyelins in Peritoneal Fluid From Ovarian Endometriosis Patients. *J Steroid Biochem Mol Biol* (2016) 159:60–9. doi: 10.1016/j.jsbmb.2016.02.023
20. Zhao YY, Wang HL, Cheng XL, Wei F, Bai X, Lin RC, et al. Metabolomics Analysis Reveals the Association Between Lipid Abnormalities and Oxidative Stress, Inflammation, Fibrosis, and Nrf2 Dysfunction in Aristolochic Acidinduced Nephropathy. *Sci Rep* (2015) 5:12936. doi: 10.1038/srep12936
21. Ried JS, Baurecht H, Stuckler F, Krumsiek J, Gieger C, Heinrich J, et al. Integrative Genetic and Metabolite Profiling Analysis Suggests Altered Phosphatidylcholine Metabolism in Asthma. *Allergy* (2013) 68:629–36. doi: 10.1111/all.12110
22. Deepinder F, Chowdary HT, Agarwal A. Role of Metabolomic Analysis of Biomarkers in the Management of Male Infertility. *Expert Rev Mol Diagn* (2007) 7:351–8. doi: 10.1586/14737159.7.4.351
23. Bogatcheva NV, Sergeeva MG, Dudek SM, Verin AD. Arachidonic Acid Cascade in Endothelial Pathobiology. *Microvasc Res* (2005) 69:107–27. doi: 10.1016/j.mvr.2005.01.007
24. Monk JM, Turk HF, Fan YY, Callaway E, Weeks B, Yang P, et al. Antagonizing Arachidonic Acid-Derived Eicosanoids Reduces Inflammatory Th17 and Th1 Cell Mediated Inflammation and Colitis Severity. *Mediators Inflamm* (2014) 2014:917149. doi: 10.1155/2014/917149


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