Profiling the male germline genome to unravel its reproductive potential

Stephanie Cheung, M.Sc., Philip Xie, B.Sc., Zev Rosenwaks, M.D., and Gianpiero D. Palermo, M.D., Ph.D.

The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine, Weill Cornell Medicine, New York, New York

Objective: To identify specific germline mutations related to sperm reproductive competence, in couples with unexplained infertility.
Design: In this retrospective study, couples were divided according to whether they had successful intracytoplasmic sperm injection outcomes (fertile) or not (infertile). Ancillary sperm function tests were performed on ejaculates, and whole exome sequencing was performed on spermatozoal DNA. Sperm aneuploidy and gene mutation profiles were compared between the 2 cohorts as well as according to the specific reasons for reproductive failure.
Setting: Center for reproductive medicine at a major academic medical center.
Patient(s): Thirty-one couples with negative infertility workups and normal semen parameters.
Intervention(s): Couples with mutations on fertilization- or embryo development-related genes were subsequently treated by assisted gamete treatment or microfluidics, respectively.
Main Outcome Measure(s): Intracytoplasmic sperm injection cycle outcomes including fertilization, clinical pregnancy, and delivery rates.
Result(s): Sperm aneuploidy was lower in the fertile group (4.0% vs. 8.4%). Spermatozoa from both cohorts displayed mutations associated with sperm–egg fusion (*ADAM3A*) and acrosomal development (*SPACA1*), regardless of reproductive outcome. The infertile cohort was then categorized according to the reasons for reproductive failure: absent fertilization, poor early embryo development, implantation failure, or pregnancy loss.

Spermatozoa from the fertilization failure subgroup (n = 4) had negligible PLCζ presence (10% ± 9%) and gene mutations (*PLCZ1, PIWIL1, ADAM15*) indicating a sperm-related oocyte-activating deficiency. These couples were successfully treated by assisted gamete treatment in their subsequent cycles.

Spermatozoa from the poor early embryo development subgroup (n = 5) had abnormal centrosomes (45.9% ± 5%), and displayed mutations impacting centrosome integrity (*HAUS1*) and spindle/microtubular stabilization (*KIF4A, XRN1*). Microfluidic sperm processing subsequently yielded a term pregnancy.

Spermatozoa from the implantation failure subgroup (n = 7) also had abnormal centrosomes (53.1% ± 13%) and carried mutations affecting embryonic implantation (*IL9R*) and microtubule and centrosomal integrity (*MAP1S, SUPT5H, PLK4*), whereas those from the pregnancy loss subgroup (n = 5) displayed mutations on genes involved in trophoblast development (*NLRP7*), cell cycle regulation (*MARK4, TRIP13, DAB2IP, KIF1C*), and recurrent miscarriage (*TP53*).
Conclusion(s): By assessing the sperm genome, we identified specific germline mutations related to various reproductive processes. This information may clarify elusive factors underlying reproductive competence and enhance treatment for couples with unexplained infertility. (Fertil Steril® 2023;119:196-206. ©2022 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Intracytoplasmic sperm injection, sperm DNA, whole exome sequencing, germline mutations, ART failure

Approximately 10%–15% of couples in the United States are affected by infertility (1). The inability to reproduce is equally attributed to the male and female partners, with the remainder being due to a combined contribution (2). Screening of the woman includes an assessment of ovarian function, tubal patency, and uterine cavity. However, infertility investigation for the male partner is limited to a semen analysis. Although the semen analysis is useful in providing information on the presence, motility, and morphology of spermatozoa, it is unable to inform on the function or fertilizing capacity of the male gamete. This is especially important in cases with unexplained infertility, wherein couples with negative infertility workups and normal sperm parameters still experience reproductive failure due to poor or absent fertilization, poor embryo

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Correspondence to: Gianpiero Palermo, M.D., Ph.D., The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine, Weill Cornell Medicine, 1305 York Avenue, Y720, New York, NY 10021 (E-mail: gdpalerm@med.cornell.edu).

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development, implantation failure, or worse, pregnancy loss (3, 4).

Over the years, genetic testing has become increasingly relevant in reproductive medicine. For instance, couples currently undergo extended carrier screening in addition to peripheral blood karyotyping (5). Genetic assessments in male infertility include testing for whole chromosomal structural aberrations, partial chromosomal defects, and monogenic diseases (6). These genetic tests are exclusively performed on peripheral blood samples, and although these tests are focused on identifying the etiology of compromised sperm production and preventing the transmission of inherited defects, they do not provide any information on gamete competence (7).

More intriguing is the identification of a subtle male factor or the detection of gamete function, particularly in individuals with normal semen parameters. There has recently been a renewed effort to identify superimposed occult factors that may impair a man's reproductive potential and affect assisted reproductive technology (ART) related clinical outcomes. For instance, localization patterns of the ganglioside monosialotetrahexosylganglioside (G_{M1}), assessed by the Cap-Score assay, can identify the percentage of spermatozoa capable of fertilization (8). In addition, G_{M1} is a key regulator of capacitation and acrosome exocytosis, and by assessing its localization, the Cap-Score has been prospectively shown to predict the probability of a man's fertility (9). Therefore, couples in which the man has a normal Cap-Score may be counseled for the less invasive intrauterine insemination, whereas a compromised Cap-Score, despite a normal semen analysis, would suggest the need for in vitro fertilization (IVF) or even intracytoplasmic sperm injection (ICSI) (10).

Sperm chromatin fragmentation assays have become widely popular and can detect elevated sperm DNA fragmentation, which is correlated with poor embryo development, low implantation, and high miscarriage rates (11, 12). However, assay protocols vary in specificity on whether they can identify overall fragmentation or more specific types of DNA breaks.

A sperm activating factor staining assay can confirm the presence of a labile protein, phospholipase-Cζ (PLCζ), stored in the perinuclear theca of the sperm head and responsible for triggering oocyte activation (13). The results of PLCζ staining can be validated by the mouse oocyte activation test (MOAT), which involves injecting a patient's spermatozoa into mouse oocytes to determine fertilization capability. However, MOAT is heterospecific in nature, so the injection of human spermatozoa into mouse oocytes may not necessarily provide the most reliable results. In addition, MOAT grading is also not very precisely defined and has a wide span of interpretation. Furthermore, it is unfeasible to perform the assessment in a setting without an animal facility and trained personnel, as mouse oocytes must be stimulated and harvested (14).

Centrosomal defects are also associated with fertilization failure and developmental arrest and can be identified by immunofluorescence staining or high magnification techniques such as transmission electron microscopy (15–18). However, this technique is costly and requires laborious sample preparation, as well as in-depth understanding of the sperm cell.

Regardless of the available ancillary assessments and genetic tests, the sperm-related reasons for poor ART outcome, occurring despite a normal semen analysis, remain largely unknown. However, we hypothesized that detectable genetic differences exist in spermatozoa obtained from men with unexplained infertility, and that these differences can be used to understand the specific aspects of their suboptimal ART outcomes. Therefore, in this study we investigate the relationship between the genetic profile of spermatozoa and the reasons for reproductive failure, after ICSI, in couples with negative infertility workups and normal sperm parameters. Furthermore, although the aforementioned ancillary assessments provide valuable information on the male gamete, they each have their limitations and can only assess a single facet of sperm reproductive potential. Therefore, we envision the possibility of a single genetic assessment, performed on the male gamete, that could unravel subtle information on its true reproductive competence and ability to sustain fertilization, zygote formation, embryo cleavage, and complete post implantation development.

MATERIALS AND METHODS

Inclusion Criteria and Study Design

This study took place between August 2019 and December 2021 at the Center for Reproductive Medicine of a major academic medical center. Couples who underwent ICSI were considered eligible. We included couples with normal karyotypes, negative infertility workups, and normal semen parameters. Those undergoing cycles with oocyte or sperm donation were excluded.

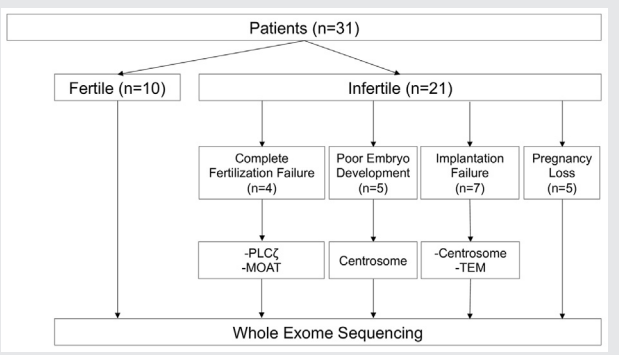
These couples were divided according to whether they had successful ART outcomes (fertile) or not (infertile). The infertile cohort was then grouped according to the reasons for reproductive failure, including absent fertilization, poor embryo development, implantation failure, and pregnancy loss. Ancillary sperm assessments were performed on ejaculates to identify specific male gamete dysfunctions. Sperm DNA sequencing was performed to assess copy number variants (CNVs) and to identify candidate gene mutations. Gene mutation profiles were compared between the fertile and infertile cohorts, as well as according to the reasons for reproductive failure (Fig. 1). Although we adopted a complete whole exome sequencing approach, for the purpose of this investigation we primarily focused on those genes that were specifically related to spermiogenesis, impaired fertilization, early embryo cleavage, as well as embryo developmental competence.

This study was approved by the *Institutional Review Board of the New York Presbyterian Hospital-Weill Cornell Medicine* (IRB 1006011085), and all participants gave their informed consent.

Infertility Workup

Female infertility evaluation consisted of a comprehensive review of the medical history, targeted physical examination, and tests focusing on ovarian reserve, ovulatory function, tubal patency, and uterine structural abnormalities. Hormone profiling and karyotyping were performed for each couple to

FIGURE 1



Study design flowchart.
We included 31 couples with normal karyotypes, negative infertility workups, and normal semen parameters, and divided them according to whether they had successful ICSI outcomes (fertile) or not (infertile). The infertile cohort was then categorized into 4 subgroups based on the reasons for reproductive failure, including absent fertilization, poor embryo development, poor implantation, and pregnancy loss. Ancillary sperm assays (PLCζ immunofluorescence, mouse oocyte activation test, centrosome, and ultrastructural sperm assessments) were performed to evaluate specific male gamete functions. Whole exome sequencing was performed on the spermatozoa from all patients to assess sperm aneuploidy by copy number variant analysis, and to identify candidate gene mutations. Gene mutation profiles were then compared between the fertile and infertile cohorts, as well as according to the reasons for reproductive failure.

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confirm the absence of any genetic alterations. Couples also did not have any family history of genetic diseases. None of the male partners were taking testosterone supplementation, nor medications to improve semen parameters before or during study participation. Ejaculates were evaluated according to *World Health Organization* standards (4), cryopreserved, and donated by the participants.

PLCζ Staining and Assessment

Couples with reproductive failure due to fertilization failure underwent a PLCζ immunofluorescence assessment, performed as previously described (13), to determine whether the absence of fertilization was caused by a sperm-related oocyte-activating deficiency. Sperm specimens were incubated overnight with polyclonal anti-PLCζ antibody, then labeled with a secondary antibody, anti-rabbit IgG, and counterstained with 4′,6-diamidino-2-phenylindole. The percentage of spermatozoa exhibiting PLCζ immunofluorescence in the acrosomal, equatorial, and post acrosomal regions of the head was recorded for ≥200 cells per specimen. In our previous study, we found that patients with low PLCζ presence in their spermatozoa yielded consistently low or unobtainable fertilization (13). Therefore, we established a 30% normal threshold by accepting the value 2 standard deviations below the mean. Each assessment was also performed against a known fertile control that consistently displayed a presence of PLCζ ranging from 80% to 90% of the spermatozoa assessed.

Mouse Oocyte Activation Test

MOAT was performed as previously described to confirm the PLCζ immunofluorescence results (13). Mouse oocytes were retrieved from B6D2 F1 hybrid mice, and piezo-actuated ICSI using patient spermatozoa was performed. Positive and negative controls were established by injecting donor spermatozoa with proven fertility or sham ICSI, respectively. The percentage of successful oocyte activation was determined by assessing the number of oocytes with 2 polar bodies and 2 pronuclei, as well as development to the 2-cell stage. A ≥85% threshold was considered normal (19–21). This protocol was approved by the *Institutional Animal Care and Use Committee of New York Presbyterian Hospital-Weill Cornell Medicine* (0605-493A).

Centrosome Assessment

Spermatozoa from men of couples with poor embryo development or poor implantation were screened for the presence of centriolar structures (22). The specimens were labeled with a primary anti-centrin mouse antibody, followed by a secondary goat anti-mouse IgG and counterstained with 4′,6-diamidino-2-phenylindole. The percentage of spermatozoa exhibiting centrosome immunofluorescence, indicated by 2 green signals at the base of the sperm head, was recorded for ≥200 cells per specimen. A normal threshold of ≥60% was used (23).

Ultrastructural Sperm Assessment

Transmission electron microscopy was performed to assess sperm organelles including acrosomes, nuclei, centrioles, and the microtubular arrangement in flagella (24). Dehydrated specimens were sliced by ultramicrotome to 100-nm sections. These sections were then viewed by an electron microscope (JEOL USA, Inc., Peabody, MA, USA) at 300,000× magnification, where a sperm ultrastructure tomography was generated. A minimum of 200 spermatozoa per slide were observed, and the percentage of cells with abnormalities were recorded.

Sperm Chromatin Fragmentation Assessment

Sperm chromatin fragmentation assessment was performed by terminal deoxynucleotidyl dUTP transferase nick-end labeling (TUNEL), using a commercially available kit (In Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's protocol (25). A minimum of 500 spermatozoa per sample were examined and the percentage of cells exhibiting positive fluorescein signals, indicating DNA breakage, was recorded. Sperm chromatin fragmentation of ≤15% was considered normal (26).

Whole Exome Sequencing

Extraction and amplification of DNA were performed on the spermatozoa from all men, using a commercial kit (Repli-G Single Cell; Qiagen, Hilden, Germany) (27). Specimens were sent to an external facility (Genewiz, Inc; South Plainfield, NJ), where they underwent 150-bp paired-end exome sequencing on an Illumina HiSeq 2500 platform. Reads

were trimmed to remove poor-quality nucleotides (error rate <0.01), and quality assessments of each indexed sample were performed by quantitative polymerase chain reaction. A high quality, average coverage of 85x was obtained for the specimens, with >90% exome coverage (Agilent SureSelect Human All Exon V6). The base calling accuracy for all samples was approximately 99.9%, as indicated by an average Phred quality score of Q38. After CNV detection was completed using CLC Bioinformatics Genomics Server 9.0, the detected variants were annotated to identify gene mutations. All genomic coordinates were based on the human genome assembly GRCh38 (hg38).

Ovarian Superovulation and Oocyte Collection

Full descriptions of the stimulation protocol and oocyte collection can be found in previous reports from our institution (24, 28).

Embryo Culture and Morphologic and Cytogenetic Evaluation of the Conceptus

Embryo biopsy and evaluation were performed for the poor embryo development, implantation failure, and pregnancy loss subgroups. The trophectoderm biopsy procedure was performed at the blastocyst stage (day 5) owing to reduced chances of harm and mosaicism compared with early stages (29). Embryos with the following morphologic grades were considered good-quality: blastocele, 1–3 (degree of expansion ≥ 50% the volume of the embryo); inner cell mass, A–B (clear inner cell mass with healthy cells); and trophectoderm, A–B (healthy and cohesive cells) (30). Only confirmed euploid embryos were recommended for transfer.

Embryo Transfer and Assessment of Clinical Outcome

The assessment of successful fertilization was performed under an inverted microscope (31). In preparation for embryo transfer, patients underwent daily progesterone supplementation, vaginally administered starting the day after ovulation. Serum β-hCG levels were measured 10–14 days after embryo transfer. Clinical pregnancy was defined as fetal heart beat (+FHB) activity, detected on ultrasound at 7 weeks gestation. Couples with +FHB were monitored until delivery, or until pregnancy loss.

Bioinformatics and Statistical Analysis

Copy number variant calling and gene mutation annotation were performed using CLC Genomics Server 9.0 modules including *Next Generation Sequencing (NGS) core tools or mapping and re-sequence analysis*. Sperm aneuploidy was assessed by calculating the proportion of chromosomal abnormalities detected by CNV analysis (32). The CNVs were then ranked according to these log-ratio values and corresponding genes annotated. Statistical thresholds of $P<.0005$ for significance and $Q<0.05$ for false positive discovery were used. Sperm genetic profiles were compiled by identifying the mutations that were commonly carried by the spermatozoa from all men within the same group or subgroup. Power analyses were performed using STATA (Stata/BE 17; StataCorp LP, College Station, TX). The Mann-Whitney *U* test and two-sample *t* test were used to compare sperm aneuploidy between the fertile and infertile cohorts (GraphPad Software, San Diego, CA). A *P* value of $<.05$ was considered to be statistically significant.

RESULTS

Thirty-one couples were enrolled in this study. All couples had normal peripheral karyotypes, negative infertility workups, and normal semen parameters (Table 1).

Ten couples, who successfully conceived by ICSI, comprised the fertile cohort (Table 1). The infertile cohort comprised 21 couples who underwent 25 ICSI cycles in which they obtained a 68.4% (91/133) fertilization rate and, while they achieved a 42.9% (6/14) clinical pregnancy rate, all clinical pregnancies ended in pregnancy losses.

Sperm chromatin fragmentation, when performed, was noted. The CNV assessment indicated lower sperm aneuploidy in the fertile (4.0%) versus the infertile (8.4%) cohort ($P<.00001$). Sperm genetic profiles for these couples identified mutations associated with sperm–egg fusion (*ADAM3A*) and acrosomal development (*SPACA1, SPATA16*), regardless of whether they achieved a successful pregnancy.

To further investigate a subtle male factor that may impair sperm reproductive potential, we categorized patients from the infertile cohort into 4 subgroups, based on the reasons for reproductive failure (Table 2).

In the first subgroup, 4 couples (maternal age, 37.7 ± 3 years; paternal age, 38.1 ± 2 years) underwent a total of 6 ICSI cycles in which an average of 4.3 oocytes were injected. Although these couples had an overall oocyte nuclear

TABLE 1

Study population demographics and overall clinical outcomes of fertile and infertile cohorts at study inclusion

Couples	31	
Maternal age (y) (mean ± SD)	37.1±3	
Paternal age (y) (mean ± SD)	38.9 ± 3	
Semen parameters		
Concentration (10^6/mL ± SD)	59.2 ± 30	
Motility (% ± SD)	44.8 ± 18	
Morphology (% ± SD)	4.1 ± 1	
	Fertile	Infertile
Couples	10	21
Maternal age (y) (mean ± SD)	37.2 ± 2	37.1 ± 3
Paternal age (y) (mean ± SD)	38.5 ± 2	38.9 ± 3
Intracytoplasmic sperm injection cycles	10	25
Oocytes retrieved	97	176
Metaphase-II oocytes (%)	69 (71.1)	133 (75.6)
Fertilization (%)	57 (82.6)[b]	91 (68.4)[b]
Cycles with ET	10	14
Clinical pregnancy (+FHB[a]) (%)	10 (100)[c]	6 (42.9)[c]
Deliveries (%)	10 (100)	—

Note: A total of 31 couples were enrolled in this study. All couples had normal peripheral karyotypes, negative infertility workups, and normal semen parameters. Ten couples, who successfully conceived by intracytoplasmic sperm injections, comprised the fertile cohort. The infertile cohort was comprised of 21 couples who underwent 25 intracytoplasmic sperm injection cycles where they obtained a 68.4% (91/133) fertilization and, while they achieved a 42.9% (6/14) clinical pregnancy rate, all clinical pregnancies ended in pregnancy losses.

[a] +FHB: Presence of at least one fetal heartbeat.

[b,c] χ^2, 2x2, 1 df, P<0.05.

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TABLE 2

Assessment for specific reproductive failure and subsequent treatment cycles

	Fertilization Failure	Poor Embryo Development	Implantation Failure	Pregnancy Loss
Couples	4	5	7	5
Sperm assessments				
PLCζ Presence (%)	10 ± 9	—	—	—
Mouse oocyte activation test positivity (%)	44.3 ± 42	—	—	—
Centrosome presence (%)	—	45.9 ± 5	53.1 ± 13	—
Ultrastructural abnormality (%)	—	—	70	—
Mutations	*ADAM15* *PIWIL1* *PLCZ1*	*HAUS1* *KIF4A* *XRN1* *SPAG17*	*MAP1S* *SUPT5H* *PLK4* *IL9R*	*NLRP7* *MARK4* *TRIP13* *POLD1* *DAB2IP* *KIF1C* *TP53*
Post assessment treatment				
Couples	4	1	0	0
Cycles	4	1	—	—
Oocytes retrieved	54	7	—	—
Metaphase-II oocytes (%)	40 (74.1)	6 (85.7)	—	—
Fertilization (%)	18 (45.0)	4 (66.7)	—	—
Cycles with ET	2	1	—	—
Clinical pregnancy (+FHB) (%)	2 (100)	1 (100)	—	—
Deliveries (%)	2 (100)	1 (100)	—	—

Note: Patients from the infertile cohort were categorized into 4 subgroups, based on the reasons for reproductive failure: fertilization failure, poor embryo development, implantation failure, and pregnancy loss. Ancillary sperm function assays and whole exome sequencing were performed on the ejaculated specimens and spermatozoal DNA, respectively. Consenting couples were treated in their subsequent cycles according to sperm assessment results. Couples from the first subgroup were treated with assisted gamete treatment by exposing both gametes to calcium ionophore to artificially induce calcium oscillations required for fertilization. Two couples have undergone embryo replacement thus far, and both have successfully achieved clinical pregnancies. One couple from the second subgroup elected to undergo a subsequent cycle, where we performed microfluidic sperm selection. They obtained 2 euploid conceptuses for transfer that resulted in clinical pregnancy. ET = embryo transfer, FHB = fetal heart beat.

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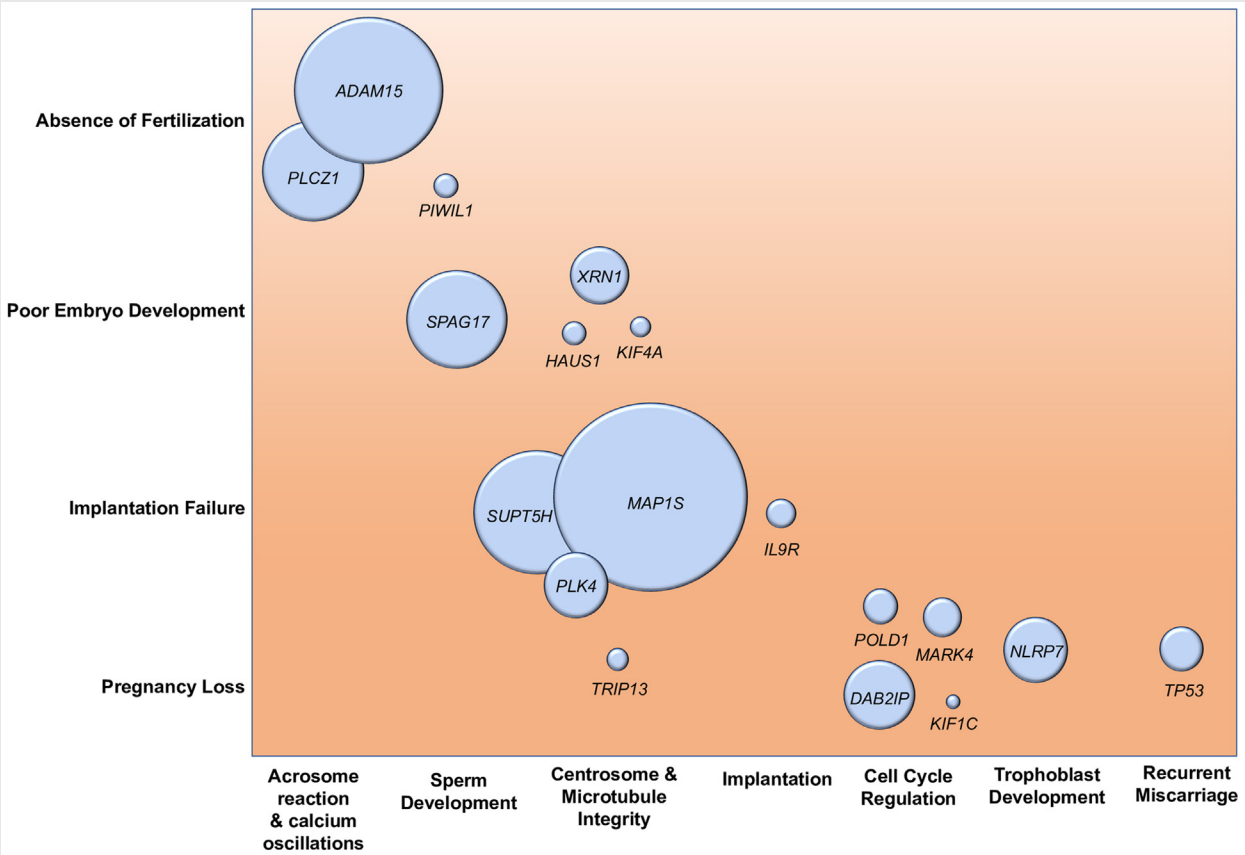
maturity rate of 76.5% (26/34), there was complete fertilization failure in all cycles. Most spermatozoa from men in this subgroup lacked PLCζ, as indicated by an average PLCζ expression of 10% ± 9%. These findings were validated by MOAT in which 44.3% ± 42% of oocytes reached the 2-cell stage, indicating a dysfunctional oocyte-activating capacity of the male gamete. A confirmatory sperm NGS assessment identified an average of 83.7 ± 56 mutations per patient on genes essential for sperm–egg binding (*ADAM15*) and the meiotic differentiation of spermatocytes (*PIWIL1*). We also identified mutations on *PLCZ1*, responsible for eliciting calcium oscillations and oocyte activation (Fig. 2). These mutations were neither identified in spermatozoa from men in the fertile cohort, nor were they present in the other infertile subgroups. Therefore, we treated these couples with assisted gamete treatment (AGT) by exposing both gametes to calcium ionophore to artificially induce calcium oscillations required for fertilization. We subsequently observed a 45% (18/40) fertilization rate. Two couples have undergone embryo replacement thus far, and both have successfully achieved clinical pregnancies.

In the second subgroup, 5 couples (maternal age, 37.4 ± 1 years; paternal age, 38.6 ± 1 years) underwent 5 ICSI cycles. An average of 7 oocytes were injected, yielding a fertilization rate of 80% (28/35). The resulting conceptuses were assessed by preimplantation genetic testing; however, none were recommended for transfer due to the presence of embryo aneuploidy. Our centrosome evaluation evidenced normal centriolar structures in only 45.9% ± 5% of cells assessed. This was corroborated by our sperm genetic assessment, which revealed an average of 52.0 ± 40 mutations per patient essential for centrosome integrity (*HAUS1*), spindle or microtubular stabilization (*KIF4A, XRN1*), and the function or structure of motile cilia (*SPAG17*). We neither identified these mutations within the fertile cohort, nor were these detected in the other infertile subgroups. One couple from this subgroup elected to undergo a subsequent cycle, where we performed microfluidic sperm selection. They obtained a 66.7% (4/6) fertilization rate, which generated 2 euploid conceptuses for transfer that resulted in clinical pregnancy.

The next subgroup consisted of 7 couples (maternal age, 37.0 ± 2 years; paternal age, 37.6 ± 2 years) who underwent a total of 8 ICSI cycles. Although all couples underwent embryo transfers, none achieved a clinical pregnancy and have yet to undergo subsequent cycles. Centrosome assessment evidenced normal centriolar structures in 53.1% ± 13% of spermatozoa assessed. Moreover, transmission electron microscopy revealed that approximately 70% of the cells were characterized by vacuolization, inclusion, and dysmorphic heads. The proximal centriole, when visible, appeared normal. Our sperm genetic assessment identified an average of 44.0 ± 13 mutations per patient on genes primarily involved in maintaining microtubule and centrosomal integrity (*MAP1S, SUPT5H, PLK4*). In addition, spermatozoa displayed mutations on *IL9R*, an interleukin receptor that has been increasingly implicated in embryonic implantation. These mutations were also exclusive to the spermatozoa from this subgroup.

FIGURE 2



Bubble plot portraying the gene mutations identified in the infertile cohort, according to the specific reasons for reproductive failure (y-axis). Bubble sizes correspond to the number of variants identified on that particular gene, and the categorization of gene functions are shown on the x-axis.

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The last subgroup comprised 5 couples (maternal age, 37.8 ± 3 years; paternal age, 38.0 ± 2 years) who were treated in 6 ICSI cycles. All patients successfully achieved clinical pregnancies; however, each resulted in pregnancy loss at 7 to 8 weeks postimplantation. The spermatozoa from these patients displayed an average of 36.3±10 mutations/patient in genes related to trophoblast development (*NLRP7*), cell cycle regulation (*MARK4, TRIP13, POLD1, DAB2IP, KIF1C*), as well as a gene linked to recurrent miscarriage (*TP53*). These genes were unaffected in spermatozoa from the other subgroups, as well as in the fertile cohort. Couples in this subgroup have not undergone subsequent cycles thus far.

DISCUSSION

A semen analysis is imperfect, yet it remains a pivotal component in the assessment of male infertility (33). Over the years, ancillary sperm assessments have gained popularity; however, each of these are able to assess only one facet of sperm reproductive potential. Attempts to query the genome and epigenome have also been made but are generally only prompted by the identification of a specific sperm defect or abnormal semen parameters, and mainly focus on the causes of spermatogenic failure and/or morphological abnormalities (34). As a result, men with normal sperm parameters are often excluded from extensive genetic testing. Moreover, current genetic assessments are performed exclusively on somatic cells. Of particular note, is the clear dichotomy between somatic cells and the germline, with the latter being regulated by crucial mechanisms that modify chromatin status without altering primary DNA sequences, therefore granting its transgenerational properties (35). Germline and somatic mutations also occur in different settings (36).

Despite the established ancillary assessments and genetic tests, the sperm-related reasons for poor ART outcome, regardless of a normal semen analysis, remain an area of uncertainty. Therefore, in this study we investigated the relationship between the sperm genetic profile and the reasons for reproductive failure in couples with negative infertility workups and normal sperm parameters.

Our comparison of the 2 study cohorts showed a significantly greater CNV incidence in spermatozoa from the infertile

group, despite there being no differences in semen parameters, further demonstrating that a routine semen analysis does not sufficiently measure sperm reproductive potential (37). Furthermore, spermatozoa from all patients, regardless of clinical outcome, carried mutations on genes involved in sperm–egg fusion and acrosomal development. This suggests that these mutations are not specific to the unexplained infertility condition, but rather to the necessity of ICSI, as defects in sperm–egg fusion and acrosomal development would affect sperm fertilizing capacity that can only be overcome by ICSI, and not by intrauterine insemination or standard IVF. Indeed, disruptions in the *ADAM* family of genes are associated with the inability of spermatozoa to migrate into the oviduct, whereas decreased *SPACA1* patterns are associated with poorer standard IVF outcomes (38, 39). Therefore, the presence of these mutations in our study population upholds ICSI as the optimal assisted reproduction technique, as it is known for being capable of overcoming sperm acrosomal dysfunction as well as other gametic defects (40).

To further evaluate the role of the male gamete in clinical outcome, we categorized the infertile couples according to the reasons for reproductive failure. Couples in the first subgroup represented rare incidences of total fertilization failure with ICSI, despite an adequate proportion of mature oocytes and normal sperm concentration. A negligible presence of PLCζ, validated by MOAT, confirmed a sperm-related oocyte-activating deficiency as the culprit for absent fertilization. Spermatozoa from all men in this subgroup also displayed mutations on genes involved in sperm–egg binding, spermatocyte differentiation, and most importantly, the elicitation of calcium oscillations for oocyte activation, which corroborated the immunofluorescence staining results. Spermatozoa that lack PLCζ can still successfully fertilize with the use of AGT, in which both gametes are exposed to calcium ionophore to artificially induce the calcium oscillations required for fertilization (13). When AGT was applied in the subsequent cycles of couples from this subgroup, fertilization was achieved, which ultimately yielded term pregnancies.

Our assessment for the second subgroup, presenting with reproductive failure due to poor embryo development, began with a sperm centrosome assessment. Aberrant centrosomes are associated with abnormal embryonic development (41). Additionally, the distal centriole in particular serves as the scaffold for the sperm flagellum and, if missing or abnormal, contributes to impaired sperm kinetics (42). We found that most of the spermatozoa lacked centrin marker expression, indicating an abnormal or absent centrosome. The mutations on *HAUS1*, involved in maintaining centrosome integrity, supported this observation. Additionally, the spermatozoa carried mutations impacting spindle and microtubular stabilization. Although it is important to consider that embryo aneuploidy ranges from mild to more severe forms, previous reports have shown that a microfluidic sperm processing technique can select spermatozoa with optimal genomic integrity and consequently improve clinical outcome, especially for couples presenting with a high incidence of embryo aneuploidy (43, 25). Therefore, we performed microfluidics sperm selection for the couple that elected to undergo a subsequent cycle, which successfully generated euploid conceptuses for transfer and ultimately led to a clinical pregnancy.

Spermatozoa from the third subgroup of couples, with reproductive failure due to poor implantation, also lacked expression of the centrin marker. Moreover, a large proportion of the sperm cells were characterized by ultrastructural abnormalities. We identified mutations on genes essential for maintaining microtubule and centrosomal integrity, which is unsurprising, given that successful implantation is partially dependent on embryo quality (44). However, mutations were also identified on the interleukin receptor gene, *IL9R*. The specific role of *IL9R* mutations, when carried by spermatozoa, on the conceptuses and their implantation remains unclear. *IL9R* is found on one of the pseudoautosomal regions at the ends of the gonosomes (Yq12/Xq28), and a pseudoautosomal region recombination deficiency has been linked to Klinefelter syndrome, suggesting their role in chromosomal segregation (45, 46).

Spermatozoa from men in the last infertile subgroup, with reproductive failure due to pregnancy loss at 7 to 8 weeks post-implantation, displayed the most mutations including those on genes related to cell cycle regulation, trophoblast development, and even recurrent miscarriage. In women, *NLRP7* mutations lead to defects in the trophoblast progenitor self-renewal process in early post implantation embryos (47). Mutations on genes involved in cell cycle regulation have been implicated in chromosomal abnormalities responsible for early pregnancy loss, and although *TP53* is a well-known tumor suppressor, variants on this gene have also been increasingly associated with recurrent miscarriages (47, 48). Our findings align with this trend, suggesting that the role of *TP53* in regulating cell proliferation extends to reproduction by influencing pathways essential for normal placentation. Indeed, a study on men from couples experiencing recurrent pregnancy loss identified polymorphisms and aberrant sperm methylation in imprinted embryo development-related genes, suggesting that epigenetic factors are involved (49). Regarding treatment options for this subgroup, microfluidics sperm selection may help decrease the risk of pregnancy loss by addressing sperm DNA damage (50). Alternatively, surgically retrieved spermatozoa are also characterized by better genomic integrity as well as a lower incidence of aneuploidy compared with their ejaculated counterparts, and may therefore be considered as an effective solution after consultation with a reproductive urologist (26, 27).

The limitations of this study mainly stem from its retrospective nature and relatively small number of subjects. Our study was aimed at infertile couples with idiopathic infertility, where a putative subtle male factor may be at play. Couples were selected according to their willingness to participate, and there was no difference in demographics between the participants and nonparticipants. This included ethnicity, socioeconomic status, age, and type of infertility, as well as semen parameters for the male partners, or hormonal profile, antimüllerian hormone level, and body mass index for the female partners. Subtle differences that were identified indicated a high total sperm motility as well as high proportion of oocyte maturity in the nonparticipant group, whereas the clinical

pregnancy rate was high among the participant cohort. However, this was a mathematical difference without any clinical relevance. Indeed, the delivery rates were ultimately comparable between the 2 cohorts. Therefore, although the study group represents approximately 2% of the population and is an obvious limitation of this study, the findings are still generalizable to the population queried. Although our post hoc analyses showed that the power to detect an effect of sperm aneuploidy on ICSI outcome was sufficient, at 0.92, results should still be prospectively validated in a larger study population, which would also allow for the identification of additional spermatozoal abnormalities that may have been previously missed. Although we attempted to control for female factors, we also cannot exclude that subtle male factors were exposed by the limited number of oocytes injected on average for these couples. The unique mutations identified in relation to the reasons for an unsuccessful ART outcome with ICSI obviously do not represent an association with unexplained infertility, but rather an attempt to unravel infertility indications that now fall under the "umbrella" of unexplained male infertility. Nevertheless, this is the first study to our knowledge that attempts to attribute the various causes of reproductive failure to mutations carried by spermatozoa. Our findings can potentially be incorporated into a single diagnostic test for men who are encouraged to pursue ICSI, replacing the need for multiple ancillary sperm assessments. However, due to the inherent variability within a spermatozoa population, it is important to determine the proportion of gametes that carry these mutations, especially for couples undergoing ICSI, where spermatozoa are individually selected. Therefore, future endeavors would include the utilization of single cell NGS to explore gamete heterozygosity. In addition, a somatic DNA analysis should be performed to determine whether any of the germline mutations identified in this study overlap with somatic mutations in the same genes. Publicly accessible databases of somatic and germline mutations consist of many shared variants primarily because DNA has basic chemical vulnerabilities that are identical in both settings (36). Although there were no abnormalities identified in the participants' karyotype analyses, several of the altered genes presented in our manuscript have also been identified in the peripheral blood of men with infertility. For instance, *PLCZ1* mutations are commonly implicated as a major cause of fertilization failure, and alterations on *PIWIL1* have been associated with impaired histone-to-protamine exchange during spermiogenesis (51, 52). There is also increasing evidence that deleterious variants on *HAUS1* are pathogenic for idiopathic nonobstructive azoospermia (53). However, these studies were exclusively conducted on somatic cells, and consequently lack information specific to the gamete genome. Therefore, in this study we focused on the exclusive description of germline mutations and their effect on reproductive outcome. Although the specific mutations identified in our study are novel, we nonetheless plan to perform a comparison between the somatic and germ cell mutations within the same individual to understand an eventual relationship between the 2 types of abnormalities and validate the diagnostic value of our spermatozoal DNA assessment. Furthermore, although spermatozoa from the fertile cohort were characterized by a low incidence of CNVs and did not appear to carry the gene mutations identified in the infertile cohort, further studies focusing on the frequency of sperm abnormalities in couples successfully treated by ICSI should still be considered. On a related note, ancillary sperm tests were not performed on the fertile cohort, and although we used normal thresholds established from our previous assessments on PLCζ presence, centrosome integrity, and ultrastructural abnormalities in known fertile individuals, we cannot exclude that the fertile cohort described in this particular study may present with some variability (13, 17, 54, 55).

The lack of assessments on the male gamete is partially attributed to the fact that spermatogenesis is a complex process, controlled by well-coordinated transcriptional and post-transcriptional regulators. Nevertheless, the spermatozoon is not simply a carrier that delivers the male genome to the oocyte and should therefore not be overlooked (56). Although an array of ancillary tests can be used to evaluate spermatozoa, they can be tedious and inaccurate, and are each capable of assessing only a single facet of sperm reproductive potential. By sequencing the sperm exome, we identified candidate genes associated with the different causes of reproductive failure in couples with normal infertility workups and semen parameters. In addition, the novel variants identified in our study population may lay the foundation for future gene therapy research. Furthermore, our findings would allow for the design of a custom gene panel for targeted sperm DNA sequencing. This clinical panel, used prospectively at the time of semen analysis and before the start of the patients' cycles, would encompass all of the altered genes identified in this study and could discern relevant genetic changes that can streamline male infertility clinical management. Most importantly, screening spermatozoa for these mutations would serve as a useful precision medicine tool to enhance the diagnosis, treatment, and prediction of clinical outcome for couples with unexplained infertility.

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Perfilar el genoma de la línea germinal masculina para descifrar su potencial reproductivo.

Objetivo: Identificar mutaciones germinales específicas relacionadas con la competencia reproductiva de los espermatozoides, en parejas con infertilidad inexplicada.

Diseño: En este estudio retrospectivo, las parejas se dividieron según si tenían resultados exitosos de inyección intracitoplasmática de espermatozoides (fértiles) o no (infértiles). Se realizaron pruebas auxiliares de función de los espermatozoides en los eyaculados, y se realizó la secuenciación completa del exoma en el ADN de los espermatozoides. Los perfiles de aneuploidía y mutación genética de los espermatozoides se compararon entre las 2 cohortes, así como de acuerdo con las razones específicas del fracaso reproductivo.

Entorno: Centro de medicina reproductiva en un importante centro médico académico.

Paciente(s): Treinta y una parejas con exámenes negativos de infertilidad y parámetros normales de semen.

Intervención(es): Las parejas con mutaciones en genes relacionados con la fertilización o el desarrollo embrionario fueron tratadas posteriormente mediante tratamiento asistido con gametos o microfluídica, respectivamente.

Principales medidas de análisis: Resultados del ciclo de inyección intracitoplasmática de espermatozoides, incluida la fertilización, el embarazo clínico y las tasas de parto.

Resultado(s): La aneuploidía espermática fue menor en el grupo fértil (4,0% vs. 8,4%). Los espermatozoides de ambas cohortes mostraron mutaciones asociadas con la fusión espermatozoide-óvulo (ADAM3A) y el desarrollo acrosomal (SPACA1), independientemente del resultado reproductivo. La cohorte infértil se clasificó de acuerdo con las razones del fracaso reproductivo: ausencia de fertilización, desarrollo embrionario temprano deficiente, fracaso de implantación o pérdida del embarazo. Los espermatozoides del subgrupo de fracaso de fertilización ($n = 4$) tenían una presencia insignificante de PLCζ (10% ± 9%) y mutaciones genéticas (PLCZ1, PIWIL1, ADAM15) que indican una deficiencia de activación de ovocitos relacionada con los espermatozoides. Estas parejas fueron tratadas con éxito mediante tratamiento asistido de gametos en sus ciclos posteriores. Los espermatozoides del subgrupo de desarrollo embrionario temprano deficiente ($n = 5$) tenían centrosomas anormales (45,9% ± 5%) y mostraban mutaciones que afectaban la integridad del centrosoma (HAUS1) y la estabilización del huso / microtubular (KIF4A, XRN1). El procesamiento microfluídico de espermatozoides posteriormente produjo un embarazo a término. Los espermatozoides del subgrupo de fracaso de implantación ($n = 7$) también tenían centrosomas anormales (53,1% ± 13%) y portaban mutaciones que afectaban a la implantación embrionaria (IL9R) y a los microtúbulos y la integridad centrosomal (MAP1S, SUPT5H, PLK4), mientras que los del subgrupo de pérdida de embarazo ($n = 5$) mostraban mutaciones en genes implicados en el desarrollo de trofoblastos (NLRP7), regulación del ciclo celular (MARK4, TRIP13, DAB2IP, KIF1C), y aborto recurrente (TP53).

Conclusión(es): Al evaluar el genoma del espermatozoide, identificamos mutaciones específicas de la línea germinal relacionadas con diversos procesos reproductivos. Esta información puede aclarar los factores elusivos que subyacen a la competencia reproductiva y mejorar el tratamiento para las parejas con infertilidad inexplicable.