

Genetic and  
epigenetic molecular  
marks providing  
information on the  
fertility com...



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## **Abstract**

### **Study question:**

Can the assessment of genetic and epigenetic molecular marks provide information on the fertility competence of the male gamete?

### **Summary answer:**

Aneuploidy assessment together with the estimation of specific gene mutations and function helps to predict the competence of the male gamete to participate in embryo development.

### **What is known already:**

Evaluation of the reproductive quality of the male gamete by standard semen analysis is often inadequate to predict ART outcome. Men may be prone to meiotic error and have a higher proportion of spermatozoa with fragmented chromatin capable of affecting the conceptus' health. In men with unexplained infertility, supplementary tests may be pivotal to gain insight into the paternal contribution to the zygotic genome.

### **Study design, size, duration:**

In a 23 month period, a total of 113 consenting men were included in the study with an additional 5 donor specimens used as a control. Among the study participants, 87 were screened for sperm aneuploidy and were ranked according to their increasing age. A total of 16 men were assessed by whole genome karyotyping and categorized according to their reproductive outcome as either fertile or infertile. Two anonymous donor specimen with proven fertility served as a control. Another set of men (n= 10) had their gene expression analyzed by RNA-seq and were profiled according to their

reproductive capacity in comparison to three couples utilizing a proven fertile donor specimen.

**Participants/materials, setting, methods:**

Specimens were obtained from consenting men. Anonymous donor specimens with proven fertility were used as a control. FISH analysis was performed on at least 1000 sperm cells with a threshold of 1.6% for chromosomes X, Y, 13, 15, 16, 17, 18, 21, and 22. Men were ranked according to their increasing age in order to compare disomy and diploidy. DNA was extracted from as few as 500 spermatozoa by PCR-based random hexamer amplification. RNA was isolated from  $7.25 \times 10^6$ /mL human spermatozoa using a spin column commercial kit, and processed by RNA-seq by next generation sequencing using an illumina platform. Expression values were calculated in fragments per kilobase of transcript per million mapped reads (FPKM). ART was carried out by ICSI where fertilization rates and pregnancy characteristics were recorded.

**Main results and the role of chance:**

FISH results revealed that the average aneuploidy rate was highest for men in the over-55 age group (9.6%). Moreover, men in the over-55 age group had the highest average disomy specifically for chromosomes 17 (1.2%) and 18 (1.3%). ART results for the entire cohort were comprised of 157 ART cycles in which outcome was stratified by paternal age. The youngest age group (25-30 years old) had a fertilization rate of 87.7% which decreased to 46.0% in the over-55 age group. Accordingly, the clinical pregnancy rate was highest in the 25-30 age group (80.0%) while no pregnancies were attained in the over-55 age group. Furthermore, the rate of pregnancy loss

was characterized by a steadily increasing trend, highest in the 51-55 age group (50.0%).

Assessment by NGS was performed on a cohort of patients classified as having recurrent pregnancy loss and compared to patients successfully treated by ART as well as a control. The average male age was  $38.3 \pm 7$  yrs and female age  $37.5 \pm 5$  yrs. Six couples in 15 ICSI cycles achieved a clinical pregnancy rate of 80% while ten infertile couples treated in 21 cycles achieved a pregnancy rate of only 23.8% however, all resulting in pregnancy loss. DNA sequencing, yielded an overall aneuploidy of 4.0% for the fertile and 8.6% for the infertile group ( $P < 0.00001$ ). DNA duplications at 2.3% in the control progressively increased from 8.4% in the fertile cohort to as high as 95% in the infertile group ( $P < 0.00001$ ). DNA deletions progressively increased from the control at 4.4% to 6.1% in the fertile and reaching 92.5% in the infertile cohort ( $P < 0.00001$ ). In addition, we were able to identify 17 genes that reported the highest mutation rate in the infertile cohort. These genes engaged in key roles of gametogenesis as well as fertilization and embryo development.

RNA data for 10 men with normal semen analyses comprised 5 men unable to attain a pregnancy after ART and 5 men who successfully sustained a term pregnancy in comparison to a control group ( $n = 3$ ). The analysis resulted in a total of 86 differentially expressed genes ( $P < 0.001$ ). Of them, 24 genes were overexpressed and 62 under-expressed in the infertile cohort when compared to the fertile cohort and control. Specifically, DNA repair genes (APLF, CYB5R4, ERCC4 and TNRF5F21) and apoptosis modulating

genes (MORC1, PIWIL1 and ZFAND6) were remarkably under-expressed ( $P < 0.001$ ).

**Limitations & reasons for caution:**

This study identified limitations of FISH aneuploidy screening in predicting male reproductive potential. As it may be expected but not undeniably documented, advancing paternal age affected spermatogenic meiosis, represented almost exclusively by non-disjunction with consequent inability to fertilize and support a successful implantation. While genomic sequencing procedures appear relevant to indicate the reproductive competence of the male gamete, the relatively wide number of genes involved and the limited observations require confirmation on a larger study population.

**Wider implications of the findings:**

In infertile couples where the female partner presents with a normal workup and the male partner with an uninformative semen analysis, the utilization of ancillary tests may offer additional insight. Indeed, sperm aneuploidy assessment supported by information on specific gene mutations may indicate the subtle dysfunctions of the spermatozoon. By assessing sperm RNA it is possible to estimate the performance of DNA repair genes as well as spermatogenesis-related genetic defects with consequence on the proper development and function of specific gamete organelles. Finally, by querying non-coding RNA we may gather knowledge on the embryo developmental competence of the male gamete, providing crucial information on the etiology of unexplained infertility and overall reproductive capacity of the infertile male.

Study funding/competing interest(s): University Hospital.

Trial registration number: N/A

## **Introduction**

About 15% of the general reproductive aged population has fertility problems. The American Society for Reproductive Medicine estimates that male and female factors contribute about equally to this condition, with approximately one-quarter likely being a combination of factors from both partners. After 12 months of unprotected intercourse without pregnancy, affected couples typically begin to seek care and explore the possibility of fertility treatments.

Typically, to establish the appropriate clinical treatment and minimize the risk of failure, an extensive evaluation of the female, and to a lesser extent the male, is undertaken. If no severe male or female factors are detected, fertility treatments such as timed intercourse (TIC) or intrauterine insemination (IUI) are recommended in combination with ovarian stimulation. After three or four unsuccessful IUI cycles particularly if a severe male or female factor is detected, in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) are typically considered.

Current clinical practice focuses on whether there are sufficient spermatozoa with satisfactory motility and morphology to reach and likely fertilize the oocyte. The utility of the semen analysis [3] in selecting the least invasive fertility treatment for idiopathic infertile couples appears limited [5].

Moreover, semen analysis rarely predict the functioning or fertilizing capacity of the male gamete. This is particularly evident in cases of unexplained  
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infertility where both the male and female partner have normal results for all conventional tests. Due to these reasons, several investigators have begun to explore the genetic basis of male infertility and therefore utilize additional tests to gain more insight toward the reproductive capacity of the individual. Other specific measures that may complement the workup include DNA fragmentation, the presence of anti-sperm antibodies, endocrine status, and detection of AZF microdeletions on the Y chromosome [6].

The difficulty in establishing male gamete competence may be due to the fact that spermatogenesis is a complex differentiation process commonly divided into three main phases: self-renewal and proliferation of spermatogonia, meiotic division of spermatocytes, and post-meiotic differentiation of spermatids into spermatozoa. These events are controlled by well-coordinated transcriptional and post-transcriptional regulators.

Spermatozoa are not just a vehicle that delivers the male genomic contribution to the oocyte. Upon fertilization, the spermatozoon provides a complete, highly structured, and epigenetically marked genome that, together with a defined complement of RNAs and proteins, plays a distinct role in early embryonic development.

Although several studies have explored the effect of genetic variants such as single-nucleotide polymorphisms (SNPs), copy number variants, differential genome packaging, differential methylation, proteomic changes, and differential sperm RNAs in infertile men, comparatively few have examined the effect of such assays within the context of the reproductive clinic.

We hypothesize that through genetic and epigenetic analysis, we will be able to identify mutations and differential expression of specific genes in men presenting with unexplained infertility compared to those successfully treated by ART and a fertile control, leading to a better insight into the etiology of male infertility.

## **Materials and Methods**

### **Patients**

From December 2014 to November 2016, a total of 113 sperm samples from male partners of couples with a history of prior ART failure or recurrent pregnancy loss were evaluated. The male partners' semen parameters were evaluated according to most recent criteria [3]. Ejaculated specimens were prepared for FISH assessment as well as DNA and RNA extraction for NGS sequencing (Repli-G Single Cell and RNEasy kits; Qiagen, Hilden, Germany). The mean male age was  $38.3 \pm 7$  years. Additionally, 5 specimens from donors with proven fertility were similarly processed and served as a control. All patients had a peripheral karyotype carried out and informed consent was obtained. This study was approved by the Internal Review Board of our institution.

### **Semen Collection and Preparation**

Ejaculates provided by masturbation were centrifuged in 1: 1 dilution with human tubal fluid medium buffered with HEPES (H-HTF; Irvine Scientific, Santa Ana, CA) at 300g for 20 minutes on a single layer density gradient (Enhance-S Plus Cell Isolation Media, 90%; Vitrolife, San Diego, CA). Motile spermatozoa were retrieved by aspirating the 90% fraction with a fire-polished Pasteur pipette, rinsed in H-HTF, centrifuged for 5 minutes at 500g, <https://assignbuster.com/genetic-and-epigenetic-molecular-marks-providing-information-on-the-fertility-competence-of-the-male-gamete/>



and resuspended in PBS preferably to a concentration of approximately  $10 \times 10^6$  spermatozoa/ml.

### **Preparation of spermatozoa for FISH analysis**

For FISH analysis, 5 $\mu$ l of washed semen or surgically retrieved spermatozoa was smeared on precleaned glass slides and allowed to dry overnight. Slides were fixed in Carnoy's fixative (3: 1 methanol: acetic acid) for 15 minutes at room temperature, and placed on a 37°C slide moat overnight. Sperm nuclei were decondensed by slide incubation for 3 minutes at room temperature in 5 mmol/L dithiothreitol (DTT; Sigma Chemical Co., St. Louis, MO) in 0. 1M tris(hydroxymethyl)aminomethane (Trizma HCl; Sigma Chemical Co.), followed by 3M Sodium Chloride and 300mM tri-Sodium citrate dehydrate (2X standard saline citrate; Vysis, Downers Grove, IL) pH 7. 0 for 1 minute at room temperature. Excessive agitation of the slides was avoided in all decondensation and washing steps in an effort to limit sperm loss, especially from smears performed with testicular spermatozoa. Decondensed slides were hybridized with probes specific to chromosomes X, Y, 13, 15, 16, 17, 18, 21, and 22 for 5 minutes at 37°C. Sperm nuclei were then counterstained with 7 ul of 4', 6-diamino-2-phenylindole (DAPI) and cover-slipped. Using an Olympus BX61 fluorescent microscope at 1000X, numerical abnormalities for these chromosomes in at least 1000 spermatozoa for each specimen as well as 2 anonymous donor controls were assessed, with a threshold of 1. 6% while maintaining a 2-3% FISH error. The incidence of disomy, nullisomy, and diploidy was recorded (Applied Imaging, CytoVysion v3. 93. 2).

**Preparation of spermatozoa for TUNEL analysis**

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was carried out according to a protocol previously reported [23]. Briefly, 5 $\mu$ l of raw semen sample was smeared on precleaned glass slides and allowed to dry overnight. Slides were then fixed in 4% paraformaldehyde for one hour and left to dry once more overnight. The slides were permeabilized in a solution of 0.1% Triton X-100 and 0.1% Sodium Citrate in PBS for two minutes, washed, and allowed to dry. The kit reagent was then added to the slides (In Situ Cell Death detection Kit; Roche Diagnostics, Rotkreuz, Switzerland), a coverslip added, placed in a humidified chamber and stored at 37°C for one hour and subsequently washed with PBS. Upon drying, 7 $\mu$ l of DAPI antifade solution was added to counterstain nuclei and coverslips applied. Slides were screened under a fluorescent microscope for green fluorescence indicating chromatin fragmentation, with a threshold of 15%.

**Whole Molecular Karyotype by NGS**

Study specimens and 2 donor controls were processed by centrifugation in human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA) at 600g for 10 minutes. After adjusting the concentration to 500 cells/mL for each sample, DNA extraction and amplification was achieved with the use of a commercial kit (Repli-G Single Cell; Qiagen, Hilden, Germany) through PCR-based random hexamer amplification. Decondensation was carried out by incubating specimens with Dithiothreitol (DTT) at 65°C for 10 minutes. Following amplification, DNA was submitted for quality control testing where a DNA concentration of 447.8 $\pm$ 198ng/ $\mu$ l and purity of 1.7 $\pm$ 0.1 was confirmed. Specimens were processed by Next Generation Sequencing

technology and aneuploidy was assessed by recording the Copy Number Variations (CNVs) with CASAVA and VarScan2 software (The Genome Institute, St. Louis, Missouri). This analysis allowed for whole genome molecular karyotyping as well as detection of specific gene mutations.

### **RNA Extraction in Human Spermatozoa**

Seven to  $25 \times 10^6$  human sperm cells were lysed by  $\beta$ -mercaptoethanol for 5 minutes, and total RNA was isolated using a hybrid protocol with warmed ( $37^\circ\text{C}$ ) TRIzol® Reagent (ThermoFisher Scientific, USA) and homogenized by vigorous vortexing. Ethanol (70%) was added to each tube of the homogenized cells and mix well by pipette. Impurities were removed with RW1 buffer (Qiagen) followed by RPE buffer. Total RNA purified using an RNeasy Mini Kit spin column (RNEasy; Qiagen, Hilden, Germany), at room temperature. Ribonucleic acid was eluted with 30ul of RNase free water directly onto the columns followed 1-minute spin at 11, 000 rpm x 1 min at  $4^\circ\text{C}$ . The nucleic acid was quantified by an Agilent 2100 bioanalyzer to determine RNA integrity number (RIN). Spermatozoal RNA concentration was calculated by a NanoDrop spectrophotometer and confirmed using Qubit RNA assay. RNA samples were then made into paired-end libraries using oligo-dT to capture mRNA poly-A tails (TruSeq RNA Library Prep Kit, Illumina, San Diego, CA). Pilot paired-end 36bp RNA-sequencing was carried out (NextSeq 500; Illumina, San Diego, CA) and expanded to 50-60 M reads at  $2 \times 75$  bp. Expression profiles were compared between the study and control groups. Sample standardization was achieved by accounting for the coefficient of variation of biological variation between the samples. To avoid the possibility of over- or under-representing FPKM, an algorithm by edgeR

(LGPL; Bioconductor) and CONTRA was implemented which is able to overcome experimental conditions such as fragmentation.

### **Ovarian Stimulation and Oocyte Preparation**

Oocyte retrieval was performed after ovarian superovulation with gonadotropins and pituitary desensitization with GnRH-agonists or antagonists. The choice of a stimulation protocol was dependent on patient age, etiology of infertility, and previous treatment history. For all patients, one of several established stimulation protocols was utilized; lupron downregulation, microflare lupron or antagonist. Human chorionic gonadotropin (hCG) was administered (3, 300–10, 000 IU) when at least two follicles had reached or exceeded 16- to 17-mm diameter as observed by ultrasound. Dosage of hCG was tailored according to estradiol (E2) level and body mass index. Oocyte retrieval was performed approximately 35 to 36 hours after hCG administration via transvaginal needle guided aspiration. These oocytes were then exposed to 40 IU recombinant hyaluronidase (Cumulase, Halozyme Therapeutics, Inc. San Diego, CA) to remove cumulus-corona cells in a previously defined manner prior to ICSI (Palermo, et al., 1999).

### **Pregnancy Assessment and Luteal Support**

Starting on the day of oocyte retrieval, methylprednisolone (16 mg/day) and tetracycline (250 mg every 6 hours) were administered for 4 days to all patients. Progesterone administration (25–50 mg I. M./day) was started on day 3 after hCG administration and was continued until the establishment of pregnancy. A serum  $\beta$ hCG assay was performed 14 days after the ovum pick-up. A biochemical pregnancy was defined as a positive  $\beta$ hCG level that

decreased prior to when an ultrasound could detect an implantation site. A clinical pregnancy was defined as the presence of a fetal heartbeat by ultrasound assessment during the 7th week of gestation.

## **Data Analysis**

The CNV assessment was done using a computer algorithm (CONTRA; GPLv3, Bioinformatics) which includes a module that compares the study group copy number gains and losses to base-level log-ratios created from the control group. The CNVs were then ranked according to these log-ratio values and the corresponding genes were noted and grouped by function. RNA analysis was performed using Cufflinks (v2. 0. 10; CBCB, College Park, MD) with specific focus on non-coding RNA and measured the abundance of small non-coding RNA (sncRNA) and long non-coding RNA (lncRNA). Gene expression values were calculated in Fragments Per Kilobase of Exon of transcript per million mapped reads (FPKM) and normalized read counts, followed by differential gene expression analysis using open-source software bioinformatics tools Tophat and Cufflinks, respectively. Statistical thresholds of  $P < 0.0005$  for significance and  $Q < 0.05$  threshold for false positive discovery were used. Chi-square analysis with the Yate's correction using the Sigma Stat program (Jandel Scientific, San Rafael, CA) was used to evaluate all hypotheses. Pearson's correlation coefficient and unpaired t-test were also used to compare the control and study groups (Graphpad Software, San Diego, CA). A P value of  $< 0.05$  was considered to be statistically significant.