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## Whole exome sequencing improves the diagnosis and care of men with non-obstructive azoospermia

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## ABSTRACT

Non-obstructive azoospermia (NOA) is a severe and frequent cause of male infertility, often treated by testicular sperm extraction followed by intracytoplasmic sperm injection. The aim of this study is to improve the genetic diagnosis of NOA, by identifying new genes involved in human NOA and to better assess the chances of successful sperm extraction according to the subject's genotype. Exome sequencing was performed on 96 NOA subjects negative for routine genetic tests. Bioinformatics analysis was limited to a panel of 151 genes selected as known causal or candidate genes for NOA. Only highly deleterious homozygous or hemizygous variants were retained as candidates. A likely causal defect was identified in 16 genes in a total of 22 subjects (23%). Six genes had not been described in man (*DDX25*, *HENMT1*, *MCMDC2*, *MSH5*, *REC8*, *TDRKH*) and 10 were previously reported (*C14orf39*, *DMC1*, *FANCM*, *GCNA*, *HFM1*, *MCM8*, *MEIOB*, *PDHA2*, *TDRD9*, *TERB1*). Seven subjects had defects in genes from piwi or DNA repair pathways, three in genes involved in post-meiotic maturation and 12 in meiotic processes. Interestingly, all subjects with defects in meiotic genes had an unsuccessful sperm retrieval, indicating that genetic diagnosis prior to TESE could help identify individuals with low/null chances of successful sperm retrieval and thus avoid unsuccessful surgeries.

**Key words:** Genetics of male infertility, non-obstructive azoospermia, whole exome sequencing, genetic diagnosis.

## INTRODUCTION

Male infertility is considered by the World Health Organization (WHO) as a global health concern affecting more than 50 million couples worldwide. Non-obstructive azoospermia (NOA), defined by the complete absence of spermatozoa in the ejaculate, even after centrifugation of the semen sample followed by microscopic examination of the pellet <sup>1</sup>, occurs in approximately 1% of men and 10% of infertile men <sup>2</sup>. It is a highly heterogeneous condition with a broad genetic basis. Spermatogenesis is a highly complex process comprising three main successive steps: spermatocytogenesis that allows the proliferation and growth of spermatogonia, meiosis (I and II) which produces haploid cells, and spermiogenesis during which round spermatids undergo numerous biochemical and morphological changes including chromatin compaction, acrosome biogenesis and flagellum assembly and elongation, to produce mature spermatozoa. Defects in any of these complex and specialized processes can prevent the production of sperm cells and induce NOA, which is expected to be extremely genetically heterogeneous. Consistent with these observations, mutations in more than 600 genes were shown to decrease fertility in animal models <sup>3</sup> and 2274 genes present an elevated expression in testis, including 474 genes that are only detected in testis <sup>4</sup>. Currently, only two genetic analyses are performed routinely for NOA individuals: a karyotype which identifies sex chromosome anomalies, in particular Klinefelter syndrome (47 XXY) and various translocations, and the search for microdeletions in the AZF region. Using these tests, a diagnosis is obtained for approximately 20% of the studied subjects <sup>5</sup> suggesting that the overwhelming majority of subjects remains undiagnosed.

Regardless of the diagnosis results or lack thereof, most couples want to initiate a pregnancy if possible with their own gametes. For NOA individuals, the only option to achieve intra-couple pregnancy is to perform testicular sperm extraction (TESE) followed by in vitro fertilization (IVF) performed using intracytoplasmic sperm injection (ICSI), but success rates remains low and sperm cells are only found in 30-50% of cases <sup>5</sup>. The whole TESE-ICSI procedure is invasive, lengthy, psychologically wounding and expensive and should be avoided when the chances of success are too low. Renouncing TESE is a difficult decision but it is the physician's responsibility to only propose a medical procedure if the expected gain outweighs the risks. In the absence of a precise diagnosis, the practitioner has no predictive information and in many cases, a TESE-ICSI will be unsuccessfully performed rather than moving faster

toward the alternative procedures that are sperm donation or adoption. In addition, infertile men, and by extension their relatives and potential offspring, are at higher risk of related adverse health issues requiring preventive follow up or specific treatments. This is well established for Klinefelter syndrome, Kallmann syndrome or primary ciliary dyskinesia but increasing evidence also points to important links between cancer-risks and infertility <sup>6</sup>. Genetic diagnosis is therefore essential, especially when associated with a prognostic value, to provide informed counselling to individuals undergoing TESE and/or endocrine therapy<sup>7</sup>. In the past decade, the overwhelming development of high-throughput sequencing and in particular the use of whole exome sequencing (WES) has permitted the identification of many pathological genes in the field of male infertility <sup>8,9</sup>. Recent cohort studies or familial studies also demonstrated the relevance of WES and highlighted the involvement of additional genes in the etiology of NOA <sup>10-12</sup>. In view of the extreme genetic heterogeneity of NOA and the increasing affordability of the technique, we believe that WES should now be included in the panel of genetic techniques proposed to infertile men.

Here, we recruited 96 infertile men with idiopathic NOA who had undergone TESE, and performed WES on each one. To facilitate data analysis and to focus on high confidence gene defects we defined a list including 151 candidate genes and we considered only highly deleterious homo or hemi-zygous variants.

## **SUBJECTS AND METHODS**

### **Study subjects**

A total of 96 unrelated men originating from North Africa affected by primary infertility and displaying non-syndromic non-obstructive azoospermia were recruited and treated at the “Clinique les Jasmins” in Tunis, Tunisia according to the established routine protocol. All subjects had a normal karyotype and no microdeletion of the Y chromosome. None declared to have any other health defect. In particular, they did not present anosmia, any disorder of sex development or abnormal secondary sex characteristics. Details on all subjects is provided in Supplementary Table 1.

Informed consent was obtained from all the individuals participating in the study according to local protocols and the principles of the Declaration of Helsinki. The study was approved by local ethics committees, and samples were then stored in the Fertithèque collection declared

to the French Ministry of health (DC-2015-2580) and the French Data Protection Authority (DR-2016-392).

All subjects had two sperm collections realized at least two months apart, to establish the diagnosis of azoospermia. Semen was collected by masturbation after three days of sexual abstinence. Semen samples were incubated for 30 min at 37°C for liquefaction and then centrifuged at 3000 g for 15 min.

### **Tissue samples collection and histological examination**

All samples were obtained during routine therapeutic testicular sperm extraction (TESE) from all subjects recruited in the study. In all cases, a micro-TESE procedure was performed using a 6-fold magnifying loupe as described previously<sup>13</sup>.

Tissues were fixed by immersion in 4% paraformaldehyde (PFA) for 14 h, embedded in paraffin, and sectioned (5 µm). For histological analysis, after being deparaffinized, slides were stained with hematoxylin and eosin. The colored sections were analyzed under light microscope.

### **Gene panel design and predictive outcome**

Two independent reviewers conducted a literature search in Pubmed using the following keywords: (azoospermia gene) OR (non-obstructive azoospermia gene) OR (genetics of male infertility) OR (meiotic gene). The MGI database describing the phenotypes of KO mice was also interrogated using the keyword “azoospermia”. The search was performed on several occasions, the last search occurring on July 1<sup>st</sup> 2021.

### **Whole exome sequencing and variants filtering**

Genomic DNA was isolated from EDTA blood using the DNeasy Blood & Tissue Kits from QIAGEN SA (Courtaboeuf, France). Genetic data were obtained from various sequencing centers, in particular Novogene and Integragen. Coding regions and intron/exon boundaries were sequenced after enrichment using SureSelect Human All Exon V6 –from Agilent.

An alignment-ready GRCh38 reference genome (including ALT, decoy and HLA) was produced using "run-gen-ref hs38DH" from Heng Li's bwakit package (<https://github.com/lh3/bwa>). The exomes were analyzed using a bioinformatics pipeline

developed in-house. The pipeline consists of two modules, both distributed under the GNU General Public License v3.0 and available on github.

The first module (URL of this primary module is indicated in the Web resource section) takes FASTQ files as input and produces a single merged GVCF file per variant-caller, as follows. Adaptors are trimmed and low-quality reads filtered with fastp 0.20.0 [FASTP]<sup>14</sup>, reads are aligned with BWA-MEM 0.7.17 [BWA-MEM]<sup>15</sup>, duplicates are marked using samblaster 0.1.24 [SAMBLASTER]<sup>16</sup>, and BAM files are sorted and indexed with samtools 1.9 [SAMTOOLS]. SNVs and short indels are called from each BAM file using strelka 2.9.10 [STRELKA]<sup>17</sup> and GATK 4.1.8.1 to produce individual GVCF files from each variant-caller. These are finally merged with mergeGVCFs.pl to obtain a single multi-sample GVCF per caller. Using several variant-callers allows to compensate for each caller's flaws.

The second module (URL of this secondary module is indicated in the Web resource section) takes each merged GVCF as input and produces annotated analysis-ready TSV files. This is achieved by performing up to 15 streamlined tasks, including the following. Low-quality variant calls (DP<10, GQ<20, or less than 15% of reads supporting the ALT allele) are discarded. Variant Effect Predictor v104<sup>18</sup> is used to annotate the variants and predict their impact, allowing to filter low-impact (MODIFIER) variants and/or prioritize high-impact ones (e.g. stop-gain or frameshift variants). Variants with a minor allele frequency greater than 1% in gnomAD v2.0 or 3% in 1000 Genomes Project phase 3 are filtered. Additional information can be found in<sup>19</sup>. Copy number variants (CNVs) were searched using the ExomeDepth software package as previously reported<sup>20,21</sup>.

### **Sanger verification of candidate variants**

Candidate variants were subjected to Sanger verification using an Applied Biosystems 3500XL Genetic Analyzer. Analyses were performed using SeqScape software (Applied Biosystems). Sequences of primers used and expected product sizes are summarized in Supplementary Table 2.

## **RESULTS:**

### **Established gene panel**

An initial search using selected keywords selected 20,595 manuscripts. Based on titles and abstracts, we excluded all manuscripts describing genes related to other spermatoc

phenotypes, genetic associations and risk factors, chromosome anomalies, AZF deletions, CNVs affecting multiple genes and syndromic NOA, leading to the selection of approximately 1000 manuscripts, which were analysed in detail. From these eligible publications, we extracted the gene names, inheritance pattern, the testicular histological information when available, animal models if studied, functional tests if performed, the ethnicity and geographic origin of the investigated subjects, sporadic cases or familial cases, nature of the identified variants. Data from the MGI database (<http://www.informatics.jax.org/>) describing the phenotypes of KO mice using the keyword “azoospermia” yielded 313 hits. The described phenotypes were analysed and correlated with the characteristics of generated knock-out mice (conditional/spontaneous mutation etc.). Candidate genes were only retained if strong evidence was available from the studied documents. Overall a total of 151 genes were selected as likely implicated in NOA (Supplementary Table 3). This list is available as an open evolving resource that can be updated by all actors of the field in a free access URL (see the link in the Web Resources section at the end of the manuscript). A STRING analysis was performed on all the selected gene products (Figure 1), highlighting numerous interactions between many of the selected proteins.

### **Exome sequencing**

After variant filtering, each person carried on average 62 homozygous and 1497 heterozygous variants. Overall, among these variants 3.3% were classified by Variant Effect Predictor as having a HIGH impact (alleles with such variants are not expected to produce a functional protein). In addition 7.2% of variants were classified as having a likely deleterious effect (L-HIGH): this includes missense variants predicted as deleterious by at least 3 methods among SIFT, PolyPhen, CADD, mutationTaster or REVEL, as well as splice-region variants predicted deleterious by both *ada* and *rfmethods* from *dbSNV*<sup>22</sup>. On average, each person carried 2.1 homozygous HIGH variants and 3.6 homozygous L-HIGH variants passing all previous filters. We identified a total of 25 heterozygous HIGH variants and 47 L-HIGH heterozygous variants in one of the 151 candidate genes. At this stage, as the confirmation of the segregation of the variants was not possible (to establish if the variants were *de novo*, mother transmitted or localized in trans), we did not investigate the potentially dominant transmission nor did we consider compound heterozygous variants. Overall we identified 22 subjects (23%) with a homozygous or hemizygous HIGH (n=14) or L-HIGH (n=8) variant in one of the 151 candidate



genes (Table 1). We focused on the variants that present a high probability of being responsible for the subjects' NOA.

### Identified gene variants

A total of 7 subjects had a homozygous loss of function variant (mainly frameshift variants) in 6 genes (*C14orf39* (MIM 617307), *FANCM* (MIM 609644), *GCNA* (MIM 300369), *HFM1* (MIM 615684), *MEIOB* (MIM 617670), *TDRD9* (MIM 617963)) previously described to be associated with NOA<sup>23-30</sup> (Table 1). In addition, two individuals harbored the same homozygous missense variants in *MCM8* (MIM 608187):p.His161Pro. Similarly, two other persons carried the same homozygous variants in *PDHA2* (MIM 179061):p.Met227Val. Both these genes have previously been associated with NOA<sup>31,32</sup>. Overall, for these 11 individuals we can consider that a near certain diagnosis has been obtained.

An additional 7 subjects were identified with a homozygous loss of function variant in 6 genes whose function was described as critical for spermatogenesis (Table 1). For each of these genes (*DDX25* (MIM 607663), *HENMT1* (MIM 612178), *MCMDC2* (MIM 617545), *MSH5* (MIM 603382), *REC8* (MIM 608193), *TDRKH* (MIM 609501)), knock out mouse models have been produced which all exhibit male sterility<sup>33-40</sup>. Some of these genes have been described to be linked with female infertility (*MSH5*)<sup>41</sup> but no deleterious variants have yet been described in men with NOA. We can therefore consider that the data presented here confirms the direct link of these genes with NOA. Last, four subjects carried missense variants in genes already described to induce NOA (*DMC1* (MIM: 602721), *HENMT1* (MIM: 612178) and *TERB1* (MIM: 617332)). *DMC1* homozygous missense variants have been described in male and female infertile siblings and KO mice present with male and female sterility<sup>42,43</sup>. Here, two subjects carried a distinct homozygous missense variant in *DMC1*. The two *DMC1* variants identified, p.Thr122Ala and p.Pro287His, are not found in gnomAD and are respectively predicted as pathogenic by 9 and 11 out of 12 prediction softwares (see varsome URL). Similarly, the *HENMT1* and *TERB1* variants are very rare in gnomAD (2 occurrences) and are respectively predicted as pathogenic by 9/12 and 7/11 prediction software. The presence of all variants was confirmed by Sanger sequencing (Supplementary Figure 1), and all variants were submitted to Clinvar (see Clinvar\_URL) under the reference SUB10823688.

Overall, we obtained a high confidence diagnosis for 18 subjects (HIGH impact variants or L-HIGH variants in genes identified in two subjects) and a likely diagnosis for 4 additional subjects (missense variant not previously published).

### **Histological phenotype and TESE outcome**

All subjects underwent surgery to perform a testicular sperm extraction (TESE). In all cases one part of the extracted tissues was fixed and colored for histological analysis and the other part was dilacerated for attempts to extract living spermatozoa. Fixed slides were carefully examined to obtain a histological diagnosis. Overall 14 subjects were classified as having hypospermatogenesis, 36 a Sertoli cell only syndrome, 11 a testicular degeneration, 6 a pre-meiotic arrest, 23 a meiotic arrest and 6 a post-meiotic arrest (Figure 2A). All individuals with hypospermatogenesis had a positive TESE (100%), subjects with testicular degeneration had 36.6% positive TESE, those with post-meiotic arrest 33.3%, Sertoli-cell only syndrome 8.3%, pre-meiotic and meiotic arrest had 16.7 and 8.7% respectively (Figure 2B, C). The positive TESE results might seem in conflict with the histological diagnoses of Sertoli-cell only syndrome and pre-meiotic and meiotic arrest. This can be explained by the fact that the tubules might be unevenly affected and some of the histological results might not be representative of the whole testis environment.

### **Genotype phenotype correlation**

Among the 22 diagnosed persons, 12 (55%) had gene defects altering a gene described to be critical for meiosis (*C14orf39*, *DMC1*, *HFM1*, *MCM8*, *MCMDC2*, *MEIOB*, *MSH5*, *REC8*, *TERB1*) (Table 2). The histological diagnosis was concordant for 10 subjects, evidencing a meiotic arrest. For all these subjects, diagnosed with genetic defect in a meiotic gene, the TESE was negative (Table2). Only two individuals with a missense variant in the meiotic *MCM8* gene were scored as having a Sertoli-cell only syndrome. Seven individuals had defects in genes described to be involved in the Piwi pathway or in DNA repair (*FANCM*, *GCNA*, *HENMT1*, *TDRD9*). Among these individuals, TESE was positive for 2 subjects (29%). Last, three subjects had defects in genes involved in post-meiotic maturation (*DDX25*, *PDHA2*) and one (33%) had a positive TESE.

## **DISCUSSION:**

The recent development of high throughput sequencing methods permitted the identification of an exponential number of candidate genes in all genetic disorders. The field of male infertility also benefited from these advances and the past years saw the identification and characterization of numerous infertility genes. For example we identified *SPINK2* as a cause for post-meiotic arrest<sup>44</sup> and a recent cohort study focusing on NOA subjects with spermatogenic maturation arrest (MA) permitted to identify 5 candidate genes and to identify a likely genetic defect in 23 out of 147 subjects (16%)<sup>10</sup>. Here we performed WES analysis on 96 NOA individuals showing different testicular histologies and identified 7 candidate genes and identified a likely genetic defect in 22 out of 96 subjects (23%). For men with NOA, the benefit of a clear molecular diagnosis following exome sequencing has however been limited as the genetic diagnosis only rarely has a direct impact on the subject's proposed treatment. Clear guidelines associated with the predicted treatment outcome are necessary to turn WES into a useful tool for the routine diagnosis and care of NOA individuals.

**Exome sequencing is a powerful tool in the framework of infertility diagnosis.**

Following exome sequencing, using our list of candidate genes and strict guidelines designed to be integrated into an automated bioinformatics process, we obtained a high confidence diagnosis for 19% of the analysed persons and an additional very likely diagnosis for 4%. Currently, the only genetic tests carried out routinely for NOA individuals are a karyotype and the search for microdeletions in the AZF region of the Y chromosome. These analyses yield a diagnosis for less than 20% of the tested individuals<sup>5</sup>. In this study, we see that exome sequencing combined with our proposed analysis method permits to double the diagnosis efficiency for this pathology. Here, all subjects were recruited at the Clinique les Jasmins in Tunis and originated from North Africa. We can estimate that half of the included individuals were born to related parents and this certainly contributed to this high diagnosis efficiency. The yield of diagnosis of a genetically more heterogeneous population might therefore be lower but diagnosis efficiency should improve rapidly for all subjects with the publication of new studies which will permit to quickly enrich the list of candidate genes. Here, we only presented the variants affecting the genes from a limited list of genes likely linked with NOA. Despite all our efforts, this list is likely incomplete and may contain mistakes, but more importantly, considering how fast the field is moving, it will certainly be incomplete by the time this article is printed. However, this list is available online and we aim to keep it updated,

to serve as a shared tool for the analysis of WES data in the context of NOA. Variants in other genes not included in the list of candidate genes appeared interesting but were not discussed in this manuscript, as additional work has to be carried out to confirm or infirm their implication in NOA. To that end we generated 4 knock-out mice lines using Crispr/Cas9 technology to gain additional information on their function. Also, we were very restrictive in the selection of the candidate variants. As we did not have the possibility to perform familial studies to assess the segregation of the variants, we only considered homozygous or hemizygous variants and did not select the individuals with two deleterious variants in the same candidate genes as we could not confirm that the variants were bi-allelic. Several variants did not fulfill our selection criteria but appeared interesting. This was the case for potential splice variants, not affecting consensus acceptor or donor site, but appearing as good candidates necessitating further functional validation.

Interestingly, some of the identified genes have been described to be linked with female infertility and primary ovarian insufficiency (C14orf39<sup>29,45</sup>, HFM1<sup>46</sup>, MCM8<sup>32</sup>, MEIOB<sup>47</sup>, MSH5<sup>41</sup>, DMC1<sup>42</sup>) highlighting common mechanisms between male and female meiosis. As many genes have been described to induce both male and female infertility, it might be relevant to produce a large list of candidate genes regrouping genes described to be associated with either and both male and female fertility.

Overall, the use of a well-established candidate gene list and strict criteria for variant selection permits a quick and easy analysis of WES results, allowing a rapid clinical diagnosis. As highlighted before, the list of candidate genes is expected to be in constant evolution, hence WES appears much preferable to a targeted sequencing approach that would very rapidly become obsolete and would not permit the discovery of new candidate genes.

### **Histological phenotype and identified gene defects**

Careful examination of histological results permitted to identify 6 types of testicular defects. The most frequent defects were SCOS observed in 36 subjects and meiotic arrest observed in 23 (Figure 2). There was a relatively good correlation between the histological phenotype and the outcome of TESE. All subjects with hypospermatogenesis had a positive TESE (100%), results were fair for subjects with testicular degeneration (36.6% positive TESE) and post-meiotic arrest (33.3%) and poor for Sertoli-cell only syndrome (8.3%), pre-meiotic and meiotic arrest (16.7 and 8.7% respectively). In some cases, this classification was difficult to make as

different tubules might present slightly different anomalies and some persons might have several overlapping phenotypes (Supplementary table 2). Histological classification of testicular tissues has always been recognized as challenging. A large study performed on 1418 samples show a matched phenotypic classification between right and left testicular biopsies in 81.2% of SCO cases whereas a matched classification was only obtained for 25-67.7% of post-meiotic arrests<sup>48</sup>. This illustrates that multiple sampling is necessary to obtain an overall view of the spermatogenesis. Here, for simplification, we retained the observed majoritarian defect. Interestingly, a diagnosis was obtained for 12 out of 22 subjects (55%) identified as having a meiotic arrest. Conversely, diagnosis efficiency was low for the other testis histological categories and a likely variant was identified in 6% of SCOS subjects, in 9% of testicular degeneration, in 21% of hypospermatogenesis, and in 17% of subjects with pre and post-meiotic arrest. One of the main reasons to explain the predominance of individuals with a genetic diagnosis among the subjects scored with a meiotic arrest might be that meiosis has been extensively studied and many meiotic genes have been identified and are present in our list of candidate genes.

We tried to group the identified genes by function. Nine of the identified genes were described to have a direct function in meiotic processes, 5 a role in piwi pathways or DNA repair (Table 2). Only 2 genes described to be involved in post-meiotic processes were identified. It is interesting to note that we observed genes regrouped in these broad functions following the string analysis of the 151 candidate genes (Figure 1). There was a relatively good correlation between the histological phenotype and what was expected from the presumed gene function. Nine out of the 16 identified genes are described to be directly involved in meiosis (Table 2) and 10 out of the 12 individuals concerned had a testis histology phenotype of meiotic arrest. The two remaining subjects had variants in *MCM8*, involved in chiasma synthesis and chromosomal pairing stabilization and were scored to have SCOS. Conversely, three individuals scored to have a meiotic arrest had variants in *HENMT1*, *TDRD9* and *TDRKH*, three genes known to be involved in the PIWI pathway. These small discrepancies were also illustrated by the fact that several persons with defects in the same genes (*HENMT1*, *TDRD9* and *PDHA2*) had a different histological subphenotype and a different TESE outcome. For *PDHA2* two individuals had the same mutation and one had a hypospermatogenesis and a positive TESE, the other a meiotic arrest and a negative TESE. Overall these observations

highlight the limit of the histological classification but also the difficulty to predict the effects of different (or even identical) gene defects. Variable expressivity and penetrance of defects in a same gene or even of the very same genetic defects are well known caveats of human genetics<sup>49</sup>. Here we see that individuals with the same gene defect (as observed for *PDHA2*) can present a different testicular phenotype and TESE outcome. It is not possible to know if these discrepancies are due to the testicular tissue heterogeneity and to the skill of the surgeon on the day of the biopsies, or to the variable expressivity or penetrance of the gene defect. The main goal for performing a genetic diagnosis for NOA subjects is to obtain for the concerned persons, a reliable negative diagnosis that may prevent the realization of a useless TESE procedure. Genes such as *PDHA2*, which do not provide a clear TESE prognostic would be classified as uncertain and would not counter-indicate TESE.

It is interesting to note that all 12 individuals with defects in meiotic genes had a negative TESE indicating that a negative prognostic can be reached with a good degree of confidence, in particular for meiotic genes. In a recent study, Krausz and colleagues studied NOA men with a maturation arrest and a negative TESE<sup>10</sup>. Interestingly, they identified a total of 12 candidate genes of which 9 can be considered to be meiotic genes (*MEI1*, *MEIOB*, *MSH4*, *RAD51L1*, *SHOC1*, *STAG3*, *SYCE1*, *TERB1*, *TEX11*), thus confirming the strong link between defects in meiotic genes and negative TESE.

## **Conclusion**

We used here a simple strategy based on a list of candidate genes to facilitate the interpretation of WES data and the identification of causal gene defects associated with a prediction of their consequence on spermatogenesis. Our results indicate that a clinical diagnosis interpreted in light of other clinical arguments can provide a strong argument against TESE. This demonstrates that exome sequencing can help the clinician to provide his patient with a more precise and relevant information which will help them adopt the most appropriate course of action, permitting in some instances to avoid hopeless surgical procedures.

## **Description of supplemental data**

Supplemental data include three tables, one figure and one online table:

Supplementary Table 3 is available online (URL provided in the Web resource section).

### **Declaration of interests**

The authors declare no competing interests.

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### **Data and Code Availability**

All identified variants were submitted to Clinvar under the reference SUB10823688.

Details of the exome analysis pipeline used in the study is available on github (URL provided in the Web resource section).

### **Web Resources**

Supplementary Table 3: List of all candidate genes defined for NOA in this study is available at:

<https://docs.google.com/spreadsheets/d/14uA4h43deSiwqdrKk81JYPOjnh-E0UI-jK9ntkyburg/edit?usp=sharing>

Online Mendelian Inheritance in Man: <http://www.omim.org>

Varsome: <https://varsome.com>

Clinvar: <https://www.ncbi.nlm.nih.gov/clinvar/>

gnomAD: <https://gnomad.broadinstitute.org/>

<https://github.com/ntm/grexome-TIMC-Primary>

and <https://github.com/ntm/grexome-TIMC-Secondary>

MGI database: <http://www.informatics.jax.org/>

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## Figure titles and legends

**Figure 1: STRING analysis of physical and functional protein–protein interactions between the proteins encoded by the 151 candidate genes including in the NOA panel.**

Analysis performed using STRING: <https://string-db.org/>. Two main clusters are highlighted in blue circles, loosely regrouping meiotic genes (left) and PIWI and DNA repair genes (right).

**Figure 2: Definition and schematic representation of the different NOA sub-phenotypes illustrated by subjects' histological sections.**

A) The testicular biopsies were categorized into different histopathological patterns, indication of the total number of individuals with each anomaly.

**Normal Spermatogenesis:** the seminiferous tubules are lined by a thin basement membrane and the germinal epithelium shows normal progression from spermatogonia to spermatozoa along with spermatocytes and spermatids..

**Hypospermatogenesis (HS):** the germinal epithelium shows all the stages of germ cells but their number is reduced.

**Sertoli cell only syndrome:** the tubules contain only Sertoli cells and no germ cell.

**Testicular degeneration:** including both seminiferous tubule hyalinization and degenerating tubules. Hyalinized tubules are characterized by a thickened basement membrane, smaller diameter devoid of epithelial cells and collagen deposition. Degenerating tubules are characterized by hypocellularity.

**Germ cell maturation arrest** (pré-méiotique arrest (PreMA), méiotique arrest (MA) and post-méiotique arrest (Post-M)): at a specific cell stage the process of spermatogenesis is arrested.

**Mixed pattern** (not shown): there is variation in the histopathological pattern in the same testicular biopsy.

B) Proportion of individuals with successful TESE according to the testis histology

C) Examples of testis sections from individuals with different testicular defects

## Tables

Table 1

Gene	Subject	Variant	Type of variant	MAF, gnomAD
<b>Loss-of-Function (LoF) Variants in previously reported candidate genes</b>				
<i>C14orf39</i>	P0142	NM_174978.3:c.204_205del ; p.His68GlnfsTer2	Frameshift	4,1E-06
<i>FANCM</i>	P0138	NM_020937.4:c.5791C>T ; p.Arg1931Ter	Stop gained	1,0E-03
<i>GCNA</i>	P0137	NM_052957.5:c.1507del ; p.Glu504LysfsTer11	Frameshift	0,0E+00
<i>HFM1</i>	P0369	NM_001017975.6:c.3588+1G>A	splice donor	0,0E+00
<i>MEIOB</i>	P0074	NM_001163560.3:c.1118_1121del ; p.Phe373SerfsTer6	Frameshift	0,0E+00
<i>TDRD9</i>	P0080	NM_153046.3:c.3483_3484dup ; p.Ser1162IlefsTer3	Frameshift	0,0E+00
	P0279	NM_153046.3:c.720_723del ; p.Ser241ProfsTer4	Frameshift	7,7E-05
<b>Deleterious missense variants previously reported in NOA candidate genes</b>				
<i>MCM8</i>	P0370	NM_001281520.2:c.482A>C ; p.His161Pro	Missense	0,0E+00
	P0281	NM_001281520.2:c.482A>C ; p.His161Pro	Missense	0,0E+00
<i>PDHA2</i>	P0253	NM_005390.5:c.679A>G ; p.Met227Val	Missense	6,0E-05
	P0144	NM_005390.5:c.679A>G ; p.Met227Val	Missense	6,0E-05
<b>Loss-of-Function (LoF) Variants in novel strong candidate genes (no mutations described in NOA men)</b>				
<i>DDX25</i>	P0283	NM_013264.5:c.1129C>T ; p.Arg377Ter	Stop gained	1,6E-05
<i>HENMT1</i>	P0272	NM_001102592.2:c.456C>G;p.Tyr152Ter	Stop gained	0,0E+00
<i>MCMDC2</i>	P0085	NM_173518.5:c.1795C>T ; p.Arg599Ter	Stop gained	1,3E-04
	P0355	NM_172166.4:c.537+1G>A	Splice donor	0,0E+00
<i>MSH5</i>	P0321	ENST00000375703.7:c.1015_2508del	CNV (homo)	0,0E+00
<i>REC8</i>	P0088	NM_001048205.2:c.860_861del ; p.Pro287ArgfsTer74	Frameshift	0,0E+00
<i>TDRKH</i>	P0110	NM_001083965.2:c.1003A>T;p.Lys335Ter	Stop gained	0,0E+00
<b>Novel deleterious missense variants in NOA candidate genes</b>				
<i>DMC1</i>	P0352	NM_007068.4:c.364A>G ; p.Thr122Ala	Missense	0,0E+00
	P0082	NM_007068.4:c.860C>A ; p.Pro287His	Missense	0,0E+00
<i>HENMT1</i>	P0109	NM_001102592.2:c.226G>A ; p.Gly76Arg	Missense	8,0E-06
<i>TERB1</i>	P0145	NM_001136505.2:c.733G>A ; p.Gly245Arg	Missense	6,5E-06

**Table 2: Predicted gene function and clinical information on affected individuals**

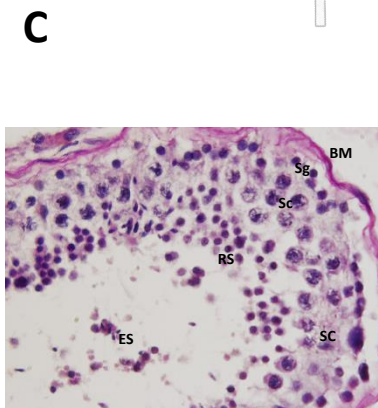
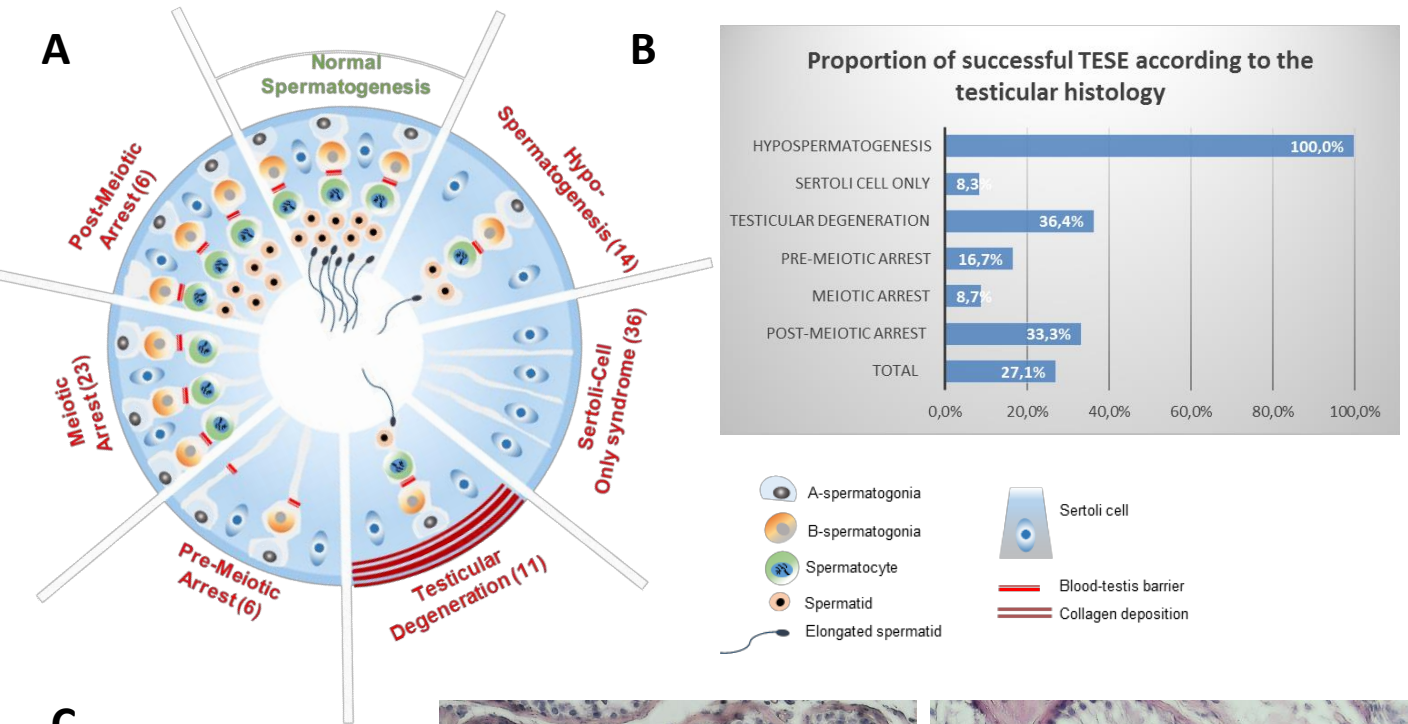
Indicated testis histology are: Sertoli cell only (SCOS), Testicular degeneration (TD), Hypospermatogenesis (HS), Spermatogenic Arrest : Pre-Meiotic (PrMeA), Meiotic (MeA), Post-Meiotic (PoMA). Sperm retrieval is either positive (POS) or negative (NEG).

Gene	Function	Patient	Left/right testis volume (mL)	FSH (IU/L)	Testis histology	Sperm retrieval
<b>Genes involved in meiosis</b>						
<i>C14orf39</i>	Synaptonemal complex	P0142	10-15/10-15	NA	MeA	NEG
<i>DMC1</i>	Chiasma Strand invasion	P0352*	>15/>15	NA	MeA	NEG
		P0082*	10-15/10-15	9	MeA	NEG
<i>HFM1</i>	Chiasma Strand invasion	P0369	5-10/5-10	5.16	MeA	NEG
<i>MCM8</i>	Chiasma synthesis and stabilization	P0370	5-10/5-10	16.7	SCOS	NEG
		P0281	<5/<5	27	SCOS	NEG
<i>MCMDC2</i>	Meiotic recombination	P0085	5-10/5-10	NA	MeA	NEG
<i>MEIOB</i>	Chiasma synthesis and stabilization	P0074	10-15/10-15	2.16	MeA	NEG
<i>MSH5</i>	Chiasma synthesis and stabilization	P0355	10-15/10-15	6.08	MeA	NEG
		P0321	>15/>15	9.65	MeA	NEG
<i>REC8</i>	Meiotic recombination	P0088	5-10/5-10	12.9	MeA	NEG
<i>TERB1</i>	Assembly of a meiotic telomere complex	P0145*	10-15/10-15	4.23	MeA	NEG
<b>Genes involved in Piwi pathway and/or DNA repair</b>						
<i>FANCM</i>	DNA repair pathway	P0138	5-10/5-10	NA	TD	NEG
<i>GCNA</i>	Regulator of genome stability	P0137	>15/>15	2.24	PrMeA	NEG
<i>HENMT1</i>	Component of the piwi pathway	P0272	10-15/10-15	3.29	HS	POS
		P0109			MeA	NEG
<i>TDRD9</i>	Piwi pathway, transposon silencing	P0080	<5/<5	16	HS	POS
		P0279	10-15/10-15	4.6	MeA	NEG
<i>TDRKH</i>	Component of the piwi pathway	P0110	5-10/5-10	27.6	MeA	NEG
<b>Genes involved in post-meiotic maturation</b>						
<i>DDX25</i>	Post-transcriptional regulation	P0283	5-10/5-10	NA	PoMA	NEG
<i>PDHA2</i>	Carbohydrate oxidation	P0253	10-15/10-15	2.87	HS	POS
		P0144	10-15/10-15	2.4	MeA	NEG





Figure



Abreviations:  
 BM = Basal membrane  
 CS = Sertoli cell  
 Sg = Spermatogonia  
 Sc = Spermatocyte  
 RS = Round spermatid  
 ES = Elongated spermatid

