

# Analysis of 29 Targeted Genes for Non-obstructive Azoospermia: the Relationship Between Genetic Testing and Testicular Histology

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## Research Article

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# Abstract

**BACKGROUND.** Few studies have evaluated the relationship between testicular histology and pathogenic variations of genes regulating spermatogenesis.

**AIM.** To analyze the presence of potentially pathogenic variants of 29 candidate genes known to cause spermatogenic failure (SPGF) in patients with non-obstructive azoospermia (NOA) who underwent testicular histology.

**PATIENTS AND METHODS.** Sixty patients with NOA referred to the Department of Transfusion Medicine and Transplantation Biology, University Hospital Center Zagreb, Croatia, for testicular biopsy were consecutively assessed for eligibility. Twelve patients were excluded from the study because they had Klinefelter syndrome (n=1), Yq microdeletions (n=6), testicular trauma (n=2), or *in-situ* germ cell neoplasia (n=3). Therefore, 48 patients were considered eligible and included in this study. They were divided into three groups: those who had cryptorchidism (n=9), those with varicocele (n=14), and those with idiopathic NOA (n=25). All included patients underwent blood withdrawal for next-generation sequencing analysis and gene sequencing.

**RESULTS.** We found a possible genetic cause in 4 patients with idiopathic NOA (16%) and in 2 with cryptorchidism (22%). No pathogenic or possibly pathogenic mutations were identified in patients with varicocele. Variants of undetermined significance (VUS) were found in 11 patients with idiopathic NOA (44%), 3 with cryptorchidism (33%), and 8 patients with varicocele (57%). VUSs of the *USP9Y* gene were the most frequently as they were found in 14 out of 48 patients (29%). In particular, the VUS *USP9Y*c.7434+14del was found in 11 patients. They showed varied histological pictures, including Sertoli cell-only syndrome, mixed atrophy, and hypospermatogenesis, regardless of cryptorchidism or varicocele. No direct correlation was found between the gene mutation/variant and the testicular histological picture.

**CONCLUSION.** Different mutations of the same gene cause various testicular histological pictures. These results suggest that it is not the gene itself but the type of mutation/variation that determines the testicular histology picture. Based on the data presented above, it remains challenging to design a genetic panel with prognostic value for the outcome of testicular sperm extraction in patients with NOA.

## 1. Introduction

Spermatogenesis is an approximately 74 day-long process that occurs in the seminiferous tubules, where diploid spermatogonial stem cells (SSCs) differentiate into haploid spermatozoa. It requires several delicately regulated molecular mechanisms which, to date, are only partially known. Consequently, the etiology of spermatogenic failure (SPGF) is identified only in a minority of infertile patients, highlighting the scarcity of our knowledge of these mechanisms. One epidemiologic study reported oligozoospermia in 20% of 8,518 male partners of infertile couples, but the cause was identified in only 25% of them [1]. Similarly, another study of 26,091 male partners of infertile couples reported a prevalence of oligozoospermia and non-obstructive azoospermia (NOA) of 49% and 11%, respectively. Noteworthy, the diagnosis could be reached in these couples in only 28%. Thus, as many as 72% of these couples remained of unknown etiology [2]. This finding highlights the need for studies that further clarify the etiology of SPGF. This need is becoming even more pressing as the prevalence of SPGF is set to increase further. Indeed, meta-regression data indicate a global trend toward decreasing sperm concentration and count [3].

Genetic screening of male infertility is of relevant importance for the diagnosis, prognosis, and decision-making clinical management of these patients. Karyotype analysis, AZF microdeletions of the long arm Y chromosome (Yq), screening of genes whose mutations are involved in central hypogonadism, and *CFTR* gene analysis are genetic tests currently used in clinical practice [4]. In recent years, a deeper understanding of the molecular factors involved in mouse spermatogenesis has led to the search for homologous factors in humans and the design of suitable gene panels [5]. Blood testing for these gene panels in patients with SPGF has yielded encouraging results. For example, analysis of *TEX11*, *NR5A1*, and *DMRT1* genes in 80 patients with NOA, normal karyotype, and without Yq microdeletions identified probable pathogenic mutations in 4 patients (5% of the whole cohort), thus increasing the diagnosis rate up to 25% [2]. Furthermore, the evaluation of a panel of 15 genes (*USP9Y*, *NR5A1*, *KLHL10*, *ZMYND15*, *PLK4*, *TEX15*, *TEX11*, *MEIOB*, *SOHLH1*, *HSF2*, *SYCP3*, *TAF4B*, *NANOS1*, *SYCE1*, and *RHOXF2*) in the blood of 25 patients with apparently idiopathic oligozoospermia or NOA led to the identification of pathogenic variants of the *NR5A1* and *TEX11* genes in 3 patients (12.0%) [6]. Similarly, the evaluation of 37 genes in 16 patients with NOA identified potential pathogenic gene variants in 6 of them [7]. These studies used a methodological approach based on next-generation sequencing (NGS) that allows for rapid and cost-effective analysis of a large number of genes.

It is known that more than 2000 genes are involved in spermatogenesis [5], and without a careful genotype-phenotype analysis, the risk of distorted conclusions or false interpretations is high. Furthermore, only a few studies have explored the association between testicular histology and pathogenic variations of SPGF genes [8,9]. Further studies, therefore, still need to be performed to have firm conclusions. Therefore, this study aimed to evaluate the presence of potentially pathogenic variants of 29 SPGF-associated genes in a cohort of patients with NOA undergoing testicular histology.

## 2. Patients And Methods

### 2.1 Patient selection

Sixty patients with NOA referred to the Department of Transfusion Medicine and Transplantation Biology, University Hospital Center Zagreb, Croatia, for testicular biopsy were consecutively evaluated for eligibility. Each patient underwent testicular biopsy (after a complete diagnostic workup with their andrologist); their clinical data were carefully assessed. Twelve patients had Klinefelter syndrome or other karyotype abnormalities, Yq microdeletions, testicular trauma, torsion, *cystic fibrosis transmembrane conductance regulator (CFTR)* gene mutations, germ cell neoplasia in situ (GCNIS), or had undergone chemotherapy were excluded from the study. Therefore, 48 patients were considered eligible and included in this study. They were divided into three groups:

those who had cryptorchidism (n=9), those with varicocele (n=14), and those with idiopathic NOA (n=25) (**Figure 1**). All included patients underwent blood draw for NGS analysis and candidate gene sequencing (**Table 1**).

## 2.2 Ethic statement

The protocol of the study has been approved by the Ethical Committee of the University Hospital Zagreb has approved the study (reference: 8.1.-21/175-1; no.: 02/21 JG). Informed written consent was obtained from each participant after a full explanation of the purpose and nature of all procedures used. The study has been conducted according to the principles expressed in the Declaration of Helsinki.

## 2.3 Testicular histology

The samples were obtained following the open testicular biopsy procedure described by Holstein and colleagues [10]. They were fixed in Bouin's fluid immediately after excision inside the operational theatre. Afterwards, the tissue was washed several times in lithium carbonate solution, dehydrated, incubated in intermedium (methyl-benzoate), benzoyl and benzoyl-paraffin and then fixed in paraffin. The blocks were cut extensively by a rotary microtome Leitz 1512 (Austria) set at a section thickness of 4 µm. The obtained serial sections were then stained in hematoxylin and eosin (H&E stain) and prepared for histological analysis (the evaluation of preservation of seminiferous epithelium). The microscopic analysis was performed using Eclipse (E600) microscope (Nikon, Japan). The digital camera Axiocam has been employed for photo documentation (Carl Zeiss, Germany). The spermatogenesis status included the following histological pictures/classification: full spermatogenesis; hypospermatogenesis; maturation arrest; Sertoli cells only syndrome (SCOS); tubular fibrosis and mixed atrophy.

## 2.4 Bioinformatics and genetic analysis

Genetic analysis was performed using the NGS approach and a custom-made gene panel designed to include the main genes involved in SPGF (**Table 1**). A MiSeq personal sequencer (Illumina, San Diego, CA) was used for NGS. Sanger sequencing was performed when target region coverage was less than 10 reads. All laboratory methods have been described elsewhere [11]. We searched international databases dbSNP ([www.ncbi.nlm.nih.gov/SNP/](http://www.ncbi.nlm.nih.gov/SNP/)) and Human Gene Mutation Database professional (HGMD; <http://www.biobase-international.com/product/hgmd>) for all nucleotide changes. *In silico* evaluation of the pathogenicity of nucleotide changes in exons was performed using the Variant Effect Predictor tool (<http://www.ensembl.org/Tools/VEP>) and MutationTaster (<http://www.mutationtaster.org>). Minor allele frequencies (MAF) were checked in the Genome Aggregation Database (gnomAD) (<http://gnomad.broadinstitute.org/>). All variants were evaluated according to American College of Medical Genetics and Genomics guidelines [12].

# 3. Results

We found a possible genetic cause in 4 patients with idiopathic NOA (16%) and 2 with cryptorchidism (22%) since the mutations of these genes were predicted to be pathogenic or likely pathogenic. No pathogenic or likely pathogenic mutations were identified in patients with varicocele. Molecular and clinical data of these patients are summarized in **Table 2**. Their testicular histology is provided in **Figures 2, 3 and 4**.

Variants of undetermined significance (VUS) were found in 11 patients with idiopathic NOA (44%), 3 with cryptorchidism (33%), and 8 patients with varicocele (57%). The molecular and clinical data of these patients are summarized in **Table 3**. VUS of the *USP9Y* gene was the most frequently found, being identified in 14 out of 48 patients (29%). In particular, the *USP9Y* c.7434+14del VUS was found in 11 patients. Five of these latter patients had testicular histology of mixed atrophy, 1 maturation arrest, 2 hypospermatogenesis, 1 SCOS in the right testis and mixed atrophy in the left one; 1 patient had hypospermatogenesis in the right testis and mixed atrophy in the left one.

## 3.1 Genetic abnormalities in Sertoli-cell only syndrome

Of the 48 included patients, SCOS was found in both testes of 5 patients with idiopathic NOA, 2 with cryptorchidism, and 1 with varicocele. The pathogenic c.6058G>T stopgain mutation in the *DNAH1* gene was found in 1 patient with varicocele (**Table 2, Figure 2, Panels A and B**). No other pathogenic mutation was found in the remaining patients. However, the *ZPBP* c.128-6del splice region and intron VUS and the *SYCP3* c.643del frameshift VUS were found in one patient with idiopathic NOA and one patient with varicocele, respectively (**Table 3**).

## 3.2 Genetic abnormalities in maturation arrest

Maturation arrest in both testes was found in only 1 patient with idiopathic NOA who carried the *USP9Y* c.7434+14del splice region and intron VUS (**Table 3**).

## 3.3 Genetic abnormalities in mixed atrophy

Overall, 4 patients with idiopathic NOA, 4 with cryptorchidism, and 10 with varicocele had mixed atrophy in both testes. The likely pathogenic *NANOS1* c.262del frameshift mutation was identified in 1 patient with cryptorchidism (**Table 2, Figure 2, Panels C and D**). No other pathogenic mutations were found in the remaining patients. The *USP9Y* c.7434+14del frameshift VUS was found in 1 patient, and the *USP9Y* c.7434+14del splice region and intron VUS was found in 2 patients with idiopathic NOA. In patients with varicocele and mixed atrophy, the *PLK4* c.17G>A missense, the *NANOS1* c.830\_833del frameshift, the *USP9Y* c.7434+14del and c.7434+14del splice region, and intron VUS were identified (**Table 3**).

## 3.4 Genetic abnormalities in hypospermatogenesis

Hypospermatogenesis was found in both testes of 5 patients with idiopathic NOA, 2 patients with cryptorchidism, and 3 patients with varicocele. The pathogenic *ZPBP* c.127+1G>A splice donor, the *PLCZ1* c.20del frameshift, and the *ZMYND15* c.1260\_1261del and c.1263del frameshift mutations were

identified in patients with idiopathic NOA (**Table 2, Figure 3, Figure 4, Panel A and B**). No other pathogenetic mutations were found. VUS were found in 3 patients with idiopathic NOA (*CFAP44* c.2202\_2204del inframe deletion, *DNAH1* c.4739C>G missense, *SUN5* c.476G>A missense, *DPY19L2* c.1025A>C missense, *TEX11* c.2186-6\_2186-3del splice region and intron, *USP9Y* c.7434+14del splice region and intron, *SYCE1* c.52G>C missense, and *ZMYND15* c.1260\_1262del inframe deletion variants), in 2 with cryptorchidism (*USP9Y* c.449T>C missense, *USP9Y* c.7434+14del splice region and intron, and *USP9Y* c.7434+14dup splice region and intron variant), and in 2 patients with varicocele (*TEX15* c.541-4\_541-3del splice region and intron, *PLK4* c.17G>A missense, and *NANOS1* c.830\_833del frameshift variants) (**Table 3**).

## 4. Discussion

The genetic diagnosis of male infertility represents a very important step, but at the same time, it is a difficult challenge. On the one hand, spermatogenesis is a complex mechanism, and various conditions can compromise it; on the other hand, more than 2000 genes are involved in this multifaceted process. Therefore, a genetic diagnosis can remain elusive in many cases [5]. Nowadays, NGS, a widely used method, allows the evaluation of a large number of genes simultaneously, rapidly, and inexpensively. However, the large number of gene variants that can be identified is virtually unlimited and may exceed the ability to validate them with functional studies. Therefore, bioinformatics is set to become an increasingly important tool.

Few studies have evaluated mutations in genes known to cause SPGF in NOA patients with known testicular histology. A recent study by Krausz and colleagues assessed a panel of genes involved in meiosis in 147 patients with NOA and maturation arrest. At least 12 genes (*ADAD2*, *TERB1*, *SHOC1*, *MSH4*, *RAD21L1*, *TEX11*, *TEX14*, *STAG3*, *MEIOB*, *DMRT1*, *MEI1*, and *SYCE*) have been found associated with maturation arrest [8]. Therefore, the authors suggested that these genes may represent a gene panel to be used before sperm retrieval by testicular sperm extraction (TESE) in NOA patients. However, mutations/variants of the same gene seem capable of causing different testicular histological pictures. For example, partial deletion of the *TEX11* gene resulted in maturation arrest in the study by Krausz and colleagues [8], but other mutations (e.g. splice region and intron or missense) of the same gene are compatible with different testicular histological findings such as hypospermatogenesis (case ID 125687856 of the present study) or with oligozoospermia [6].

The present study was designed to understand whether variations of a homemade panel of genes involved in spermatogenesis can be associated with specific testicular histology in patients with NOA. The panel was developed based on our previous review study [5]. Only SPGF-associated penetrating genes were selected. Furthermore, only those with more significant negative consequences (splicing, frameshift, missense, start loss, deletions) were chosen to reduce the possibility of benign variants. In contrast, those with a frequency greater than 0.05 in the general population were excluded. We found pathogenic variants of the following five genes in six patients (12.5%) using this panel.

*DNAH1* encodes for a protein that belongs to the dynein family of proteins. These are microtubule-associated motor protein complexes. Specifically, the DNAH1 protein, an inner arm heavy chain dynein, has been identified in the full length of the sperm flagellum, and the gene mutations have been classically associated with multiple morphological abnormalities of sperm flagella [13]. However, *DNAH1* mutations have been recently suggested deserving investigation not only in patients with asthenozoospermia but also in those with azoospermia [14]. Indeed, an observational study has analyzed *DNAH1* gene mutations in a cohort of 200 patients with NOA, reporting the presence of pathogenic variants in 3 of them (1.5%) [14]. Accordingly, mutation of the *DNAH5* gene, encoding for another component of the dynein family of proteins, has already been reported in a patient with NOA [15, 16]. On this basis, the possible role of the *DNAH1* gene in the pathogenesis of NOA needs to be further elucidated. We herein report a pathogenic c.6058G>T stopgain mutation of the *DNAH1* gene in a patient with SCOS, borderline serum FSH levels, low testicular volume, and cryptorchidism. These data support the role of this gene in NOA.

*NANOS1* encodes for a transcript involved in the modulation of germ cell proliferation and, therefore, acts in the very early phase of spermatogenesis [5]. Few reports have already suggested the link between mutations of this gene and SPGF. The heterozygous deletion of two single amino acids (p.Ser78del or p.Ala173del) has been reported in Polish patients with NOA, low testicular volume (6-10 ml), and elevated serum FSH levels (15.4-18.2 IU/l). The testicular histology was available only in one patient and showed SCOS [17]. In addition, the pArg246His and Arg276Tyr *NANOS1* missense mutations have been described in patients with severe oligozoospermia [17]. Here we show a pathogenic frameshift *NANOS1* mutation in a patient with cryptorchidism, low testicular volume, borderline FSH values, and mixed atrophy. Our findings expand the knowledge on the testicular histological features that can associate with *NANOS1* mutations.

*PLCZ1* encodes a protein of 608 amino acids expressed in spermatozoa and, specifically, in the acrosomal and post-acrosomal regions, in the intermediate tract, and the main part of the tail [18]. This protein represents the molecular trigger for the oocyte activation during fertilization, as human PLCZ1 has been observed to stimulate the activation of the mouse oocyte and the embryo's development up to the blastocyst stage [19]. Furthermore, PLCZ1 has been identified as a marker of human sperm health, and this protein's total expression levels have been correlated with sperm parameters [20]. We found the c.20del frameshift pathogenic mutation of the *PLCZ1* gene in a patient with apparently idiopathic NOA. He has normal testicular volume and serum FSH levels and hypospermatogenesis. There are no other studies that relate PLCZ1 to patients with NOA, most probably because the mutation of this gene is not usually searched in these patients. Further studies are needed to clarify better whether mutations of this gene are associated with hypospermatogenesis.

*ZPBP* encodes for a protein of 351 amino acids involved in acrosome compaction. The absence of this protein leads, in mice, to the fragmentation of the acrosome and the disruption of the Sertoli-spermatid junctions. These structural changes result in dysmorphic spermatozoa with a decreased ability to penetrate the zona pellucida [21]. However, disruption of the Sertoli-spermatid junction can also lead to abnormal spermatogenesis, resulting in azoospermia [22]. Despite this, no studies have described *ZPBP* gene mutations in patients with NOA. For the first time, we report *ZPBP* gene mutations in two patients with NOA, one with hypospermatogenesis and one with maturation arrest/mixed atrophy. This finding suggests that *ZPBP* gene mutations may play a pathogenic role in patients with NOA.

*ZMYND15* encodes for a protein expressed in the testis, containing a zinc finger MYND motif and a nuclear localization signal. It is predicted to be a transcriptional repressor controlling normal temporal expression of haploid cell genes during spermiogenesis [23]. Mutations of this gene have already been

associated with azoospermia due to maturation arrest [24] and in patients with severe oligozoospermia [25]. Herein, we show the presence of a pathogenic mutation of this gene in a patient with hypospermatogenesis. Interestingly, the expression of ZMYND15 mRNA in the seminal plasma may predict successful sperm retrieval with high sensitivity and specificity. Indeed, its expression was significantly decreased in patients with NOA and no sperm retrieval compared with NOA and successful sperm retrieval [26].

We found several VUS of the SPGF genes evaluated in patients with NOA. The majority of these VUSs are compatible with their testicular histological features but cannot be described as pathogenic because of the lack of functional studies. At least some of them will likely be considered pathogenic, thus further raising the diagnostic rate found in the present study. Among the genes we tested, we would like to underline the high prevalence of VUSs of the *USP9Y* gene and, in particular, the c.7434+14del variant. Furthermore, very interestingly, the *USP9Y* VUSs were found associated with different testicular histological features. These include SCOS, mixed atrophy, and hypospermatogenesis regardless of the presence of cryptorchidism or varicocele since they were also found in patients with idiopathic NOA. These findings lead to at least two major considerations: 1) the *USP9Y* gene may play a role in the pathogenesis of NOA; 2) there is not a direct correlation between a gene and specific testicular histology. More likely, the mutation/variation of a specific gene involved in spermatogenesis may cause different testicular histological features. This concept challenges the construction of custom-made gene panels for TESE prognostic purposes.

The *USP9Y* gene encodes for the ubiquitin-specific protease 9 and maps within the AZFa region of the Y chromosome. The hypothesis suggesting the role of the *USP9Y* gene in spermatogenesis failed when a case report documented normal spermatogenesis in a man with the complete deletion of this gene [27]. However, this is in sharp contrast with the mild testicular phenotype reported in two cases with complete *USP9Y* deletion [28]. Hence, still today, the role of this gene in spermatogenesis is a matter of debate. The data of the present study may suggest an association between VUSs of this gene and NOA.

The results of the present study must be interpreted with caution because of the absence of a control group consisting of fertile men and the relatively low sample size. However, the stringent inclusion criteria, the exclusion of all possible acquired causes of NOA, and the availability of the testicular histology for each patient included in this study represent its main strengths.

In conclusion, the results of the present study showed that a relevant percentage of patients with apparently idiopathic NOA have mutations of SPGF genes. Furthermore, the data, taken together with those of other studies [6,8,9,17], suggest that not the gene itself but the type of mutation is associated with testicular histology. In fact, different mutations of the gene at stake cause various testicular histological features. This challenges the design of a gene panel with predictive value for sperm retrieval by TESE in patients with NOA.

## Declarations

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## Tables

**Table 1.** Spermatogenic failure genes sequenced in this study.

Gene	Inheritance	OMIM number	OMIM phenotype	Mutation detection frequency	HGNC gene number
<i>AURKC</i> ( <i>STK13</i> )	AR	603495	SF5	98.5% (72/87)	Aurora kinase C
<i>BRDT</i>	AR	602144	SF21	1 consanguineous family	Bromodomain testis associated
<i>CATSPER1</i>	AR	606339	SF7	Two 4-generation consanguineous families	Cation channel, sperm associated 1
<i>CFAP43</i>	AR	617558	SF19	13% (4/30)	Cilia and flagella associated protein 43
<i>CFAP44</i>	AR	617559	SF20	–	Cilia and flagella associated protein 43
<i>DNAH1</i>	AR	603332	Sf18	28-44%	Dynein axonemal heavy chain 1
<i>DPY19L2</i>	AR	613893	SF9	66.7% (36/54)	Dpy-19-like 2 ( <i>C. elegans</i> )
<i>HSF2</i>	AD	140581	-	<1%(1/766)	Heat shock transcription factor 2
<i>KLHL10</i>	AD	608778	SF11	1.3% (7/556)	Kelch-like family member 10
<i>MEIOB</i>	AR	617670	SF22	1 consanguineous family	Meiosis specific with OB domains
<i>NANOS1</i>	AD	608226	SF12	2.6% (5/195)	Nanos homolog 1 ( <i>Drosophila</i> )
<i>NR5A1</i>	AR	184757	SF8	2% (7/315)	Nuclear receptor subfamily 5, group A, member 1
<i>PICK1</i>	–	605926	–	–	Protein interacting with PRKCA1
<i>PLCZ1</i>	AR	608075	Sf17	2 family	Phospholipase C zeta 1
<i>PLK4</i>	AD	605031	-	1.2% (1/81)	Polo like kinase 4
<i>RHOXF2</i>	-	300447	-	<1% (1/250)	Rhox homeobox family member 2
<i>SEPT12</i>	AD	611562	SF10	1.3% (2/160)	Septin 12
<i>SLC26A8</i>	AD	608480	SF3	2% (3/145)	Solute carrier family 26 (anion exchanger) member 8
<i>SOHLH1</i>	-	610224	-	2% (2/100)	Spermatogenesis and oogenesis specific basic helix-loop-helix 1
<i>SPATA16</i>	AR	609856	SF6	1 consanguineous family	Spermatogenesis associated 16
<i>SYCE1</i>	AR	611486	SF15	1 consanguineous family	Synaptonemal complex central element protein 1
<i>SYCP3</i>	AD	604759	SF4	10.5% (2/19)	Synaptonemal complex protein 3
<i>SUN5</i>	AR	613942	SF16	47% (8/17)	Sad1 and UNC84 domain containing 5
<i>TAF4B</i>	AR	601689	SF13	1 consanguineous family	TAF4b RNA polymerase II, TATA box binding protein (TBP)-associated factor
<i>TEX11</i>	XLR	300311	SF, X-linked, 2	1-2.4% (7/289) azoospermia; 15% azoospermia with meiotic arrest	Testis expressed 11
<i>TEX15</i>	AR	605795	-	2 family; 1 proband	Testis expressed 15, meiosis and synapsis associated
<i>USP9Y</i>	YL	400005	SF, Y-linked, 2	3 proband (4-db DEL; DEL incl. entire gene)	Ubiquitin specific peptidase 9, Y-linked
<i>ZMYND15</i>	AR	614312	SF14	1 consanguineous family	Zinc finger, MYND-type containing 15
<i>ZPBP</i>	–	608498	–	2% (7/381)	Zona pellucida-binding protein

**Abbreviations:** AD, autosomal dominant; AR, autosomal recessive; SF, spermatogenic failure.

**Table 2.** Pathogenic or likely pathogenic variants in genes identified in patients with non-obstructive azoospermia and their clinical characteristics.

Gene symbol (OMIM number)	Patient code	Notes	FSH (IU/l)	Testis histology	Left/right testicular volume (ml)	Nucleotide change	Amino acid change
<i>DNAH1</i> (603332)	160585017	Cryptorchidism	12.9	SCOS	8.74/9	NM_015512.5:c.6058G>T	NP_056327.4:p.Glu202C
<i>NANOS1</i> (608226)	141879319	Cryptorchidism	11.3	Mixed atrophy	Smaller than normal	NM_199461.4:c.262del	NP_955631.1:p.His88T
<i>PLCZ1</i> (608075)	265952375	Idiopathic	N	Hypospermatogenesis	N	NM_033123.4:c.20del	NP_149114.2:p.Leu7Cy
<i>ZPBP</i> (608498)	119526696	Idiopathic	11.48	Hypospermatogenesis	NA	NM_007009.3:c.127+1G>A	/
	122836561	Idiopathic	10.1	Mixed atrophy (left) Maturation arrest (right)	N	NM_007009.3:c.4del	NP_008940.2:p.Glu2Arg
<i>ZMYND15</i> (614312)	163680875	Idiopathic	N	Hypospermatogenesis	NA	NM_001136046.3:c.1260_1261del	NP_001129518.1:p.Gly
						NM_001136046.3:c.1263del	NP_001129518.1:p.Gly

**Abbreviations:** *DNAH1*, *Dynein, axonemal, heavy chain 1*; FSH, follicle stimulating hormone; *NANOS1*, *NANOS C2HC-type zinc finger 1*; NA, not assessed; N, normal; *PLCZ1*, *Phospholipase C, zeta-1*; SCOS, Sertoli cell-only syndrome; *ZPBP*, *Zona pellucida-binding protein*.

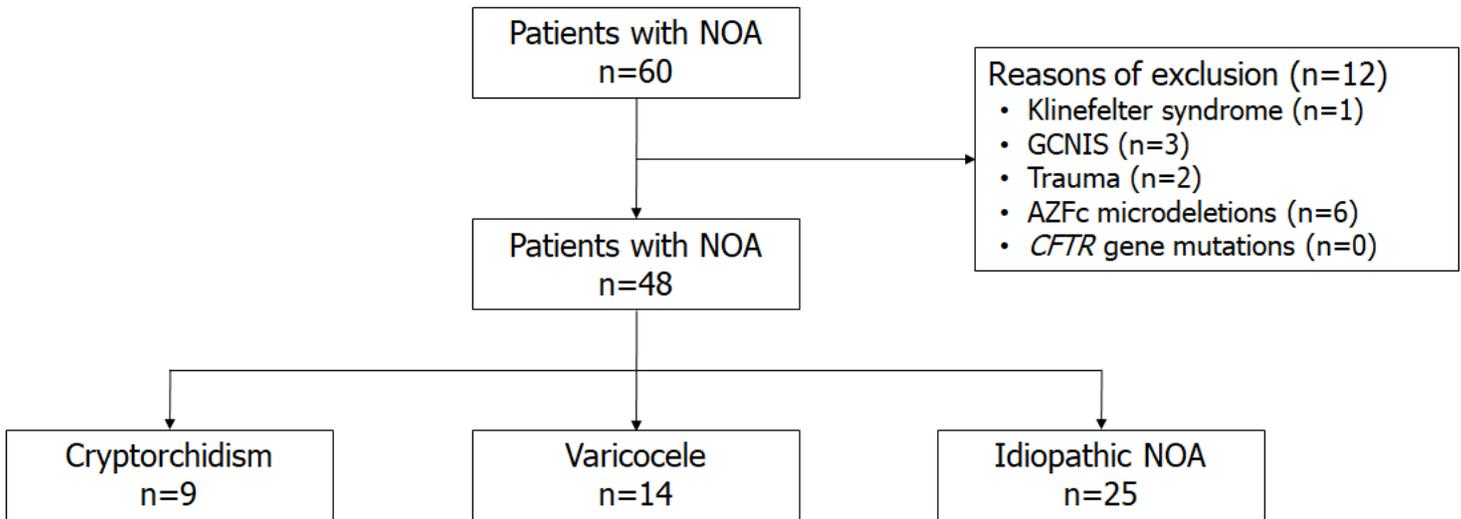
**Table 3.** Variants with uncertain significance in genes identified in patients with non-obstructive azoospermia and their clinical characteristics.

Gene symbol (OMIM number)	Patient code	Notes	FSH (IU/l)	Testis histology	Left/right testicular volume (ml)	Nucleotide change	Amino acid
<i>CATSPER1</i> (606389)	143808128	Varicocele	NA	Mixed atrophy	NA	NM_053054.4:c.1270T>C	NP_444282.
<i>CFAP44</i> (617559)	115748838	Idiopathic	N	Hypospermatogenesis	NA	NM_018338.3:c.2202_2204del	NP_060808.
<i>DNAH1</i> (603332)	111008936	Idiopathic	7.9	Hypospermatogenesis	NA	NM_015512.5:c.4739C>G	NP_056327.
<i>DPY19L2</i> (613893)	125687856	Idiopathic	N	Hypospermatogenesis	NA	NM_173812.5:c.1025A>C	NP_776173.
<i>NANOS1</i> (608226)	105816704	Varicocele	8.7	Hypospermatogenesis	NA	NM_199461.4:c.830_833del	NP_955631.
	131919035	Varicocele	26.05	Mixed atrophy	NA	NM_199461.4:c.830_833del	NP_955631.
<i>PLK4</i> (605031)	100132259	Varicocele	6.3	Hypospermatogenesis	NA	NM_014264.5:c.17G>A	NP_055079.
	113090176	Varicocele	18	Mixed atrophy	3.5/3.5	NM_014264.5:c.17G>A	NP_055079.
<i>SYCE1</i> (611486)	163680875	Idiopathic	N	Hypospermatogenesis	NA	NM_130784.3:c.52G>C	NP_570140.
<i>SYCP3</i> (604759)	130059718	Varicocele	27.3	SCOS	7.5/10	NM_001177948.1:c.643del	NP_001171.
<i>SUN5</i> (613942)	111008936	Idiopathic	7.9	Hypospermatogenesis	NA	NM_080675.4:c.476G>A	NP_542406.
<i>TEX11</i> (300311)	125687856	Idiopathic	N	Hypospermatogenesis	NA	NM_001003811.2:c.2186-6_2186-3del	/
<i>TEX15</i> (605795)	148743930	Idiopathic	23	SCOS	NA	NM_001350162.2:c.541-4_541-3del	/
	100132259	Varicocele	6.3	Hypospermatogenesis	NA	NM_001350162.2:c.541-4_541-3del	/
<i>USP9Y</i> (400005)	122354337	Idiopathic	NA	Maturation arrest	NA	NM_004654.4:c.7434+14del	/
	125687856	Idiopathic	N	Hypospermatogenesis	NA	NM_004654.4:c.7434+14del	/
	128766983	Idiopathic	N	Mixed atrophy	NA	NM_004654.4:c.6574del	NP_004645.
	128766983	Idiopathic	N	Mixed atrophy	NA	NM_004654.4:c.7434+14del	/
	130424034	Idiopathic	28.9; 37	Mixed atrophy	12.6/7	NM_004654.4:c.7434+14del	/
	148743930	Idiopathic	23	SCOS	NA	NM_004654.4:c.7434+14del	/
	151184989	Idiopathic	28.9	SCOS/mixed atrophy	N	NM_004654.4:c.7434+14del	/
	156833670	Idiopathic	NA	Hypospermatogenesis/mixed atrophy	N	NM_004654.4:c.7434+14del	/
	125938310	Cryptorchidism	NA	Hypospermatogenesis	NA	NM_004654.4:c.449T>C	NP_004645.
	125938310	Cryptorchidism	NA	Hypospermatogenesis	NA	NM_004654.4:c.7434+14del	/
	141879319	Cryptorchidism	11.3	Mixed atrophy	Smaller than normal	NM_004654.4:c.7434+14del	/
	142201659	Cryptorchidism	26	SCOS/Mixed atrophy	N/ Smaller than normal	NM_004654.4:c.7434+13_7434+14del	/
	143296311	Cryptorchidism	NA	Hypospermatogenesis	NA	NM_004654.4:c.7434+14dup	/
114516827	Varicocele	N	Mixed atrophy	NA	NM_004654.4:c.7434+14del	/	
114709776	Varicocele	N	Mixed atrophy	NA	NM_004654.4:c.7434+14del	/	
143808128	Varicocele	NA	Mixed atrophy	NA	NM_004654.4:c.7434+14dup	/	

<i>ZMYND15</i> (614312)	101797172 163680875	Idiopathic Idiopathic	6.39 N	SCOS Hypospermatogenesis	NA NA	NM_001136046.3:c.1262G>A NM_001136046.3:c.1260_1262del	NP_001129: NP_001129:
<i>ZPBP</i> (608498)	120151485	Idiopathic	20	SCOS	Smaller than normal	NM_007009.3:c.128-6del	/

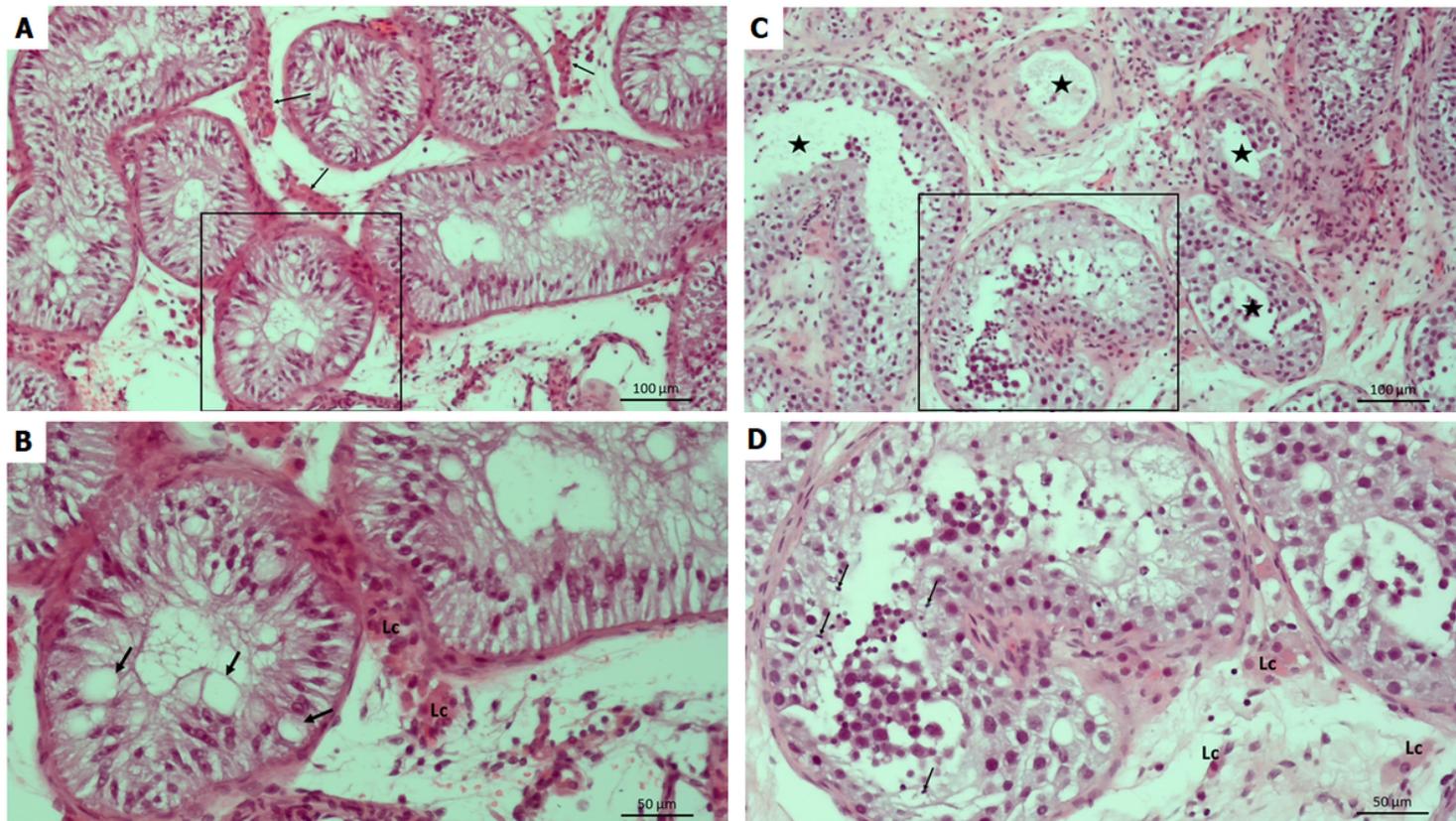
**Abbreviations:** *CATSPER1*, Cation channel, sperm-associated, 1; *CFAP44*, Cilia- and flagella-associated protein 44; *DNAH1*, Dynein, axonemal, heavy chain 1; *DPY19L2*, *DPY19-like 2*; FSH, follicle stimulating hormone; *NANOS1*, *NANOS C2HC-type zinc finger 1*; NA, not assessed; N, normal; *PLK4*, *Polo-like kinase 4*; SCOS, Sertoli cell only syndrome; *SYCE1*, Synaptonemal complex central element protein 1; *SUN5*, *SAD1 and UNC84 domain-containing protein 5*; *TEX11*, *Testis-expressed gene 11*; *TEX15*, *Testis-expressed gene 15*; *USP9Y*, *Ubiquitin-specific protease 9, Y chromosome*; *ZMYND15*, *Zinc finger mynd-containing protein 15*; *ZPBP*, *Zona pellucida-binding protein*.

## Figures



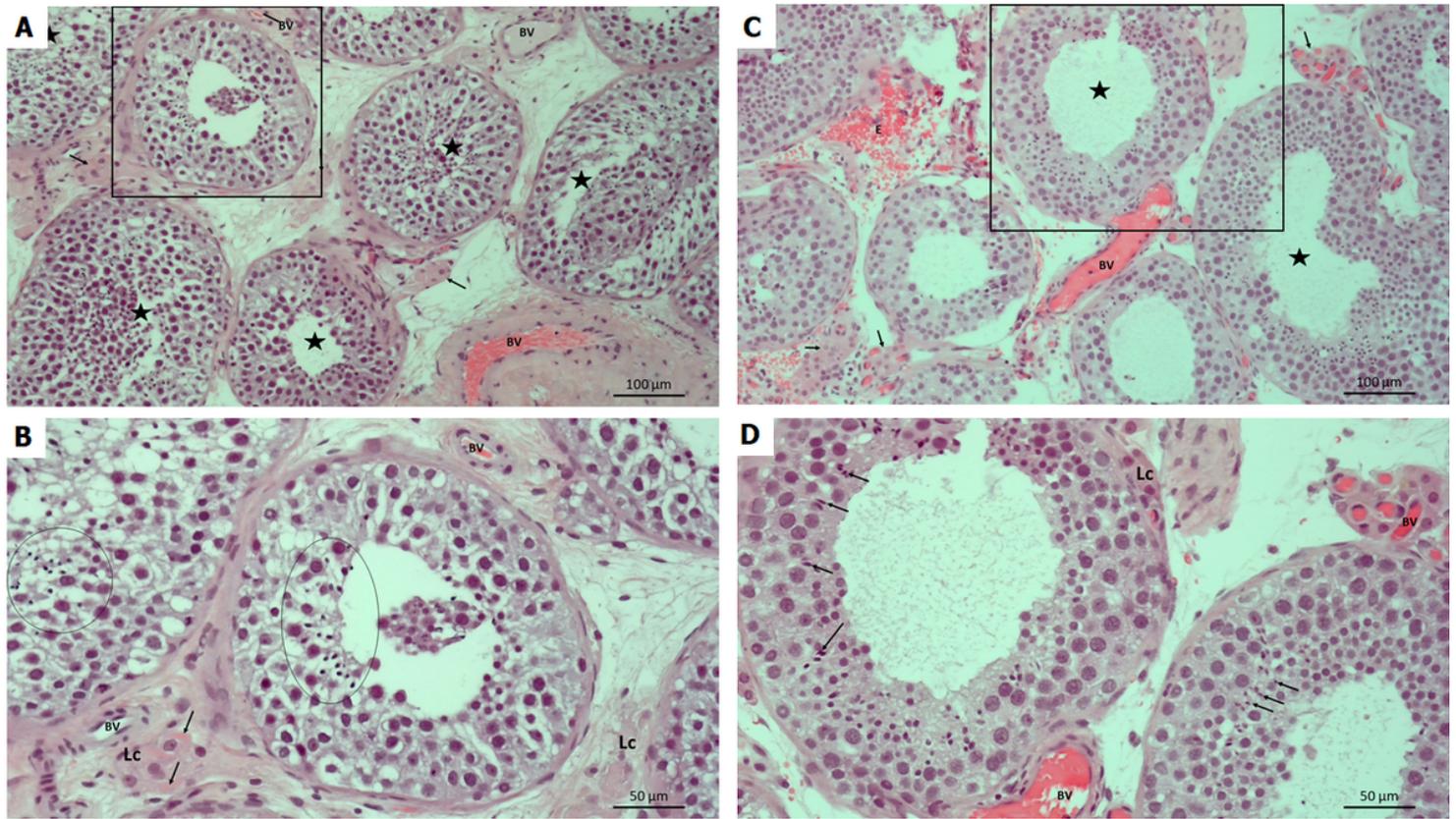
**Figure 1**

Flowchart of the patients included in the study. Out of the 60 patients assessed for eligibility, 48 were finally included. These were divided into 3 groups, those with cryptorchidism, varicocele, and idiopathic NOA. Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; GCNIS, germ cell neoplasia in situ; NOA, non-obstructive azoospermia.



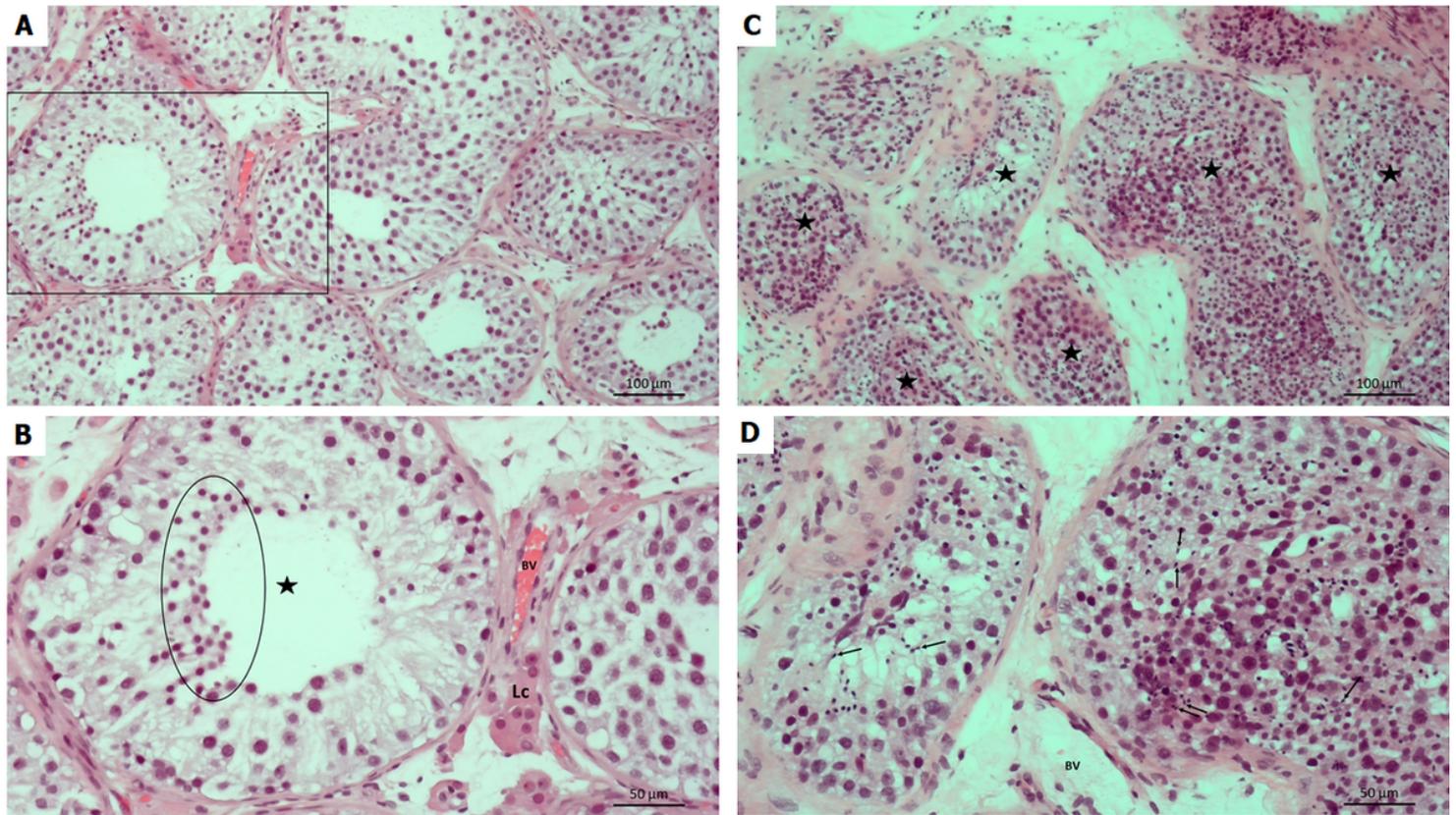
**Figure 2**

Testicular histology of patients code 160585017 and 141879319. Panel A. Testicular biopsy from an azoospermic patient. Seminiferous tubules display narrow lumen and are lined with Sertoli cells exclusively. Leydig cells clusters are seen between the seminiferous tubules (→). Patient 160585017. Panel B. Detail from Panel A. Due to the depletion of spermatogenic cells, Sertoli cells display giant vacuoles in their cytoplasm (→). Lc, clusters of Leydig cells. (H&E, x200, scale = 50 μm) Patient 160585017. Panel C. An azoospermic patient with mixed atrophy of seminiferous tubules. Seminiferous epithelium displays a variable status of spermatogenesis (\*). Patient 141879319. Panel D. Detail from Panel C. In one of the seminiferous tubules, late spermatids (→) are clearly visible, despite the disorganisation of the seminiferous epithelium. Lc, Leydig cells (H&E, x200, scale bar = 50 μm) Patient 141879319.



**Figure 3**

Testicular histology of patients code 265952375 and 119526696. Panel A. Testicular biopsy from a patient with hypospermatogenesis. In most tubules, (\*) seminiferous epithelium is slightly disorganised but bears late spermatids. Within the loose connective tissue, there are clusters of Leydig cells of moderate size (→). BV, blood vessels. Patient 265952375. Panel B. Detail from Panel A. Despite changed stratification of spermatogenic cells, seminiferous tubules preserved the capacity to produce late spermatids (circled areas). The interstitial compartment bears small blood vessels (BV) and Leydig cells (Lc). Some Leydig cells are rich in Reinke's crystals (→). (H&E, x200, scale bar = 50 μm) Patient 265952375. Panel C. Testicular parenchyma sample from an azoospermic man. Seminiferous tubules display heterogeneous morphology of spermatogenesis (hypospermatogenesis, maturation arrest), including some tubules rich in late spermatids (\*). Clusters of Leydig cells (→) have an abundant network of accompanying capillaries. As a consequence of surgery, extravasated erythrocytes (E) are frequently encountered in the bioptic material. BV, blood vessel. Patient 119526696. Panel D. Detail of Panel C. These two seminiferous tubules have maintained a regular diameter and morphology of spermatogenesis. Some late spermatids (→) are in the final stage of spermiogenesis and will be released as spermatozoa in the lumen. Peritubular Leydig cells (Lc) are found adjacent to the lamina propria of the tubule. The interstitium is rich in blood vessels (BV). (H&E, x200, scale = 50 μm) Patient 119526696.



**Figure 4**  
 Testicular histology of patients code 122836561 and 16368087. Panel A. Testicular biopsy from an azoospermic patient with maturation arrest. The tubules are lined with Sertoli cells and all types of spermatogenic cells, apart from late spermatids. Patient 122836561. Panel B. Detail of Panel A. Seminiferous tubule (\*) with the "spermatid stop" (maturation arrest). Spermatogenesis progresses only to the stage of round (early) spermatids (circled area). Lc, Leydig cells. BV. (H&E, x200, scale = 50 µm) Patient 122836561. Panel C. A testicular tissue sample from an azoospermic patient with hypospermatogenesis. The stratification of seminiferous epithelium (\*) is not maintained. However, all spermatogenic cells, including late spermatids, are visible. Patient 163680875. Panel D. Detail of Panel C. Despite the disorganised seminiferous epithelium, many late spermatids (→) can be identified. BV, blood vessel. (H&E, x200, scale = 50 µm) Patient 163680875.