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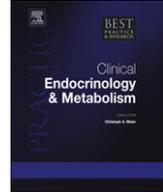


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# Male infertility: Pathogenesis and clinical diagnosis

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Infertility affects about 7% of all men. The etiology of impaired sperm production and function can be related to factors acting at pre-testicular, post-testicular or directly at the testicular level. Primary testicular failure accounts for about 75% of all male factor infertility. Genetic factors can be identified in about 15% of cases (congenital hypogonadotrophic hypogonadism, congenital absence of vas deferens, primitive testicular failure). Despite progresses, mainly in the field of genetics, the etiology is still unknown in about 50% cases and it is termed "idiopathic infertility". A part from few exceptions, the only available therapy for male factor infertility is assisted reproduction which allows conception also in severe male factor, including azoospermia following testicular sperm extraction. The complete diagnostic workup is important for: i) the identification of treatable/reversible or health-threatening conditions; ii) selection of patients for assisted reproductive techniques; iii) for appropriate genetic counselling including preventive measures (preimplantation or prenatal diagnosis) to safeguard the health of future offspring.

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## Background

About one out of seven European couples suffer from reproductive health disorders in the form of infertility or sterility. As male causes for infertility are found in half of involuntarily childless couples, it must be assumed that approximately 7% of all men are confronted with fertility problems.<sup>1</sup> The etiology of impaired sperm production and function can be related to different congenital or acquired factors acting at pre-testicular, post-testicular or directly at the testicular level (Table 1). Genetic factors can be identified in each etiologic category and some of them are currently part of the diagnostic workup of selected groups of patients (Table 2).

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**Table 1**

List of etiological factors involved in male factor infertility.

<i>Congenital factors</i>	
-Anorchia	
-Cryptorchidism	
-Congenital Absence of Vas Deferens	
-Genetic abnormalities (caryotype anomalies including Klinefelter syndrome; Y chromosome microdeletions; Kallmann syndrome, mutations in genes involved in Hypothalamus–pituitary–gonadal axis, Partial/Mild Androgen Insensitivity syndrome)	
<i>Acquired factors</i>	
-Testis trauma	
-Testicular torsion	
-Post-inflammatory forms (orchitis, epididymitis)	
-Obstruction, subobstruction of proximal and/or distal urogenital tract	
-Recurrent urogenital infections, prostatitis, prostatovesiculitis	
-Exogenous factors (medications, cytotoxic drugs, irradiation, heat etc)	
-Systemic diseases (liver cirrhosis, renal failure etc)	
-Varicocele (depending on the grade)	
-Surgeries that can damage vascularisation of the testes	
-Erectile, ejaculatory dysfunction	
-Acquired hypogonadotropic hypogonadism or endocrine factors	
<i>Idiopathic forms</i>	
-Unknown etiology (about 50%)	

## General clinical approach

It is generally accepted that the diagnostic workup of infertile couple should be initiated after 12 months of regular unprotected intercourse. However it must be taken into consideration that about half of the couples which do not conceive during the first year will do so during the second year. Earlier evaluation may be performed if known male or female risk factors exist. The diagnostic workup of the infertile male should include careful medical and reproductive history, physical examination and semen analysis followed by second level exams.

**Table 2**

Diagnostic genetic testing in male infertility and therapeutic options. IHH: Idiopathic Hypogonadotropic Hypogonadism; ART: Assisted Reproductive Techniques; IUI: Intrauterine Insemination; IVF: In Vitro Fertilization; ICSI: IntraCyttoplasmic Sperm Injection; sdr: syndrome; spzoa: spermatozoa.

Gene or region	Indication for testing	Therapeutic option
<i>KAL-1</i>	<i>Hypogonadotropic Hypogonadism</i>	<i>Hormone therapy or ART</i>
<i>KAL-2 (FGFR1)</i>	Kallmann sdr	
<i>PROK2/PROK2R</i>	Kallmann sdr or normosmic IHH	
<i>FGF8</i>	Kallmann sdr or normosmic IHH	
<i>GnRH1/GNRHR</i>	IHH (normosmic)	
<i>KISS1/GPR54</i>	IHH (normosmic)	
<i>TAC3/TAC3R</i>	IHH (normosmic)	
<i>FSH</i>	Isolated FSH deficiency	
<i>LH</i>	Isolated LH deficiency	
<i>CFTR</i>	<i>Congenital Post-testicular forms</i>	<i>ART (IVF or ICSI)</i>
	Congenital Absence of Vas Deferens (uni/bilateral)	
	Idiopathic epididymal obstruction	
<i>Chromosomal anomalies</i>	<i>Primary testicular dysfunction</i>	<i>ART (IUI, IVF or ICSI)</i>
<i>Y chromosome microdeletions (AZFa, AZFb, AZFc)</i>	Azoo or <10 millions spzoa/ml	
<i>gr/gr deletion (partial AZFc)</i>	Azoo or <5 millions spzoa/ml	
<i>AR mutations</i>	Oligozoospermia (<20 millions spzoa/ml)	
	Hypoandrogenized infertile man	

*Medical and reproductive history* is focused on the identification of risk factors or behavioral patterns that could affect fertility. Systemic diseases, previous chemo/radiotherapy, alcohol abuse, gonadal toxicant exposure, intake of anabolic steroids and toxic drugs should be ruled out. The patient should be asked about i) familiarity for infertility, recurrent abortion and malformations; ii) duration of infertility and prior fertility with previous or current partner; iii) impaired libido, reduction of the volume of ejaculate, erectile or ejaculatory dysfunction, coital frequency and timing iv) orchitis, testicular trauma, inguinal surgery, cryptorchidism and varicocele; iv) sexually transmitted diseases, prostatitis, prostatovesiculitis and recurrent urogenital infections.

*Physical exam* should include evaluation of secondary sex characteristics such as hair distribution and escutcheon, body proportions, voice and gynecomastia. Particular focus should be given to the genitalia: i) examination of the penis including the location of urethral meatus; ii) measurement of testicular volume by Prader orchidometer; iii) palpation of the testes, epididymides for cysts and consistency, vas deferens for total or segmental absence; iv) palpation of scrotum for varicocele; v) digital rectal exam. Certain physical features such as hypoandrogenization, hypospadias may be due to mutations in the androgen receptor gene. Extremely small firm testis, with typical eunocoid features are indicative for Klinefelter syndrome. On the other hand, an eunocoid habitus with infantile genitalia, sparse or nearly absent body hair, gynecomastia and low testicular volume is typical of congenital gonadotrophin deficiency (the presence of hypo/anosmia orients towards Kallmann syndrome).

*Semen analysis* is of fundamental importance to diagnose and define the severity of the male factor. However, it must be kept in mind, that although the definition of male factor is based on abnormal semen parameters, other male factors (rare functional defects) may play a role even when the semen analysis is normal. Conversely, severe disturbances of semen parameters may still be compatible with the couple's fertility since a highly fertile female partner may compensate for male subfertility. Semen analysis should be performed according to the WHO manual in a standardized way.<sup>2</sup> This analysis will provide sperm density, total number, motility, morphology and semen parameters such as semen volume, pH, viscosity. Reference ranges for sperm parameters have been recently updated and provided by the last version of the WHO manual.<sup>2</sup> In case abnormal sperm parameters are found semen analysis should be repeated. The nomenclature used in case of pathological findings is reported in Table 3. In general, diagnosis should be based on at least 2 semen analyses and if they are discordant a third exam is requested. Pre-analytical factors able to interfere with the reliability of the analysis are: i) inappropriate collection or transport of semen (the specimen should be kept at body temperature during transport; the abstinence period should be between 2–5 days, no loss of ejaculate during collection should occur) ii) antibiotic therapy or fever before semen collection (N.B. spermatogenesis takes almost 3 months in human and the effect of an exogenous noxae may persist over 2–3 months). Analytical factors may also alter the reliability of the exam and for this reason laboratories should participate at an external quality control program. In the era of second level assisted reproductive techniques, such as Intra Cytoplasmic Sperm Injection (ICSI) for which few spermatozoa are sufficient for fertilization, it is important that the laboratory performs the analysis of pellet after centrifugation of the semen specimen, in order to distinguish between azoospermia (complete absence of spermatozoa both in the ejaculate and in the pellet) and cryptozoospermia (absence of spermatozoa in the ejaculate but detection of spermatozoa in the pellet). Similarly, the distinction between necrozoospermia (immotile and dead) and immotile spermatozoa (living but immotile) by supravital staining is

**Table 3**

Nomenclature related to pathological semen quality according to WHO (2010).<sup>2</sup>

Oligozoospermia	Sperm concentration $<15 \times 10^6/\text{ml}$ ; total sperm number $<39 \times 10^6/\text{ml}$
Asthenozoospermia	$<32\%$ progressively motile spermatozoa
Teratozoospermia	$<4\%$ morphologically normal spermatozoa
Oligo-asteno-teratozoospermia	Disturbance of all three parameters
Azoospermia	No spermatozoa in the ejaculate
Cryptozoospermia	Spermatozoa absent from fresh preparation but observed in a centrifuged pellet
Aspermia	No ejaculate
Leucospermia (leucocytospermia)	$>1 \times 10^6/\text{ml}$ leucocytes in the ejaculate

important before ICSI is advised, since in the former case the likelihood of successful embryo development is virtually zero.

*Second level exams* should be performed to further elucidate the etiology and are selected on the basis of clinical suspicion and semen phenotype. Some of these analyses may also provide prognostic values for testicular sperm retrieval in azoospermic men. The most relevant second level exams are: hormone measurement (FSH, LH, total testosterone and SHBG), genetic testing, microbiological exam of semen and urine, urethral swab, scrotal and testicular ultrasound, scrotal color Doppler and transrectal ultrasound.

## Etiology of male factor infertility

### *pre-testicular causes*

This category of infertility includes mainly two types of pathological conditions: hypogonadotropic hypogonadism (HH) and coital disorders (erectile dysfunction, ejaculatory disorders such as ejaculatio precox, retrograde ejaculation).

#### *Hypogonadotropic hypogonadism*

The deficit of LH and FSH secretion may be due to congenital or acquired factors which may be related to a primary lesion in the pituitary gland or could be secondary to insufficient hypothalamic GnRH production.<sup>3,4</sup> A detailed description of the **congenital** forms of HH is already provided in this issue by Dr Rey and Dr Grinspon. The list of candidate genes (Table 2) both for normosmic HH and Kallmann syndrome is constantly growing and genetic testing should be based on the patient's gender, family history (if any), mode of disease inheritance, and the presence of additional clinical anomalies. In about 30–40% of patients mutation in one (or more than one) candidate gene(s) can be now identified.<sup>4</sup>

The diagnosis of **acquired** HH, is of importance not only with respect to infertility but also for its potential implications to general health (in some cases it may represent a life-threatening condition). In fact, acquired HH can be related to tumours (both benign or malignant), and may affect the secretion of more than one pituitary hormone leading to their deficit or excess. Common causes of acquired HH are tumours (secreting or non secreting pituitary adenomas, craniopharyngioma, meningioma etc), infections, infiltrative diseases, empty sella, radiation treatment and autoimmune hypophysitis.

The phenotypic presentation of congenital and acquired forms are substantially different. **Congenital** conditions are usually associated with delayed puberty and related signs (eunuchoid habitus, sparse or nearly absent body hair, gynecomastia and very low testicular volume). However, in some cases reduced spermatogenesis and mild hypoandrogenism may be the only symptoms and thus the diagnosis may be delayed until adulthood. **Acquired** forms in adulthood usually manifest with few symptoms (if only gonadotrophins are affected) such as the reduction of the volume of ejaculate, of beard growth, impaired libido and asthenia.

The diagnosis of HH is based on hormone measurement showing low plasma levels of LH, FSH and testosterone but in certain forms other pituitary hormones may be increased (for example PRL or GH) or decreased depending on the etiology. Brain imaging (MR or CT) and of sella turcica completes the diagnostic workup in acquired cases. Anabolic steroid abuse may also lead to pseudo-central form of hypogonadism and patients should be explicitly asked about the use of such drugs when no sign of hypoandrogenization is present with very low LH values.

HH is a treatable form of infertility and the treatment with gonadotrophins will allow natural conception in the large majority of cases (even with relatively low sperm count, if the female partner is fertile). The identification of the involved gene in the congenital forms can facilitate a more accurate genetic counselling i.e. a risk estimation for transmission to the offspring. It is also interesting to note, that in some cases of congenital HH, long term testosterone treatment has lead to spontaneous reversibility of reproductive function.<sup>5,6</sup> It is therefore plausible that in the future, the identification of mutations will have not only diagnostic but also prognostic value for the treatment options and responsiveness.

### Coital disorders

Erectile dysfunction and ejaculatory disorders are very rare cause of infertility. A careful andrological diagnostic workup is mandatory in order to exclude organic forms of erectile dysfunction (vascular, neurogenic) and other co-morbidities. Anejaculation or retrograde ejaculation may occur in diabetic patients as well as after retroperitoneal lymph-node dissection, spinal cord injury,<sup>7</sup> bladder neck surgery and multiple sclerosis.<sup>8</sup>

The diagnosis of retrograde ejaculation is based on the absence of spermatozoa in the seminal fluid but their presence in the urine after masturbation. Assisted reproductive techniques (mainly in vitro fertilization with embryo transfer, IVF) are the only reliable options in case of retrograde ejaculation. In subjects affected by anejaculation due to spinal cord injury both vibrostimulation and electro-ejaculation are effective methods for sperm retrieval and pregnancy can be obtained via IntraCytoplasmic Sperm Injection (ICSI). Medical treatment is widely available for most patients affected by erectile dysfunction and premature ejaculation. Psychosexual therapy (individual or of the couple) is a valid therapeutic option especially for premature ejaculation and psychogenic erectile dysfunction.

### Post-testicular causes

This etiological category includes all obstructive/subobstructive lesions of the seminal tract (distal or proximal), infections and inflammatory diseases of accessory glands and autoimmune infertility. In case of bilateral obstruction the semen phenotype is azoospermia (absence of spermatozoa in the ejaculate) while in the other post-testicular conditions a different degree of impairment of the three major sperm parameters (sperm count, motility and morphology) can be observed. Diseases affecting the accessory glands are typically associated with low volume of ejaculate due to the fact that about 90% of the ejaculate originates from the seminal vesicles and prostate. Low semen volume with high pH and viscosity is characteristic for prostatitis or ejaculatory duct obstruction due to prostatic cyst. The presence of leucocytes over 1 millions/ml is typical for inflammation of accessory glands. The most common pathogens causing infections of the urogenital tract are *Ureaplasma urealyticum*, *Enterococcus faecalis*, *Escherichia Coli* and are usually associated with typical irritative urinary tract symptoms and prostatodynia.<sup>9,10</sup> The presence of micro organisms and/or leucocytes in the seminal fluid may affect sperm motility and the fertilizing capacity of spermatozoa due to the production of reactive oxygen radicals by activated leucocytes.<sup>11</sup> An autoimmune reaction against the spermatozoa as an isolated abnormality is seen in <5% of infertile males. The presence of antisperm antibodies can be the consequence of previous infections or inflammatory diseases of the testis or epididymis able to disrupt the haematotesticular barrier. If the percentage of motile spermatozoa coated by antisperm antibodies is >80%, a pure immunological factor is likely and the only therapeutic option is in vitro fertilization.

### Congenital absence of the vas deferens

This is a congenital post-testicular disease which may affect one or both vas deferens and is typically associated with agenesis of the seminal vesicles, and epididymal malformations. The Congenital Bilateral Absence of the Vas Deferens (CBAVD) with agenesis of seminal vesicles can be suspected after scrotal examination (absence of vas deferens) and on the basis of semen analysis: semen volume <1.0 ml with an acid pH (<7) and absence of spermatozoa and of immature germ cells in semen smears. Transrectal ultrasound and eventually the measurement of seminal markers of the seminal vesicles (fructose) or epididymis (alpha-glucosidase) will provide further elements for the final diagnosis. The diagnosis of CBAVD together with the unilateral form is of particular interest since this pathology is considered a mild form of cystic fibrosis (CF) and transmitted as an autosomal recessive disease.<sup>12,13</sup> The most widely diffused mutation both in CF and CAVD is the severe delta F508 (p.Phe508del) mutation (about 70% of the total CF mutations in patients). In case of CAVD the second mutation is “mild” whereas in case of CF it is usually a “severe” mutation. The role of intron 8 variants (IVS8-5T, IVS8-7T, IVS8-9T) in the phenotypic expression of mutations is now well established. The three variants include different numbers of thymidines within the acceptor splice site of intron 8 i.e. 5, 7 and 9, respectively. The length of the T tract affects the splicing efficiency of exon 9 and thus the

percent of normal CFTR mRNA. The 5T tract is the less efficient one and allows about 8–10% of CFTR mRNA to be completed with exon 9. The lack of exon 9 leads to a non-functional Cl channel and thus the combination of 5T with other mutations (severe or mild) may cause the development of CF or CAVD, respectively. There is a five- to six-fold increase in the frequency of the 5T variant among CAVD chromosomes.<sup>14</sup>

Some studies from the late 90's suggested an association between CFTR mutation and defective testicular function, however latest data based on an extensive CFTR analysis in infertile individuals versus well selected fertile population definitively excludes the involvement of the CFTR gene variants in sperm production.<sup>15</sup> In a mouse model, Xu et al.<sup>16</sup> demonstrated that CFTR protein is also involved in HCO<sub>3</sub>-membrane transport which is essential for sperm capacitation and thus for sperm fertilizing ability. These data suggest a possible role for CFTR mutations in the etiology of unexplained couple infertility but it requires further confirmation.

All men with CAVD should be screened for CFTR mutation with the exception of those who present renal agenesis/malformation which is likely to be related to an other not yet identified gene defect. Given that the frequency of a particular CFTR mutation is variable between different geographic areas and shows important ethnic differences, the routine mutation screening is based on a panel of mutations (in average 30 mutations) which are the most common for a given population. Patients affected by CAVD may have sperm in their ejaculate (monolateral absence of vas deferens) or be azoospermic (bilateral absence of vas deferens). Since azoospermia is due to obstruction, patients with CBAVD are assumed to have normal testicular function. Thanks to the possibility to combine testis biopsy<sup>14</sup> with ICSI both CF or CBAVD patients may now generate their own biological children, but they can transmit their CFTR mutations to their descendents. Since the carrier frequency of CFTR mutations in persons with European descent is generally high (1:25), the screening for CF gene mutations in the female partners of men with CAVD without congenital kidney anomalies or with CF should be performed before assisted reproduction. If mutations are detected in both partners, the risk of an offspring with CF (or mild forms of CF such as CAVD, depending on the type and combination of mutations) is very high and Preimplantation Genetic Diagnosis (PGD) should be advised to the couple, although this diagnostic procedure is not allowed in some countries. However, it remains in most cases difficult to make precise risk estimates due to different degree of penetrance of the same genotype between different individuals.<sup>17</sup>

*The diagnosis of post-testicular forms* is based on physical exam (testis volume is typically normal, signs of epididymal or vas deferens obstruction or cyst or agenesis), normal FSH values and scrotal and/or transrectal ultrasound (to better localize the level of obstruction and the accessory glands). In addition, microbiological exams should be performed in case of suspected prostatitis or prostate-vesiculitis or epididymitis or of history of recurrent urogenital tract infections in the patient or in his partner. The search for pathogens such as Chlamydia and Ureaplasma should be performed in urethral swab, while other germs can be analyzed in the seminal fluid and urine. As stated above, in case of CAVD (without kidney malformations) and congenital epididymal malformations screening for CFTR gene mutations is mandatory not only in the patient but also in his partner.

### **Primitive testicular dysfunction**

A large number of pathologies may lead to primary testicular failure (see Table 1). Among them cryptorchidism (especially bilateral forms), orchitis, testis trauma, torsions, iatrogenic forms (gonadotoxic medications, chemo/radiotherapy, previous inguinal surgery), some systemic diseases and genetic factors such as caryotype anomalies and Y chromosome microdeletions are well defined causes of impaired sperm production.

### *Varicocele*

The association of varicocele with infertility is not clear-cut as it can be observed also in about 10% of normozoospermic men. On the basis of the WHO data<sup>18</sup> varicocele is related to semen abnormalities, decreased testicular volume and decline in Leydig cell function and therefore it can be considered as a co-factor of impaired sperm production.

Despite this potential negative effect, which is largely dependent on the grade of varicocele, the large WHO infertility study focusing on varicocele<sup>19</sup> indicated that there was an excess of couples in which both partners had factors associated with reduced fertility compared with the expected rate of coincidence in the general population. This implies that a minor cause of impaired fertility, such as varicocele, will manifest more likely in couples in which the female partner also has reduced fertility. The diagnosis of varicocele is made by clinical examination and can be confirmed by colour Doppler analysis. Diagnostic procedure and classification of a varicocele has been defined by the WHO.<sup>20</sup> Reviews of randomised clinical trials have raised doubts about the benefit of varicocele treatment in infertile men, and therefore the couple should be informed about the uncertainties of treatment benefit.<sup>21,22</sup> According to the EAU guidelines 2010,<sup>23</sup> varicocele treatment is recommended for symptomatic cases and for adolescents who have progressive failure of testicular development documented by serial clinical examinations.<sup>24,25</sup>

### *Cryptorchidism*

Cryptorchidism is the most frequent congenital birth defect in male children and can occur as an isolated disorder or in association with other congenital anomalies (syndromic cryptorchidism).<sup>26</sup> The incidence varies between 2 and 9% at birth (with some geographical variations across Europe) and decreases to 1–2% by 3 months of age due to delayed spontaneous descent.<sup>27–29</sup> However, cryptorchidism is found not only as a congenital disorder, but also as an acquired disorder diagnosed during infancy and childhood.<sup>29,30</sup> The so called “acquired cryptorchidism” is defined as the ascent of the testis into a cryptorchid position after normal scrotal position at birth and its cumulative incidence by age 24 months can be even higher than that observed at birth (in the UK congenital forms have a prevalence of 5.7% while “acquired” forms 7%).<sup>31</sup>

The interaction of genetic and environmental (mainly endocrine disrupters) factors acting on the fetal testis has been proposed as the major determinant of the progressive increase during the past 50 years of pathologies such as cryptorchidism, testis cancer, hypospadias and impaired spermatogenesis in Western countries. This observation led to the theory of the testicular dysgenesis syndrome which includes all the four above mentioned pathologies.<sup>32</sup> Congenital and acquired cryptorchidism are both very common but may have different etiology and consequences for fertility may also be different.

Two major hormone/receptor systems are involved in testis descent: testosterone and its receptor AR (Androgen Receptor) and the INSL3/RXFP2 (former LGR8) system.<sup>27,33</sup> Insulin-like factor 3 (INSL3) and its G-protein-coupled receptor, RXFP2 (relaxin family peptide 2) are essential for the first phase of testicular descent whereas androgens are involved in the inguinal phase. Mutations in the Androgen Receptor (AR) gene are associated predominately with syndromic forms of cryptorchidism.<sup>33</sup> Another well known syndromic form is related to a karyotype anomaly, the Klinefelter syndrome.

The *INSL3* gene has been extensively screened for mutations but mainly polymorphisms and only two missense, the P49S and V18M mutations have so far been reported.<sup>34–36</sup> The role of these two mutations (all heterozygous) in the pathogenesis of cryptorchidism remains to be established. The *RXFP2* gene has been sequenced only in a small number of subjects (approx 160 patients) and one missense mutation at codon 222 (T222P) has been reported. Subsequent studies have been all focusing on the T222P variant and data for more than 4000 subjects are now available (Ref. 37 and references therein). The distribution of this variant shows marked geographic differences, and it is mainly present in the Mediterranean area due to a “founder effect”.<sup>37–40</sup> Given that this variant has been reported also in controls with normal testis descent (1–2%), it cannot be considered a clear-cut cause of cryptorchidism and should not be included in the list of causative genetic alterations of this disease. In Italy it seems to confer a mild risk to cryptorchidism but its diagnostic role is greatly limited by the fact that 1.7% of controls are carrier of this variant.<sup>37</sup> At the moment, no genetic test is available for non-syndromic cryptorchidism.

### *Genetic factors influencing spermatogenesis*

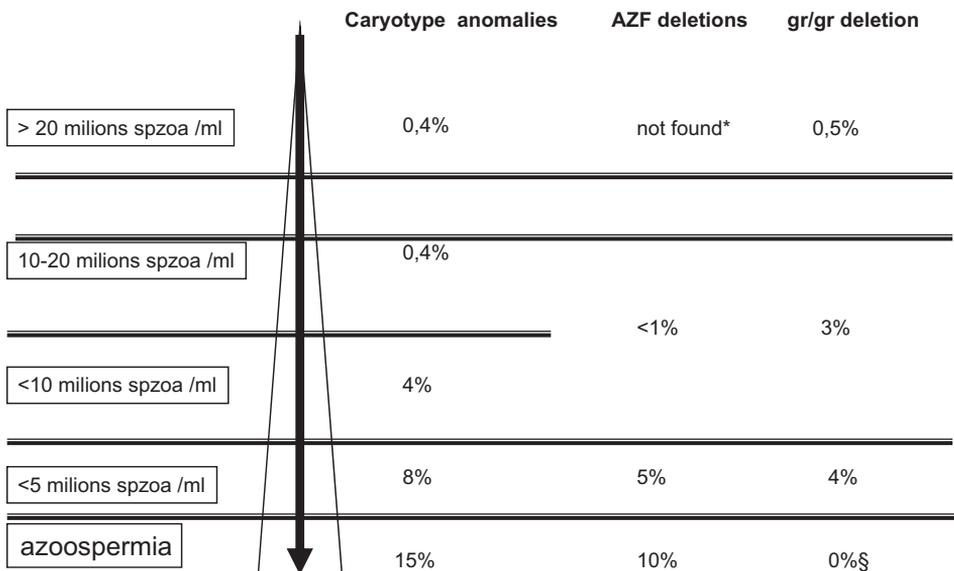
Two routine genetic tests are performed during the diagnostic workup of oligo/azoospermic men: karyotype analysis and Y chromosome microdeletion analysis. Mutation analysis of the AR gene should be performed only in selected cases since evidence for a routine testing is still lacking.

### Chromosomal abnormalities

Caryotype abnormalities occurs in about 0.4% of the general population and can affect the number or the structure of chromosomes. The majority of chromosome abnormalities are generated during meiosis.<sup>41</sup> Severely impaired sperm production is associated with a significantly higher frequency of both numerical and structural chromosomopathies.<sup>42</sup>

The more severe is the testicular phenotype the higher is the frequency of chromosomal abnormalities (Fig. 1). Patients with less than 10 million spermatozoa/ml show already a 10 times higher incidence (4%) of mainly autosomal structural abnormalities in respect to the general population. Among severe oligozoospermic men (<5 million spermatozoa/ml) the frequency increases to 7–8%, whereas in non-obstructive azoospermic men it reaches the highest values, 15–16%.<sup>42</sup>

Klinefelter syndrome (47, XXY) represents the most common caryotype abnormality in azoospermia and severe male factor infertility, followed by Y chromosome terminal deletions (Yq-) and structural autosomal abnormalities. About 80% of patients bear a 47,XXY caryotype whereas the other 20% represented either by 47,XXY/46,XY mosaics or higher grade sex chromosomal aneuploidy or structurally abnormal X chromosome. A detailed clinical description of the Klinefelter syndrome is provided in this issue by Dr Wikstrom and Dr Dunkel. Concerning reproduction, the large majority of subjects affected by this syndrome are azoospermic and would not be able to naturally conceive. However, Testicular Sperm Extraction (TESE) and especially microsurgical TESE (micro-TESE) followed by ICSI with an average of 30–50% of testicular sperm recovery rate may allow Klinefelter patients to generate their own genetic children.<sup>43–47</sup> Moreover spermatozoa can even be found in the ejaculate of mainly mosaic patients or in non mosaic but young patients,<sup>43</sup> indicating the potential importance of an early diagnosis which would allow a preventive cryopreservation of ejaculated spermatozoa to preserve fertility. The genetic integrity of gametes has been questioned by FISH studies (for review see Martin RH<sup>48</sup> and references therein) and a study based on ICSI combined with PGD reporting a significant fall in the rate of normal embryos for couples with Klinefelter syndrome in respect to controls (54% versus 77.2%).<sup>49</sup> However, according to recent reviews children born from Klinefelter fathers are healthy and only one 47,XXY fetus has been reported so far (for review see Staessen et al.,<sup>49</sup> Lanfranco et al.,<sup>50</sup> Fullerton et al.<sup>47</sup> and references therein). Despite this encouraging data, due to the



**Fig. 1.** Frequency of genetic anomalies in primitive testicular failure. The % of caryotype anomalies is based on Ref. 35; AZF deletions on Ref. 48,51 and gr/gr deletion on Ref. 53 (the largest caucasian population). § The frequency of gr/gr deletion varies in different geographic areas and this deletion type was found also in azoospermic men by other authors \*Clinically relevant, complete AZF deletions were not found in normozoospermic men. Spzoa: spermatozoa

significant increase of sex chromosomal and autosomal abnormalities in the embryos of Klinefelter patients, ICSI+PGD should be an appropriate preventive option.<sup>49</sup>

Oligozoospermic men with <10 million spermatozoa have a higher risk to have autosomal abnormalities than azoospermic men.<sup>42</sup> Robertsonian translocations, reciprocal translocations, paracentric inversions and marker chromosomes are the most frequently found abnormalities. The importance of the detection of these structural chromosomal anomalies is related to the increased risk of aneuploidy or unbalanced chromosomal complements in the fetus. In case of Robertsonian translocations a special risk is represented by uniparental disomies which are generated through a mechanism called “trisomy rescue” (repairing of trisomy) during the first division of the zygote. For chromosome 14 (the most frequently involved chromosome) and 15, both paternal and maternal uniparental disomies are pathological and give rise to severe disease such as Angelman or Prader–Willi syndromes despite an apparently normal or balanced karyotype.

#### *Y chromosome microdeletion analysis*

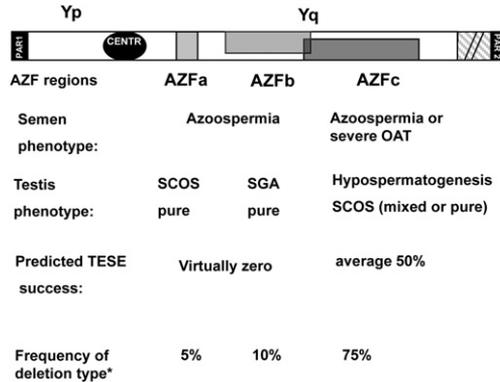
Clinically relevant deletions occur on the long arm of the Y chromosome and remove partially or in the large majority of cases completely one or more AZF regions (classically divided into three AZoospermia Factor regions, AZFa, AZFb and AZFc<sup>51</sup>). Five recombination hot spots have been identified on the Yq11 which are responsible for the formation of deletions through a mechanism called non-allelic homologous recombination.<sup>52</sup> Following the definition of the five deletion breakpoints, it became evident that the AZFb and AZFc regions are overlapping, which implies that “complete” AZFb deletion always removes part of the AZFc region.<sup>53</sup> However, since the currently used screening strategy is based on markers which are unable to distinguish between the 3 subtypes involving the AZFb+c regions, for clinical practice we continue to refer to the three “classical” deletion intervals AZFa, AZFb and AZFc. In each AZF region a number of candidate genes have been identified, however their function in spermatogenesis remains largely unknown.<sup>52</sup> Since deletions occur in block-removing more than one gene – the role of a single AZF gene cannot be extrapolated from the AZF deletion phenotype and thus it is unclear if they are all participating in spermatogenesis. Gene specific deletions removing a single gene has been reported only in the AZFa region, these studies suggest that *USP9Y* gene is not essential for spermatogenesis and it is most likely a “fine tuner” of sperm production.<sup>54</sup>

Y microdeletion is the most frequent known molecular genetic cause of severe impairment of spermatogenesis. Its frequency is about 10% in non-obstructive azoospermic and 3–5% in idiopathic severe oligozoospermic men.<sup>55</sup> The identification of Yq microdeletions is not only relevant for the diagnosis but it may have prognostic value prior testicular biopsy (TESE).<sup>56,57</sup> In this regard, in case of complete AZFa and AZFb deletions of the Y chromosome testicular biopsy is not advised because the chance of finding spermatozoa is virtually zero (Fig. 2). Those Y deletions which are compatible with the presence of spermatozoa in the testis or in the ejaculate are obligatorily transmitted to the male offspring, therefore genetic counselling is mandatory. The extent of spermatogenic failure of the son may vary substantially, however given the strict cause–effect relationship between AZF deletions and impaired spermatogenesis, normal spermatogenesis cannot be expected.

*Indications* for AZF deletions screening are based on sperm count and include azoospermia and severe oligozoospermia (<5 million spermatozoa/ml). Thanks to the EAA guidelines<sup>58</sup> and EAA/EMQN external quality control programme (<http://www.emqn.org/emqn/>) Yq testing has become more homogeneous and reliable in different routine genetic laboratories. The EAA guidelines provide a set of primers (two markers for each region) which is able to detect virtually all clinically relevant deletions. The initial large variability of deletion frequencies was more likely the consequence of technical problems and the use of unreliable markers rather than the existence of true ethnic differences.

#### *gr/gr deletion*

A different type of Yq deletions has been described in the AZFc region and termed “gr/gr deletion”.<sup>59</sup> This deletion removes half of the AZFc region gene content affecting the dosage of multicopy genes mapping inside this region (such as *DAZ*, *CDY1*, *BPY2*). A 7 times higher risk (OR = 7.9 with 95% CI (1.8–33.8);  $p < 0.001$ ) for developing oligozoospermia has been reported in gr/gr deletion carriers



**Fig. 2.** Schematic representation of the Y chromosome with the AZF regions. Genotype/phenotype correlation and the prognosis for testicular sperm retrieval in azoospermic carriers of “complete” AZF deletions is shown. \*The frequency of different types of AZF deletions are calculated on total number of deletion carriers. The missing 10% of deletions are deletions including more than one AZF regions such as AZFa+b+c, or AZFb+c (these large deletions called “terminal deletions” can be diagnosed by caryotype analysis as well).

TESE: Testicular Sperm Extraction; AZF: AZoospermia Factor; OAT: Oligo Astheno Teratozoospermia; PAR1 and PAR2: Pseudoautosomal Region 1 and 2; CENTR: centromere

in the largest Caucasian study population (>1000 subjects) published to date.<sup>60</sup> The frequency in oligozoospermic patients is about 4%. However, both the frequency and phenotypic expression may vary in different ethnic groups, depending on the Y chromosome background (in specific Y haplogroups, such as D2b and Q3, common in Japan and certain parts of China, the deletion is fixed and apparently does not have any negative effect on spermatogenesis). A recent meta-analysis which considers only those studies which are free of methodological and selection biases reports an overall 2.4 fold risk for reduced sperm production in *gr/gr* deletion carriers.<sup>61</sup> In the same study the authors also demonstrated that the deletion affects the spermatogenic potential even in the normal range. *gr/gr* deletion has also been reported as a potential risk factor for testicular germ cell tumours,<sup>62</sup> however, this data needs further confirmation in an ethnically and geographically matched case-control study setting.

The screening for *gr/gr* deletion is based on a PCR method described by Repping et al. (2003).<sup>59</sup> However, given a 5% false deletion rate detected in a recent multicenter study,<sup>63</sup> deletions should be confirmed by gene dosage analysis.<sup>63,64</sup>

There are mainly two reasons for *gr/gr* deletion testing in infertile men: i) the deletion contributes to the etiopathogenesis of impaired sperm production since it is able to influence the spermatogenic potential of the carrier; ii) the couple should be aware that the deletion (i.e. a genetic risk factor for impaired sperm production) will be obligatorily transmitted to their male offspring and the deletion may become complete AZFc deletion (i.e. a clear cut causative factor for spermatogenic impairment) in the next generation.<sup>65,66</sup>

#### Mutation analysis of the AR gene

The AR gene is situated on the X chromosome and its screening should be performed only in suspected mild form of Partial Androgen Insensitivity (PAIS). Patients with mild PAIS have male infertility as their primary or even sole symptoms. This condition can be suspected on the basis of hormone profile especially high Androgen Sensitivity Index (ASI) and hypoandrogenization. Since the frequency of mutations in the AR gene in unselected infertile men (including both normal and high ASI) varies between 0–1.7%<sup>67,68</sup> a routine screening is not advised.

#### Diagnosis of primary testicular dysfunction

In case of severe impairment of spermatogenesis FSH level is usually increased above the normal range (>8 IU/L)<sup>69</sup> and testis volume is reduced (<15 ml). Although both FSH values and testis

volume reflect the degree of spermatogenic failure (the more severe forms are associated with the highest FSH values and lowest testis volume), there is no absolute FSH value or testis size to predict the presence/absence of spermatozoa in the testis of an azoospermic man. Moderate/mild oligozoospermia and azoospermia due to spermatogenic arrest (meiotic or post-meiotic phases) are typically associated with normal FSH and normal testis volume. Scrotal Color Doppler ultrasound is usually performed in the case of varicocele for grading. Given that the incidence of testis cancer is higher in patients with history of cryptorchidism and in those affected by severe impairment of spermatogenesis (especially if testicles are severely hypotrophic), testis ultrasound in these patients is advised for preventive purposes. In about 2.4% of infertile men testicular microlithiasis (TM) can be detected by ultrasound. Although TM can be seen in various benign and malignant processes, it has a remarkable association with testis cancer (especially with its pre-invasive state). According to a recent review<sup>70</sup> the presence of TM is an indication for testicular biopsy if the patient has more than one risk factor for testis cancer (e.g. cryptorchidism and poor semen quality or contralateral tumour). The biopsy might reveal Carcinoma in Situ (CIS) which is considered a precursor lesion of testis cancer.

Genetic testing should be performed according to the semen phenotype (caryotype and Y deletions) and level of androgenization (AR gene mutation screening).

## Conclusions and future directions

Despite major advances in the diagnostic workup of infertile males, the etiopathogenesis of testicular failure remains undefined in about 50% of cases and are referred to as “idiopathic infertility”. These “idiopathic” cases are likely to be of genetic origin since the number of genes involved in human spermatogenesis is probably over thousands<sup>71</sup> and only a small proportion of them has so far been identified and even fewer has been analyzed. Thanks to the routine genetic tests listed in this article, a genetic factor can be diagnosed in about 15% of cases. Mutational analyses of a few spermatogenesis candidate gene have been performed mainly in research context and up to now translation of results into clinical practice is lacking.<sup>72,73</sup> Several Single Nucleotides Polymorphisms (SNPs) associated with various classes of male infertility have also been reported, but associations are weak, and replication studies have often failed to validate the initial findings.<sup>74,75</sup> A major advancement in the identification of genetic factors is expected by the use of large scale whole genome analysis (WGA). To date only a single pilot WGA<sup>76</sup> and one “follow up” study on 172 SNPs have been performed.<sup>77</sup> Although the two studies provided evidence for some SNPs as potential risk factors and identified new candidate genes, data needs to be confirmed on much larger study populations. Besides SNPs structural variations such as copy number variations in regions or in multicopy genes relevant to spermatogenesis could also contribute to infertility. Up to now only two CNVs have been correlated to infertility: i) AZFc gene dosage variation due to complete/partial removal or duplication of multicopy AZFc genes; ii) *TSPY1* copy number variation on Yp.<sup>78</sup>

Apart from few exceptions, such as central hypogonadism and some post-testicular forms, the only available treatment option for the large majority of male factor infertility is medically-assisted reproductive technology (ART), especially in vitro fertilization (IVF) or ICSI.<sup>79,80</sup> However, ART is a symptomatic therapy which does not address the underlying cause for infertility with the risk of transmitting both identified and not yet identified genetic anomalies. An increased incidence of malformations and chromosomal anomalies was reported especially when the reason for performing ART was severe male factor infertility.<sup>81,82</sup> Apart from the above mentioned health consequences of the offspring fathered by a man with severe spermatogenic failure, there is still very little known about the long term health condition of both the infertile man and of his offspring.

There is an urgent need for an intense research in the field of genetics and epigenetics of male infertility (with special interest towards gene-environmental interaction) not only to provide the missing etiologic factors but also to ensure appropriate genetic counselling and a rational basis for the development of future etiology-based prevention and therapies.

### Practice points

- Male factor infertility (abnormal sperm parameters) affects about 7% of the general male population
- Full diagnostic workup includes medical/reproductive history collection, physical examination, semen analyses (at least two) and second level exams such as hormone measurement, genetic testing, microbiological exams and scrotal/testicular/transrectal ultrasound (in selected cases)
- Genetic factors can be identified in about 15% of cases and the most relevant are caryotype abnormalities, Y chromosome microdeletions and *CFTR* gene mutation in CAVD. Their diagnosis is important for appropriate genetic counselling (risk estimate for transmission, health consequences and prevention)
- The aim of the diagnostic workup is to identify reversible/treatable (central hypogonadism, some coital disorders, some post-testicular forms) and non-reversible forms (the large majority) suitable for symptomatic therapy such as assisted reproductive techniques
- The etiology of spermatogenic failure remains undefined in about 50% of cases (“idiopathic infertility”) and these cases are likely caused by genetic factors (thousands of genes are involved in spermatogenesis and only a minority of them has been studied so far).

### Research agenda

- Infertility is a polygenic multifactorial disease with heterogeneous phenotype. It is likely that a proportion of spermatogenic disturbance is due to gene–environmental interaction and genetic background may create variability in sensitivity to environmental factors. The identification of environmental factors with negative effect on testis function and the analysis of polymorphisms in genes involved in metabolism/action of environmental factors is of importance for prevention.
- New approaches such as Whole Genome Analysis for genetic polymorphisms (SNPs, CNVs), whole genome sequencing for the identification of causative mutations in exceptionally large study populations will likely provide a major advancement in the field. Besides genetic studies, epigenetic analysis of the male gamete is also warranted.
- The identification of new genetic and epigenetic factors involved in spermatogenesis will provide a better understanding of their impact on the general and reproductive health of the carrier and his future children and a basis for etiology-based therapy.

### Conflicts of interest

The author declares that she has no conflicts of interest.

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