Genetic and molecular diagnostics of male infertility in the clinical practice

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TABLE OF CONTENTS

1. ABSTRACT

Male infertility represents one of the clearest examples of complex phenotype with substantial genetic basis. It is indeed well established that genetic causes account for 10-15% of infertility cases, including chromosomal abnormalities and single-gene mutations. However, a large proportion of infertile males does not receive a clear diagnosis and thus they are reported as idiopathic or unexplained. Male (in)fertility is commonly based on standard semen analysis, which, however, cannot clearly distinguish fertile from infertile populations and therefore fails to detect any abnormality in many cases. Abnormal sperm function or specific molecular defects can be hypothesized in these cases. This review considers practical genetic and molecular diagnostic tests for male infertility, reporting on the most frequent genetic causes of male infertility and on the pros and cons of most commonly used techniques for genetic, molecular and functional sperm evaluation. Finally, this review will discuss recent advances in pharmacogenetics and new developments on sperm analysis that will form the basis for future research.

2. INTRODUCTION

A considerable number of couples of reproductive age are infertile. A male factor is responsible, alone or in combination with female factors, in about half of the cases (1-3). Several risk factors and causes might affect male fertility, including lifestyles, diabetes, obesity, hormonal diseases, testicular trauma, cryptorchidism, varicocele, genitourinary infections, ejaculatory disorders, chemo/radio or surgical therapies (4-6). Moreover, mutation screening and association studies performed on numerous male mouse models in the last few years definitively demonstrate the high prevalence of genetic causes of spermatogenic impairment. Indeed, male infertility represents one of the clearest examples of complex phenotype with substantial genetic basis. It is well

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Molecular diagnostic in male infertility

Table 1. Major genetic causes of male infertility

Chromosomal abnormalities	Gene(s) implication
Karyotype abnormalities	47,XXY (Klinefelter Syndrome)
	47,XYY
	46,XX
	Y chromosome aberration
Y Chromosome microdelations	Yq, AZFa, AZFb, AZFc
X-linked mutations	KAL1 (Kallmann Syndrome)
	AR (Androgen Insensitivity Syndrome)
Autosomal gene mutations	CFTR
	INSL3
	LGR8
	dNAI1, DNAH5, DNAH1
	GnRH, GnRHR
	FGFR1 (KAL2)
	FGF8 (KAL6)
	HS6ST1
	PROKR2/PROK2 (KAL3/KAL4)
	KISS1R/KISS1
	TACR3/TAC3
	CHD7
	NELF
	EBF2

established that genetic causes account for 10-15% of infertility cases (7), including chromosomal abnormalities (identifiable by karyotype analysis) and single-gene mutations (identifiable by molecular diagnostic techniques) that influence at different levels many physiological processes involved in male reproduction, such as hormonal homeostasis, spermatogenesis and sperm quality (8). However, a large proportion of infertile males does not receive a clear diagnosis and thus they are reported as idiopathic or unexplained, suggesting a poor understanding of the basic mechanisms regulating spermatogenesis and sperm function. Male (in)fertility is commonly based on standard semen analysis, which, however, cannot clearly distinguish fertile from infertile populations and fails to detect any abnormality in many cases (9). This is particularly evident in cases of infertility or repeated assisted reproduction failure with normal routine semen parameters. Abnormal sperm function or specific molecular defects can be hypothesized in these cases, triggering further research aimed at identifying new potential genetic markers of fertilizing ability and male fertility. For example, sperm nuclear DNA damage has received growing attention in recent years, both as an independent risk factor for infertility and in relation to poor reproductive outcome (10-13). Although contrasting data has been reported, it is increasingly evident that the integrity of sperm DNA is of vital importance for normal sperm function and embryo development (14, 15). Damaged DNA in sperm that fertilise an oocyte can have a negative impact on foetal development and on the health of offspring throughout adult life and might also be responsible for abnormal blastocyst development, failed implantation and spontaneous miscarriages (16-18). Different alterations are included in the definition of sperm DNA damage, including defective sperm chromatin packaging, apoptosis, oxidative stress, DNA fragmentation, gene mutations and aneuploidy. As a consequence, several techniques for the assessment of sperm DNA have been described (19-22). This review

focuses on the most widely used assay of genetic and molecular diagnostics. Furthermore, we took into account possible future developments in male infertility.

3. GENETIC DIAGNOSTICS OF MALE INFERTILITY

Genetic tests are routinely included in the diagnostic work-up of infertile males, and different guidelines have been proposed to correctly use these tests in cases of low sperm count and/or motility, or before entering an assisted reproduction program (23). Genetic causes of spermatogenetic impairment include chromosomal aberration and single-gene mutations (24). Although we are still far from understanding the molecular basis of many forms of male infertility, this aspect has generated increasing interest in recent years. In fact, if natural selection prevents the transmission of mutations causing infertility, it is also true that this protective mechanism might be overcome by assisted reproduction techniques. Consequently, the identification of genetic factors of male infertility is important for the appropriate assistance of infertile couples (25). In table 1 are reported the major genetic causes of male infertility and the respective involved genes.

3.1. Chromosomal abnormalities

Chromosomal abnormalities are present in about 5% of infertile patients, with an increase to about 15% in azoospermic males. This includes both numerical and structural aberrations of chromosomes (26, 27). The prevalence of karyotype anomalies is directly related to the severity of spermatogenic impairment, but also normozoospermic men might be carriers of chromosomal disorders (28, 29). Chromosomal abnormalities can be homogeneous or in mosaicisms and might involve the sex chromosomes and the autosomes.

Klinefelter syndrome (KS) represents the most frequent karyotype abnormality detected in infertile subjects, especially in azoospermic men (30). The genetic characteristic is the extra X-chromosome, which might be inherited from both parents. The percentage of KS is estimated to be 0.1-0.2% of newborn boys and the most prevalent form is the classic 47,XXY karyotype. The prevalence of KS in azoospermic and severely oligozoospermic men is 5-10% (31, 32), and infertility is the prevailing symptom of most KS patients. The main features of KS are represented by hypogonadism with testicular hypotrophy and high levels of gonadotropins. In azoospermic KS patients residual spermatogenesis can be found both in classic forms and in mosaic (46,XY/47,XXY) forms.

The karyotype 47,XYY is the second most frequent aneuploidy of the sex chromosomes and, although some studies by fluorescent *in situ* hybridization (FISH) showed an increase in aneuploidy in sperm samples, an increased risk of aneuploidy in the offspring has not been documented. The ensuing effect on spermatogenesis is variable or even null. Furthermore, normozoospermic men might exhibit this karyotype (33, 34).

A 46,XX karyotype might be rarely found in infertile patients with male phenotype, both with and without detection of the SRY gene (in these cases testes formation is guaranteed by another gene, autosomal or X-linked, involved in sex determination). 46,XX male subjects are invariably azoospermic with testicular atrophy. (35).

Further alterations of the sex chromosomes include structural aberrations of the Y chromosome, such as deletions, rings, isochromosomes, inversions and translocations (36). Among these, the most frequent alterations are translocations between the Y chromosome and autosomal chromosomes. The frequency in the general population is 0.02%, but the frequency increases to 0.2% in oligozoospermic patients and 0.9% in subjects undergoing Intracytoplasmic sperm injection (ICSI) (30).

Many autosomal genes are involved in normal sexual development, testis determination, spermatogenesis and fertility (37). Autosomes aberrations detected by karyotype anaylsis include translocations, inversions, other structural abnormalities such as extra satellite marker chromosome, and clinical syndromes including trisomy 21 and partial duplications. Autosomal translocations are 4-10 times more common in infertile patients when compared with normozoospermic ones (38), and the most frequent of these translocations are robertsonian translocations or centric fusions (39). These translocations can affect male fertility and pregnancy outcome and, in particular, males with reciprocal translocations have a high rate of unbalanced spermatozoa due to meiotic segregation errors. (40). Therefore, genetic counselling and analysis of chromosomal constitution in sperm of translocation carriers is mandatory in order to assess the risk of transmission of unbalanced forms.

In conclusion, karyotype analysis should be performed in azoospermic and severely oligozoospermic men with primary testiculopathy, and in all cases (including normozoospermia) before assisted reproduction techniques (ART) and/or after repeated ART failure (41).

3.2. Y chromosome microdeletions

Hundreds of genes distributed on the Y chromosome, the X chromosome and the autosomes are necessary for normal sexual development, for the formation and the descent of the testes and for spermatogenesis (42). Among them, those located on the long arm of the Y chromosome have gained in last years particular attention given the high frequency with which genetic alterations of this chromosome are found in male infertile patients. Indeed, microdeletions in the Y chromosome long arm (Yq) represent the most frequent molecular genetic cause of severe infertility, being detected in 10-15% of non-obstructive azoospermic and in 5-10% of severely oligozoospermic patients (43). Virtually n patient with Yq microdeletions has >5 million sperm/mL and generally these microdeletions are found in men with a sperm count < 2 million/mL (44). Although the genetic pathways and mechanisms of spermatogenic impairment are still largely unknown, three regions, referred to as "azoospermic

factors" (AZFa, b and c from proximal to distal) have been identified on Yq (45), but different classifications have also been proposed (46). Deletions of these regions are identified by molecular techniques, especially PCR, following specific guidelines (47). The most frequent microdeletion involves the AZFc region (about 60-70% of the deletions) and produces the loss of several genes. Different degrees of spermatogenetic alterations might be found, but, in general, the most part of patients with this alteration have sperm in the ejaculate or in the testes (48). ART techniques allow the transmission of Yq microdeletions, and male offspring of men with this genetic alteration will therefore also carry the deletion and will posses an impairment of spermatogenesis (49). Moreover, with Yq microdeletions could produce a higher percentage of sperm with sex chromosome aneuploidies (50). Genetic diagnostic and counseling are therefore particularly recommended for oligozoospermic patients who undergo *in vitro* fertilization.

3.3. X-linked and autosomal gene mutations

The genetic abnormalities of this group are rarer and their role in the pathogenesis of infertility is not yet fully understood. The main conditions associated with the X chromosome are represented by mutations in the KAL1 gene causing the Kallmann syndrome and mutations in the androgen receptor (AR) gene causing varying degree of androgen insensitivity. Kallmann syndrome has a frequency of 1:10.000 and is characterized by isolated hypogonadotropic hypogonadism (low serum level of testosterone, luteinizing hormone and follicle-stimulating hormone), infertility and anosmia (51). The most frequent genetic alteration is a mutation in the X-linked gene KAL1, although recent evidence has shown a highly heterogeneous genetic component associated with anosmia or normosmia (52). Mutations in the AR gene cause a variety of defects known as androgen insensitivity syndrome, with phenotypes ranging from female appearance of external genital in the complete forms, to various kinds of genital abnormalities in the partial forms and to isolated male infertility in milder forms (53). Mutations in the AR gene have been described in 2-3% of azoospermic and severely oligozoospermic men and might be suggested by high testosterone and luteinizing hormone levels (53-55).

Numerous autosomal genes have been suggested to play a role in male reproductive function, but mutations in only a few of them have routine clinical importance, namely the CFTR (cystic fibrosis transmembrane conductance regulator) gene, the INSL3 (insulin-like factor 3) gene and RXFP2 (relaxin family peptide receptor 2, formerly known as LGR8, leucine-rich repeat-containing G-protein coupled receptor 8) gene. Mutations in the CFTR gene are associated to congenital bilateral or unilateral absence of the vas deferens (56), therefore presenting as obstructive azoospermia, mild oligozoospermia or even normozoospermia (57). Mutations in this gene are very frequent in the general population (1/25 subject is carrier in the western countries) (58) and patients with CFTR mutations are frequently candidates for *in vitro* fertilization. Therefore, screening for CFTR gene mutations is frequently recommended by international guidelines on

Table 2. Main methods assessing sperm quality and function

Detection	Method
Protamination	Aniline blue
	Toludine blue
	Chromomycin A3
DNA integrity and fragmentation	Acridine orange
	SCSA
	COMET
	Halo test
	TUNEL
	γH2AX
	In situ Nick Translation
Aneuploidies	FISH
Mitochondrial function and apoptosis	JC-1
	Annexin-V

both partners before assisted reproduction techniques are employed.

INSL3 is a member of the relaxin-like hormone family produced by the Leydig cells and is a major determinant for the transabdominal phase of testicular descent (59, 60). Beside the role in testicular descent, INSL3 seems to cause endocrine and paracrine actions in adults, and deficiency of this hormone might represent a sign of functional hypogonadism (61). Mutations in INSL3 and its receptor RXFP2 represent a cause or risk factor for cryptorchidism (62), although their role in infertile men without a history of cryptorchidism is still unknown.

Finally, some authors have reported a possible association between mutations in dynein genes and isolated asthenozoospermia with consequent implications for male infertility (63). Mutations in dynein genes cause a rare disorder (prevalence of 1:16 000–1:60 000), primary ciliary dyskinesia (PCD). To date, three of the several dynein arms subunits have been found to be mutated in individual patients and/or in several families affected by PCD. These genes encode for three proteins: dNAI1 (axonemal dynein, intermediate chain 1) (64), DNAH5 (axonemal dynein, heavy chain 5) (65) and DNAH11 (axonemal dynein, heavy chain 11) (66).

Further genes involved in hypogonadotropic hypogonadism are: GnRH and GnRHR located respectively on 8p21–8p11.2 and 4q21.2 chromosomes, FGFR1 gene, also called KAL2, located on chromosome 8p12, FGF8 which is located on chromosome 10q24, also called KAL6, HS6ST1 on chromosome 2q21, PROKR2 and PROK2 called KAL3/4 and located on 20p13 and 3p21.chromosome respectively, KISS1R (19p13.3) and KISS1 (1q32), TACR3 (4q25) and TAC3 (12q13–q21), CHD7 (8q12.1), NELF and EBF2 (67).

4. MOLECULAR AND GENETIC SPERM DIAGNOSTICS

A number of methods have been proposed in recent years to assess sperm quality and function, as well as DNA integrity, such as protamination and DNA packaging, DNA fragmentation and chromosome aneuploidy (68). The usefulness of these methods in the evaluation male infertility and as prognostic markers for natural fertility and assisted reproduction are still debatable, partly because

standardization has not been reached for some of these methods, because normal values are yet under investigation, and because prospective studies have not been performed (69). Nevertheless, they might still provide important information in selected forms of male infertility. In particular, they can be used as additional, second level sperm tests after standard semen analysis in idiopathic and non-idiopathic cases of oligozoospermia. Furthermore, they can give additional information on sperm function and fertilizing ability in infertile males with normal standard semen parameters and in couples experiencing repeated abortion, implantation failure, and recurrent early pregnancy loss during assisted reproduction techniques. In table 2 are summarized the main methods assessing sperm quality and function.

4.1. Protamination
4.1.1. Aniline blue
Aniline blue is an acidic dye which has a greater affinity for the basic groups of the nucleoprotein in the loose chromatin of sperm nucleus. In human spermatozoa, the protamination process (the substitution of the nuclear proteins histones with protamines during the transition from spermatids to mature sperm) is not complete and a fraction of DNA (10-15%) remains bound to histones, (70). Sperm nuclei with normal chromatin packaging are nearly colourless, while increased aniline blue staining indicates loose chromatin packing (defective histone-protamine substitution).

Pro: The technique is simple, inexpensive and requires a simple bright field microscope for the analysis.

Con: Inter-observer subjectivity in establishing classification groups and the heterogenous slide staining are a prominent drawback of this technique.

4.1.2. Toludine blue
Toludine blue is a basic stain that evaluates phosphate residues of the sperm DNA with loosely packed chromatin and fragmented ends. The sample can be analyzed using an ordinary microscope or a flow-cytometre. Sperm heads with normal chromatin packaging are light blue whereas in sperm with defective protamination the stain attaches with lysine rich regions of histone and produces a violet-bluish intense coloration (71).

Pro: The technique is simple, inexpensive and requires an ordinary microscope for the analysis. This technique results very precise using cytometre.

Con: Inter-observer subjectivity is a prominent drawback of this technique. The method is very expansive using cytometre evaluation.

4.1.3. Chromomycin A3
Chromomycin A3 (CMA3) is a fluorimetric assay to indirectly measure the amounts of protamines present in sperm nucleus. It is a specific GC-rich sequences dye and it interacts at the same site at which protamine binds to the DNA. Chromatin structure can be easily assessed by indirectly determine the protamine presence.

Therefore, greater intensity of CMA3 staining indicates protamine deficiency or aberrant chromatin packing (72).

Pro: CMA3 staining is a simple and not expensive techniques and it requires a simple fluorescence microscope for the analysis.

Con: Inter-observer subjectivity is a prominent drawback of this technique.

4.2. DNA integrity and fragmentation
The integrity of sperm DNA can be evaluated with different techniques: the acridine orange staining assay, sperm chromatin structure assay (SCSA), COMET assay, Spem Chromatin Dispersion (HALO) test, Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling assay (TUNEL) test, γH2AX evaluation, and *in situ* nick translation test,

4.2.1. Acridine orange
Acridine orange is a metachormatic dye that has different fluorescent property in the presence of single- or double-stranded DNA. In the case of completely matured spermatozoa nuclei, which are rich in disulphide bond (S-S), DNA is kept in double-stranded form (native DNA). (73). Green fluorescence is emitted from the sperm nuclei when acridine orange binds to native DNA as a monomer. In the case of immature sperm nuclei with a large number of histones or S-S-poor protamine or in the presence of fragmented DNA, there are more single stranded regions after denaturation and the dye produces red florescence.

Pro: Easy and fast, this method can be used as a diagnostic tool to assess the quality of human- spermatozoa, in particular DNA fragmentation.

Con: Inter-observer subjectivity and rapidly fading of the fluorescence are prominent drawback of this technique.

4.2.2. Sperm chromatin structure assay
Sperm chromatin structure assay (SCSA), is the flow cytometric version of acridine orange test. The assay measures the susceptibility of sperm DNA to breakage after mild acid treatment. The proportions of spermatozoa having single-stranded (abnormal) and double-stranded (normal) DNA are determined by cytometry allowing the fast analysis of a high number of sperm (74).

Pro: It is a simple and less time consuming method allowing analysis of high numbers of cells.

Con: It does not give much information about the amount of DNA damage in the single sperm, but only a percentage of sperm with higher susceptibility to DNA breaks.

4.2.3. Comet assay
Comet assay is a single cell gel electrophoresis, performed under neutral or alkaline conditions. Sperm cells are lysed and then subjected to horizontal agarose electrophoresis and DNA is visualized with the help of a DNA-specific fluorescent dye. Fragmented DNA migrates away from the central nucleus, creating a "comet image". The shape is due to the migration and accumulation of the short DNA fragments and the intensity of the tail represents the amount of DNA fragments (75). The DNA damage is quantified by measuring the displacement between the genetic material of the nucleus comet head and the resulting tail by specific image analysis software.

Pro: The percentage of single and double DNA breaks for single spermatozoa are easily detectable, and with one assay it is possible to analyse numerous cells at the same time.

Con: DNA damage can be overestimated because of the presence of residual RNA which creates background during analysis, or because of proteins that hamper the movement of fragments during gel electrophoresis. The technique setup is industrious and a dedicated software is necessary to analyze the results (76).

4.2.4. Sperm chromatin dispersion (Halo) test
Sperm chromatin dispersion (Halo) test , similarly to the COMET test, involves agarose gel electrophoresis of single sperm that are then treated with an acid or alkaline denaturating solution to remove nuclear proteins and create single-stranded DNA were there are breaks. After cell lysis a halo around the head is generated in cells with low levels of DNA breaks, while the cells with more extensive DNA breaks show a small halo or no halo because the DNA loops do not diffuse. Even though the results obtained using this technique have been correlated with other DNA fragmentation techniques, it is not a direct measure of DNA breaks but a reflection of the overall chromatin structure (77).

Pro: The number of DNA breaks for single spermatozoa are easily detectable, and it is possible to analyse numerous cells at the same time.

Con: As with the comet assay test, the technique setup is not so easy, and a dedicated software is necessary to analyse the results. DNA damage can be overestimated by the presence of residual RNA (76).

4.2.5. Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling
Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling (TUNEL), is based on the TdT-mediated incorporation of fluorescent labelled nucleotides at the 3'-OH ends of single- and double-strand DNA breaks to create a signal which increases with the number of DNA breaks (78). The signal can be detected by fluorescence microscopeor by flow cytometry (19). Using microscope analysis, spermatozoa are classified as 'positive' or 'negative' for DNA damage, while in a flow cytometer analysis, the fraction of positive sperm is represented by the cells above a threshold channel value on a relative fluorescence intensity scale.

Pro: It analyses both single- and double-strand DNA breaks, and cytofluorimetric analysis allows the analysis of thousand of cells in few times.

Con: The technique is not standardized and normal values are laboratory dependent.

4.2.6. γH2AX

γH2AX, plays a major role in the mechanisms of recognition and repair of DNA double strand breaks. When double-strand breaks exist, some protein-kinase, like ATM (Ataxia Telangiectasia Mutated protein), phosphorylate and activate the H2AX histone. This phosphorylation involves a Serine residue in position 139 of the extreme C-terminus, leading to the formation of a modified form called γH2AX (phosphorylated H2AX) (79-84). This reaction has a rapid kinetics and its functional role is to recruit a multi-purpose DNA-protein complex devoted to the repair of DNA damage, called *focus*, at the site of DNA breakage (85). In mature sperm, these aspects are still poorly investigated and standardized although it has been demonstrated that of H2AX is activated during the remodelling process of chromatin that occurs in spermatids during the final stages of spermatogenesis and the expression and phosphorylation of H2AX histone has been demonstrated also in human mature sperm after induction of oxidative stress with hydrogen peroxide or after incubation of ejaculated sperm with mutagens such as Adriamycin (86-89). The quantification of the number of *foci* is considered a sensitive method for measuring DNA damage in terms of double strand breaks.

Pro: It is a measurement of DNA double-strand breaks and it can be evaluated by a fluorescence microscopy or by flow cytometer.

Con: Few studies have been published and standardization and normal value are still under consideration

4.2.7. *In situ* nick translation

In situ nick translation is similar to TUNEL because it is an enzymatic labelling method that incorporates biotinylated dUTP at single strand DNA breaks with template-dependent DNA polymerase-I. In this procedure one strand of the double-stranded DNA is nicked with deoxyribonuclease (DNase-I). Subsequently, a 5'–3' exonuclease, DNA polymerase-I, extends the nicks to gaps and the polymerase replaces the excised nucleotides with fluorescent-labelled substitutes on the basis of the complementary strand (90). The measured parameter is proportional to fluorescent spermatozoa with incorporated dUTP.

Pro: The technique is simple, inexpensive and requires only a fluorescence microscope for analysis.

Con: Since it uses a template dependent polymerase it has low sensitivity as compared to other techniques and identifies only single-strand breaks.

4.3. Aneuploidies
4.3.1. Fluorescence *in situ* Hybridization (FISH)

The emerging technology of FISH analysis using chromosome-specific DNA probes to detect numerical chromosomal abnormalities in decondensed sperm, offers the possibility to obtain data on aneuploidy frequencies in large populations of sperm. FISH is a multicolour analysis that allows screening of improper chromosome segregation during the first or second meiosis. Usually, five-colour FISH is performed to detect aneuploidies for the sex chromosomes and chromosomes 13, 18 and 21, which represent the most common aneuploidies detected at birth in humans (Klinefelter syndrome, Turner syndrome, trisomy 13, 18 and 21) and are the most important cause for congenital abnormalities, developmental disabilities, mental retardation and infertility in humans (91). Furthermore, chromosomal aneuploidies in general are responsible for a great deal of pregnancy loss and assisted reproduction failure (92). In order to standardize the analysis and minimize inter-individual differences, automated systems can be used instead of manual score.

Pro: A large number of spermatozoa can be analyzed, especially with automated systems.

Con: Only few chromosomes can be analysed and normal values (percentage of sperm with specific aneuploidy) are still dabatable.

4.4. Mitochondrial function and apoptosis
4.4.1. JC-1

The mitochondrial stain JC-1 (5,59,6,69-tetrachloro-1,9,3,39-etraethylbenzimidazolylcarbocyanine iodide) allows distinguishing between spermatozoa with poorly and highly functional mitochondria. Mitochondrial status is an important trait of sperm physiology, as they generate a major part of the ATP required for sperm metabolism, membrane function and motility. Furthermore, the loss of mitochondria membrane potential is one of the earliest apoptosis mechanisms in the cell systems. In healthy spermatozoa, JC-1 accumulates in the cytosol as a green-fluorescence monomer whereas in the presence of high mitochondrial membrane potential the monomers accumulates as aggregates inside the mitochondria, emitting red fluorescence. When spermatozoa are dying, and the mitochondrial membrane potential is no more persistent (collapses), JC-1 exists only in monomeric form and emits green fluorescence (93, 94).

Pro: Fast and not complex, this method can be used as a diagnostic tool to assess the quality of human ejaculated spermatozoa and represents the only method available clinically available for evaluating mitochondrial function.

Con: JC-1 staining may be affected by many variables and it required careful preparation and controls for adjusting the cytometer.

4.4.2. Annexin-V

One of the early steps during apoptosis is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Here it can be detected by annexin V labelling dye indicating an early plasma membrane degeneration.The combination of Annexin V-FITC with propidium iodide (PI) to detect sperm vitality in citofluorimetry is able to simultaneously
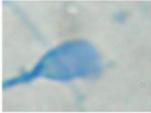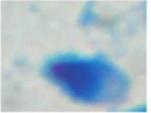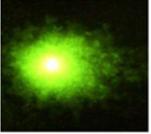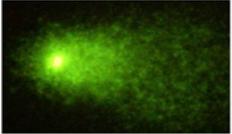
Test (Nuclear evaluation)	Normal	Pathologic
ANILINE BLUE (DNA pakaging- histones evaluation)		
COMET ASSAY (DNA integrity - DS and SS breaks evaluation)		
HALO TEST (Chromatine evaluation and DNA integrity)		
TUNEL (DNA integrity - DS and SS breaks evaluation)		
ACRIDINE ORANGE (Chromatine evaluation-nondenatured or denatured DNA quantification)	Double Strand	Single Strand
FISH (Aneuploidies evaluation)	(18 / y) (18 / x)	(18 / x,x,y)
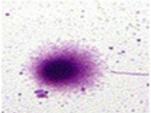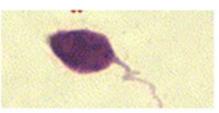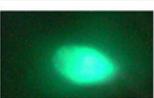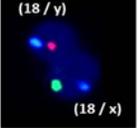
Figure 1. Representative images obtained by different methods used to the sperm nuclear evaluation.

distinguish live spermatozoa from those in apoptotisis and from those dead (95, 96).

Pro: Cytofluorimetric analysis allows analysing thousand of cells in few times. It is easy and fast.

Con: It requires the use of adequate controls for adjusting the cytometre.

Among all these methods there is not a gold standard as no one is able to give all the information about the quality and quantity of the damage on the individual sperm. Furthermore, have not yet been identified threshold values of normality and /or pathology and therefore each laboratory should build by itself. Figure 1 shows some examples of the above described methods.

5. NEW DEVELOPMENT AND PHARMACOGENETICS

Molecualr and genetic aspects of spermatogenesis and male infertility have gained increasing interest in last years and new possible diagnostic techniques are continuously proposed, especially with the aim of detecting and selecting the best "ideal" sperm for fertilization. Recent studies have proposed scientific evidence and proposals for diagnostic tests that, although not yet into the routine clinical practice,

most likely will play a role in the diagnostic workup of infertile patients in a near future.

5.1. Sperm genetics
5.1.1. Telomeres

Telomeres are non-coding DNA sequences, composed of highly conserved hexameric tandem nucleotide repeats (TTAGGG) located at the ends of chromosomes and confer chromosome stability and genome integrity. They undergo progressive shortening with each cell division and when telomeres reach a critical minimum length, cells can not divide and the cell enters cell-cycle arrest or undergoes apoptosis. Telomere length (TL) is maintained by telomerase, a ribonucleoprotein complex that is maximally expressed in highly proliferative cells such as germ and neoplastic cells. Telomere length is a complex trait that is determined by normal cell division, reactive oxygen species, genotoxic insults, genetic predisposition, aging, lifestyle factors, psychological stress and the age of the father at the time of conception (97). Although the role of sperm telomeres is not clearly known, recent studies documented intriguing findings in different aspects of male reproduction that merit further investigation. In fact, although sperm and leukocyte telomere lengths tend to be strictly correlated in the same individual, leukocyte telomere length decreases and sperm telomere length increases with age (98). This finding is not

yet fully understood, but it is supposed to be related to the high activity of the telomerase reverse transcriptase (TERT), the catalytic subunit of the telomerase, in germ cells, or to a selective cellular attrition leading to death of sperm stem cells with shorten telomere length and therefore selection of a subset of sperm with longer telomeres. Consistent with the elongation of sperm telomere length with age, a positive correlation has been found between paternal age at birth and offspring leukocyte telomere length (99). The paternal contribution to offspring leukocyte telomere length is apparently stronger than the maternal contribution, and the paternal age effect is cumulative across multiple generations. Intriguingly, one recent study analysed sperm telomere length in small groups of fertile and infertile subjects with normal sperm counts and found lower telomere length in sperm of this latter group (100). The most commonly method to evaluate telomere length is a quantitative PCR method. Telomere length analysis might therefore be a new potential biomarker of sperm, but further studies are needed to clarify the physiopathological link between sperm telomere length and damaged spermatogenesis, and the effect on offspring telomere length of assisted reproduction techniques performed on couple of advanced age and oligozoospermic men.

5.1.2. Comparative Genomic Hybridization array

Comparative Genomic Hybridization array (CGH-array), has been looked as a potential methodology to improve chromosome analysis of sperm cells, up to single spermatozoa, allowing simultaneous evaluation of all chromosomes in one experiment. (101). The aCGH procedure involves screening of the entire chromosome complement by DNA microarray allowing having a molecular karyotype of the cells because it analyzes the entire genome, detecting both aneuploidies and structural chromosomal alterations (102). It is currently used in research and in diagnostic clinical practice, such as prenatal diagnosis and pre-implantation genetic diagnosis, and we recently reported its application in the analysis of single human sperm, showing that even normozoospermic donors have a relevant percentage (8%) of sperm with different genetic anomalies, including aneuploidies and gains and losses in different chromosomes (unbalanced sperm) (103). Future application of this method might therefore give important information on the biology and pathophysiology of spermatogenesis and sperm chromosome aberrations in normal subjects and in patients at higher risk of producing unbalanced sperm, such as infertile men, carriers of karyotype anomalies, men with advanced age, subjects treated with chemotherapy, and partners of couples with repeated miscarriage and repeated failure during assisted reproduction techniques. The limiting aspect of this technique is currently related to the high cost of the array.

5.1.3. Raman microspectroscopy I

Raman microspectroscopy s based on the principle of inelastic scattering, which results from the interaction between light and matter. The unique pattern of changes in the vibrational state originating from each molecule and its environment, collectively, constitutes a chemical "fingerprint" that can be used to identify and characterize biomolecules, cells or tissues (104). Raman spectroscopy can provide information about DNA packaging at the single sperm cell level in living cells. It can be used to obtain images of the spermatozoa cell shape together with a chemical analysis of the sperm cell contents. It offers detailed information about the conformation, composition and intermolecular interactions of macromolecules such as DNA and proteins in sperm. This technique is noninvasive and nondestructive at moderate photon energies and it can work *in vitro* and *in vivo* under a wide range of environmental conditions (105). Raman spectroscopy combined with the use of image analysis could represent a possible label-free and rapid identification of normal sperm cell, however, to date there are only preliminary data and more studies are needed to validate the efficacy of this technique.

5.2. Pharmacogenetics

One of the most exciting aspects of the genetics of male infertility is related to the recent advances in pharmacogenetics. Although studies are still limited, it is presumable that the identification of genetic markers that can be used to select infertile patients to be treated and to personalize pharmacological treatment will change in a near future the clinical approach to these patients. In particular, single nucleotide polymorphisms (SNPs) in the FSHB and FSH receptor (FSHR) genes have been correlated with serum FSH and testicular function, and have been recently reported to play a role in determining the response to treatment with FSH (106). The FSHR genotype influences serum FSH levels because the c.2039A>G polymorphism (resulting in Ser at amino acid position 680) confers relative insensitivity to FSH action, while the FSHB genotype determined by a polymorphism in the promoter of the gene (-211 G>T allele) modifies the transcriptional activity of the FSHB promoter and therefore the amount of FSH produced by the pituitary gland (107-108). These SNPs have been used to identify responders patients to FSH treatment in two studies, which suggested that men with the Ser680 allele in the FSHR gene or those with the -211 T allele in the FSHB gene had a significant increase in spermatogenesis after treatment with respect to carriers of the other genotypes (109-110). If these studies will be confirmed, these SNPs could be useful both as diagnostic and prognostic parameters in selected infertile men, represented by oligozoospermic men with normal FSH levels.

6. CONCLUSIONS

With the increasing of male infertility rate and the continuous development of assisted reproduction techniques, the attempt to clarify the cause and to better evaluate sperm quality is necessary, in particular for idiopathic infertile couples and those undergoing ART cycles. A better understanding of the genetic causes of damaged spermatogenesis and the molecular mechanisms of sperm damage, and the refinement of the genetic and molecular techniques for sperm evaluation and selection are important advances that will lead to the optimization of the diagnostic and therapeutic management of male and couple infertility. Faced with such a pletora of new

proposed techniques and diagnostic tests, it is fundamental to know which tests are already routinely used in the clinical practice and those that are likely to be used in the near future.

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