

## Review

# Male infertility: role of genetic background



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## Abstract

Male infertility represents one of the clearest examples of a complex disease with a substantial genetic basis. Numerous male mouse models, mutation screening and association studies reported over the last few years reveal the high prevalence of genetic causes of spermatogenic impairment, accounting for 10–15% of severe male infertility, including chromosomal aberrations and single gene mutations. Natural selection prevents the transmission of mutations causing infertility, but this protective mechanism may be overcome by assisted reproduction techniques. Consequently, the identification of genetic factors is important for appropriate management of the infertile couple. However, a large proportion of infertile males are diagnosed as idiopathic, reflecting poor understanding of the basic mechanisms regulating spermatogenesis and sperm function. Furthermore, the molecular mechanisms underlying spermatogenic damage in cases of genetic infertility (for example Yq microdeletions) are not known. These problems can be addressed only by large scale association studies and testicular or spermatozoal expression studies in well-defined alterations of spermatogenesis. It is conceivable that these studies will have important diagnostic and therapeutic implications in the future. This review discusses the genetic causes of male infertility known to date, the genetic polymorphisms possibly associated with male infertility, and reports novel results of global gene expression profiling of normal human testis by microarray technology.

**Keywords:** *aneuploidy, Klinefelter, male infertility, microarray, polymorphism, Y chromosome*

## Introduction

Infertility affects about 15% of couples trying to conceive in western countries (De Kretser, 1997), and genetic causes may be identified in a large proportion of them. In about 15% of male and 10% of female infertile subjects, genetic abnormalities may be present, including chromosome aberrations and single gene mutations. Genetic risks for couples undergoing IVF and intracytoplasmic sperm injection (ICSI) are related to transmission of constitutional genetic abnormalities, genetic alterations present only in sperm, or de-novo generated genetic disorders. Therefore, the identification of genetic factors has become good practice for appropriate management

of the infertile couple (Foresta *et al.*, 2002). Besides known genetic causes of male infertility, recent studies analysed the possible involvement of genetic polymorphisms as risk factors for spermatogenic impairment.

This paper discusses the most important genetic causes of male infertility (**Table 1**) and the role of genetic polymorphism. The results of a novel study aimed at identifying genetic defects of human spermatogenesis by means of testicular expression microarray analysis are then presented.

**Table 1.** Frequency and associated phenotypes of the most common genetic abnormalities associated with male infertility. The prevalence shown is the frequency found in the associated phenotype.

<i>Genetic abnormality</i>	<i>Phenotype</i>	<i>Prevalence (%)</i>
Chromosomal aberrations	From azoospermia to normospermia	2–10
Klinefelter's syndrome	Azoospermia–severe oligospermia	5–10 azoospermia, 2–5 severe oligospermia
Other sex chromosome alterations	From azoospermia to normospermia	0.1–0.2
Robertsonian translocations	Azoospermia–severe oligospermia	0.5–1.0
Reciprocal translocations	Azoospermia–severe oligospermia	0.5–1.0
Y chromosome deletions	Azoospermia–severe oligospermia	5–10
<i>AZFa</i>	Azoospermia–SCOS	0.5–1.0
<i>AZFb</i>	Azoospermia–spermatogenic arrest	0.5–1.0
<i>AZFc</i>	Azoospermia–severe oligospermia	3–7
<i>AZFb-c</i>	SCOS/spermatogenic arrest	0.5–1.0
Partial <i>AZFc</i> deletions	From azoospermia to normospermia	3–5
Gene mutations		
<i>CFTR</i>	Obstructive azoospermia	60–70 (5% in infertile men)
<i>AR</i>	Azoospermia-oligospermia	2–3
<i>INSL3-LGR8</i>	Cryptorchidism	4–5

SCOS: Sertoli cell only syndrome.

## Genetic causes of male infertility

### Chromosomal abnormalities

The prevalence of chromosome abnormalities is higher in infertile men, this figure being inversely related to the sperm count. Based on the largest published series, it could be estimated that the overall incidence of a chromosomal factor in infertile males ranges between 2 and 8%, with a mean value of 5%. This value increases to about 15% in azoospermic males, largely comprising patients with 47,XXY Klinefelter's aneuploidy. Sex chromosome abnormalities are predominant, but a wide range of structural autosomal anomalies are also found. The most common type of karyotype abnormality detected in infertile subjects is represented by Klinefelter's syndrome (KS) (Foresta *et al.*, 2002). In a series of 750 consecutive severe oligozoospermic men (sperm count < 5 (10<sup>6</sup>/ml) (Foresta *et al.*, 2005), chromosomal aberrations were present in 42 subjects (5.6%) of which 29 (69.0%) were of the Klinefelter's type.

KS is the most frequent sex chromosome aneuploidy in human males, occurring in approximately 0.1–0.2% of newborn boys. The prevalence of KS among infertile men is very high, up to 5% in severe oligozoospermia and 10% in azoospermia (Foresta *et al.*, 2005). KS is a form of primary testicular failure with testicular hypotrophy and elevated gonadotrophin plasma concentrations, and it represents the most common form of male hypogonadism.

It has been always assumed that almost 100% of non-mosaic 47,XXY males are azoospermic. However, in the present series, 72 of 94 (76.6%) non-mosaic KS males had complete azoospermia, whereas the remaining had spermatozoa in the ejaculate.

Although clear data are not available, it is also possible that a fraction of azoospermic KS men might actually have residual spermatogenesis in some seminiferous tubules (Foresta *et al.*, 2005). Mosaic 47,XXY/46,XY patients produce spermatozoa in variable numbers. Although the exact percentage of men with spermatozoa in the ejaculate is not known, in the present series, 20 of 27 (74.1%) were azoospermic.

Before the introduction of ICSI, the fertility outlook for the vast majority of KS patients was bleak. To date, 54 normal children have been born from 122 men with KS by ICSI with testicular (48 children, 118 patients) or ejaculated spermatozoa (six children from four patients) (reviewed in Ferlin *et al.*, 2005a). Although the great majority of children born to fathers with KS are chromosomally normal, the risk of producing offspring with chromosome aneuploidies is significant, particularly the risk of fathering a 47,XXY or 47,XXX child (Reubinoff *et al.*, 1998; Ron-El *et al.*, 2000). In fact, the incidence of aneuploid spermatozoa (particularly of disomies) is increased in KS (Ferlin *et al.*, 2005a). Aneuploid spermatozoa are probably the result of meiosis of a few 47,XXY spermatocytes and of meiotic abnormalities occurring in normal 46,XY germ cells present in a compromised testicular environment (Ferlin *et al.*, 2005a).

Other sex chromosome aneuploidies detected with higher prevalence in infertile men are represented by 47,YYY, 46,XX and Y chromosome aberrations (inversions, Yq deletions, etc.). Furthermore, translocations involving the sex chromosomes (X-autosomal and Y-autosomal translocations) are also frequent (Mau-Holzmann, 2005).

Robertsonian translocations are the most frequent structural chromosomal abnormalities in humans and can affect fertility,

with varying degrees of sperm alteration in men. Robertsonian translocations occur when two acrocentric chromosomes (13–15, 21, 22) fuse together. The resulting single abnormal chromosome, generally dicentric, contains most of the long arms of the original two, and subsequent loss of their short arms. The incidence of Robertsonian translocations is about 1 in 1000 newborns (Therman and Susman, 1993). The most common combinations are between chromosomes 13 and 14 and between chromosomes 14 and 21. Carriers of the Robertsonian translocation generally have normal phenotypes. However, the translocation can affect fertility and/or pregnancy outcome due to possibly impaired gametogenesis and/or production of gametes with an unbalanced combination of the parental rearrangement. Fertility problems in Robertsonian translocation male carriers are due to varying degrees of spermatogenic defects directly related to the disturbance of the meiotic process. In populations of infertile males, 0.8% were carriers of a Robertsonian translocation (De Braekeleer and Dao, 1991); this is up to nine times higher than in the general population.

Reciprocal translocations are found with a frequency of 0.9/1000 newborns. A translocation consists of a mutual exchange of chromosomal segments between two chromosomes. In general, there is no apparent alteration to the carrier's phenotype. However, in couples experiencing repeated pregnancy losses, the incidence of chromosomal translocations is higher than the incidence present in a newborn series (De Braekeleer and Dao, 1991). On the other hand, there is also evidence which indicates that the presence of translocations alters the spermatogenic process. Summarizing the findings from different series of studies on infertile, oligozoospermic and azoospermic males, the incidence of reciprocal translocation carriers is seven times higher than in a newborn series.

## Y chromosome microdeletions

Microdeletions in the Y chromosome long arm (Yq) represent the most frequent molecular genetic cause of severe infertility, observed with a prevalence of 10–15% in non-obstructive azoospermia and 5–10% of severe oligozoospermia (Foresta *et al.*, 2001). Most of the deletions are found in men with a sperm count below  $2 \times 10^6/\text{ml}$  (Ferlin *et al.*, 2007). Three regions, referred to as 'azoospermia factors' (AZFa, b and c from proximal to distal), have been defined as spermatogenesis loci (Vogt *et al.*, 1996). The genetic pathways and mechanisms of spermatogenic impairment in men with Yq microdeletions are unknown. The function of AZF genes in spermatogenesis is also not clear, and the molecular mechanisms altered in cases of AZF deletions are completely unknown. The majority of Y microdeletions produce the simultaneous loss of several genes mapped within AZFb and AZFc loci (Kuroda-Kawaguchi *et al.*, 2001; Stuppia, 2001; Repping *et al.*, 2002; Ferlin *et al.*, 2007). AZFa deletions are less frequent and involve only two genes, *USP9Y* (ubiquitin specific peptidase 9, Y-linked) and *DBY* (DEAD box polypeptide 3, Y-linked). Most of the AZF microdeletions are generated by intrachromosomal homologous recombination between repeated sequence blocks organized into palindromic structures showing a nearly identical sequence (Kuroda-Kawaguchi *et al.*, 2001; Repping *et al.*, 2002). The complete AZFc deletion, b2/b4 deletion, removes eight gene families including all members of the *DAX* (deleted in azoospermia) gene family, which represents the strongest candidate responsible for the AZFc phenotype (Foresta

*et al.*, 2001; Kuroda-Kawaguchi *et al.*, 2001; Repping *et al.*, 2002). Deletions in the AZFa region usually lead to Sertoli cell-only syndrome, complete deletions of AZFb or AZFb+c lead to azoospermia associated with Sertoli cell-only syndrome or pre-meiotic spermatogenic arrest (Vogt *et al.*, 1996; Foresta *et al.*, 2001; Ferlin *et al.*, 2007). The most frequent AZFc deletion leads to azoospermia or severe oligozoospermia, associated with different spermatogenic phenotypes in the testis. In general, 60–70% of these men have spermatozoa in the ejaculate or in the testis (Ferlin *et al.*, 2007).

Most men with Yq microdeletions require ICSI to overcome their infertility. Since all spermatozoa from Y-deleted men harbour the same microdeletions, ICSI allows the transmission of such microdeletions. Male offspring of men with Yq microdeletions will therefore also carry the deletion and will have spermatogenic impairment in adulthood. However, a recent discovery is that men with AZFc also produce a higher percentage of spermatozoa with aneuploidies. In fact, it was recently reported that patients with AZFc deletions had a significant reduction in the percentage of normal Y-bearing spermatozoa compared with normozoospermic control men, a concomitant increase in nullisomic spermatozoa and a significant increase of XY-disomic spermatozoa (Foresta *et al.*, 2005; Ferlin *et al.*, 2007). Therefore, AZF microdeletions can be considered as 'pre-mutations' for a subsequent complete loss of the Y chromosome in the AZF-deleted patients' spermatozoa, increasing the risk of embryonic XO cells (Vogt, 2004).

Although no genital abnormalities or other somatic defects in the ICSI-AZFc offspring are reported, genetic counselling should take into account the observations of sperm sex chromosome aneuploidies in these men, and the possible increased risk of generating 45,X (Turner's syndrome) or 47,XXY embryos (Klinefelter's syndrome). It has to be noted, however, that these risks are more theoretical because in the 31 children already born from men with AZF deletion (Jiang *et al.*, 1999; Page *et al.*, 1999; Cram *et al.*, 2000; Komori *et al.*, 2002; Stouffs *et al.*, 2005), no consequences other than transmission of the Yq deletion have been reported. Nevertheless, clear information regarding implantation rate and incidence of spontaneous abortion for the partners of men with Yq microdeletions is not yet available.

## Gene mutations

Several hundreds of genes are necessary for normal sexual development, testis determination, testis descent, and spermatogenesis. However, only a few have routine clinical importance. These include the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, whose mutations cause cystic fibrosis and absence of vas deferens, the androgen receptor gene, whose mutations cause the androgen insensitivity syndrome and spermatogenic damage, and the *INSL3* (insulin-like factor 3) and *LGR8* (leucine-rich repeat-containing G-protein coupled receptor 8) genes, whose mutations have been associated with abnormalities in testis descent (cryptorchidism).

There is general agreement that 60–70% of patients with congenital bilateral absence of the vas deferens (CBAVD) have mutations in the *CFTR* gene, with no other clinical symptoms of cystic fibrosis. In a series of unselected severely oligozoospermic men (Foresta *et al.*, 2005), a prevalence of 1.2% (9/750) of *CFTR* gene mutations was found. A recent survey in Italy found

*CFTR* mutations in 37.5% of CBAVD individuals and 6.6% in males with non-obstructive azoospermia (Stuppia *et al.*, 2005). Furthermore, the 5T allele was found with high prevalence both in males with non-obstructive azoospermia (9.9%) and in those with CBAVD (100%). All together, 11.6% of subjects entering assisted reproductive techniques had either a *CFTR* mutation or the 5T allele. Subjects with *CFTR* mutations are good candidates for ICSI, using spermatozoa retrieved from the ejaculate, testis or epididymis. Spermatogenesis in these patients is assumed to be normal, and aneuploidy rate is not increased in the sperm of affected patients. However, because of the risk of cystic fibrosis in the offspring of couples in whom the female partner is heterozygous for a *CFTR* mutation, screening for *CFTR* mutations should be considered before assisted reproduction techniques (Foresta *et al.*, 2002).

Mutations in the androgen receptor (*AR*) gene on the X chromosome cause a variety of defects known collectively as androgen insensitivity syndrome (AIS). Patients with mild AIS (MAIS) have male infertility as their primary or even sole symptoms. In a recent screening of 1517 azoo-oligozoospermic individuals, 26 patients carrying *AR* mutations (20 different mutations) (1.7%) were found, and none in the control group (Ferlin *et al.*, 2006a). Importantly, of the 26 men with *AR* gene mutations, two had cryptorchidism, one cryptorchidism and hypospadias, one gynecomastia, whereas 22 did not show signs of androgen insensitivity other than spermatogenic impairment. Furthermore, only a minority of infertile males with elevated testosterone and LH (suggestive for androgen insensitivity) had mutations in the *AR* gene, even though the higher the ASI (androgen sensitivity index, product of LH (testosterone), the more likely a mutation in the *AR*. Therefore, *AR* gene mutations might play a role as genetic causes of male infertility and are found with a prevalence of about 2% in unselected infertile men, with similar prevalence in azoospermia, severe oligozoospermia and moderate oligozoospermia (Ferlin *et al.*, 2006a). No clear hormonal or clinical data could be used to preselect patients at higher risk of mutations. Although mild signs of androgen insensitivity may be present in some cases, most men with *AR* abnormalities do not differ from the vast majority of infertile males.

INSL3 is a member of the relaxin-like hormone family produced by the Leydig cells. Research on INSL3 in humans has expanded in recent years following the identification in rodents of a role for this peptide in the transabdominal phase of testicular descent by acting on gubernaculum (Nef and Parada, 1999; Zimmermann *et al.*, 1999). A further impulse has been given to this research by the description of the related receptor, LGR8 (Overbeek *et al.*, 2001; Bogatcheva *et al.*, 2003). A role in human cryptorchidism has been suggested, since several mutations in INSL3 and LGR8 leading to amino acid substitution were found. A review of the literature found a prevalence of mutations of 4–5% in men with cryptorchidism or ex-cryptorchidism (Ferlin and Foresta, 2005). Some of these mutations represent common polymorphisms found with similar frequency in both patients and controls, whereas seven of them were detected exclusively in men with history of maldescent (P49S, R73X, P93L, R102C, R102H, N110K in INSL3 and T222P in LGR8). Moreover, a significant association of *INSL3* gene mutations was found in men presenting one or more signs of testicular dysgenesis syndrome (Ferlin *et al.*, 2006b). However, a causative role for some of these mutations is not clearly supported by functional

analyses. Therefore, although a role for mutations of *INSL3* and *LGR8* genes in cryptorchidism is reasonable, additional studies are needed to establish an association between the disruption of the INSL3 pathway and higher risk of infertility or testicular cancer. Finally, the identification of high concentrations of circulating INSL3 in adult males and the expression of LGR8 in many tissues raises new questions on the endocrinological role of this hormone (Foresta *et al.*, 2004; Bay *et al.*, 2005; Ferlin *et al.*, 2006c). Apart from the role in testicular descent and cryptorchidism, INSL3 therefore has possible important yet unidentified endocrine and paracrine actions in adults, and deficiency of this hormone may represent an important sign of functional hypogonadism (Foresta *et al.*, 2004; Ferlin and Foresta, 2005; Ferlin *et al.*, 2006c,d).

## Genetic polymorphisms and male infertility

The analysis of polymorphisms in genes involved in spermatogenesis represents one of the most exciting areas of research in genetics of male infertility. Polymorphisms or genetic variants in these genes are considered potential risk factors which may contribute to the severity of spermatogenic failure. Several polymorphic variants have been described in association with male infertility. However, these association studies often do not report unique results. This is mainly due to different important aspects: the size and the composition of the study population, the type of polymorphism analysed and the techniques used, the multifactorial condition and heterogeneity of male infertility phenotype, the variability between individuals in the phenotypic effect of causes acting at the testicular level, and the ethnic and geographical differences that contribute to genetic variations.

The phenotypic effects of gene polymorphisms are modulated by other genetic factors or genetic background and environmental factors, providing an important example of the gene-environment interaction in phenotype development. Therefore, it is likely that polymorphisms only in association with a specific genetic background and/or with environmental factors can lead to spermatogenic impairment or testicular dysfunction. Polymorphisms in different genes have been studied for possible association with male infertility, but many of them have not been replicated and therefore definitive data are not available. Therefore, only for some polymorphisms sufficient, although not conclusive, data have been produced.

One example is represented by partial AZFc deletions. As mentioned above, AZFc deletions including all members of *DAZ* gene family represent the most frequent molecular cause of spermatogenic impairment. More recently, smaller palindromes were discovered within the large amplicons in AZFc region and it has been shown that intrachromosomal recombinations within AZFc could produce partial deletions in AZFc. In fact, apart from initial studies reporting partial *DAZ* deletions, performed by fluorescence in-situ hybridization and Southern blotting, a number of studies recently reported partial AZFc deletions analysed by AZFc-specific sequence tagged sites, *DAZ*-specific SNVs or gene dosage analysis. Partial AZFc deletions are associated with variable clinical and histological phenotypes, and could be associated with an increased risk of spermatogenic failure. Different partial AZFc



deletions have been identified (Repping *et al.*, 2003, 2004); gr/gr, which removes 1.6 Mb, b1/b3 and b2/b3, which remove 1.8 Mb, and others more infrequent. Even if definitive data are still missing, partial AZFc deletions, particularly the gr/gr subtype, might be considered a risk factor for spermatogenic failure, at least in some populations (Repping *et al.*, 2003; Machev *et al.*, 2004; de Llanos *et al.*, 2005; Ferlin *et al.*, 2005b; Giachini *et al.*, 2005; Huclenbroich *et al.*, 2005). However, some of these partial AZFc deletions, including gr/gr, can also be found in fertile men with normal spermatogenesis, so it remains to be resolved whether these aberrations may play a role alone or in combination with other genetic or environmental factors.

Other polymorphisms largely studied in association with male infertility are related to the *AR* gene. In fact, the *AR* gene exhibits two polymorphic sites in exon 1, characterized by different numbers of CAG and GGC repeats resulting in variable lengths of polyglutamine and polyglycine stretches in the N-terminus of the *AR* protein, which seem to modulate *AR* function. The number of CAG and GGC repeats ranges from about 10 to 35 (with a mean of  $21 \pm 23$ ) and 4 to 24 (with a mean of  $16 \pm 17$ ) respectively in normal men. A number of recent studies have examined the possible link between the length of the CAG repeat and male infertility. The basis for these investigations has been the finding that longer CAG repeat lengths result in reduced *AR* transcriptional activity both *in vivo* and *in vitro*. This observation has led to the hypothesis that longer polyglutamine tracts might possibly be considered a risk factor for male infertility. This is consistent with the finding that polymorphisms in CAG tract length are related to sperm concentration in normal men (von Eckardstein *et al.*, 2001). However, previous studies examining CAG repeat number in infertile men have reported conflicting results, with some showing no expansion, and others reporting increased length (but still within the normal range) compared to fertile control males (reviewed in Ferlin *et al.*, 2004). In particular, studies involving Singaporean, Australian, North American and Japanese subjects found an association between CAG length and male infertility, whereas this was not evident in studies from Europe. These discordant results may reflect the patient ethnicity selection, as the distribution of the number of CAG repeats is lowest in African-Americans, intermediate in whites, and highest in Asians. Furthermore, additional bias may be related to sample size restrictions, choice of the control group and patient inclusion criteria. Only two studies reported the distribution of GGC lengths among the infertile men and found no difference from that in the general population (Tut *et al.*, 1997; Lundin *et al.*, 2003). Recently, the effects of variation in length of both the CAG and the GGC repeat have been examined, and it has been shown that some haplotypes might modulate *AR* function and might increase an individual susceptibility to infertility (Ferlin *et al.*, 2004). A similar study in Swedish men confirmed that *AR* haplotypes might increase the risk of infertility (Ruhayel *et al.*, 2004).

Y chromosome haplogroups have also been studied for possible association with Yq microdeletions or infertility. The presence of variable phenotypes, ranging from oligozoospermia to azoospermia, in patients showing apparently identical Yq microdeletions suggests the presence of other modifier genes able to produce different genetic backgrounds or particular Y chromosome constitution, which in turn could balance or enhance the molecular effect produced by the loss of genes mapped within AZF loci. Absence of significant association

between Y haplogroups and Y microdeletions was found in a European sample (Paracchini *et al.*, 2000) and in a northwestern European sample (Quintana-Murci *et al.*, 2001). However, in this latter study, an association was found in the Danish population and an association between certain Y haplogroups and AZFc deletions has recently been described in northern Italy (Arredi *et al.*, 2007), showing that studies on microgeographically controlled population are desirable and more informative. The gr/gr deletions were observed on several haplogroups, among which the D2b haplogroup contained only deleted chromosomes (Repping *et al.*, 2003). This same branch of the Y-phylogeny was suggested to be associated with infertility in Japanese men (Kuroki *et al.*, 1999), although this result has not been confirmed (Carvalho *et al.*, 2003). Instead, the b2/b3 deletion and the more precisely defined *DAZ3/DAZ4* deletions seem to be completely associated with the haplogroup N, an ancient lineage widespread in northern Europe and in Asia (Fernandes *et al.*, 2004; Repping *et al.*, 2004). Two works highlighted a possible association between haplogroups and infertility: the D2b haplogroup in the Japanese and the haplogroup hg 26+ (or the K\*(xP) according to the YCC nomenclature) in a Danish sample (Krausz *et al.*, 2001). Another study (Previdere *et al.*, 1999) on Italian populations highlighted a significant difference between the control and infertile samples, although the variability in frequency due to differences among subpopulations was not taken into account.

Studies of polymorphisms in many other genes have been published in recent years. Some genes seemed to be more promising (*MTHFR*, *DAZL*, *POLG*, *FSHR*, *ER-α*), but also in these cases conflicting results have been obtained. The enzyme 5-methylenetetrahydrofolate reductase (*MTHFR*) is involved in the conversion of homocysteine to methionine. A point mutation in its coding region (C677T) decreases the activity of the enzyme by about 30% in heterozygotes (CT) and 80% in homozygotes (TT). Possible negative effects of the *MTHFR* (C677T) mutation on male fertility might be due to an alteration in the expression of spermatogenesis genes induced by undermethylation, or spermatozoa might be damaged by a higher production of toxic reactive oxygen metabolites causing DNA damage. To date, six studies have reported the possible association between *MTHFR* C677T polymorphism and male infertility, but firm conclusions cannot be drawn, mainly because of non-homogenous population selection and ethnic differences. In fact, studies from Germany (Bezold *et al.*, 2001), India (Singh *et al.*, 2005) and Korea (Park *et al.*, 2005) showed an association between both homozygous (TT) and heterozygous (CT) *MTHFR* polymorphism and azoospermia-oligozoospermia, whereas studies from the Netherlands (Eibisch *et al.*, 2003) did not find such association and studies from Italy (Stuppia *et al.*, 2003; Paracchini *et al.*, 2006) obtained conflicting results.

*DAZL* (deleted in azoospermia-like) is an autosomal homolog of the Y-linked *DAZ* gene, and it is expressed in the germ cells where it encodes for an RNA-binding protein. Two single nucleotide polymorphisms (SNP) in exons 2 (A260G) and 3 (A386G) have been reported. Initial report from Taiwanese men (Teng *et al.*, 2002) showed that the SNP at position 386 (T54A) was significantly associated with male infertility. However, subsequent studies from Italy (Bartoloni *et al.*, 2004; Becherini *et al.*, 2004), Germany (Tschanter *et al.*, 2004) and Japan (Yang *et al.*, 2005) did not confirm these results. However, four novel

missense mutations in *DAZL* have been associated with male infertility in a recent study (Tung *et al.*, 2006), highlighting the need for further biochemical and genetic investigation.

DNA polymerase (is responsible for replication and repair of the mitochondrial genome. Human DNA polymerase ( is composed of a catalytic subunit and an accessory subunit. Mutations in the gene for the catalytic subunit (*POLG*, polymerase gamma) have been shown to be a frequent cause of mitochondrial disorders. The human *POLG* gene contains a 10-unit CAG trinucleotide repeat encoding a poly-glutamine stretch near the N-terminus of the mature protein. Initial studies suggested that alteration of the CAG repeat could be associated with loss of sperm quality and to contribute to 5–10% of the male infertility cases in the European population (Rovio *et al.*, 2001). However, more recent studies from Italy and France reported that alterations in the CAG trinucleotide repeat of *POLG* was found at the same frequency in both normal and infertile men (Krausz *et al.*, 2004; Akinin-Seifer *et al.*, 2005; Brusco *et al.*, 2006).

Recently, SNP have been assigned to the FSH receptor (*FSHR*) gene. These SNP give rise to different *FSHR* haplotypes that modify the action of FSH. In exon 10 of the *FSHR* gene, two SNP are found corresponding to amino acid positions 307 and 680 of the mature protein. These two polymorphisms result in two major, almost equally common allelic variants in the Caucasian population: Thr<sup>307</sup>–Asn<sup>680</sup> and Ala<sup>307</sup>–Ser<sup>680</sup>. The *FSHR* polymorphism at position 680 influences serum FSH concentrations in women and the sensitivity of the *FSHR* to FSH *in vivo*. For example, a different need for FSH is seen in women during ovarian stimulation for IVF (Gromoll and Simoni, 2005). Another SNP is located at position (29 of the *FSHR* gene promoter (resulting in G→A exchange), whose impact, alone or in combination with exon 10 SNP, is less clear. In men, the impact of the *FSHR* SNP is still debatable. In particular, a preliminary study (Simoni *et al.*, 1999) showed no differences in the distribution of *FSHR* polymorphisms between normal and infertile men, whereas a more recent study (Ahda *et al.*, 2005) reported a different allelic frequency in azoospermic with respect to normozoospermic men. Recently, no significant difference was found in allelic variant frequency and genotype distribution between male infertility and *FSHR* exon 10 SNP alone and in combination with the SNP at position -29 (Pengo *et al.*, 2006). It is possible, therefore, that *FSHR* haplotypes represents one of the gene polymorphisms that, alone or in combination, might influence spermatogenesis, but the significance of this association needs to be verified by further studies.

Genetic screening of the *ERα* (oestrogen receptor alpha) gene locus has revealed the existence of several polymorphic sites. The most widely studied are the *PvuII* and *XbaI* restriction fragment length polymorphisms in intron 1, the (TA)<sub>n</sub> variable number of tandem repeats within the promoter region of the gene and the C→G polymorphism at codon 325 in exon 4. Some studies suggested an association between *ERα* polymorphisms and male infertility, particularly severe oligozoospermia. In the Greek population an association has been found with the intronic SNP *XbaI* and not with *PvuII* (Kukuvitis *et al.*, 2002), whereas in Spanish men an association was found only for the *PvuII* polymorphism (Galan *et al.*, 2005). The only study analysing exon 4 SNP reported a significantly different allelic distribution between azoospermic and control men in

Japanese men (Suzuki *et al.*, 2002). Furthermore, the (TA)<sub>n</sub> polymorphism in the Italian population, although not differently distributed between infertile and control men, showed an effect on sperm count (Guarducci *et al.*, 2006). Finally, the AGATA haplotype, recently described as a possible new risk factor for cryptorchidism in the Japanese population (Yoshida *et al.*, 2005), was not associated with severe male factor infertility in two independent European populations (Galan *et al.*, 2007). Therefore, although it is possible that different haplotypes of *ERα* may contribute to the male infertile phenotype, additional studies are warranted.

## Expression profiling of normal human testis

Despite the large amount of data collected in the last 10 years about the genetic basis of male infertility, the biological mechanisms leading to the disruption of spermatogenesis in patients who are carriers of genetic abnormalities are still largely unknown. In particular, although several genes involved in male infertility have been detected, the molecular pathways affected by their loss of function have not yet been clarified. The identification of these pathways could provide new information about the biological basis of the process of spermatogenesis and a more rational basis for treatment of male infertility. A breakthrough in this complex feature could derive from a post-genomic, rather than genomic, approach to the genetics of the male infertility.

The functional characterization of genes related to spermatogenesis and the study of their expression patterns is hampered by the complex nature of the testis, consisting of different cell types, each contributing to the total testicular transcriptome. However, in recent years microarray technology, able to simultaneously analyse in a single experiment thousands of genes expressed in a biological sample, has provided a powerful tool in the analysis of genome-wide expression profiles of different human tissues.

Few studies concerning human testis global gene expression profiles obtained by microarray technology have been reported to date (reviewed in He *et al.*, 2006). Some of these studies were principally focused on the identification of genes involved in the different steps of testis development. For example, Sha *et al.* (2002), analysing expression patterns in both human and mouse testes, identified 256 genes with significant differential expression at different development stages. Among these, 101 genes were identified as critically related to testis development and spermatogenesis regulation. Cheng *et al.* (2003) and Xu *et al.* (2003), identified novel human genes, *NYD-SP16* and *NYD-SP12*, the testicular expression of which seems to be developmentally regulated.

On the other hand, a limited number of studies aimed at the analysis of pathological versus normal testis and the identification of novel genes associated with male infertility have been reported. Fox *et al.* (2003) studied gene expression in testicular biopsies from men with Sertoli cell-only syndrome (SCOS) compared with subjects with normal histology of the testis. Three distinct groups of genes were identified: those expressed most abundantly in SCOS biopsies, those expressed most abundantly in normal testis, and those showing

similar expression in both tissues. Among transcripts more abundantly detected in normal testis, genes required for cell cycle progression, morphogenesis and development, as well as genes specifically implicated in spermatogenesis were observed, while the list of up-regulated genes in SCOS samples was mostly represented by genes with ubiquitous expression patterns. Yang *et al.* (2004) identified 128 differentially expressed genes possibly related to azoospermia, of which 56 were up-regulated and 72 down-regulated in azoospermic testes with respect to normal testes. Lin *et al.* (2006) investigated gene expressions of testicular RNA from Taiwanese men with normal spermatogenesis, maturation arrest and SCOS. In this study, the microarray analysis identified a list of genes differentially expressed in the three samples and among these 10 novel genes related to infertility were identified.

Due to the extreme variability typically observed in the results provided by experiments carried out by microarray technology, different replicas of expression profiling on normal human testis are required in order to gain consistent knowledge of the complex pathway regulating the production of spermatozoa.

In order to achieve this knowledge, it was decided to analyse global gene expression patterns of testicular biopsies from men with normal spermatogenesis. Analysis is made against a home-made RNA universal reference composed of a commercial human purified RNA pool (brain, liver, muscle, lung). The analysis is carried out using a 21,329 spotted 70-mer oligonucleotide microarray designed on Human Unigene clusters (Microcribi Padova, Italy). Each oligonucleotide is spotted in two replicates (for a total of 42,658 spots) on a MICROMAX glass slide SuperChip provided by PerkinElmer Life Sciences Inc. The goal is to identify the list of up- and down-regulated testis-specific genes directly linked to testis metabolism and function with respect to general metabolism in humans.

Testis biopsies consisting of 50-mg specimens were submerged in RNAlater solution (Ambion, Austin, TX, USA) immediately after removal and stored at 4°C. Total RNA was extracted using the SVtotal RNA Isolation System kit (Promega, Madison, WI, USA). In order to obtain a sufficient amount of RNA for microarray analysis, the RNA was amplified using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion). The obtained aRNA was labelled with Cy3 (normal testis) (Amersham, Pharmacia Biotech, UK) and hybridized on the microarray. Fluorescent signals were captured by a ScanArray 5000 Packard laser scanning and analysed using the ScanArray Express Microarray Analysis System software version 3.0. Finally, the data obtained were statistically analysed using the SAM system (Significance Analysis of Microarray). In each experiment, a 1.4-fold change in the signal of each spot was considered as the evidence of a different expression of the specific transcript.

Preliminary research using SAM analysis revealed a group of 3300 genes up-regulated in testis as compared with the reference RNA pool, of which 2718 were annotated UniGene. These annotated genes were grouped together using the EASE software (Expression Analysis Systematic Explorer version 2.0), which allowed identification of a list of biological processes in which the identified genes are involved. Based on their GeneOntology number, a total of 152 different biological

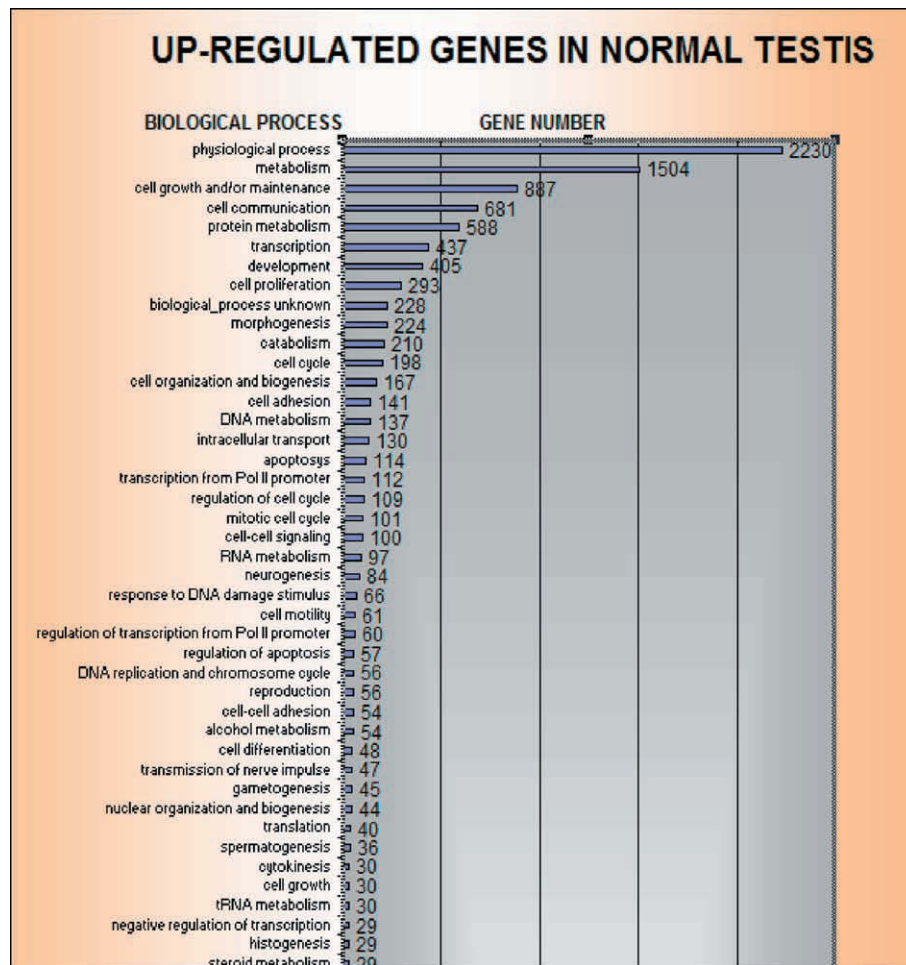
processes were identified. In this kind of analysis, the same gene can be reported in more than one class, being involved in different biological processes. The most abundant categories were those related to physiological process and metabolism, which are very unspecific classes, presenting 2230 and 1504 genes respectively. Other abundant but not specific classes were those related to cell growth and maintenance (887 genes), cell communication (681), protein metabolism (588), transcription (437), development (405) and cell proliferation (293) (**Figure 1**). Interestingly, 228 genes showed unknown biological process. Other less represented classes were those containing genes specifically involved in functions related to human testis, such as reproduction (spermatogenesis, sex differentiation, fertilization, spermatid development), meiosis (regulation, synaptonemal complex organization), imprinting (epigenetic regulation of gene expression), steroid metabolism and acrosome reaction (**Table 2**). Among these, of interest is the presence of genes mapped within the AZF loci of the Y chromosome such as *DAZ*, *BPY* (basic charge, Y-linked), *DBY*, *SMCY* (Smcy homolog, Y-linked), as well as the presence of the *DAZL* gene, the autosomal homologue of the human *DAZ*.

Among genes down-regulated in testis compared with the reference RNA pool, 3400 genes were identified, of which 2654 were annotated UniGene. Grouping together these genes by EASE software, a list of the same biological processes shown after grouping up-regulated genes was identified, except for those specifically related to testis function (**Figure 2**).

These preliminary results clearly demonstrate that expression profiling using microarray technology is able to evidence genes specifically involved in testicular function, and to provide a general pattern of the human normal testis transcriptome. Based on this knowledge, the comparison among these expression profiles and those obtained from testis of patients with different forms of idiopathic and genetic male infertility will provide useful information about the biological basis of each different condition and about genes involved in the pathogenesis of spermatogenesis failure.

## Conclusions

Male infertility represents one of the clearest examples of a complex disease with a substantial genetic basis. Numerous male mouse models, mutation screening and association studies performed in the last few years definitively demonstrate the high prevalence of genetic causes of spermatogenic impairment. However, a large proportion of infertile males are diagnosed as idiopathic, reflecting poor understanding of the basic mechanisms regulating spermatogenesis and sperm function, hence the inability properly to diagnose the aetiology. Furthermore, the molecular mechanisms underlying spermatogenic damage in cases of genetic infertility (for example Yq microdeletions) are not known. These problems can be addressed only by large scale association studies and testicular or spermatozoal expression studies in well-defined alterations of spermatogenesis. It is conceivable that these studies will provide in the future important diagnostic and therapeutic implications.



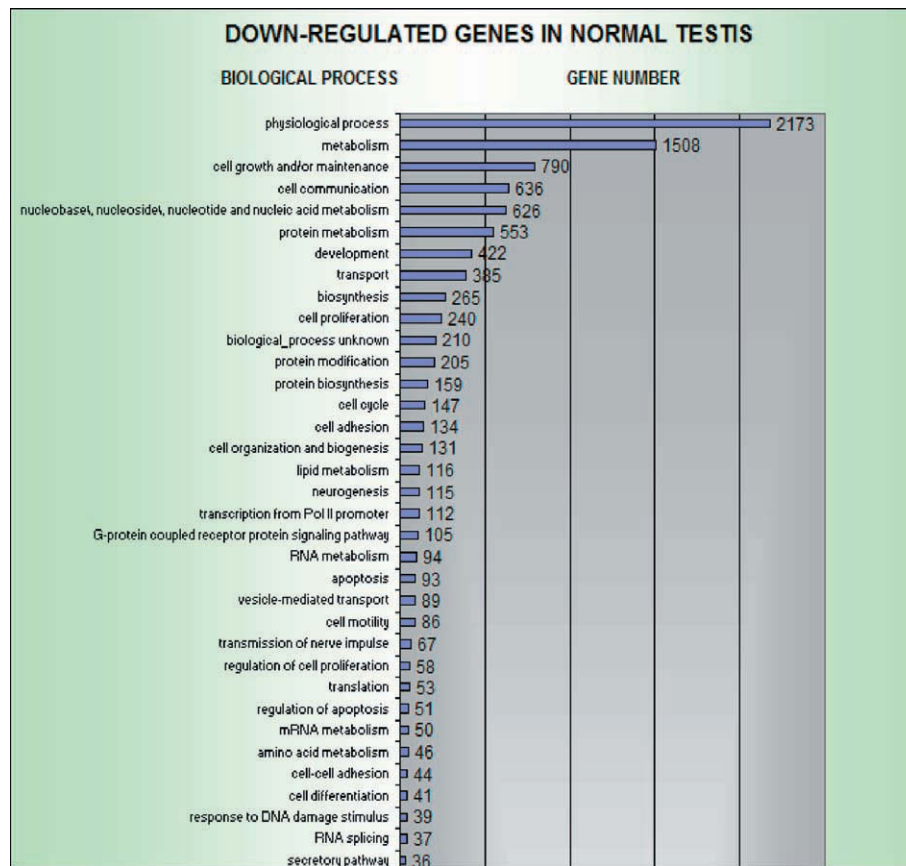
**Figure 1.** Gene classes derived by grouping up-regulated transcripts in normal human testis according to their biological processes using Expression Analysis Systematic Explorer (EASE) analysis.



**Table 2.** Genes specifically involved in male reproduction as identified by global expression profile of normal testis by microarray technology.

<i>GO biological process</i>	<i>No. of genes</i>	<i>Genes</i>
Reproduction	56	<i>ADAM2; ADAM20; AKAP3; BAX; BCL2L10; BMP15; BRD2; C15ORF2; C8ORF1; CNA1; CCT6B; CSNK2A2; CYLC1; D8S2298E; DAZ; DAZL; DBY; FLJ13057; LJ20080; FOXJ1; FSHB; FSHPRH1; HSF2BP; HYAL4; LOC56926; MAGOH; MAK; MCSP; MOV10L1; NR6A1; ODF1; OR7C1; OVOL1; PCMF; PIWIL1; PPAP2A; PRKAG1; PRM1; PRM2; PROL1; RFP; SECP43; SMCY; SPAG1; SPAM1; SPINLW1; STK22B; STK22C; STRBP; STRIN; SYCP1; TCFL5; TESK2; TNP1; TXNDC2; ZP2</i>
Gametogenesis	45	<i>BAX; BCL2L10; BMP15; BRD2; C15ORF2; C8ORF1; CCNA1; CCT6B; CSNK2A2; CYLC1; DAZ; DAZL; DBY; FLJ13057; FOXJ1; FSHB; FSHPRH1; HSF2BP; MAGOH; MAK; MOV10L1; NR6A1; ODF1; OR7C1; OVOL1; PCMF; PIWIL1; PPAP2A; PRKAG1; PRM1; PRM2; PROL1; RFP; SECP43; SMCY; SPINLW1; STK22B; STK22C; STRBP; STRIN; SYCP1; TCFL5; TESK2; TNP1; TXNDC2</i>
Spermatogenesis	36	<i>BCL2L10; BRD2; C15ORF2; CCNA1; CCT6B; CSNK2A2; CYLC1; DAZ; DAZL; DBY; FOXJ1; FSHPRH1; HSF2BP; MAK; MOV10L1; NR6A1; ODF1; OR7C1; OVOL1; PIWIL1; PRKAG1; PRM1; PRM2; PROL1; RFP; SECP43; SMCY; SPINLW1; STK22B; STK22C; STRBP; SYCP1; TCFL5; TESK2; TNP1; TXNDC2</i>
Steroid metabolism	29	<i>ABCA1; AKR1D1; APOF; CETP; CYP11B2; CYP7A1; FDPS; FLJ13352; HSD3B2; LSS; MVK; NR1I2; OSBPL10; OSBPL2; OSBPL5; OSBPL9; PRKAA2; PRKAG2; RODH; SC4MOL; SCAP; SOAT1; SQLE; SR-BP1; SREBF2; STAR; STS; TM7SF2; TSTA3</i>
Steroid biosynthesis	16	<i>CYP11B2; CYP7A1; FDPS; HSD3B2; LSS; MVK; PRKAA2; PRKAG2; RODH; SC4MOL; SCAP; SQLE; SR-BP1; STAR; TM7SF2; TSTA3</i>
Meiosis	15	<i>C8ORF1; CCNA1; CSPG6; GAJ; MSH4; RAD51L3; RAD52; RBM7; SMC1L1; STAG3; SUV39H2; SYCP1; UBE2D3; XRCC2; ZW10</i>
Imprinting/regulation of gene expression/epigenetic	14	<i>ARHI; DNMT3B; FLJ21103; FLJ21940; FLJ23024; KIAA0913; MBD3; PAXIP1L; PRO1853; SIRT4; SIRT7; SUV39H2; TIP120A; TSSC3</i>
Fertilization	11	<i>ADAM2; ADAM20; AKAP3; D8S2298E; DAZ; HYAL4; MCSP; SPAG1; SPAM1; TNP1; ZP2</i>
Sex differentiation	7	<i>CDKL2; DMRT3; DMRTA2; DMRTB1; FSHPRH1; IDE; PROL1</i>
Steroid hormone receptor signalling pathway	7	<i>CRSP6; EGLN2; ESR1; NCOA6; PPAP2A; PPARBP; RBM14</i>
Meiosis I	7	<i>GAJ; MSH4; RAD51L3; RAD52; STAG3; SYCP1</i>
Spermatid development	4	<i>CSNK2A2; STK22C; STRBP; TNP1</i>
Male meiosis	3	<i>CCNA1; SUV39H2; UBE2D3</i>
Germ-cell migration	3	<i>PCMF; PPAP2A; STRIN</i>
Binding of spermatozoa to zona pellucida	2	<i>SPAM1; ZP2</i>
Synaptonemal complex formation	2	<i>STAG3; SYCP1</i>
Acrosome reaction	1	<i>AKAP3</i>
Sex determination	1	<i>MAGOH</i>
Sperm motility	1	<i>MCSP</i>
Reproductive behaviour	1	<i>PI3</i>
Androgen biosynthesis	1	<i>RODH</i>

GO: gene ontology.



**Figure 2.** Gene classes derived by grouping down-regulated transcripts in normal human testis according to their biological processes using Expression Analysis Systematic Explorer (EASE) analysis.

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