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·Review·

Genetic and epigenetic risks of intracytoplasmic sperm injection method

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Abstract

Pregnancies achieved by assisted reproduction technologies, particularly by intracytoplasmic sperm injection (ICSI) procedures, are susceptible to genetic risks inherent to the male population treated with ICSI and additional risks inherent to this innovative procedure. The documented, as well as the theoretical, risks are discussed in the present review study. These risks mainly represent thatconsequences of the genetic abnormalities underlying male subfertility (or infertility) and might become stimulators for the development of novel approaches and applications in the treatment of infertility. In addition, risks with a polygenic background appearing at birth as congenital anomalies and other theoretical or stochastic risks are discussed. Recent data suggest that assisted reproductive technology might also affect epigenetic characteristics of the male gamete, the female gamete, or might have an impact on early embryogenesis. It might be also associated with an increased risk for genomic imprinting abnormalities. *(Asian J Androl 2006 Nov; 8: 643–673)*

Keywords: genetic risks; epigenetic risks; intracytoplasmic sperm injection; testis; male infertility

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1 The importance of evaluation of microscopic and macroscopic consequences of intracytoplasmic sperm injection (ICSI) techniques

ICSI represents a revolutionary technique of *in vitro* fertilization (IVF) developed during the past decade. It might represent the laboratory method of choice for the treatment of severe cases of male infertility. This method has become popular through the years and has been an invaluable stimulator for the development of novel approaches and applications along with the standard IVF. The use of ICSI resulted in the application of sympromatic (i.e. non-etiological) modes of treatment of severe cases of male infertility. In addition, ICSI has been a successful procedure for the fertilization of *in vitro* matured human oocytes [1]. Nevertheless, reservations for the effect of ICSI on the genetic constitution of the offspring derived from this technology have been raised [2].

Until the introduction of ICSI procedures in human assisted reproduction, the lack of an adequate number of competent spermatozoa for the performance of assisted reproduction methods (i.e. IVF) was a barrier for the achievement of pregnancies in cases where genetic deficiencies affected the male reproductive potential. However, nowadays, because ICSI techniques bypass several barriers in the natural fertilization process, there is much concern on the safety of ICSI and the probable transmission of reproductive deficiencies (of genetic etiology) or other genetic abnormalities to the offspring. Furthermore, the rapid employment of these methods in humans and the lack of organized experimental and clinical trials prior to the wide application of ICSI procedures have raised some additional concerns. One negative consequence of the use of ICSI techniques is the shift away from research on micro-insemination systems. Thus, there might be a need to develop new research directions. One new target might be the development of more stringent spermatozoal selection/preparation methods to reduce the risk of transmission of male genetic factors that have been associated with genetic risks for the ICSI offspring to the female gamete.

In order to appreciate the potential genetic risks of ICSI techniques, it is necessary to analyze the causes of male infertility, particularly those that have a genetic basis. The use of ICSI procedures for the therapeutic management of infertile males with a genetic defect might overrun the limitations for transfer of this particular defect to the next generation. Thus, ICSI techniques might be responsible for the transmission of a genetic defect to the next generation. Therefore, ICSI procedures might propagate (i.e. maintain and increase) the incidence of a genetic defect related to the development of impaired spermatogenesis within a male population.

Furthermore, because gametes and early embryonic genomes undergo an epigenetic reprogramming, ICSI techniques might interfere with the establishment of normal parental imprinting, resulting in embryonic or fetal abnormalities [3, 4].

2 Strong evidence proves a genetic basis of several spermatogenic defects

During the past decade, there has been a dramatic expansion in the number of genes involved in spermatogenesis, sexual differentiation and reproductive deficiencies. The development of differential display reverse transcriptase-polymerase chain reaction (RT-PCR) procedures has led to the identification of many genes that are differentially regulated in various cell and tissue types [5]. Anway *et al.* [5] used the above technique to identify genes that are expressed in isolated mouse testicular type A spermatogonia and in more advanced germ cells. The authors identified cDNA fragments for *mDEAH9*, *RanBP5*, *GC3*, *GC12*, and *GC14* genes in the testis and type A spermatogonia from wild type mice but not in samples from mutant sterile W/Wv mouse testis. RT-PCR analyses of isolated spermatogonia, pachytene spermatocytes and round spermatids found that *mDEAH9*, *RanBP5*, *GC3*, *GC12* and *GC14* genes were expressed in all three cellular populations. *RanBP5* expression appeared to be regulated during the cycle of the seminiferous epithelium with the highest expression in stages III through VII. Expression of *GC14* was greatest in the meiotic germ cellular subpopulations. In addition, Anway *et al.* [6] identified a murine testis complementary DNA encoding a homolog to human A kinase anchoring protein-associated sperm protein (ASP). Northern blot and RT-PCR analyses did not detect ASP mRNA in mouse spleen, brain, liver, lung, heart, kidney, skeletal muscle, ovary or Sertoli cells. In contrast, the above techniques localized ASP mRNA to the germ cell compartment of the seminiferous tubules in the testis. In addition, Anway *et al.* [7] provided strong evidence that the effects of endocrine disruptors on spermatogenetic capacity in subsequent (F1 and F2) generations might be the result of altered DNA methylation patterns in the male germ line. The latter study showed the ability of environmental factors to reprogram the genes in the male germ line and to promote a transgenerational disease state [7]. Other studies by Anway and Skinner [8] confirmed the transfer of abnormal phenotypes (through epigenetic actions on the male germ line) to subsequent generations analyzed.

Mouse models with reproductive defects as a major phenotype have been created and now hold over 200 [9]. These models are helping to define mechanisms of reproductive function, as well as identify potential new genes involved in the pathophysiology of reproductive disorders. Mouse models for the study of reproductive defects have been produced by spontaneous mutations, transgene integrations, retroviral infection of embryonic stem cells, ethylnitrosurea mutagenesis and gene targeting technology. Several genes required for vertebrate fertility are highly conserved in evolution with orthologues in *Drosophila melanogaster* (i.e. *DDX4*), fat facets (*DFFRY*), and boule (*DAZ*) [10–12]. Defects in sexual differentiation pathways can cause infertility in mice and humans of both sexes. It has been pointed out by Matzuk and Lamb [9] that several gene defects or gene-related pathophysiologies leading to defects in sex determination or development (i.e. pseudohermatidism, sex reversal, Denys-Drash syndrome, pseudovaginal perineoscrotal hypospadias**,** cryptorchidism or congenital bilateral absence of vas deferens), defects in sperm production and function (i.e. myotonic dystrophy, Nooman syndrome, sickle cell anemia, β-thalassemia, Kartagener syndrome, primary ciliary dyskinesia, Fanconi anemia or ataxia telangiectasia) and endocrinopathies lead to human male infertility. In addition, numerical/structural chromosomal abnormalities result in human male infertility as well. Knockout animal models have provided strong evidence supporting the genetic basis of human male infertility in subpopulations of infertile men.

Of major importance are research efforts focused on the genes of sex chromosome Y and also on genes associated with certain genetic syndromes having the development of male infertility as an inherent component of their phenotype. Consequently, these studies provide evidence for the molecular basis of the genetic risks of ICSI procedures.

Today, a significant percentage of spermatogenic abnormalities can be studied and classified according to genetic criteria. In fact, 30% of spermatogenic abnormalities are considered to have a genetic basis-related etiology [13–15]. A clinical classification of spermatogenic disorders alone cannot directly associate a phenotype with a particular genetic abnormality. Excluding the genetic syndromes/pathophysiologies showing infertility as one of the characteristics of their clinical phenotype, in the vast majority of infertile males the clinical diagnosis of infertility is not associated with any other clinically important phenotypic manifestations/characteristics.

In most infertile males, the aetiology of infertility is unknown (i.e. idiopathic). This is the reason the majority of fertility specialists recommend the clinical and laboratory evaluation of infertile males before the application

of ICSI techniques. A major objective of the current communication was to associate the genetic defects of infertile males with their semen quality and reproductive potential. Another objective was to emphasize the probability of the transmission of major or minor paternal genetic defects to the embryo/offspring when ICSI procedures are applied. Major genetic or epigenetic defects in the male XY-embryo might be manifested at the fetal or neonatal stage as profound and severe manifestations [9, 16]. In contrast, minor genetic defects in the male XY-embryo might not affect the early embryonic development directly but might play a significant detrimental role in the reproductive potential of the affected newborns.

3 Genetics of male infertility

3.1 Single gene disorders

A subpopulation of patients that present to IVF clinics for treatment of male factor infertility might have incomplete penetrance of a single gene genetic disorder. Another population might show some clinical manifestations characterizing the disorder that is the cause for the development of infertility.

3.1.1 Congenital bilateral absence of vas deferens due to cystic fibrosis transmembrane conductance regulator gene mutations

Most of the congenital bilateral absence of vas deferens (CBAVD) cases (60–90%) and some cases of unilateral absence of the vas deferens are to the result of mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. This gene is responsible for the underlying genetic defect in cystic fibrosis (CF), a genetic recessive disorder with an incidence of carriers between 5–6% in the Caucasian population. Among infertile patients with CBAVD, the incidence of *CFTR* mutation-carriers is estimated to be 20-fold greater than that in the general population [17]. Mutations in *CFTR* are classified as severe or mild. The association between the genotype and the phenotype is complex. In general, the mild mutations result in mild alterations in phenotypes restricted in the male reproductive tract and are characterized by obstructive azoospermia.

More than 700 mutations in *CFTR* gene spanning (approximately 230 kb) have been described [18]. CBAVD patients have either two mild *CFTR* mutations or a mild mutation in combination with a severe one. The most frequent severe mutation is the ΔF508 representing the majority (60–70%) of the CF mutations in carriers and patients. In addition, polymorphisms reducing the production of the CFTR protein (5T, 7T) have been shown. In particular, the homozygous or heterozygous presence of the 5T allele is a frequent finding in CBAVD patients with incomplete penetrance. The identification of this allele, corresponding to an inefficient acceptor splice site with a 90% reduction of the CFTR protein synthesized, is associated with a spectrum of presentations of phenotype from healthy fertile males to CBAVD patients [19]. Compound heterozygotes carrying the 5T allele but showing a *CFTR* mutation might present with atypical or typical clinical phenotypes of CF. At least seven other mutations commonly related to CBAVD have been described and they are almost all related to defective CFTR protein processing [17]. In addition, the missence *R117H* mutation in exon 4 is also related to CBAVD in association with the 5T variant [20]). Thus, testing for *R117H* and 5T/7T/9T polymorphism is important in the infertility setting.

Recovery of epididymal or testicular spermatozoa and subsequent employment of ICSI techniques are essential to assist reproduction in the group of CBAVD male patients. This approach has the risk of producing affected offspring when the female partner is a carrier. Consequently, at least the most common *CFTR* mutations (up to 90%) should be screened (see above paragraph). Genetic counselling is strongly recommended for these patients (Table 1). Testing the obstructed azoospermic men for the most common mutations and associated polymorphisms (28 in total) is the appropriate first step. Preimplantation genetic diagnosis (PGD) is recommended for couples who are both positive for CF mutations and wish to integrate ICSI and genetic diagnosis at early stages of the embryonic development [21, 22].

Josserand *et al.* [23] detected *CFTR* mutations on 56 alleles of 50 males with congenital bilateral absence of vas deferens. A total of 15 (30%) were compound heterozygote and 26 (52%) heterozygote. In all, 38% of the patients had a positive sweat test. It appears that congenital absence of vas deferens can be seen in male heterozygote carriers of one *CFTR* mutation or compound heterozygotes with two mutations, one of which might not be detected by the mutation analysis. This is important, as it will affect counselling of couples especially if the female partner carries a *CFTR* mutation.

Table 1. Suggested basic genetic testing before intracytoplasmic sperm injection treatment. PGD, preimplantation genetic diagnosis; PND, prenatal diagnosis; *CFTR*, cystic fibrosis transmembrane conductance regulator.

	Screening	Major risk for	Partner at risk	Counselling	Needed test	Considerations
Strongly recommended	Male and female karyotype	Implantation failure, spontaneous abortion, abnormal offspring	Both partners	Karyotype in both partners. PGD/PND if one partner's karyotype is abnormal	Karyotype in both partners	To identify chromosomal abnormalities and avoid embryonic implantation failure and affected offspring
	Cystic Fibrosis	Affected offspring (variable phenotype)	Male (obstructive azoospermia)	Screening of both partners. PGD/PND if both partners are carriers	DNA test for *CFTR* mutations	To identify carriers and avoid affected offspring
	Y micro-deletions	Affected male offspring with Y-microdeletions or birth of 45,X offspring	Male (non-obstructive azoospermia or crypto-azoospermia)	Possibility of infertile male offspring. The role of PGD should be discussed	Molecular analysis for Y chromosome microdeletions	To avoid a needless testis biopsy and birth of infertile male offspring or 45,X offspring
Optional	Kallman syndrome	Carrier female offspring	5% of infertile males with hypogonado-trophic hypogonadism	PGD	Genetic analysis for KAL-1 mutations/ deletions	Consultation of a carrier-female offspring when puberty is reached to avoid X linked-related disorders in her offspring
	Kennedy disease	Offspring with spinobulbar muscular atrophy	Man with defect in spermato-genesis	PGD/PND	Molecular Screening of the androgen receptor gene	To avoid transmitting trinucleotide repeat disorders
	Sperm aneuploidy	Offspring with chromo-somal abnormalities/ spontaneous abortions	Male partner with a history of chemo-therapy or exposure to toxic agents	PGD	Sperm fluorescent *in situ* hybridization techniques using at least one probe for an autosomal chromosome and two probes for the two sex chromosomes	To explain implantation failures and recurrent abortions

3.1.2 Kartagener syndrome and other monomorphic anomalies of spermatozoa

Primary akinesia or dyskinesia of the cilia is a gene-ral term used to describe disorders of the structure of the cilia mainly in the airways and the sperm tail resulting in impaired sperm motility [24]. Affected individuals have chronic manifestations (as a result of the above disorder) in their airways. Males are usually infertile as a result of the sperm tail defects. There are structural anomalies in the proteins forming the bridging links of the dynein in the axoneme [25]. The co-existence of sinusitis, bronchiectasia, immotile spermatozoa and situs inversus characterizes Kartagener syndrome. The prevalence of situs inversus of any etiology appears to be in a range between 1 in 25 000 and 1 in 8 000. Twenty to 25% of these individuals with complete mirror-image situs inversus have ciliary dyskinesia and respiratory symptoms (Kartagener syndrome) as associated findings [26]. The prevalence of Kartagener syndrome in the general population is approximately 1: 40 000.

Earlier linkage analyses in a large number of primary ciliary dyskinesia families showed extensive heterogeneity [26]. No single genomic region harbouring a common primary ciliary dyskinesia locus was identified. However, several potential chromosomal regions that could harbour genes for primary ciliary dyskinesia were localized [26]. To date, mutations in two genes have been associated with a minority of primary ciliary dyskinesia/Kartagener syndrome cases. These are genes coding for the dynein axonemal heavy chain 5 and the dynein axonemal intermediate chain 1.

A considerable number of additional monomorphic human sperm defects have been described. Most appear to be exceedingly rare and they might only be detectable through electron microscopy [27]. For the '9 + 0' axoneme defect [28] and globozoospermia (round head defect), evidence from family studies suggests that these are genetically determined disorders [29]. The mode of inheritance of monomorphic human sperm defects is most likely to be autosomal recessive or X-linked [13]. No mapping data for the responsible genes are available yet [13]. Thus, monomorphic anomalies of spermatozoa represent a defined entity with distinct genetic background and variable characteristics as, for example, globozoospermia [13, 24] (see the section 3.13). Globozoospermia is found in less than 0.1% of infertile male partners [30]. Although these pathophysiologies of sperm motility and morphology are heterogenous, the genetic diagnosis is based on the clinical and laboratory examination, and the appropriate genetic tests (see the section 3.13). In a recent study, no mutation was found among six patients with globozoospermia [30]. Counseling is of paramount importance to inform the couples about the risk of transmitting these disorders to their offspring.

3.1.3 Genetic disorders with endocrine or neurologic implications

Kallman syndrome is implicated in approximately 5% of the infertile males with hypogonadotrophic hypogonadism. Anosmia is a result of deletions in the Xp22 region or mutations of the *KAL-1* gene. The syndrome phenotype varies from normogonadotrophic fertile patients to the total absence of the gonadotrophins (FSH and LH) as a result of insufficiency of GnRH. The full abnormal phenotype is due to the inefficient migration of the hypothalamic olfactory neurons and those producing GnRH. When the serum testosterone profiles are sufficient to support sexual differentiaton, the male phenotype is normal and spermatogenesis can be stimulated by gonadotrophins to permit subsequent use of ICSI procedures [31].

GnRH receptor gene mutations (autosomal recessive inheritance) result in hypogonadotropic hypogonadism with oligospermia. In addition, FSH receptor gene mutations are associated with variable degrees of spermatogenic defects. Activating mutations of the same gene have been described. Furthermore, mutations in genes encoding the LH receptor, 5α-reductase 2, or CYP 21 might cause defects in spermatogenesis [32]. Affected males might be treated with ICSI and, therefore, are at risk to transmit the underlying defect to the offspring.

A form of Kennedy disease characterized by androgen resistance and a molecular defect in the androgen receptor gene is associated with male infertility and defects in spermatogenesis [33–35]. The main feature of this condition is spinobulbar muscular atrophy (SBMA) with neurodegenerative phenotype. The gene responsible for the expression of androgen receptor is located on the X chromosome (Xq11-q12, OMIM #313700). The latter men might be candidates for ICSI techniques before the full onset of their disease, and they should also be informed that the consequences of their disease might be considered much more devastating than the infertile phenotype and that their disease might result in severe clinical manifestations. Nevertheless, as we have previously reported, couples with female SBMA carriers might

request PGD in order to assure the birth of an unaffected offspring [36]. Myotonic dystrophy and fragile X syndrome, similarly as the Kennedy disease, represent disorders characterized by dynamic trinucleotide repeat expansions. Decreased sperm function or azoospermia are common in patients with myotonic dystrophy [37–39]. In cases of myotonic dystrophy of intermediate clinical severity, the use of combined ICSI and PGD procedures might assist to prevent the transmission of the defect to the offspring [40]. The X chromosome is not transmitted directly through a male carrier of an X-linked disorder to his male offspring, nevertheless it can be transmitted via a daughter to a male grandchild. Sermon *et al.* [40] have described their experience with fluorescent PCR and automatic fragment analysis for the clinical application of pre-implantation genetic diagnosis of myotonic dystrophy.

The prevalence of the fragile X syndrome (FRAXA) premutation carriers is 1/1 000 in males and 1/350 in females, whereas the prevalence of full mutation is 1/4 000 males or females [41]. Carriers of premutations have mild or no symptoms, whereas male patients with full mutation of the FRAXA syndrome have moderate to severe mental retardation, behavioural problems and spermatogenic impairment including abnormal tubular morphology and excessive number of malformed spermatids. The overall result is decreased fertility probably as a result of the fact that the gene that is responsible for the phenotype is expressed in the male gonads [42, 43]. The use of ICSI procedures as a treatment for males with FRAXA syndrome mutations, or even permutations, is definitely susceptible to serious ethical considerations. Couple counseling, written consent forms and, probably, National Authority Permission is necessary. Platteau *et al.* [44] claimed that PGD work-up for FRAXA syndrome couples should include a determination of the premutation or mutation carrier status and the paternal or maternal origin of the premutation/mutation. Fragile X-premutation carriers should be advised not to postpone reproduction.

Female premutation carriers have up to 50% (depending on CGG repeat size) risk of fragile X syndrome in their offspring and a risk (15–20%) of premature ovarian failure [41, 45]. Up to 30% of females with a full mutation can be symptomatic depending on the X-inactivation status. Female premutation carriers belonging to families with fragile X syndrome should ask for PGD or prenatal diagnosis (PND) in order to prevent transmission of the disease [46]. Sermon *et al.* [46] reported for the first time in the literature a method for PGD for FRAXA syndrome based on the amplification of the CGG triplet in the normal allele.

The above-mentioned single gene genetic disorders indicate the risks of transmitting genetic abnormalities via ICSI procedures and stress the need for systematic genetic testing in familial or sporadic infertility cases (Table 1).

3.2 Chromosomal abnormalities

Chromosomal abnormalities have been associated with infertility or subfertility in males. The incidence of chromosomal abnormalities in the karyotypes of infertile males is 5.8%, with a predominance of sex chromosomal abnormalities according to a review of pooled data from 11 surveys (9 766 men with azoospermia or oligospermia were evaluated) [2, 47]. The phenotypic consequences of the sex chromosomal abnormalities are usually mild compared with the consequences of autosomal chromosomal abnormalities in males [14]. In addition, the incidence of chromosomal aneuploidies, especially those shown in the sex chromosomes, is higher in spermatozoa from men with non-obstructive azoospermia [48]. Mateizel *et al.* [49] have shown that aneuploidy for chromosome 18 is more frequent in men with spermatogenic failure. Furthermore, sperm concentrations smaller than 20×10^6 spermatozoa/mL are associated with significantly higher percentage of *de novo* chromosomal anomalies in prenatal samples in successful pregnancies [50, 51]. Numerical abnormalities of the sex chromosomes might be found either in immature testicular germ cells (germline defects) or in spermatozoa of men whose peripheral blood cytogenetics indicate non-mosaic Klinefelter syndrome (gonadal mosaicism) [52].

If ICSI procedures are scheduled for the therapeutic management of male infertility associated with chromosomal abnormalities of the male partner, it is important to discuss with the couple the option of PGD or PND (Tables 1, 2).

3.2.1 Autosomal translocations

Autosomal translocations are 4–10 times more frequent in infertile (subfertile) males compared with fertile individuals [53, 54]. Mendelian Cytogenetic Network has approximately 265 entries of balanced reciprocal tranlocations from infertile males [55]. Among balanced chromosomal rearrangements in male infertility, half of the identified autosomal breakpoints (5/10) were found

Table 2. Type of Y-chromosome microdeletions and testicular pathology (for additional information see references 95, 97, 98, 105, 107, 109 and 110). SCOS, Sertoli cell-only syndrome; PS, primary spermatocyte; PGD, preimplantation genetic diagnosis.

Region of microdeletion	Type of deletion	Testicular Phenotype	Considerations
AZFa	Entire	SCOS	No reason to perform testicular biopsy
AZFb	Entire	PS arrest	No reason to perform testicular biopsy
AZFc	Entire	Ranging from hypospermatogenesis to SCOS	Testicular biopsy may be performed; In case of presence of spermatozoa, sperm cryoprservation is recommended; if ICSI procedures result in fertilization and early embryonic development, PGD is recom mended to avoid transfer of 45,X embryos
AZFa	Partial	hypospermatogenesis to SCOS	Testicular biopsy may be performed.
AZFb	Partial	hypospermatogenesis to SCOS	Testicular biopsy may be performed.
AZFc	Partial	hypospermatogenesis to SCOS	Testicular biopsy may be performed; In case of presence of spermatozoa in either the ejaculate or the testicular tissue sperm cryoprservation is recommended; if ICSI procedures result in fertilization and embryonic development, PGD is recommended to avoid transfer of 45X embryos.

to be located on chromosome 1, suggesting a clustering of male specific loci on this chromosome. The above breakpoints along chromosome 1 have been found to be in excess in infertile males (from the Mendelian Cytogenetics Network) compared with the karyotypes of a cohort [56].

In general, reciprocal or non-reciprocal autosomal chromosomal translocations and complex chromosomal rearrangements (involving three or more chromosomes) are associated with subfertility. This is the result of inappropriate pairing of the homologous chromosomes during meiosis, leading to meiotic disturbance or chromosomal imbalance in the male gametes [2, 57, 58].

3.2.2 Robertsonian translocations

Translocations between acrocentric chromosomes (Robertsonian) are frequent in humans, but their impact on spermatogenesis varies from the absence of spermatogonia to the development of normal spermatogenesis. The therapeutic management of Robertsonian translocations associated with infertility depends on the presence of spermatozoa and the success of ICSI procedures. In these cases, ICSI procedures raise risks for chromosomal abnormalities in the generated embryos [21, 22, 59].

The reproductive risks for the newborn, as a result of the presence of Robertsonian translocations in the infertile couple, depend on the chromosomes involved and the sex of the carrier. The most common risks are related to newborn translocation trisomies of chromosomes 13, 14, 21 or 22. An increased proportion of carriers of robertsonian translocations (usually t[13q;14q]) has been reported among oligozoospermic (1.6%) and azoospermic (0.09%) men attending infertility clinics or among the male partners in couples with recurrent spontaneous abortions [2, 60]. Therefore, there is a strong indication for the performance of PGD in combination with the ICSI procedures [61]. For the evaluation of the chromosomal composition of spermatozoa, fluorescent *in situ* hybridization (FISH) techniques are recommended with additional (to the probes for sex chromosomes) specific probes for chromosomes participating in probable reciprocal or Robertsonian translocations [62–64].

Van Assche *et al.* [63] carried out PGD and sperm analysis by FISH for the most common reciprocal translocation t (11:22). By choosing probes lying on both sides of the breakpoints and by using a combination of subtelomeric or locus-specific probes and centromeric probes, the use of three-color FISH enabled detection of all the imbalances in sperm and/or cleavage stage em-

bryos in the patients.

3.2.3 Klinefelter syndrome

Non-mosaic Klinefelter (47,XXY) and mosaic Klinefelter syndrome (46,XY/47,XXY) are the most common chromosomal abnormalities observed in azoospermic males. Adult males with non-mosaic Klinefelter syndrome (47,XXY) have hypogonadism and infertility. Disruption (arrest) in spermatogenesis is shown. Spermatogonia in these patients usually do not further differentiate beyond the stage of primary spermatocyte, but occasionally testicular focal advanced spermatogenesis up to the spermatozoon stage is observed. FISH analysis of spermatogonia and spermatocytes from men with non-mosaic Klinefelter syndrome show a variable frequency of aneuploidy of the sex chromosomes (either 47,XXY or 46, XY profiles are shown indicating gonadal mosaicism) [52, 65, 66]. Spermatozoa recovered from testicular biopsies of men with karyotypes indicating non-mosaic Klinefelter syndrome have been used to fertilize oocytes by ICSI techniques. Preimplantation blastomere-FISH analysis should be carried out with X and Y probes to confirm that the sex chromosomal complement of the embryos that are going to be transferred is normal. The birth of normal offspring has been reported after ICSI techniques using testicular spermatozoa recovered from men with non-mosaic Klinefelter syndrome [52, 65, 67-69; among others]. We can speculate that the risk of transmitting additional X chromosomes to the offspring might be related to the percentage of the 24,XY testicular spermatozoa in the recovered testicular sperm population. It appears logical to speculate that a man with a non-mosaic Klinefelter syndrome and a large percentage of abnormal 24,XY spermatozoa in his testicular biopsy sample he may have a large probability to generate a 47,XXY embryo after ICSI techniques. A number larger than 20 human offspring have been fathered by men with non-mosaic Klinefelter syndrome [52, 65]. Although all the latter offspring are normal (46,XY or 46,XX), PGD or PND are strongly recommended. Ron-El *et al.* [70] have reduced a 47,XXY embryo implanted after ICSI and embryo transfer techniques in a couple with Klinefelter syndrome. Previous studies in our laboratory have shown that among men with non-mosaic Klinefelter syndrome, those with larger secretory function of Sertoli cells have a higher probability to be positive for testicular foci for spermatogenesis up to the spermatozoon stage [52, 65]. In addition, we have previously shown that within a population of men with non-moaic Klinefelter syndrome, the larger the testicular telomerase profiles are the higher the probability of finding testicular spermatozoa is [52, 65]. In a recent study, Akashi *et al.* [71] reported a male patient with mosaic Klinefelter syndrome whose ejaculated spermatozoa were identified as being haploid by FISH before ICSI leading to the successful pregnancy of his wife and the birth of a healthy baby girl. When semen samples in men with either mosaic or non-mosaic Klinefelter syndrome are negative for spermatozoa, testicular biopsy should be carried out to recover haploid male gametes [52]. Although testicular fine needle aspiration has been used as a diagnostic tool in a general group of non-obstructed azoospermic men [72], its role in men with Klinefelter syndrome has not been evaluated.

A subpopulation of men with non-mosaic Klinefelter syndrome has both 46,XY spermatogonia/primary spermatocytes and 47,XXY spermatogonia/primary spermatocytes in their seminiferous tubuli [52]. A previous study in our laboratory has not indicated sex chromosomal nondisjunctions during the meiotic divisions of the 46,XY spermatogonia/primary spermatocytes in men with non-mosaic Klinefelter syndrome [52]. Subsequently, similar numbers of testicular 23,X round spermatids and 23,Y round spermatids are thought to have been produced from the meiosis of the normal 46,XY spermatogonia/primary spermatocytes in the above men. To explain the larger proportion of 23,X round spermatids compared with the 23,Y round spermatids within a population of men with non-mosaic Klinefelter syndrome, an attractive speculation is that an XX pairing and a univalent Y chromosome type of pairing occurs in the great majority of 47,XXY primary spermatocytes that undergo regular meiosis [52]. In contrast, an XY pairing and a univalent X chromosome type of pairing might occur in a minority of 47, XXY primary spermatocytes that undergo regular meiosis. This speculation can explain a) the increased proportion of the hyperhaploid 24,XY round spermatids compared with the hyperhaploid 24,XX round spermatids within a population of men with non-mosaic Klinefelter syndrome [52], and b) the larger proportion of testicular 23,X round spermatids compared with testicular 23,Y round spermatids within a population of men with Klinefelter syndrome [52, 65]. XX pairing and a univalent Y type of pairing in 47,XXY primary spermatocytes that undergo meiosis is expected to result in increased proportions of 23,X round spermatids/spermatozoa and 24,XY round spermatids/spermatozoa (post-meiosis) in the testicles of

men with Klinefelter syndrome [73]. This is because a regular meiosis in a 47,XXY spermatogonium with an XX pairing and a univalent Y should lead to the production (from one 47,XXY spermatogonium) of two 23,X spermatids and two 24,XY spermatids [73]. Increased proportions of 24,XY round spermatids compared with 24,XX round spermatids within a population of men with Klinefelter syndrome and larger proportion of 23,X round spermatids compared with 23,Y round spermatids have been found, indeed, within a population of men with non-mosaic Klinefelter syndrome in our laboratory [52]. In contrast, if an XY pairing and a univalent X had been present in the majority of 47,XXY primary spermatocytes, regular segregation of the sex chromosomes would have resulted in increased proportions of a) 23,Y round spermatids/spermatozoa (compared with 23,X round spermatids/spermatozoa) and b) 24,XX round spermatids/spermatozoa (compared with 24,XY round spermatids/spermatozoa) in the testicles of men with Klinefelter syndrome [73]. In fact, if a XY sex vesicle is formed and the extra X chromosome is free, regular segregation of the sex chromosomes would produce (from one 47,XXY primary seprmatocyte) two 24,XX spermatids/spermatozoa and two 23,Y spermatids/spermatozoa [73]. It appears that the findings of our previous study demonstrating an increased proportion of 24,XY round spematids compared with 24,XX round spermatids and a larger proportion of 23,X round spermatids compared with 23,Y round spermatids suggest an XX pairing a Y univalent in the majority or in all of the 47,XXY primary spermatocytes that undergo meiosis [52]. Therefore, we might suggest that an XX pairing and a univalent Y chromosome type of pairing occurs in the great majority of 47,XXY primary spermatocytes that undergo meiosis.

3.2.4 47,XYY

Paternal non-disjunction of the sex chromosomes during meiosis is the underlying cause for the presence of an extra Y chromosome. Although some 47,XYY males are fertile and produce normal gametes, a limited subpopulation of 47,XYY males might have severely impaired sperm production [74]. Although the additional Y chromosome might be spontaneously corrected during meiosis, there is a high incidence of disomic spermatozoa with 24, XY or 24,YY constitution [75]. Post-fertilization, the risk of aneuploidy of the sex chromosomes in the derived embryos might be expected to depend on the frequency of the aneuploid spermatozoa in the testicular tissue of the ICSI participants. It appears logical to speculate that the larger the percentage of sperm aneuploidies is within a population of testicular spermatozoa recovered from a testicular biopsy sample of a man with 47,XYY syndrome syndrome, the larger the probability is that the embryologist will aspirate and process for ICSI an aneuploid spermatozoon, with an overall result a larger probability to generate an aneuploid embryo. ICSI procedures are applicable with the reservation of a higher genetic risk for aneuploid embryos. PGD or PND are strongly recommended.

3.2.5 Structural abnormalities of the X chromosome

Structural abnormalities of the X chromosome, such as minor deletions or reciprocal translocations involving the chromosome X and an autosomal chromosome, are occasionally the cause of male infertility [76]. Deletions of a large part of the X chromosome of the female gamete results in the loss of one or more genes and is incompatible with the development of a male embryo after ICSI procedures because males have only one X chromosome and the loss of any genes normally located on the X chromosome is not compensated [14].

The results of an X-autosome translocation vary considerably depending on the sex of the carrier of such an aberration and the position of the translocation break points. Female carriers of a balanced X-autosome translocation generally are phenotypically normal. An important exception is evident in those women in whom the break points in the X chromosome involve the critical region Xq13-q26. These women are always infertile because of gonadal dysgenesis [77]. Reciprocal X-autosome translocations affect male fertility. A possible hypothesis is that reciprocal X-autosome translocations might interfere with X chromosome inactivation [77, 78]. Thus, it has been proposed that X-autosome translocations interfere with the process of X chromosome inactivation resulting in meiotic arrest at the primary spermatocyte stage. A probable hypothesis is the reactivation of the X chromosome, which is supposed to remain transcriptionally silent during spermatogenesis and the overall result, might be azoospermia [79, 80]. Information on the percentage of male germ cells with X-autosomal translocations in the above men is not available in the literature today. ICSI procedures might be applied in these cases (using testicular spermatozoa from testicular foci of advanced spermatogenesis) [14], however, there is a risk of transmission of either balanced or un-

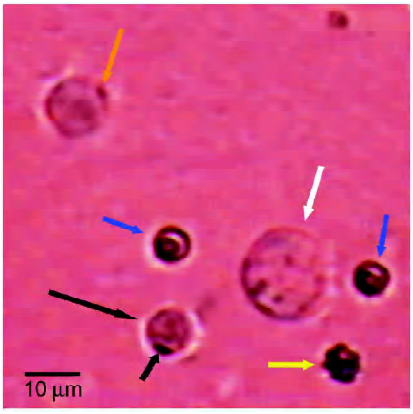

Figure 1. Human male round germ cells in minced testicular tissue recovered from a non-obstructed azoospermic man. Observation via a confocal scanning laser microscope. A primary spermatocyte is indicated by a white arrow. A secondary spermatocyte is indicated by an orange arrow. A round spermatid is indicated by a long black arrow. The acrosomal cap of the round spermatid is indicated by a short black arrow. Blue arrows indicate red blood cells. A white blood cell is indicated by a yellow arrow (For further information on morphometric and morphological differences of male round germ cells under confocal scanning laser microscopy, please see references 16 and 52).

balanced chromosomal translocations in the resulting embryos.

Production of secondary spermatocytes and spermatids (Figure 1) depends on the X chromosome inactivation driven by an X-linked gene acting at the primary spermatocyte stage. The X and Y chromosome form a single mass in the zygotene stage during pairing of the chromosomes at meiosis I [78, 81]. The pyruvate dehydrogensa 1 gene is silent in spermatocytes and spermatids [80]. The inactivation of the X chromosome is essential to prevent the recombination between X and Y chromosomes during meiosis [80]. It is not clear why the X-chromosome should be inactivated during spermatogenesis. Because there is no evidence that products of the X-chromosome are not permissive for spermatogenesis, it might be suggested that inactivation of the X-chromosome might reflect not the metabolic needs of the testicular germ cells but specific meiotic events such as chromosomal pairing and recombination. X-chromosome inactivation might be directed by an X-linked gene during the primary spermatocyte stage [14]. Thus, the existence of translocations involving the chromosome X might have a considerable effect in spermatogenesis, impairing the capacity of primary spermatocytes to enter meiosis [80]. In some cases, spermatogenesis progresses to the stage of elongated spermatids but this process is extremely inefficient and only a small number of spermatozoa is produced [14]. In patients having spermatids or few spermatozoa in testicular biopsies, the probability of chromosomal abnormalities in the embryos derived by ICSI techniques cannot be excluded. PGD might help to avoid transfer of the affected embryos [21, 22].

3.2.6 Chromosomal Inversions

Inversions (peri- and paracentric) of chromosomes 1, 3, 5, 6, 9, 10 and 21 have been described in infertile men [60, 82–84]. The impact of chromosomal inversions in the development of impairment in spermatogenesis in infertile males is variable. Arrest at the primary spermatocyte stage has been described for a particular pericentric inversion on chromosome 1, whereas pericentric inversions of other chromosomes have been associated with azoospermia or oligospermia [60, 82]. The couples should be informed about the probability of spontaneous abortion if pregnancy is achieved via assisted reproduction [85].

3.3 Deletions of the Y chromosome

Abnormalities in the Y chromosome are discussed separately in the present review study because the structural abnormalities of this chromosome have a direct effect on sexual differentiation and fertility. Various structural abnormalities of the Y chromosome are distinguishable at the molecular or the cytogenetic level. Translocations and microdeletions are the most frequently observed structural abnormalities.

The Y chromosome is a complex chromosome that contains heterochromatin located among repeated genes, gene families and palindromic motifs. The non-recombining region of the Y chromosome contains three classes of euchromatic sequences [86], including: i) those that are transposed from the X chromosome during the process of the evolution of the Y chromosome (X transposed); ii) those sequences that are somewhat similar to sequence information from the X (X degenerate); and iii) those sequences that are repeated across the proximal short arm of the Yp and across most of the Yq.

Translocations between the Y chromosome and autosomal chromosomes [87–89] appear to be more common and have a detrimental influence on spermatogenesis. Ooplasmic injections have been applied in such cases after testicular biopsy and recovery of spermatozoa. A risk of developmental delay as the result of chromosomal

imbalance in the offspring has been suggested [90]. It has also been suggested (by a limited number of studies) that dicentric Y chromosomes do not allow spermatogenesis to proceed further than primary spermatocyte stage (early maturation arrest) [91, 92]. Therefore, ICSI procedures cannot be taken into consideration for the therapeutic management of these couples.

In the Yq11.21-23 region, where the azoospermia factor (AZF) is located, there are three loci related to spermatogenesis (AZFa, AZFb and AZFc). These loci have been clustered in tandem and contain putative or candidate genes detrimentally affecting spermatogenesis when they are absent. In a general population of ICSI participants, the frequency of deletions is 2–3%, whereas in infertile males with azoospermia, the frequency of deletions is 6–12% [15, 93]. Deletions are present in 5.8% of men with severe oligozoospermia. Katagiri *et al.* [86] have shown an incidence of Y chromosome microdeletions equal to 16% in a population of azoospermic men and equal to 4% in a population of severe oligospermic men. In the above study, Y chromosome microdeletions were absent when sperm concentration was larger than 5 000 000 spermatozoa/mL. AZFa region harbors the genes *DFFRY*, *USP9Y* and *DBY* that are important for spermatogenesis. However, the most common deletions occur in AZFc and AZFb regions involving the *DAZ* and *RBM* multiple copy genes and other genes such as *CDY1*, *PRY*, *TTY2* and *EIF1AY* expressed solely in the human testis [94, 95]. There is no clear association between the length of the deletion and the semen quality or the testicular histology. The phenotype varies from oligospermia to azoospermia with/or without testicular foci of spermatogenesis up to the spermatozoon stage. All patients with complete deletion of AZFa region or complete deletion of the AZFb region are azoospermic and negative for foci of testicular spermatozoa [96]. A strict genotype-phenotype correlation is observed only for the deletion of the entire AZFa and AZFb regions, which are associated with Sertoli cell-only syndrome and arrest at the primary spermatocyte stage, respectively [97]. On the contrary, the deletion of the most distal AZFc is associated with a heterogenous phenotype in different individuals ranging from the absence of germ cells in the testis to a severe reduction of the sperm number/motility/morphology in the ejaculate [98]. This phenomenon suggests that although spermatogenesis might start without AZFc genes, their presence is crucial to obtain quantitatively and qualitatively normal spermatogenesis. This region contains a total of eight gene families: *BPY2*, *CDY1*, *DAZ*, *TTY3.1*, *TTY4.1*, *TTY17.1*, *CSPG4LY* and *GOLGA2LY*. The classical AZFc deletion, which removes 3.5 Mb between the b2/b4 amplicons, is the most frequent type of deletion. A partial deletion termed gr/gr has been described in infertile men with varying degrees of spermatogenic failure. This deletion removes half of the AZFc region content. Another deletion with the name b2/b3 appears to have no effect on fertility status in association with a certain Y chromosome background commonly present in northern European populations [99]. The first multicopy gene identified in this region (i.e. AZFc) was the *DAZ,* which belongs to a gene family that consists of the two autosomal single copy genes *BOULE* and *DAZL* gene and the Y specific *DAZ*. No mutations for the *DAZL* and *BOULE* genes have been reported so far, except two single nucleotide polymorphisms in the *DAZL* gene [100]. Katagiri *et al.* [86] have reported surgical retrieval of epididymal spermatozoa from a man with partial deletion in AZFb region. His son had an identical deletion. Patients with AZFc deletions are either azoospermic (with or without testicular foci of spermatozoa) or have spermatozoa in the ejaculate. Additional studies confirmed that azoospermic men with complete deletions of either the AZFa or AZFb regions never demonstrated testicular spermatozoa after testicular biopsy procedures [101]. Testicular spermatozoa of men with (either complete or partial) AZFc deletions or partial AZFb deletions are anticipated to successfully fertilize oocytes and generate offspring at the same rate as non-deleted infertile men. In addition, a subpopulation of men with AZFc deletions has a certain degree of oligospermia that requires ICSI. The pathogenetic role of Y-chromosome deletions in male infertility has been questioned by reports describing microdeletions in proven fertile men [97]. However, male fertility is not a synonym for normozoospermia [97]. The pathogenetic significance of Y chromosome microdeletions is spermatogenic failure and not infertility. In rare cases, transmission of an AZFc deletion has been reported via natural conception from a subfertile younger father to an infertile son [102]. Kuhnert *et al.* [103] reported natural transmission of an AZFc Y chromosome microdeletion from a father to his sons. Rolf *et al.* [104] have reported natural transmission of partial AZFb deletion over three generations. Kamische *et al.* [105] reported transmission of a Y-chromosomal deletion involving the *DAZ* and *CDY1* genes from father to son through ICSI. Men with Y chromosomal microdeletions who are

positive for spermatozoa will almost certainly pass the deletion to male offspring generated by ICSI procedures [106–109].

A progressive decrease in testicular spermatogenetic activity over time has been reported in some infertile men with AZFc microdeletions. Thus, testicular or ejaculated spermatozoa cryopreservation might be recommended for the latter men.

Patsalis *et al.* [110] have suggested that there might be a potential risk of chromosomal aneuploidy for male offspring born to fathers with Y-chromosome microdeletions. This risk might include not only 45,X/46,XY offspring but also 45,X offspring. In addition, the above investigators recommended that PGD should be offered when men have ICSI for hypospermatogenesis caused by Y chromosome microdeletions to avoid transfer of 45X embryos.

Data by Sofikitis *et al.* [111] using the testicular androgen-binding protein activity as a marker of Sertoli cell secretory function, does not show a defect in Sertoli cell secretory function in men with Y chromosome microdeletions. We have previously hypothesized that in the future, it might be possible to achieve survival and differentiation of germ cells from non-obstructed azoospermic men (without genetically based causes of azoospermia) into the seminiferous tubuli of recipient human individuals (with AZFc microdeletions) who are negative for testicular spermatozoa [111]. The attractive hypothesis is that the recipient human Sertoli cells and the intratubular biochemical environment will support the donor human germ cells to differentiate. The above hypothesis is supported by studies in animals showing that the intratubular environment from infertile recipients can support the differentiation of donor germ cells from infertile subjects [111]. Some azoospermic couples who have considered using donor spermatozoa might be attracted by the idea of achieving pregnancy via sexual intercourse, even if the male partner ejaculates donor rather than his own spermatozoa into the reproductive tract of the female partner.

Even in Sertoli cell-only syndrome testicular histology (in sections stained by hematoxylin–eosin) from subpopulations of men with Y chromosome deletions, there is a probability that spermatids or spermatozoa can be identified in seminiferous tubules. It has been estimated that spermatozoa (either in the ejaculate or the testicular tissue) can be found in approximately 50% of azoospermic men with microdeletions in the AZFc region of the Y chromosome.

Because AZF microdeletions are transmitted from the father to the male offspring, genetic evaluation for Y chromosomal deletions is recommended in non-obstructed azoospermic men or severely oligoasthenospermic individuals. In addition, large microdeletions of the tip of the Yq chromosome might cause chromosomal instability and might be responsible for chromosomal rearrangements or even Y chromosome loss. Issues, such as testicular mosaicism of Y chromosomal deletions, expansion of the Y chromosome deletions in the offspring, lower fertilization rates post-ICSI and familial basis of Y deletions represent the target of several investigations but the results are still inconclusive [112].

Because ICSI techniques are commonly used in patients with Y chromosome microdeletions, thus posing a considerable risk of passing the deletion on to the offspring [113], proper genetic counseling followed by detailed family history and specific molecular or cytogenetic assays are recommended.

3.4 Evaluating chromosomal abnormalities in the gametes of males participating in ICSI programs

Males with severe oligospermia, obstructive azoospermia or non-obstructive azoospermia with testicular foci of spermatogenesis up to the spermatozoon stage represent the majority of candidates for ICSI. Several studies have been focused on the chromosomal constitution of spermatozoa of fertile and infertile men using FISH procedures [114, 115]. Although there is a remarkable variability in the methodology of these studies (i.e. regarding the number of FISH probes used or the selection of the patients), the findings of all these investigations indicate chromosomal abnormalities in the spermatozoa of ICSI participants (either oligospermic or azoospermic with testicular foci of spermatozoa). These abnormalities are mainly diploidy, autosomal disomy and nullisomy or aneuploidies of the sex chromosomes [114].

Spermatozoa recovered from non-obstructed azoospermic men (with testicular foci of advanced spermatogenesis) do have a higher incidence of chromosomal aneuploidy patterns among which sex chromosomal aneuploidy is the most common [48–50]. Mateizel *et al.* [49] have shown that the frequency of aneuploidy for chromosome 18 was higher in a group of azoospermic men with spermatogenic failure than in a group of azoospermic men with normal spermatogenesis. Huang *et al.* [116] reported an increase in the frequency of sex

chromosomal abnormalities in testicular spermatozoa of non-obstructed azoospermic men. In another study, Viville *et al.* [117] showed that in obstructed azoospermic men (with or without *CFTR* mutation), there have not been significant differences in the chromosomal constitution of testicular spermatozoa compared with normal semen samples.

In subpopulations of infertile men with primary testicular damage as a result of non-mosaic Klinefelter syndrome, there is a significant increase in the proportion of spermatids/spermatozoa with chromosomal aneuploidies. However, the majority of spermatids/spermatozoa (if they are present in testicular biopsy material) in the latter men have the normal haploid constitution of the chromosomes [52].

In a recent study, there was no significant difference in the incidence of aneuploid embryos between couples with obstructive azoospermia and couples with non-obstructive azoospermia [118]. Nevertheless, in both groups of the above study, the percentage of aneuploid embryos was relatively high (53–60%), indicating the potential risks of the employment of testicular spermatozoa for ICSI treatment. These patients would require a systematic monitoring of spontaneous abortions or implantation failures. In addition, the ICSI treatment should be coupled with PGD or PND for early identification of chromosomally abnormal embryos.

3.5 Mitochondrial aberrations of spermatozoa and ICSI

The presence of mitochondrial abnormalities in spermatozoa has been proposed to be a cause of male infertility; mitochondrial abnormalities have been associated with asthenospermia [119]. Low sperm motility might be associated with deformations of the mitochondrial sheath containing functional mitochondria. The combination of fluorescence microscopy and flow cytometry with electron microscopic investigations is a sensitive, precise and comprehensive examination which helps discover sperm mitochondrial abnormalities that cause asthenozoospermia [119]. Successful ICSI in a case of severe asthenozoospermia that is the result of non-specific axonemal alterations and abnormal or absent mitochondrial sheaths has been reported [120]. The application of ICSI procedures in such patients implies introduction of the whole spermatozoon into the ooplasm and raises the question of potential risks for the derived embryo attributable to the transmission of paternally inherited abnormal mitochondrial DNA into the ooplasm of the oocyte. One study has evaluated the risk of heteroplasmy (mosaicism of paternal and maternal mitochondria) in 27 newborns born after ICSI procedures. Heteroplasmy was shown in a frequency of 0.1–1.5% (which is considered to be normal and so far does not appear to be alarming) [121].

3.6 Reported congenital abnormalities and neurophychiatric development in children born after ICSI

Given the concerns from what has been already discussed in the present communication, it is important to analyze the outcome of some prominent ICSI programs and that of the ESHRE ICSI Task Force. The reported results from prenatal diagnoses in pregnancies achieved by ICSI techniques, indeed, showed a tendency for a higher frequency of aneuploidy of the sex chromosomes when compared with naturally conceived children [51, 67, 122–125].

Prospective data from Brussels have addressed the genetic consequences of the use of ICSI techniques in two consecutive studies evaluating 1 987 and 2 889 infants born after ICSI trials [51, 123, 126]. The outcome of ICSI techniques concerning the karyotypes, the existence of congenital abnormalities and the somatic or mental development was recorded. In total, 1.66% *de novo* chromosomal abnormalities of the autosomes and the sex chromosomes in equal proportions were found with an additional 0.92% of inherited structural chromosomal abnormalities (eight balanced and one inbalanced) from the father. Major congenital abnormalities were shown in a percentage equal to 2.3% of the total number of the children delivered. Fetal deaths were observed in a frequency of 1.1% after the 20th week of pregnancy. The second study compared the data between ICSI (n = 2 889) and IVF infants (n = 2 995) born in the periods 1991–1999 and 1983–1999, respectively. Using the same criteria and follow-up period, the ICSI group did not show an increased risk for major malformations or complications in comparison with the IVF group [51, 123]. Other studies comparing IVF with ICSI or ICSI-children versus children in a general population did not show any excess risk for ICSI children with the exception of the appearance of hypospadias (compared with the lower frequency of hypospadias in the general population), probably related to the paternal subfertility or to the hormones the mother received during the beginning of pregnancy [127, 128].

Although there is a subpopulation of non-obstructed azoospermic men where the etiology of azoospermia has

a genetic basis [115, 129], there is no evidence for significantly higher risks for congenital abnormalities in infants born after ICSI procedures with epididymal or testicular spermatozoa (compared with naturally conceived offspring) [123,126,130–132]. Furthermore, replacement of frozen/thawed embryos generated by ICSI was not accompanied by a significantly higher incidence of congenital abnormalities in the newborns. In another report from Sweden, data concerning 1 139 children born after ICSI procedures were reviewed [127]. A considerable frequency of 7.6% of congenital abnormalities was observed and less than half of these abnormalities were minor. In that study, the relative risk of ICSI children to show a congenital abnormality was 1.75% but when this risk was corrected for twins or triplets it dropped to 1.19%. The only congenital abnormality with the alarmingly high relative risk of 3% was hypospadias. In other studies, the somatic development of children delivered post-ICSI techniques has been shown to be normal, whereas evaluation of mental development and fertility of the offspring need longer and more pervasive studies [125].

In order to reduce the potential risks of ICSI procedures for the fetus/newborn, cytogenetic analysis in haploid male gametes (recovered either from ejaculates or testicular biopsy samples) might be recommended before ICSI procedures are carried out in men with low sperm counts or in azoospermic men. Counseling and PGD or PND are of paramount importance.

Mental and neuropsychiatric development in children delivered after ICSI techniques have been addressed in two successive reports. Both reports lacked a conclusion that supported a major abnormality in ICSI children or a significant deviation from the normally naturally conceived population apart from a) the findings concerning the presence of hypospadias [127, 128], or b) the complications related to multiple gestations [125, 130]. In a recent study [133], it was shown that singleton ICSI and IVF 5-year-olds are more likely to need health care resources than naturally conceived children. In addition, in that study, it was found that ICSI children presented with more major congenital malformations and both ICSI and IVF children were more likely to need health care resources than naturally conceived children. In another study [134], apart from a few interaction effects between mode of conception and and demographic variables, no differences were found when ICSI, IVF and naturally conceived scores on the WPPST-R and MSCA Motor Scale were compared. Nevertheless, the aforementioned interaction effects could indicate that demographic variables, such as maternal age at the time of birth and maternal educational level, play different roles in the cognitive development of IVF and ICSI children compared with naturally conceived children.

3.7 Risks and consequences of chromosomal abnormalities in ICSI children

Pooled data from a survey of results of international trials point towards a slightly elevated frequency of sex chromosome abnormalities in ICSI children (compared to the general population). Overall ICSI results (in terms of percentages of chromosomal abnormalities in fetus karyotypes) do not appear to be significantly different compared with those of IVF [51, 123].

In general, the outcomes of IVF and ICSI trials are similar [51, 123]. The incidence of *de novo* numerical sex chromosomal anomalies in ICSI children ranges from 0.23–0.83%, which appears to be slightly higher compared with the 0.19% reported in the literature for the general population. *De novo* numerical autosomal chromosome abnormalities in ICSI children range from 0.5–1.4%. The latter percentage is 3 to 10 times higher than that in the general population (0.14%). Concerning the percentage of *de novo* structural chromosomal rearrangements, there is a significant (3 to 4 times) increase from 0.07% in the general population to 0.23–0.27% in ICSI children [51, 123, 130–132]. In children born after ICSI techniques are carried out, most of these rearrangements are reciprocal and therefore do not have phenotypic consequences in the carriers. Nevertheless, these rearrangements might be responsible for the generation of abnormal male gametes by meiotic malsegregation leading to chromosomally abnormal offspring postfertilization [130, 131, 135]. Male carriers of numerical or structural chromosomal abnormalities might father offspring with abnormal and meiotically incompetent cell lines at the age of reproduction after ICSI techniques [75, 136]. There are reports of low pregnancy rates in couples with primary testicular damage (after assisted reproductive technology), probably as a result of a generalized tendency of chromosomal nondisjunction [16]. In addition, ICSI with testicular spermatozoa has been proven to be less successful in men with nonobstructive azoospermia compared with men with obstructive azoospermia [137]. The increased chromosomal aneuploidy in testicular spermatozoa from men

with non-obstructive azoospermia might explain the lower fertilization and pregnancy rates observed in that study [137]. Consistently, Aytoz *et al.* [138] have shown, after ICSI techniques, that within a group of couples that underwent ICSI techniques with ejaculated spermatozoa, the rate of intrauterine death was higher in a severely defective sperm subgroup than in better quality sperm subgroups.

The higher percentage of chromosomal abnormalities in ICSI-children compared with the general population is probably related to the parental chromosomal abnormalities (mainly in the father) [51, 123, 125, 139]. This increase in chromosomal aberrations after ICSI procedures might also result from the selection of spermatozoa, which would otherwise be unable to naturally fertilize an oocyte [117, 126, 130–132]. In a study comprising a large number of prenatal tests carried out on pregnancies that were the result of ICSI techniques, a sixfold increase in sex chromosomal aberrations and a twofold increase in autosomal chromosomal aberrations was reported [130–132]. In additional studies, a significantly higher rate of *de novo* chromosomal abnormalities in amniocentesis was observed in ICSI offspring relating mainly to a higher number of sex chromosomal abnormalies and partly to a higher number of autosomal structural abnormalities [51, 123]. This finding was related to sperm concentration and motility of the ICSI participants. The significantly higher rate of observed inherited abnormalities in the ICSI prenatal tests compared with prenatal tests in the general population was related to a higher rate of constitutional chromosomal anomalies, mainly in the fathers [51, 123]. In addition, post-ICSI increases in sex chromosomal aberrations might be a result of non-random chromosomal positioning and defects in male gamete nuclear decondensation after the ooplasmic injections of non-acrosomally reacted spermatozoa [140].

In a recent study, Bonduelle *et al.* [141] carried out a medical follow-up study of 5-year-old ICSI children and compared the findings with a population of children born after natural conception. Growth assessed as stature at follow-up was similar in the two groups despite a higher rate of preterm birth and low birthweight in the ICSI children. Common diseases and chronic illnesses occurred at similar rates in both groups. More ICSI children underwent surgical intervention and required other therapies.

3.8 Exogenous DNA and HIV transmission risks from use of ICSI procedures

HIV infection or gamete contamination by exogenous DNA do not belong to genetic or epigenetic risks. However, they represent an issue of major concern in ICSI procedures. Transmission of viral elements, especially retroviruses which have the ability to integrate and transpose in the human genome, might represent a considerable risk.

In more than 1 000 insemination cycles, artificial insemination involving HIV-seropositive males did not appear to be accompanied by transmitting the virus and 250 successful pregnancies were reported [142]. In addition, ICSI procedures using HIV-positive frozen semen samples have resulted in the generation of embryos free from the HIV virus [143–145].

Although *in vitro* preparation of semen samples by washing and gradient separation before the ICSI techniques are carried out appear to block the transmission of viruses, there is a potential risk of exogenous DNA transmission to the embryo. This hypothetical risk is based on studies in Rhesus Macaque monkeys showing that exogenous DNA bound to spermatozoa can be transferred by ICSI to the embryos and, thus, it might confer some new genetically transmitted characteristics [146]. Consequently, hypothetical binding of exogenous DNA on human spermatozoa processed for ICSI might alter the germline genetic constitution of the human offspring. A cautious manipulation of semen samples and use of strict safety procedures to exclude sources of DNA contamination during sperm manipulation are recommended in ICSI laboratories. For this reason in assisted reproduction programs, PGD procedures (using PCR) should be carried out in isolated facilities and thermal cyclers with UV decontaminators (that are separated from the ICSI laboratories) to eliminate the risk for transmission of exogenous DNA during ICSI procedures.

3.9 Genetic and epigenetic risks from the intraooplasmic injection of in vivo produced spermatids

The introduction of the intracytoplasmic injection of spermatids or secondary spermatocytes as an alternative mode of therapy of non-obstructed azoospermic men who are negative for testicular foci of spermatozoa raised several concerns for probable genetic risks associated with the immaturity of the early haploid male gamete [16, 147–149]. The genetic risks of ooplasmic injections of human round spermatids might be a) inherent to the popu-

lation of men this procedure is applied to (i.e. transferring chromosomal abnormalities/gene deletions to the offspring); or b) inherent to the procedure per se. The latter risks might be associated with abnormalities in the a) centrosomal components of the early haploid male gamete (defects in the reproducing element of the centrosome might cause zygotic spindle abnormalities after ooplasmic injections of spermatids) [16]; b) nuclear proteins; or c) spermatid oocyte-activating factor (i.e. the male gamete substance that triggers the cascade of ooplasmic events that result in the resumption of meiosis of the female gamete post-ooplasmic injections) [150–152]. In addition, it is particularly tempting to investigate in humans whether the process of genomic imprinting has been completed at the round spermatid stage [153]. This hypothesis has been evaluated in experimental mammals (*Mus musculus*) reproduced through ooplasmic injections of spermatids. The results have shown that there is no difference in the genomic imprinting establishment process between normally reproduced animals and animals generated from spermatids [154]. Studies in animals suggest that mouse genomic imprinting (Figure 2) is complete at/prior to the primary spermatocyte stage [155, 156]. The results of studies in our laboratory indicate that the genomic imprinting process in the rabbit and the rat has been completed at/before the round spermatid stage [157, 158]. It should be emphasized that even if genomic imprinting has not been completed at the round spermatid stage, the genomic imprinting process might be completed postfertilization (during early embryonic development) [16, 148, 159]. Regarding genomic imprinting abnormalities-related diseases after ooplasmic injections of spermatids, there is no evidence today of imprinting defects in the offspring [16]. However, because methylation of some imprinted genes is supposed to occur during spermatogenesis or during early embryonic development [16, 159, 160], additional studies are necessary in order to evaluate the methylation status of genes in children delivered after ooplasmic injections of spermatids.

Data on congenital and chromosomal abnormalities in children born after intracytoplasmic injection of spermatids are not sufficient to draw safe conclusions. Nevertheless, one report is alarming and indicates major abnormalities in children delivered after ooplasmic injections of spermatids [161]. Other studies on larger series did not detect an increased incidence of malformations after ooplasmic injections of spermatids [162–164]. However, considering that the number of human pregnancies achieved after ooplasmic injections of spermatids is limited, no definite conclusions can be drawn on the safety of ooplasmic injections of early haploid male gametes. Ejaculated round spermatids in the rat appear to have a lower reproductive capacity than testicular round spermatids [158]. This might be attributable to morphological defects in the ejaculated round spermatids (Figure 1).

Another alteration the male gamete undergoes during

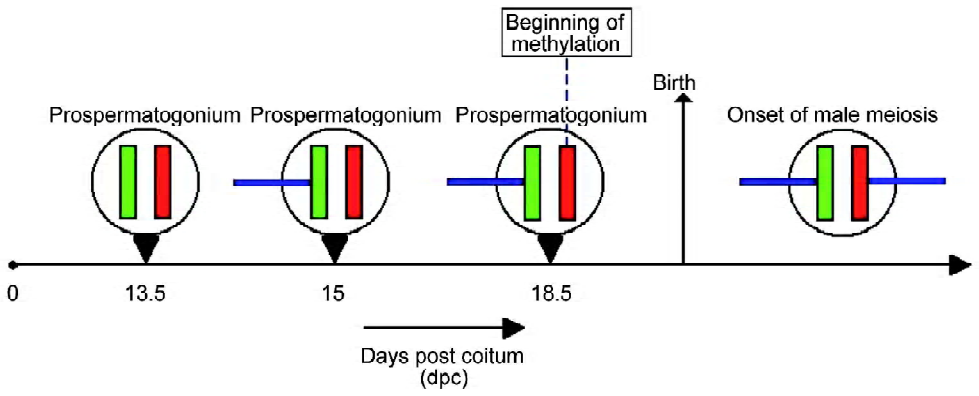

Figure 2. Methylation patterns of a maternally expressed gene during male germ cell development (see reference 226). 13.5 dpc: Prospermatogonia demonstrate an absence of methylation on both the maternal (red color) and the paternal (green color) alleles; 15 dpc: The paternal allele scquires hypermethylation, in contrast, the meternal allele remains unmethylated. The blue line indicates methylation of the respective allele; 18.5 dpc: Maternal alleles begin to acquire methylation just prior to birth; After birth: Both parental alleles are fully methylated.

spermiogenesis *in vivo* is the replacement of the nuclear histones (low disulphide bond proteins) by protamines (high disulphide bond proteins). Histones are protecting the early haploid male gamete DNA (within the cytoplasm of the oocyte) after ooplasmic injections. The presence of low disulphide bond proteins around the round spermatid DNA after round spermatid nuclei injections (ROSNI) or after round spermatid injections (ROSI) has been considered to be a factor responsible for the low outcome of these techniques. In contrast, post-ICSI, protamines are protecting the spermatozoal DNA within the ooplasm. In the case of ooplasmic injections of early spermatids, the survival of the injected spermatid DNA within the ooplasm might be detrimentally affected by the absence of protamines [16].

Post-ICSI, the resumption of meiosis of the female gamete depends on/is facilited by the presence of the oocyte-activating factor present in mouse, rabbit and human spermatozoa [150–152, 157, 165]. Defects in the expression/functionality of the oocyte activating factor after ooplasmic injections of early spermatids might account for their lower fertilization and pregnancy rates (comparatively with those after ICSI procedures). Although Kimura and Yanagimachi [150–152] and Sofikitis *et al.* [158] have shown that the oocyte-activating factor has not been expressed in mouse and rat round spermatids, respectively, several studies suggest that the oocyte activating factor has been expressed in the round spermatid in the human or the rabbit [16, 166–168].

Healthy offspring have been delivered after predecondensed sperm or even spermatid head injections into the female pronuclei of preactivated rat oocytes [169]. The latter study might suggest that novel methods of assisted syngamy have been developed and such a technology in the future might have a role in cases of human ICSI failure as a result of lack of development of male pronucleus (post-ICSI) or inability of the male and female pronuclei to fuse.

3.10 Genetic risks after assisted reproduction techniques using in vitro generated male haploid germ cells

Although induction of human meiosis and spermiogenesis in an *in vitro* culture system represents an attractive alternative solution for the therapeutic management of men who are positive for spermatogonia/spermatocytes but negative for haploid cells in their testes, the application of diploid germ cell *in vitro* culture technique might be limited by ethical considerations or safety-related factors. For instance, application of ooplasmic injections of human haploid cells generated in *in vitro* culture systems containing xenogeneic Sertoli cells [111, 164, 170] is susceptible to ethical considerations and risks regarding contamination of the human germ cells by animal viruses or animal molecules. Similarly, a major drawback for application of ooplasmic injections of haploid male gametes derived in *in vitro* co-culture systems of human diploid germ cells with supporting animal feeder somatic cells, such as Vero or STO cells, concerns the risks of transmitting infectious agents to the human germ cells [164]. The growth phase of Vero cells is usually achieved in the presence of newborn calf serum, which still poses the risk of virus or animal molecule transmission to the cultured human cells [171]. In addition, performance of assisted reproduction procedures using immature haploid germ cells derived or cultured *in vitro* is susceptible to genetic and epigenetic risks.

Kimura *et al.* [156] attempted to induce both male meiotic divisions *in vitro* within the cytoplasm of oocytes injected with primary spermatocytes. They observed a high frequency of abnormalities in male meiotic chromosomal behavior when mouse primary spermatocytes were injected into the ooplasm of MII oocytes. It seems that most primary spermatocytes have not acquired the competence for normal chromosomal segregation within the ooplasm and/or that the ooplasm does not provide adequate factors required to segregate the spermatocyte chromosomes that are still synapsed.

In humans, Sousa *et al.* [163] reported that most of the embryos, produced after ooplasmic injections of spermatids that had been generated *in vitro*, showed sex chromosomal abnormalities. The high abnormal genetic constitution of the derived human embryos might have been to the result of: a) a deficient male meiotic process *in vitro*; or b) the immature DNA-status of the *in vitro* generated haploid cells. Tesarik *et al.* [166–168] showed a very rapid progression of meiosis and/or spermiogenesis during *in vitro* culture of human primary spermatocytes and/or round spermatids, respectively. It is possible that the action of multiple checking mechanisms, which control/coordinate the male gamete morphogenetic and molecular transformations during spermatogenesis *in vivo*, cannot be completed (totally or partially) during the *in vitro* culture of spermatogenic cells. The overall result might be a high percentage of abnormal products of meiosis and/or spermiogenesis in *in vitro* culture systems. This is consistent with the fact that an increase in DNA deg-

radation of round spermatids during *in vitro* culture has been observed [168]. Thus, it appears that the clinical employment of ooplasmic injections of *in vitro* derived haploid germ cells might be associated with genetic risks attributable to the completion of meiosis or a part of the spermiogenetic process under *in vitro* conditions.

3.11 Epigenetic risks related to assisted reproduction techniques

Genomic imprinting abnormalities might also have an impact on assisted reproductive techniques in which spermatozoa are injected into oocytes. Only one copy (paternal or maternal) of an imprinted gene is active (Figure 2) and the other, the inactive one, is epigenetically "marked" by histone modification, cytosine methylation or both [172]. It has been shown that the mammalian primordial male germ cell genome undergoes extensive epigenetic reprogramming, namely demethylation (i.e. erasure of the previous imprint), to assure later at the gamete stage the establishment/consolidation of the maternal or the paternal imprint. Epigenetic marks originating from the parental cells must be erased at an early stage. Both copies of an imprinted gene are marked *de novo* during spermatogenesis according to the sex they originate from. After the consolidation of the new imprint, one of the two copies remains silent. After fertilization, imprinted genes maintain their methylation status and they escape the reprogramming (demethylation and remethylation) process. In contrast, it has been suggested that the methylation process in the unmethylated genes continues postfertilization [16, 159].

Alarming reports have recently raised concerns regarding the increased incidence of children with rare imprinting disorders, namely Angelman and Beckwith-Wiedemann syndromes (BWS), among children conceived by assisted reproduction. Two independent groups from USA and Europe have reported cases of Angelman syndrome conceived by ICSI techniques with sporadic imprinting defects [4, 173]. The mosaic methylation pattern detected in one of the patients and the absence of imprinting center mutations might support the evidence of a postzygotic epigenetic defect [174]. Furthermore, the analysis of chromosome 15 methylation pattern in a limited number of ICSI children (n = 92) did not show methylation abnormities [175].

BWS, a rare genetic condition (1/15 000), has also been reported to show a more frequent incidence among ICSI children [176–178]. It is worth mentioning that the study of DeBaun *et al.* [176] was prospective and identified an incidence of BWS equal to 4.6% among the children delivered after assisted reproduction techniques versus the background rate of 0.8% in the USA. Imprinting mutations of two BWS related genes were found in 5/6 children with BWS syndrome born after assisted reproduction [176]. The identification of Angelman syndrome and BWS syndrome among ICSI children indicate the need for additional prospective studies.

In the above mentioned reports concerning BWS and AS patients, the epigenetic defect was found in the maternal allele suggesting that the abnormality might not be related to the spermatozoa used for ICSI. Whether or not imprinting defects are related to the culture conditions, media used to the hyperstimulation protocols or other epigenetic defects related to the development of male infertility but yet unidentified remains to be elucidated [179].

As we have recently mentioned [164], achievement of the induction of meiosis of male diploid germ cells and partial completion of spermiogenesis under *in vitro* conditions might not be accompanied by all the epigenetic modifications the male gamete normally undergoes during the respective stages of spermatogenesis under *in vivo* conditions. Additional epigenetic modifications, such as DNA methylation, genomic imprinting, RNA silencing and modification of histones, are important for the *in vitro* derived haploid male gamete nucleus in order to survive within the ooplasm and trigger the cascade of events that lead to normal embryonic development [174]. Acceleration of the cytoplasmic and nuclear maturation events that occur *in vitro* in cultured male germ cells might cause a disturbance of epigenetic reprogramming resulting in aberrant gene expression, abnormal phenotypic characteristics, and defects in the male gamete capacity to fertilize the oocyte and induce normal embryonic development.

As we have emphasized in the above paragraphs, an important issue is whether genomic imprinting establishment has been completed in immature diploid or haploid male gametes. Kerjean *et al.* [180] showed that the methylation patterns of *H19* and *MEST/PEG1* genes are established as early as spermatogonial differentiation in humans. In contrast, Ariel *et al.* [181] showed that spermatogenesis-specific genes undergo late epigenetic reprogramming at the level of epididymis. Hajkova *et al.* [182] have shown that mouse PGC exhibit dynamic changes in epigenetic modifications between days 10.5 and 12.5 post coitum. PGC acquire genome-wide *de*

novo methylation during early development and migration into the genital ridge. However, following their entry into the genital ridge there is a rapid erasure of DNA methylation of regions within imprinted and non-imprinted loci. Thus, there is an active demethylation process initiated upon the entry of PGC into the gonadal anlagen. The time of reprogramming of PGC is of paramount importance, because it ensures that germ cells in the males acquire a certain epigenetic state prior to the differentiation of the definitive male germ cells in which new parental imprints are then established [182]. Defects in the epigenetic reprogramming in any cultured (*in vitro*) immature diploid germ cell population might result in the inheritance of epimutations in the haploid cells generated from the culture of the immature germ cells. The fact that DNA methyltransferase is present in spermatids might be an argument against the hypothesis that genomic imprinting is complete at the round spermatid stage. Another hypothesis is that even if the genomic imprinting has not been completed at the round spermatid stage, the male gamete genomic imprinting might be completed after the transfer of immature haploid spermatogenic cells within the ooplasm [150, 151], or even during the early embryonic development [16, 159]. This hypothesis is supported by the fact that waves of DNA methylation have been shown during early embryonic development, the blastocyst stage and the time of implantation [159]. There are several studies providing evidence for the presence of activity of the DNA methyltransferase during early embryonic development [16, 159]. In addition, from a limited data available, it appears that the imprint establishment has been completed in humans by the time the spermatid stage is reached [154, 174]. Although most of the above studies tend to suggest that the genomic imprinting process in humans has been completed prior to the spermatid stage *in vivo*, it is unknown whether the rapidly proceeding meiosis and early spermiogenesis occurring under conditions present in *in vitro* culture systems allow the completion of genomic imprinting process within these relatively short periods. This is a question of clinical importance because abnormalities in the completion of genomic imprinting during *in vitro* gametogenesis may be manifested (postfertilization) as tumor susceptibility or/and tumorgenesis.

There are epigenetic differences (Figure 2) between the parental genomes during the evolution of genomic imprinting in mammals. These epigenetic differences between the parental genomes are enhanced in the zygote by means of DNA demethylation of the paternal genome soon after fertilization, whereas the maternal genome shown *de novo* methylation [183]. Such opposite effects on the parental genomes within the same oocyte cytoplasm might be achieved by the differential binding of stored cytoplasmic factors to the parental genomes [184]. Arney *et al.* [184] have shown a preferential interaction of HP1beta protein with the maternal genome immediately after sperm entrance into the mouse oocyte. Paternal genome binding of HP1beta is only detected at the pronuclear stage. Considering that it is unknown whether oocytes at the two pronuclei plus second polar body stage that have been fertilized by *in vitro*-generated human haploid male gametes (generated from the culture of human primary spermatocytes of men with primary testicular damage) [164, 185] show normal paternal genome-binding of HP1beta, it appears that the probability that ooplasmic injections of *in vitro*-derived early haploid male gametes being accompanied by epigenetic risks related to a lack of or abnormalities in the pattern of binding of HP1beta protein with the paternal genome cannot be ruled out.

It should be emphasized that in the theoretical case of injecting an imprint-free immature male germ cell nucleus into an oocyte, fertilization might be anticipated but it should lead to embryonic lethality. Transplantation of imprint-free PGC nuclei into oocytes has resulted in embryonic lethality, partly as a result of abnormal extraembryonic tissues resulting from the inappropriate silence or activation of imprinted genes [186]. So far, imprinting during passage through at least some stages of spermatogenesis is essential because a male genome devoid of imprints cannot acquire all of them within a mature oocyte [186].

In addition to the above described epigenetic factors, defects in other epigenetic factors might contribute to the abnormal characteristics of embryos produced by ICSI procedures [16, 163]. Abnormalities/defects in the expression of oocyte-activating factor in spermatozoa (see above paragraphs) might result in defects in the capacity of the male gamete (after its entrance into the ooplasm) to activate the cascade of ooplasmic events that result in resumption of meiosis of the female gamete, fertilization and normal embryonic development. Furthermore, deficiency in the functionality of the reproducing element of the centrosome [187], or the presence of an abnormal number of centrioles in spermatozoa, might cause aberrant spindle formation after ICSI techniques resulting in ab-

normal embryonic development. Defects in the paternally inherited centrosomic components are known to represent a reason for ICSI failure (to induce appropriate embryonic development) after the entrance of the male gamete into the ooplasm [187]. In addition, Luetjens *et al.* [188] showed that abnormalities in the male gamete nucleus condensation could retard the sperm X chromosome decondensation resulting in embryonic aneuploidy through zygotic mitotic errors. Thus, we cannot rule out the probability that a) abnormalities in the nuclear condensation status of spermatozoa or b) abnormalities in the capacity of spermatozoa to decondense at an appropriate chronological order within the ooplasm (post-ICSI) might cause chromosomal abnormalities in the embryos.

3.12 Risks concerning transgenerational transmission of an acquired genetic or epigenetic defect

Apart from the genetic and epigenetic risks already described (which are substantiated by the abnormalities found in the offspring of patients treated with assisted reproduction procedures), there are also other less obvious risks. These risks may be called "risks concerning transgenerational transmission of an acquired genetic or epigenetic defect" and are mainly of two types: a) those resulting from the action of aggressive cancer treatment on gametes with overall genetic and teratogenetic consequences; and b) those that are anticipated in the future generations of ICSI offspring and concern defects in tumor suppression genes and increased susceptibility of ICSI-children for tumor development. It has been reported that there is a higher incidence of retinoblastoma among children conceived after assisted reproduction technology [189].

Although male gamete DNA damage might be inevitable during cancer treatment (i.e. chemotherapy, radiation) there is no evidence today of increased frequency of genetic defects or congenital malformations among children (either naturally conceived or conceived after ICSI techniques) fathered by men who have undergone chemotherapy. Nevertheless, DNA breaks are induced by reactive oxygen species produced either by aggressive cancer therapy or during sperm preparation techniques for carrying out assisted reproductive technology or by microorganisms contaminating the lower genitourinary tract [190, 191]. Furthermore, DNA denaturation and fragmentation are strongly correlated with a decreased reproductive potential [192]. Fertilization of an oocyte (using ICSI techniques) with a DNA-damaged spermatozoon might be accompanied by a risk for a genetic disease in the offspring.

3.13 Risks related to mutations of genes regulating the spermiogenesis process

The process of spermiogenesis is very sensitive to genetic alterations. Alterations in the expression of molecular agents in the testicular tissue as a result of defects in gene expression (null mutations, gene overexpression, exogenous gene expression and gene misexpression) could lead to a deficiency in the completion of specific steps of spermiogenesis. These defects in gene expression might result in spermatogenic arrest at the round spermatid stage or in the production of few spermatozoa with anatomical or functional defects. Although men with arrest at the round spermatid stage or oligozoospermic men with anatomically or functionally deficient spermatozoa do not have reproductive potential under *in vivo* conditions, ICSI procedures or ooplasmic injections of spermatids might offer the latter men the probability to father their own children. However, the bypassing via assisted reproductive technology of biological barriers related to defects in the spermiogenesis process is accompanied by risks for transferring gene defects to the male assisted reproductive technology offspring. The expression of phenotypic characteristics (i.e. defects in spermiogenesis) in the offspring (generated by assisted reproductive technology) depends on the chromosomal location of the respective mutated gene, the pattern of the inheritance of this gene and/or the presence of any type of mutations/alterations in the expression of this gene in the mother's genotype. To emphasize the importance of mutations in genes regulating spermiogenesis, we are describing below some genes playing a role in the spermiogenesis process.

Histone replacement by transition proteins (TP) and protamines during spermiogenesis might be affected by disruption of the *Tarbp2* gene, resulting in infertility and oligospermia [193]. A partial or complete failure to synthesize the protamines results in delayed replacement of TP and the spermatids show abnormal nuclear morphogenesis, developmental arrest and degeneration [193]. Premature translation of *Prm1* (pre-existing protamine 1) mRNA cause precocious condensation of spermatid nuclear DNA and abnormal head morphogenesis [194]. Successful interaction of mature protamine-2 with chromatin is required for displacement of TP2 [195]. Step-15 sperma-

tids in Camk4$^{-/-}$ mice show a loss of protamine-2. These animals are characterized by prolonged retention of TP2. Mice lacking the major TP1 have been obtained after targeted deletion of the Tnp1 gene. Tnp1$^{-/-}$ mice show a normal sperm production quantitatively, but only 23% of the spermatozoa show any movement and most of these spermatozoa do not show forward progression [195,196]. In these animals, sperm heads with a blunted or bent tip are seen in 16% of epididymal spermatozoa, possibly generated by the abnormal chromatin condensation that could reduce the rigidity of the fine apex of the spermatozoon [195,196]. Tnp1 contains a cAMP-responsive element (CRE) that serves as a binding site for the CRE modulator (CREM). CREM is involved in the regulation of Tnp1 gene expression and human CREM protein is synthesized in steps 1–3 round spermatids. This might explain why a reduction in Crem expression and a lack of both CREM and TP1 have been shown in human spermatids arrested at step 3 [197]. Mice with deletion in Crem presented a spermatogenesis arrest at the round spermatid step [198].

Deficiencies in intratesticular molecular factors as a result of genetic defects affect the organization and reorganization of the cytoskeleton during spermiogenesis. Thus, homozygous c-ros knockout mice are sterile and the epididymal spermatozoa have bent tails and compromised flagellar vigour within the uterus [199]. Testicular haploid expression gene (THEG) is expressed in round and elongated spermatids. The molecular products of this gene appear to play a role in the spermiogenesis because abnormal or absent flagella in mice with THEG dysruption have been shown and might be to the result of an impairment of the assembly of cytoskeletal proteins such as the tubulins [200]. A specific block in spermiogenesis was observed in homozygous JunD$^{-/-}$ mice. A lack of molecular factors encoded by the latter gene results in an absence of flagella in spermatids in the lumen of the seminiferous tubules [201, 202]. The absence of JunD led to sperm flagellar growth impairment. Additional defects in sperm nuclear and cytoskeletal morphology, and in mitochondrial localization can be observed in *nectin*-null mutant mice. Nectin-2 is a component of cell-cell anchoring junctions, playing a role in the connection of the cytoskeletal elements of neighbouring cells. Thus, this molecular system participates in the regulation of cell shape and differentiation through signalling pathways [203]. Further interesting observations on the male gamete cytoskeleton are shown in the null mutant for the zinc-finger transcription factor Egr4. In the latter animals, the flagella is often fragmented, sharply kinked or have tightly coiled distal ends. Spermatozoa with heads that are either separated entirely or bent sharply back on the flagella are observed [202, 204].

In null mice for *Sla12a2* gene (normally expressing the Na^+-K^+-$2Cl^-$ co-transporter), few spermatids are present but defects are striking when spermatids gradually acquire the features of spermatozoa [202]. Defects in the molecular system of Na^+-K^+-$2Cl^-$ co-transporter result in morphological abnormalities of spermatids. Spermatids show abnormalities in the cap-phase acrosomal vesicle and in the nuclear shape [205]. Other morphological abnormalities of the male gamete are the result of the lack of the factors that are normally expressed by the *CsnK2a2* gene. *CsnK2a2* could be a candidate globo-zoospermia gene. Mice with defects in the expression of the *CsnK2a2* gene show abnormalities in spermatid nuclear morphogenesis. Further abnormalities are observed in the nuclear and acrosomal shape.

Robertson *et al.* [206] have shown that deficiency in the production of aromatase enzyme cyp19 as a result of targeted disruption of the cyp19 gene in ArKO mice results in maturation arrest at early stages of spermiogenesis. Round spermatids do not complete elongation and spermiation. Furthermore, morphological defects in round spermatids are seen in tubules exhibiting spermiogenic arrest. Furthermore, abnormalities in the acrosomal structure are observed.

Deficiency in the production of an epithelial, microtubule-associated protein due to defects in the expression of the *E-MAP-115* gene results in abnormal shape and progressive degeneration in all condensed spermatids. Abnormalities in the microtubular manchette and in nuclear shape are also observed [202, 207]. Subnormal expression of the molecular products of the gene *Tg737* that encodes the components of the raft protein complex, designated Polaris in the mouse and *IFT88* in both *Chlamydomonas* and mouse, results in defective ciliogenesis and abnormalities in flagellar development in spermatids as well as asymmetry in left-right axis determination [208]. Polaris/*IFT88* is detected in the manchette of mouse and rat spermatids. Intramanchette transport has the features of intraflagellar transport machinery. In addition, it facilitates nucleocytoplasmic exchange activities during spermiogenesis [208].

3.14 Preimplantation genetic diagnosis (PGD)-biopsy techniques and risks

Monogenic and chromosomal abnormalities can be diagnosed using genetic material obtained from polar bodies (PB), blastomeres or trophectoderm cells [209]. Couples who have had previous unsuccessful assisted reproductive trials or have a risk of transmitting to the offspring a genetic disorder related or unrelated to their infertility status might benefit from the application of PGD. PND is the most widely applied procedure, however, it is often followed by iatrogenic termination of a pregnancy associated with a fetal recessive or dominant disorder or with a fetus numerical or structural chromosomal abnormality [210, 211].

The clinical application of PGD has a number of limitations concerning: a) its diagnostic value; b) the availability of oocytes, zygotes or embryos for biopsy; and c) the implantation or pregnancy rates after the healthy embryo transfer [212]. Embryonic biopsy, as an invasive method, might also have risks related to the post-PGD embryonic development and, furthermore, to the health of newborns [22]. For instance, there is evidence that acid Tyrode solution (commonly used to carry out PB biopsy) affects the quality and the development of the embryos that have undergone biopsy, despite the fact that the aspiration of both PB does not cause a detrimental effect on the cleavage of the zygote [213]. Currently, the usage of acid Tyrode solution is gradually being replaced by laser drilling of the zona pellucida and, thus, the utilization of chemical substances is substituted by the use of a high-energy beam. Studies comparing the two methods have been in favor of the laser drilling in terms of implantation and pregnancy rates post-biopsy [214].

Analysis of data regarding the carrying out of PGD has indicated that the PB biopsy is not used as often as the blastomere biopsy and is practically limited to cases of oocyte selection (to carry out ICSI) in female carriers of chromosomal translocations [211, 212, 215]. To carry out blastomere biopsy, at least one blastomere is aspirated from all day-3 embryos. A second (additional to the first) blastomere biopsy offers reassurance of the validity and reliability of the diagnosis, although it increases the workload of the clinical PGD procedure. The implantation and pregnancy rates related to the two cell (blastomeres) biopsy are similar to the one cell biopsy. Thus, it appears that the aspiration of a second blastomere does not have a detrimental affect on further embryonic development [216]. In addition, there is evidence proving that transfer of blastocysts (on day 5) that have been generated from embryos that had undergone biopsy on day-3 embryos does not compromise the implantation process [217].

Regarding the potential risks arising from the blastomere biopsy at the 6–8 cell stage embryo, there is concern originating from the evidence that the X chromosome inactivation process is initiated at this developmental stage [218]. Biopsy of one or two blastomeres from a limited pool of cells might disrupt the 50%/50% ratio of the random X chromosome inactivation balance [219].

Pediatric evaluation of children born after ICSI plus PGD did not show significant differences compared with children born after the use of ICSI trials [22, 220].

Biopsy of the trophectoderm is an alternative method to the blastomere biopsy with a limited experience to date [209]. However, it should be emphasized that blastocysts provide a sufficient amount of genetic material for reliable diagnosis. In addition, blastocysts that have undergone biopsy have an acceptable capacity for implantation [221].

Couples with infertility related genetic abnormalities might benefit from the use of PGD. For these couples the balance between risks and benefits supports the role of PGD to select genetically competent embryos and to avoid the PND during the pregnancy period.

4 Guidelines and conclusions

According to the guidelines suggested by a group of clinical and research experts from 12 national scientific societies, there are two types of genetic tests for ICSI candidates: a) the recommended tests; and b) the optional tests according to the clinical indications [222]. The genetic profiles of both members of each couple participating in assisted reproductive technology programs has to be carefully assessed and proper genetic counseling and a basic genetic evaluation (Table 1) will assist all couples to make informed decisions. The highly recommended diagnostic tests for infertile males participating in assisted reproductive programs include the karyotype, microdeletions of the Y chromosome and the *CFTR* mutation analysis. Additional genetic tests for KAL 1 mutations, androgen receptor, 5 α-reductase 2, hemoglobinopathies and sperm-aneuploidy analysis might be additionally suggested for selected subpopulations of infertile males (Tables 1, 2).

Data regarding thousands of children evaluated in independent studies, pooled data form surveys of world results and the ESHRE ICSI task force, as well, show

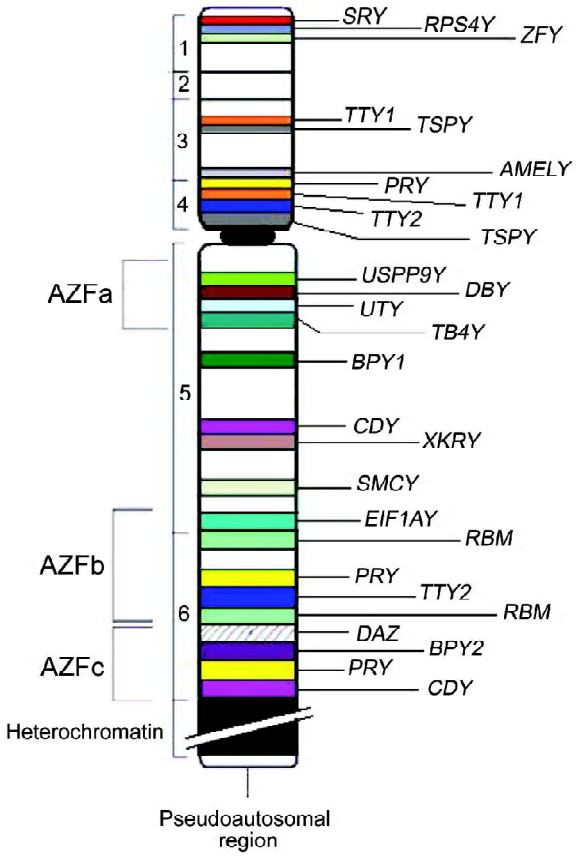

Figure 3. Genes in the human Y chromosome.

that the proportions of the most common congenital abnormalities in children delivered after ICSI techniques are not significantly different compared with those in the general population, with the exception of hypospadias [223–226]. Reservations concern the definitions of major and minor abnormalities, and the abnormalities that should be evaluated in a long run, such as deficiencies in mental development.

The genetic profiles and constitution of gametes from males treated with ICSI are variable, however, it appears that a relationship exists between the severity of the spermatogenic impairment and the chromosomal defects in the spermatozoa (either testicular or ejaculated samples) [48, 114]. In addition, germline genetic defects in spermatogenesis have to be taken into consideration when ICSI is suggested [65].

Genetic counseling by experienced scientists should emphasize that even mild or isolated phenotypic defects in the father may lead to more severe and clinically important abnormalities in the offspring. Non-obstructed azoospermic men with complete deletions of AZFa or AZFb region of the Y chromosome (Table 2, Figure 3) should not be advised to undergo testicular biopsy.

The development of ICSI as a widely applied and prominent reproductive technology has intensified the need for thorough evaluation and laboratory investigations towards two directions. The first direction is the follow-up of children derived by ICSI techniques and the second target is the analysis/study of the genetic causes underlying male infertility. Results from both directions might give rise to conclusions regarding the pathogenesis and the role of male infertility/primary testicular damage in the generation of male gamete (and subsequently embryonic) chromosomal abnormalities.

Today, ICSI might be additionally applied as a result of other indications, for example PGD. PGD can assist the genetic safety of ICSI. The effective collaboration of fertility specialists and geneticists, taken together with the introduction of thorough genetic evaluation in assisted reproduction programs, is essential to reduce the genetic risks from the application of modern reproductive technology.

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