Meiotic nondisjunction and sperm aneuploidy in humans

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Abstract

Infertility is relatively common affecting approximately 1-in-6 couples. Although the genetic basis of infertility is increasingly being uncovered, the contribution of male infertility often remains unexplained. The leading cause of pregnancy loss and cognitive impairment in humans is chromosome aneuploidy. Sperm aneuploidy is routinely evaluated by fluorescence *in situ* hybridization. The majority of studies have reported similar findings, namely: (1) all men produce aneuploid sperm; (2) certain chromosomes are more prone to undergo chromosome nondisjunction; (3) infertile men typically have significantly higher levels of sperm aneuploidy compared to controls and (4) the level of aneuploidy is often correlated with the severity of the infertility. Despite this, sperm aneuploidy screening is rarely evaluated in the infertility clinic. Within recent years, there appears to be renewed interest in the clinical relevance of sperm aneuploidy. We shall examine the gender differences in meiosis between the sexes and explore why less emphasis is placed on the paternal contribution to aneuploidy. Increased sperm aneuploidy is often perceived to be modest and not clinically relevant, compared to the female contribution. However, even small increases in sperm aneuploidy may impact fertility and IVF cycle outcomes. Evidence demonstrating the clinical relevance of sperm aneuploidy will be discussed, as well as some of the challenges precluding widespread clinical implementation. Technological developments that may lead to widespread clinical implementation will be discussed. Recommendations will be suggested for specific patient groups that may benefit from sperm aneuploidy screening and whether preimplantation genetic testing for aneuploidy should be discussed with these patients.

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Introduction

Primary infertility in humans is relatively common affecting around 15% of couples trying to conceive (De Kretser 1997). In some instances, mechanical and physiological defects are identified (e.g., tubal blockages, disturbances in the HPG axis etc.). However, the underlying cause of infertility in couples often remains unexplained (Hotelling & Carrell 2014). A diagnosis of unexplained infertility is often difficult for patients and leaves limited options for treatment, the majority of which relies on expensive and emotionally draining assisted reproductive technology (ART). Male factors contribute to approximately 50% of cases, solely or in combination with female factors (Chandra *et al.* 2005). In some instances, perturbations in the male partner are identified such as abnormal semen parameters. In many instances, the cause of reduced semen parameters fails to be identified (e.g., *CFTR* mutations that lead to absence of the vas deferens causing obstructive azoospermia). Despite the fact that male factors significantly contribute to infertility, the clinical work-up and available diagnostic tests for male factor infertility is underwhelming, with much of the clinical and diagnostic

work-up focusing on the female partner. In males, the work-up is usually restricted to family history, physical exam and assessment of semen parameters as per the WHO guidelines (World Health Organization 2010, Ioannou *et al.* 2016). Current testing in the male partner is only able to determine the underlying cause of the infertility in around 20% of cases, which leaves around 80% of male factor infertility unexplained (Hotelling & Carrell 2014). Assessment of semen parameters provides some fundamental information and may indicate the need for further genetic testing (e.g. karyotyping, *CFTR* mutation analysis and/or Y chromosome microdeletion analysis). However, the clinical validity of the semen assessment has been strongly questioned (Lewis 2007, Hotelling & Carrell 2014). Furthermore, none of the aforementioned tests assesses the genetic integrity of a semen sample (Ioannou *et al.* 2016). Several tests that assess the genetic integrity of sperm have been developed and are available clinically, including tests that evaluate levels of DNA fragmentation, epigenetics and sperm aneuploidy. Sperm aneuploidy has been assessed using the technically challenging human sperm hamster oocyte fusion assay and more recently

by fluorescence *in situ* hybridization (FISH) (Fig. 1) (Shi & Martin 2000, Tempest & Griffin 2004, Templado *et al.* 2013). Despite their availability, the clinical utilization of these tests has been very low. In this review, we shall discuss the gender differences in meiosis and the role of aneuploidy in infertility, focusing on the paternal contributions to aneuploidy.

The paternal contribution to aneuploidy is often overlooked in the ART field and is rarely considered clinically relevant. However, we shall present data that suggest increased sperm aneuploidy may negatively impact fertility and ART outcomes and can result in aneuploid offspring. We will explore the challenges to widespread clinical application of sperm aneuploidy screening, identify patients who may benefit the most from screening and potentially preimplantation genetic testing for aneuploidy (PGT-A). In addition, we shall discuss future technological advancements that may lead to widespread clinical implementation of sperm aneuploidy screening.

The paternal contribution to infertility is frequently overlooked

The sperm cell is an essential prerequisite to fertilization and embryogenesis. Nevertheless, our understanding of the role of sperm in embryogenesis

is poorly understood and largely overshadowed by the maternal oocyte (Ioannou & Tempest 2015). The gametes differ in terms of their known functions in these processes, which likely explains why there is a focus on the maternal rather than the paternal contribution. Following fertilization, the oocyte remodels the paternal genome, replacing the protamines with maternally derived histones and repairing any DNA damage to the paternal genome (Mclay & Clarke 2003). Moreover, the oocyte drives embryogenesis by contributing the cytoplasmic environment, enzymes, energy and the majority of support organelles required for the first embryonic divisions (Carrell 2013). Thus, spermatozoa are often considered to be a silent vessel whose sole function is to deliver the haploid paternal genome to the haploid oocyte. The relative perceived insignificance of spermatozoa when compared to the oocyte may, in part, explain the lack of interest to develop sperm tests and the scarcity of relevant clinically validated tests for male factor infertility (Kavitha & Malini 2014).

Chromosome aneuploidy and its relationship to infertility

Increasingly, genetic factors are being identified as the underlying basis of infertility. Perhaps one of the best known genetic factors that clearly contribute to infertility is chromosome aneuploidy. Chromosome aneuploidy is the presence of extra or missing chromosomes from the normal chromosome complement and is the leading cause of pregnancy loss and cognitive impairment in humans. Interestingly, the prevalence of chromosome aneuploidy seems to be largely restricted to humans. Thus, we do not have adequate animal models to study the mechanisms leading to a high incidence of chromosome aneuploidy in humans. The vast majority of chromosome aneuploidies in humans are not viable in a non-mosaic state and frequently lead to spontaneous abortions, often before a clinical pregnancy is even realized. Although several chromosomes (13, 18, 21, X and Y) are potentially viable in an aneuploid state, a significant proportion of these are also spontaneously aborted (Nagaoka *et al.* 2012). The most established risk factor for aneuploid conceptions is advanced maternal age (AMA) which is defined as women aged 35 years or older. The clinical relevance of the paternal contribution to chromosome aneuploidy is frequently overlooked, despite the fact that aneuploid conceptuses are paternally derived in 5–10% of autosome aneuploidies and 5–100% of aneuploidies involving the sex chromosomes (Hassold *et al.* 1993). This is likely due to the milder phenotypes seen in sex chromosome aneuploidies compared to other viable chromosome aneuploidies. However, there remains a risk of spontaneous abortion, mild cognitive impairment, infertility, and other phenotypes associated with sex chromosome aneuploidies, as well as more severe

Figure 1 Fluorescence *in situ* hybridization (FISH) to detect sperm aneuploidy. FISH in human sperm nuclei utilizing FISH probes against chromosomes 18 (aqua), 21 (green), X (gold) and Y (red). Sperm nuclei are DAPI counterstained and have been pseudo colored gray to facilitate visualization of FISH probes. Panels A, B, C and D provide examples of normal and aneuploid sperm for the aforementioned chromosomes tested. Panel A: normal haploid Y bearing sperm; B: YY disomy; C: disomy 21 and XY disomy; and D: XY disomy.

clinical phenotypes for autosomal aneuploidies that are paternally derived.

Meiosis: differences between males and females

There are fundamental biological differences between meiosis in males and females, particularly regarding timing and outcome. In females, meiosis begins *in utero*, arrests during prophase I at around 20 weeks of gestation, and only resumes when oocytes are selected from the pool for ovulation following the onset of puberty. Meiosis I in females is only completed in ovulated follicles, and meiosis II is only completed if the ovulated oocyte is fertilized. In contrast, males undergo the process of spermatogenesis, in which spermatogonial stem cells differentiate into spermatozoa over a period of 74 days (Samplaski *et al.* 2010). In males, spermatogonia undergo constant mitotic proliferation at the onset of puberty and these primordial germ cells differentiate, undergo meiosis without arrest and eventually form mature spermatozoa. Another significant difference between males and females is found in the number of haploid gametes produced at the end of meiosis. Meiosis results in the generation of four haploid gametes in males, but only one haploid gamete and two polar bodies in females. Moreover, there is a progressive and significant atresia of oocytes throughout *in utero* development until menopause in females, whereas in males there is constant proliferation of spermatogonial stem cells following puberty, which leads to the production of millions of sperm each day throughout their lifetime. Based on the intrinsic differences between meiosis in males and females, it is perhaps not surprising that chromosome aneuploidy in gametes is higher in females than in males and that the risk for aneuploid offspring is associated with AMA, given that oocytes have been arrested in meiosis I for decades. The postulated mechanisms of chromosome nondisjunction have been extensively reviewed elsewhere (Nagaoka *et al.* 2012, Webster & Schuh 2017).

Meiotic studies

Given the biological differences between males and females, it stands to reason that the biological mechanisms that go awry leading to the generation of chromosomally aneuploid gametes are likely to be different. Studies evaluating chromosome pairing and meiotic recombination during the prophase I stage of meiosis in males and females are predominantly performed using immunofluorescence staining. Typically, fluorescent antibodies against SCP1 and SCP3 of the synaptonemal complex are used, which detects proteins involved in the pairing of homologous chromosomes, antibodies against MLH1 detects sites of meiotic recombination, and CREST antibodies detect chromosome centromeres (Fig. 2). Some studies

have utilized FISH following immunofluorescence to identify individual chromosomes. For the most part, human studies have primarily focused in males due to the difficulty in obtaining female meiotic preparations. Because studies in males require an invasive testicular biopsy, the majority of published data come from infertile patients with either obstructive- or non-obstructive azoospermia (NOA) undergoing a testicular biopsy as part of ART treatment, or patients with presumably normal spermatogenesis, but undergoing a vasectomy reversal. Thus, the data obtained in these studies may not be entirely representative of the normal testicular environment.

Figure 2 Immunofluorescence of human pachytene spermatocytes. Chromosome synapsis is detected using antibodies against the synaptonemal complex (SCP1 and SCP3) labeled in red and sites of meiotic recombination by antibodies against MLH1 labeled in yellow. In addition, CREST antibodies labeled in blue are used to detect chromosome centromeres. Panel A displays a complete pachytene spermatocyte spread, note synaptonemal pairing defects resulting in splits in the synaptonemal complex (indicated by arrows). In panels B and C individual bivalents from pachytene spreads are displayed. Panel B includes bivalents from four different chromosomes (1, 8, 13, and 21). MLH1 recombination foci are indicated by yellow lines to the left of the synaptonemal complex and the location of the centromere of each chromosome is indicated by the blue line to the right of each synaptonemal complex. Note the differences in synaptonemal complex length between the chromosomes going from the largest (chromosome 1) to the smallest (chromosome 21). The larger the chromosome the more sites of recombination, with chromosome 21 possessing a single recombination foci. Each p- and q-arm possesses at least one site of recombination (chromosomes 13 and 21 are acrocentric chromosomes so do not have a p-arm), and we can see evidence of positive interference of recombination (foci are not located in close proximity to each other). Panel C displays two bivalents with meiotic defects indicated by arrows, the top bivalent is achiasmate (lacking recombination) and the bottom bivalent has a defect in chromosome pairing resulting in a gap in the synaptonemal complex. Images provided by Renee H Martin.

Lessons learned from meiotic prophase I studies

Several key consistent themes have emerged from various published studies investigating chromosome pairing and meiotic recombination in prophase I (Tempest 2011). Studies of male and female meiotic preparations reiterate the differences between sexes. Females possess more sites of recombination than males (~70 vs ~50, respectively per meiotic spread) (Gruhn *et al.* 2013). The number of recombination foci per chromosome is correlated with chromosome size; in essence, the larger the chromosome the more sites of recombination. For example, chromosome 1 is the largest and contains an average of five recombination sites, whereas chromosome 21 is the smallest and has a single recombination site in males (Martin 2008, Sun *et al.* 2005, 2008). In addition to chromosome size, gene density may also play a role. For example, even though chromosomes 18 and 19 are similar in size, they differ dramatically in gene density (Boyle *et al.* 2001, Babariya *et al.* 2017). Chromosome 19 is one of the most gene-rich chromosomes, whereas chromosome 18 is one of the gene poorest chromosomes. Chromosome 19 has a longer synaptonemal complex, and consequently, more recombination foci on average when compared to chromosome 18 in males (Sun *et al.* 2004b). With the exception of the acrocentric chromosomes, the p- and q-arms of all chromosomes possess at least one site of recombination in the normal situation (Hassold *et al.* 2000). There is also clear evidence of suppression of recombination in close proximity to other sites of recombination (Fig. 2) (Sun *et al.* 2004b). Furthermore, the preferential localization of sites of recombination along the synaptonemal complex demonstrates a more distal localization in males, when compared to females (Gruhn *et al.* 2013).

The relationship between perturbations in male meiosis and infertility

Clear differences in meiosis are observed between fertile and infertile men. The vast majority of meiotic preparations in fertile men tend to be in the pachytene stage, since this is the longest stage of meiotic prophase I (Gonsalves *et al.* 2004). This is in sharp contrast to many infertile patients. For example, NOA patients tend to have significantly reduced numbers of cells in the pachytene stage or complete absence of pachytene cells due to a partial or complete block at the zygotene stage of meiosis (Gonsalves *et al.* 2004). Additionally, a significant reduction or lack of recombination foci (achiasmate bivalents) (Fig. 2) has been observed in some NOA patients compared to fertile controls (Ferguson *et al.* 2007, Sun *et al.* 2008). FISH studies following immunofluorescence have reported that achiasmate bivalents most frequently involve chromosomes 21, 22, X and Y (Ferguson *et al.* 2007,

Sun *et al.* 2008). In males, these four chromosomes typically possess a single site of recombination, whereas all other chromosomes possess two or more. Meiotic recombination may be crucial to prevent the premature separation of homologous chromosomes, which can lead to chromosome nondisjunction in gametes. If this were the case, it would suggest the chromosomes that possess a single site of recombination have the highest risk to be achiasmate, and thus, the highest rates of chromosome nondisjunction, compared to chromosomes with multiple sites of recombination. Indeed, several studies have identified a correlation between the presence of achiasmate bivalents in NOA patients and increased levels of sperm aneuploidy, predominantly involving the aforementioned chromosomes (Ferguson *et al.* 2007, Sun *et al.* 2008). This finding is further corroborated by the majority of sperm FISH studies, which report significantly higher levels of sperm aneuploidy for these chromosomes (21, 22, X and Y) in both fertile and infertile subjects (Shi & Martin 2000, Tempest & Griffin 2004, Templado *et al.* 2013). Sequencing of single spermatozoa also demonstrated a lower frequency of recombination in aneuploid sperm (Lu *et al.* 2012). Indirect evidence of the association between aberrant recombination and sperm aneuploidy was also provided by determining the parental origin of trisomic conceptuses and liveborns (Hassold *et al.* 1993). This study demonstrated that the majority of paternally derived 47,XXY offspring lacked recombination at either the p- or q-arm pseudoautosomal regions of the sex chromosomes (Hassold *et al.* 1993). Additional evidence to suggest lack of recombination may be a contributing factor to aneuploidy is also suggested from SNP array data in cleavage-stage embryos (Rabinowitz *et al.* 2012).

In addition to disturbances in recombination, perturbations in chromosome pairing have also been observed between fertile and infertile patients. The incidence of synaptonemal complex pairing disturbances is more frequently observed in infertile than fertile patients (Sun *et al.* 2004a, 2007, Martin 2006). These pairing disturbances can include gaps (absence of synaptonemal complex formation in regions) or splits (formation of the synaptonemal complex, but failure of the synaptonemal complex to join) (Fig. 2), all of which may contribute to chromosome nondisjunction. It is important to note that meiotic studies in infertile men are usually restricted to patients with azoospermia. Azoospermia is a rare cause of male infertility and represents the most severe category of male infertility; thus, the discussed findings may not be widely applicable in patients with milder semen parameter disturbances. Nevertheless, evidence of the contribution of achiasmate bivalents and chromosome aneuploidy has been observed in multiple studies using various techniques in both fertile and infertile patients, cleavage-stage embryos and aneuploid offspring. This suggests that the production

of achiasmate bivalents is strongly associated with an increased risk of chromosome nondisjunction.

Mechanisms of chromosome aneuploidy in males

The underlying mechanism(s) for increased aneuploidy observed in some men remains to be elucidated. However, defects in the meiotic machinery leading to chromosome nondisjunction have been identified in some patients. This includes perturbations in chromosome pairing and recombination (e.g., achiasmate bivalents) and/or defects in an as of yet unidentified meiotic checkpoint that prevents aneuploid sperm from completing meiosis (Harton & Tempest 2012). Evidence of a male meiotic checkpoint comes from studies that have assessed the sperm aneuploidy frequencies in 47,XXY and 47,XYY patients. In the absence of gonadal mosaicism, the expected frequency of sex chromosome aneuploidies in the sperm of these patients would be 50%; however, studies report significantly lower levels of sperm aneuploidy (1–25%) in these patients, concomitant with a general reduction in sperm count (Ferlin *et al.* 2005, Harton & Tempest 2012). These studies provide indirect evidence that there is likely a ‘leaky’ meiotic checkpoint that prevents the completion of meiosis for a significant proportion of aneuploid sperm, and may also contribute to the low sperm counts in these patients. The existence of a meiotic checkpoint in males is further supported by studies showing that the frequency of aneuploidy in meiosis II spermatocytes is 14%, similar to the levels reported in young females (Garcia-Cruz *et al.* 2010, Uroz & Templado 2012). This finding suggests that the differences in aneuploidy levels found in gametes between males and females may have less to do with differences in chromosome nondisjunction, but rather, could be due to a meiotic checkpoint that may be more efficient in males than in females (Andreescu *et al.* 2016).

Despite the known genetic contribution, the hunt to identify the genes and mutations that may contribute to male infertility and increased sperm aneuploidy remains elusive. Studies including genome-wide association studies have been largely unsuccessful in implicating the genes involved or reproducing study findings, suggesting that male infertility is likely attributed to rare, low-frequency variants of small effect size, precluding their identification (Hotaling & Carrell 2014). Given the considerable phenotypic heterogeneity of male infertility, the complexity of spermatogenesis, the more than 2300 genes involved and the likely multifactorial mode of inheritance, it is perhaps not surprising that we have been unsuccessful in identifying SNPs, copy number variants (CNVs) or mutations associated with male infertility (Hotaling & Carrell 2014). In summary, we have a very limited understanding of the genetic mechanisms contributing to male infertility and increased sperm aneuploidy.

Human sperm aneuploidy

The advantages and disadvantages of FISH to assess sperm aneuploidy frequencies

Prior to the widespread use of FISH, a few studies utilized the technically challenging and laborious approach of karyotyping sperm chromosomes through the sperm hamster oocyte fusion assay. Thus, the vast majority of studies have predominantly utilized FISH to assess sperm aneuploidy levels. Prior to assessing the use of FISH to assess sperm aneuploidy frequencies, it is beneficial to review some of the major advantages of FISH that led to its widespread for molecular cytogenetic applications. FISH is a pivotal cytogenetic tool used to detect genetic abnormalities that has been used by researchers and clinical diagnostic labs worldwide for decades. FISH has a high sensitivity and specificity if appropriate probes are selected and applied to relevant target cells or tissues. FISH has several key advantages: (1) it is relatively rapid to perform, (2) it can be performed in interphase cells and thus, it does not require metaphase chromosomes, (3) it can be multiplexed by utilizing multiple fluorochromes up to five (or more when fluorochromes are used in combination) and (4) robust high-throughput automated systems exist for some FISH applications, which permit automated slide loading, image capture, and analysis with minimal user intervention. These advantages led to the widespread use of this very versatile molecular cytogenetic tool for a wide variety of research and clinical diagnostic applications.

Despite the numerous advantages of FISH, there are several major drawbacks for the utilization of FISH to assess sperm aneuploidy. Due to the lack of commercially available FISH probes in different fluorochromes, the majority of sperm FISH studies only utilize three to five probe targets at any one time. Furthermore, sperm aneuploidy screening requires the use of a strict scoring criteria and unlike the majority of other FISH applications, the accurate assessment of sperm aneuploidy requires the scoring of thousands of sperm nuclei due to its relatively low incidence. Therefore, sperm aneuploidy screening is laborious and costly in terms of technician time, and precludes analysis of semen samples in patients with very low sperm counts. Although FISH probes are commercially available for a wide variety of targets, they can be costly on a per sample basis, especially for less commonly used fluorochromes as these may need to be custom made. The cost is potentially increased further due to the need of a larger probe volume (compared to other FISH applications) to score the large number of cells. Typically, aneuploidy screening for three to five chromosomes may cost patients in the region of \$500–700, which also precludes the widespread implementation of this test clinically. The vast majority of sperm FISH aneuploidy studies often restrict the chromosomes analyzed to those involved in clinically viable chromosome aneuploidies

(chromosomes 13, 18, 21, X and Y). Consequently, evaluation of sperm aneuploidy by FISH only assesses a handful of chromosomes and does not provide a genome-wide assessment of aneuploidy for all 24 chromosomes in a single cell. In order to expand to more than five targets in a single experiment, FISH probes need to be mixed with different ratios of fluorochromes, which can be detected by specialized software used for spectral karyotyping applications; yet, this is not widespread amongst laboratories. Moreover, as FISH is performed in small highly condensed interphase nuclei assessing increasing targets is challenging due to the risk of overlapping signals and inability to rapidly score nuclei manually. Typically, studies that assess more than five chromosomes achieve this by performing additional rounds of FISH. Due to the thousands of sperm nuclei scored, it is technically challenging to re-probe the same sperm nuclei to increase the number of chromosomes assessed in each cell. One feasible approach to bypass many of these challenges is to utilize automated high-throughput systems.

To date, we and others largely agree that automated systems can reliably assess sperm aneuploidy (Netten *et al.* 1997, Baumgartner *et al.* 2001, Perry *et al.* 2007, 2011, Carrell & Emery 2008, Molina *et al.* 2009, Tempest *et al.* 2010, Martinez *et al.* 2013, Branch *et al.* 2017). Some studies have reported substantial reductions in analysis time (Branch *et al.* 2017), but it is important to consider some challenges. One challenge is the unique highly condensed chromatin packaging found in sperm cells, which sees the majority of histones replaced with protamines. This unique packaging requires sperm cells to be treated with dithiothreitol, lithium diiodosalicylate, pepsin or heparin to facilitate access of FISH probes to the DNA (this step is not required in any other cell type). An additional challenge is that FISH signals are often found on very different focal planes, requiring the capture of multiple Z stacks for each fluorochrome. Of note, a similar challenge is faced by manual scoring, requiring the scorer to carefully examine multiple focal planes for each cell. Thus, some studies have noted that the analysis time for automated systems may not be reduced *per se*, when compared to manual scoring. However, manual intervention is significantly less, and restricted to performing the FISH and reviewing aneuploid cells (Molina *et al.* 2009, Tempest *et al.* 2010). Careful attention is required for slide preparation (e.g., sperm density) and to optimize the software to exclude cells that do not fulfill the scoring criteria used by highly trained cytogeneticists (e.g., overlapping cells and various FISH signal criteria) (Molina *et al.* 2009, Tempest *et al.* 2010). This may in part explain some of the differences reported for some samples between manual and automated scoring (Molina *et al.* 2009, Tempest *et al.* 2010). However, once established and validated, automated approaches can be used to score more cells, chromosomes and patients to achieve

increased statistical power and importantly reduce costs (Branch *et al.* 2017). Automated systems are widely used in high-throughput diagnostic labs particularly in the field of oncology. The major limitation for its use to evaluate sperm aneuploidy is cost, and the requirement for high sample volumes to warrant purchasing such a system. Thus, most published studies have utilized manual scoring. With increasing clinical demand for sperm aneuploidy testing, however, it is likely that the use of automated approaches will increase. In the next sections, we will focus on the clinical relevance of sperm aneuploidy testing, and which patients would most likely benefit.

Sperm aneuploidy levels in men with proven fertility or with normal semen parameters

Over 50 publications have demonstrated that all men produce a proportion of aneuploid sperm (Shi & Martin 2000, Tempest & Griffin 2004, Templado *et al.* 2013). In males with proven fertility or normozoospermia (semen parameters within the normal WHO reference ranges), extrapolations from tested chromosomes suggest that approximately 3–5% of sperm are aneuploid (Pang *et al.* 1999, Tempest & Griffin 2004, Lu *et al.* 2012). If we look at chromosomes individually, around 0.1–0.15% of sperm are aneuploid for each of the 24 chromosomes (Pang *et al.* 1999, Shi & Martin 2000, Tempest & Griffin 2004). However, chromosomes such as 21, 22, X and Y have a two- to three-fold higher incidence of aneuploidy than the other chromosomes, with a frequency of around 0.3% per chromosome (Pang *et al.* 1999, Shi & Martin 2000, Tempest & Griffin 2004). Studies have shown that sperm aneuploidy frequencies are largely consistent over time in fertile or normozoospermic males with little intraindividual variation (Robbins *et al.* 1993, Rademaker *et al.* 1997, Rubes *et al.* 2005, Tempest *et al.* 2009). However, significant differences in sperm aneuploidy can occur at single time points in some individuals, suggesting aneuploidy frequencies can be altered, perhaps by transient factors, lifestyle changes or environmental exposures (e.g., diet, infection, exercise, smoking or air pollution etc.) (Tempest *et al.* 2009). Furthermore, there are clearly interindividual differences, with some males consistently producing higher levels of sperm aneuploidy (Rademaker *et al.* 1997, Rubes *et al.* 2005, Tempest *et al.* 2009).

To date, a handful of studies have examined whether advancing paternal age contributes to sperm aneuploidy. The majority of these provide little evidence to support a correlation between advancing paternal age and increased levels of sperm aneuploidy (Fonseka & Griffin 2011, Andreescu *et al.* 2016, Donate *et al.* 2016). There may be a very small effect for a handful of chromosomes in much later years, typically decades after what would traditionally be considered 'reproductive age' (Griffin *et al.* 1995, Fonseka & Griffin 2011, Garcia-

Ferreyra *et al.* 2018). However, as spermatogonia undergo mitotic proliferation, they are prone to replicative errors such as insertions and deletions, SNPs, point mutations and epigenetic alterations. As sperm cells differentiate, there is limited DNA repair, and thus, a higher risk of transmitting alterations to future offspring. A link between advanced paternal age and increased risk of sperm aneuploidy has not been established (most likely due to the biological differences in meiosis between males and females). Nevertheless, increasing evidence suggests an association with advanced paternal age and an increased risk of single-gene and multifactorial disorders (e.g., achondroplasia, apert syndrome, autism, and schizophrenia amongst others) (Yatsenko & Turek 2018).

Sperm aneuploidy as a biomarker to monitor environmental and occupational exposures

Multiple studies have demonstrated an association between environmental factors and increased sperm aneuploidy frequencies. Associations that have been reported include smoking, air pollution, benzene, chemotherapy, parabens, polychlorinated biphenyl and scrotal heating, amongst others (Robbins *et al.* 2005, Tempest *et al.* 2005, 2008, Perry 2008, Xing *et al.* 2010, Ji *et al.* 2012, Mcauliffe *et al.* 2012, Figueroa *et al.* 2015, Governini *et al.* 2015, Radwan *et al.* 2016, Perry *et al.* 2016, Beal *et al.* 2017, Jurewicz *et al.* 2017, Rives *et al.* 2017, Zhang *et al.* 2018). It is important to note that such studies in humans are notoriously difficult to design, compare and reproduce, and it is extremely difficult to prove causation given the multitude of confounding factors that exist in such studies. Furthermore, the increases in sperm aneuploidy reported in these studies although significant, rarely reach the levels observed in infertile patients considered in subsequent sections. This holds true even in the case of highly cytotoxic chemotherapeutic drugs administered to cancer patients. These patients often present with significant but transient levels of sperm aneuploidy that may or may not reach the levels reported in infertile men (Tempest *et al.* 2008). Increasingly, spermatozoa are considered as potential biomarkers to assess reproductive toxicity, as invasive procedures are required to obtain other male or female germ cells (Macgregor *et al.* 1995, Anton & Krawetz 2012). Studies involving rodent model systems have frequently shown that exposure to germinal mutagens can have significant consequences on reproductive outcomes including embryonic loss and increases in mutations and chromosomal abnormalities (Wyrobek 1993). Genotoxic and mutagenic studies frequently assess chromosome aberrations in somatic cells to determine whether compounds have carcinogenic potential through multiple techniques, including FISH, comet assay and the micronucleus assay, amongst others. Inclusion of sperm aneuploidy

in such studies could also provide valuable information on reproductive toxicants and will ultimately provide a more comprehensive evaluation of carcinogenicity and reproductive toxicity (Mandrioli *et al.* 2016). In addition to investigating chromosome aneuploidy in sperm, other biomarkers should be considered to monitor exposure to environmental hazards, including sperm DNA integrity (DNA fragmentation), chromatin modifications (epigenetics) and nuclear organization amongst others (Anton & Krawetz 2012). Emerging evidence suggests environmental factors can alter the establishment and maintenance of epigenetic modifications (Feil & Fraga 2012). Such perturbations could have significant consequences on gene expression, health and disease and fertility (Zalensky & Zalenskaya 2007, Hammoud *et al.* 2011, Aston *et al.* 2012, Millan *et al.* 2012, Hotelling & Carrell 2014, Ioannou *et al.* 2017, Ioannou & Tempest 2018). Assessment of the sperm epigenome is likely to play an increasingly important role in assessing reproductive toxicology and may be clinically relevant in the work-up of infertile men. Already commercially available tests are available to interrogate the sperm epigenome.

Sperm aneuploidy levels in infertile men

Virtually all published studies have reported higher frequencies of sperm aneuploidy in infertile males, compared to fertile or normozoospermic males (Shi & Martin 2000, Tempest & Griffin 2004, Templado *et al.* 2013), with the exception of a handful of studies (Miharu *et al.* 1994, Guttenbach *et al.* 1997). In general, similar aneuploidy frequencies are reported across studies. However, direct comparisons between studies can be challenging due to the heterogeneity of infertile patient phenotypes or controls used (proven fertile controls, normozoospermic patients, or both). Additionally, not all studies provide detailed semen parameter information. Differences also exist between studies in terms of the number of sperm scored, chromosomes analyzed, FISH probes utilized and scoring criteria used. The use of relatively standardized stringent scoring criteria attenuates the variability between studies considerably. However, not all studies provide detailed scoring criteria, numbers of raters or interrater variability (increased use of automated approaches will likely reduce many of these factors significantly). Additionally, studies often report levels of aneuploidy differently. For example, some may include or exclude nullisomy or diploidy, others may report total gonosomy aneuploidy, whereas others report XX, XY and YY disomy individually. Sperm aneuploidy is also sometimes reported as a fold increase without providing raw numbers or percentages and different statistical methods are used to determine significance. Thus, direct comparisons between studies can be problematic, and these factors should be considered when looking

at reported aneuploidy frequencies (Fig. 3). Despite these inherent challenges, the vast majority of studies reassuringly report comparable findings. The levels of sperm aneuploidy reported in different studies has been previously reviewed extensively (Shi & Martin 2000, Tempest & Griffin 2004, Templado *et al.* 2011, 2013). Overall, there is a two- to three-fold increase in sperm aneuploidy levels in infertile men compared to controls (Fig. 3). Yet, interindividual differences exist, with ten-fold or higher frequencies reported in some infertile men (Tempest & Griffin 2004, Harton & Tempest 2012, Templado *et al.* 2013, Ioannou & Tempest 2015). The existence of interindividual variation has been reported in fertile and infertile men and should be taken into account when considering reported global levels of sperm aneuploidy levels (Fig. 3). A correlation between the severity of semen parameters perturbations and the levels of sperm aneuploidy has been reported in the literature (Fig. 3). For example, NOA and severe oligoasthenoteratozoospermic (OAT) males often have significantly higher frequencies of sperm aneuploidy, compared to men with normal or milder semen parameter disturbances (Shi & Martin 2000, Tempest & Griffin 2004, Tempest *et al.* 2004). Data in NOA, and severe OAT, is often based on very low numbers due to insufficient sperm available for analysis. Notably, male partners of patients with recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF) following ART are reported to have significantly higher levels of sperm

aneuploidy (these patients will be considered in more detail in the subsequent section). Patients with numerical chromosome aberrations (e.g., 47,XXY and 47,XYY) and structural chromosome aberrations also exhibit very high levels of sperm aneuploidy for the chromosomes involved. Sperm aneuploidy levels range from 1 to 25% and 3 to 81% in individuals with numerical and structural chromosome aberrations, respectively (Fig. 3) (Harton & Tempest 2012, Ioannou & Tempest 2015). Since these studies often only test a handful of chromosomes (those involved in the aberration), sperm aneuploidy levels are likely to be even higher. Reported sperm aneuploidy levels in patients with numerical chromosome aberrations are high, but are lower than would be expected following meiotic segregation of three sex chromosomes (50%), further supporting the existence of an unidentified male meiotic checkpoint. The huge variation in sperm aneuploidy frequencies in carriers of structural chromosome aberrations is likely to be affected by the type of aberration, size and location of the aberration and chromosome(s) involved. The large variation reported in these patients makes it extremely challenging to provide accurate risk assessments to patients regarding the risk of producing chromosomally unbalanced conceptions without sperm aneuploidy testing. Clearly, a patient with 3% aneuploid sperm would be counseled differently than a patient with 80% aneuploid sperm, for the tested chromosomes. Additionally, studies also report an

Figure 3 Overview of increased sperm aneuploidy levels in infertile patients and indication of which patient groups may benefit from preimplantation genetic testing for aneuploidy (PGT-A). Included in this figure are the major categories of infertile patients who are at risk of producing significantly higher levels of sperm aneuploidy compared to fertile or normozoospermic males (controls). Patients are split into three categories and six subcategories; for each subcategory, the average fold increase in sperm aneuploidy compiled from numerous studies is presented compared to control levels. The reduction in semen parameters group is split into three patient subcategories: mild semen parameter disturbances, severe oligoasthenoteratozoospermic (OAT), and rare severe teratozoospermic, as defined by the World Health Organization (WHO). Asterisks (*) represent patient groups who may potentially benefit from sperm aneuploidy screening, if high levels of sperm aneuploidy are found, these patients may benefit from genetic counseling and being offered PGT-A. The three subcategories with the highest levels of sperm aneuploidy include specific types of severe teratozoospermia, numerical and structural chromosome aberrations. Sperm aneuploidy screening in these groups can identify the patients with extremely high levels of aneuploid sperm. These patients should receive appropriate genetic counseling and be offered PGT-A so they can make informed decisions regarding the risks to their future offspring and to potentially improve their reproductive outcomes. Note: This figure should be interpreted with caution due to interindividual differences in sperm aneuploidy frequencies and challenges associated with cross study comparisons as outlined in the text.

interchromosomal effect in patients, suggesting they may have a generalized increase in sperm aneuploidy for all chromosomes not just those involved in the chromosome aberration (Harton & Tempest 2012). Some of the highest sperm aneuploidy frequencies have been reported in a handful of patients with severe teratozoospermia (e.g., semen samples containing high levels microcephalic and macrocephalic sperm), these rare patients have aneuploidy levels exceeding 50% for the few chromosomes investigated (Fig. 3) (Hotaling & Carrell 2014).

Sperm aneuploidy and recurrent pregnancy loss (RPL) and ART recurrent implantation failure (RIF)

Loss of a clinically recognized pregnancy is relatively common occurring in 15–25% of pregnancies, however, a subset of pregnant women (less than 5%) will experience RPL (Asrm 2012). RPL is defined by the American Society of Reproductive Medicine as two or more failed clinically recognized pregnancies. In about half of these cases, the underlying cause of RPL is idiopathic, and patient work-ups often focus on maternal factors (genetic, chromosomal, AMA, hormonal disturbances, uterine abnormalities, metabolic, infectious or immune disorders etc.) (Zidi-Jrah *et al.* 2016). Even less is currently understood about the underlying basis of RIF in some ART patients. Evaluation of the male partner in RPL and RIF is typically restricted to karyotype analysis and a basic semen parameter assessment. Karyotype analysis is important in both the male and female partner to rule out structural chromosome aberrations. Balanced carriers of structural aberrations are typically phenotypically normal, but they have a high risk of producing chromosomally unbalanced gametes following meiotic segregation of the structural aberration, which could result in RPL, RIF or congenital malformations (Stern *et al.* 1999). In recent years, the paternal contribution to RPL and RIF beyond semen parameters and karyotyping is being increasingly examined. Multiple studies suggest that increased levels of sperm aneuploidy may be a significant contributory factor in RPL and/or RIF (Rubio *et al.* 2001, Carrell *et al.* 2003, Bernardini *et al.* 2004, Collodel *et al.* 2009, Rubio *et al.* 2009, Neusser *et al.* 2015, Ramasamy *et al.* 2015, Zidi-Jrah *et al.* 2016, Esquerre-Lamare *et al.* 2018). These studies have reported a two- to seven-fold increase in sperm aneuploidy for the investigated chromosomes in these patients (Fig. 3) (Collodel *et al.* 2009, Kohn *et al.* 2016, Zidi-Jrah *et al.* 2016, Esquerre-Lamare *et al.* 2018). The majority of the aforementioned studies have focused on males with reduced semen parameters; however, a large study reported that up to 40% of male partners of RPL with normal semen parameters also had significantly increased sperm aneuploidy levels (Ramasamy *et al.* 2015). These findings suggest that the production of increased sperm aneuploidy in

male patients with normal semen parameters may also contribute to RPL and RIF.

Clinical consequences of increased sperm aneuploidy

Infertile men with perturbations in their semen parameters frequently exhibit modest, but significant increases in sperm aneuploidy levels compared to their fertile/normozoospermic counterparts. The clinical relevance of increased levels of sperm aneuploidy in infertile males has not been fully elucidated. We and others argue that there is mounting evidence to suggest inclusion of sperm aneuploidy testing may be beneficial as part of the diagnostic work-up of select patients (Griffin *et al.* 2003, Gianaroli *et al.* 2005, Nagvenkar *et al.* 2005, Carrell 2008, Nicopoullos *et al.* 2008, Sanchez-Castro *et al.* 2009, Rodrigo *et al.* 2010, Harton & Tempest 2012, Zidi-Jrah *et al.* 2016). Indirect evidence of a correlation between higher levels of chromosome aneuploidy and aneuploid offspring is provided by retrospective studies. These studies have identified high levels of sperm aneuploidy in the fathers of paternally derived aneuploid offspring (Blanco *et al.* 1998, Martinez-Pasarell *et al.* 1999, Moosani *et al.* 1999, Soares *et al.* 2001, Arnedo *et al.* 2006). Several studies have also reported correlations between high levels of sperm aneuploidy and recurrent intracytoplasmic sperm injection (ICSI) or ART failure (Burrello *et al.* 2003, Petit *et al.* 2005, Kahraman *et al.* 2006, Nicopoullos *et al.* 2008), increased levels of aneuploidy in preimplantation embryos (Gianaroli *et al.* 2005, Magli *et al.* 2009, Sanchez-Castro *et al.* 2009, Rodrigo *et al.* 2010, Coates *et al.* 2015) and lower pregnancy rates and live births (Nagvenkar *et al.* 2005). Moderate increases in sperm aneuploidy in infertile men have also been postulated to contribute to the higher levels of aneuploid offspring conceived after ICSI (Egozcue *et al.* 1997, Griffin *et al.* 2003). ICSI is the ART procedure used to treat severe male infertility and has enabled many of these patients to have biological children; ICSI was rapidly adopted in IVF clinics worldwide and is now often used in ART even in the absence of a male factor (Boulet *et al.* 2015). Despite the widespread use of ICSI, concerns have been raised regarding the genetic quality of the sperm used, and that it bypasses the 'fittest sperm' selection process present in natural conceptions or conventional IVF (Griffin *et al.* 2003). These concerns may be valid, as studies have reported an approximate three-fold increase in *de novo* chromosome aberrations in ICSI derived offspring, which mirrors the three-fold increase observed in sperm aneuploidy frequencies in infertile men (Aboulghar *et al.* 2001, Bonduelle *et al.* 2002, Van Steirteghem *et al.* 2002, Devroey & Van Steirteghem 2004). The findings of these studies suggest that aneuploid sperm are equally

capable of fertilizing an oocyte than chromosomally normal sperm, which may lead to RIF, spontaneous abortions, RPL or viable aneuploid offspring.

Why is sperm aneuploidy screening rarely considered in ART?

Clearly, we can identify individuals who are at risk of producing high levels of sperm aneuploidy including those with: (1) structural or numerical chromosome aberrations; (2) azoospermia; (3) severe teratozoospermia; (4) severe OAT and (5) RPL and/or RIF. Then, why are these patients not routinely offered sperm aneuploidy screening as part of their diagnostic work-up? One issue is that ART providers may not be aware of the patients who are at an increased risk of producing higher levels of sperm aneuploidy and may benefit from screening. There are also technical challenges to consider that have perhaps limited the implementation of sperm aneuploidy screening. Typically only three to five chromosomes are analyzed by FISH. Thus, a genome-wide perspective of aneuploidy levels is not provided. Due to the relatively low levels of sperm aneuploidy, thousands of sperm are required to be scored, which is labor intensive. This issue is readily solvable with the advent of various automated systems that have the capability to accurately and reliably assess sperm aneuploidy (Netten *et al.* 1997, Baumgartner *et al.* 2001, Perry *et al.* 2007, 2011, Carrell & Emery 2008, Molina *et al.* 2009, Tempest *et al.* 2010, Martinez *et al.* 2013, Branch *et al.* 2017). In reality, we are unable to test the individual sperm that will be used in ART; thus, it can only provide a generalized overview of the levels of aneuploidy in a semen sample. Perhaps one of the greatest challenges when considering the clinical relevance and utilization of sperm aneuploidy screening is the lack of a clearly established and validated sperm aneuploidy threshold (Kohn *et al.* 2016). These need to be developed by each individual laboratory performing sperm aneuploidy testing with large numbers of individuals. Critically, it also requires clinics to share ART outcome data with these labs in order to develop and assess clinical recommendations for sperm aneuploidy screening. With increasing sperm aneuploidy screening and ART outcome data, better correlations will be able to be made regarding the consequence of increased sperm aneuploidy, which will ultimately facilitate more accurate genetic counseling and risk assessments for patients at an increased risk of RIF, RPL, spontaneous abortions and aneuploid offspring. Often, when any type of genetic testing is undertaken, the result provides some quantifiable modified risk. Unfortunately, we are not there yet with sperm aneuploidy screening, which poses significant challenges for clinicians, genetic counselors and consequently patients. Several valid questions remain: (1) what is a clinically significant level of sperm aneuploidy, and (2) how does this

translate to risk of chromosome aneuploidy in future offspring? Addressing these questions is complicated by the large maternal contribution to aneuploidy and the fact that PGT-A in embryos using SNP arrays have only reported paternally derived aneuploidies that arose post-zygotically (i.e., were mitotic rather than meiotic in origin) (Rabinowitz *et al.* 2012). However, the numbers of published embryos analyzed by SNP arrays remains relatively small especially given the large maternal contribution to chromosome aneuploidy. Thus, it is likely that the sample size may not yet be large enough to detect paternal trisomies that were meiotic in origin. Moreover, the PGT-A SNP array technology needs to be applied specifically in patient cohorts with an increased risk of sperm aneuploidy to determine whether this translates to an increase in paternally derived meiotic aneuploidies in preimplantation embryos.

Sperm aneuploidy is perhaps more straightforward to manage clinically in patients who exhibit very high levels of sperm aneuploidy, such as patients with constitutional structural or numerical chromosome aberrations or severe teratozoospermia. These patients should be counseled on the availability of PGT-A, prenatal genetic testing and the possibility of using donor sperm. The question remains, how should we clinically manage a two- to three-fold increase in sperm aneuploidy which is commonplace in infertile patients? This lack of clarity alongside the technical challenges discussed previously has precluded the widespread use of sperm aneuploidy testing as part of the work-up for those patients who might benefit. We also need to consider which chromosomes should be tested when assessing sperm aneuploidy frequencies clinically, given that typically only up to five chromosomes are tested. In most cases, the chromosomes tested focus on the clinically viable trisomies. Yet, these may not always be the most appropriate chromosomes to test. For example, in patients with structural or numerical chromosome aberrations, additional chromosomes not involved in the aberration should be also tested to identify any interchromosomal effect. Additionally, focusing on chromosomes that are not viable in an aneuploid state may be more informative in patients with RIF or RPL, as arrested embryonic development and recurrent spontaneous abortions are associated with this group. Given the potential clinical impact of sperm aneuploidy, we ought to also consider whether application of PGT-A may lead to better outcomes in patients with high levels of sperm aneuploidy undergoing ART.

Preimplantation genetic testing for aneuploidy (PGT-A) in patients with increased sperm aneuploidy

The principle behind the clinical application of PGT-A is that given the high levels of aneuploidy, the ability to identify euploid embryos will lead to increased rates

of clinical pregnancies, reduced rates of miscarriages, reduced time to pregnancy and critically improved live birth rates following ART. Theoretically, PGT-A should not only improve outcomes but also reduce the financial burdens of multiple embryo transfers, emotional stress of miscarriages and alleviate the complications associated with multiple gestations through single embryo transfers. In practice, however, the PGT field has been fraught with challenges (Chen *et al.* 2018). Some of these challenges have been mitigated by changing the technology from FISH to array comparative genomic hybridization (aCGH), and more recently next generation sequencing. Additionally, many clinics have moved from day 3 blastomere biopsy to day 5 trophectoderm biopsy. However, these advances have also added more complexity to the field, including the detection of chromosome mosaicism and segmental aneuploidies, both of which appear to be common biological phenomena in the early preimplantation embryo (Fragouli *et al.* 2010, Babariya *et al.* 2017). Segmental aneuploidies can be inherited following the meiotic segregation of a balanced structural chromosome aberration resulting in unbalanced gametes, or they can arise due to a *de novo* error in meiosis or through a mitotic error during early gametogenesis (Vera-Rodriguez *et al.* 2016). *De novo* chromosome aberrations in natural conceptions as detected through amniocentesis are found at a low frequency (Warburton 1991), with segmental aneuploidies found in ~6% of miscarriages (Martinez *et al.* 2010) and ~0.05% of liveborns (Wellesley *et al.* 2012). A huge variation in the incidence of segmental aneuploidies in preimplantation embryos has been reported, ranging from 5 to 70%, with most studies reporting an incidence of around 10–15% (Voullaire *et al.* 2000, Wells & Delhanty 2000, Vanneste *et al.* 2009, Rabinowitz *et al.* 2012, Vera-Rodriguez *et al.* 2016, Babariya *et al.* 2017). These studies suggest that the majority of segmental chromosome aneuploidies detected by PGT are not compatible with fetal development and will likely spontaneously abort, fail to implant or if viable could be associated with congenital abnormalities in the affected offspring (Wellesley *et al.* 2012). It is noteworthy that segmental aneuploidies are not correlated with AMA and appear to be predominantly mitotic in origin. However, those that are meiotic in origin are twice as likely to be paternally, rather than maternally derived (Konstantinidis *et al.* 2016). This confirms some of the early human sperm hamster oocyte fusion karyotyping data that identified up to a six-fold increase in structural chromosome aberrations in sperm compared to oocytes (Martin 2008). This indicates that meiotic processes specific to males and/or the presence of DNA damage that is unable to be repaired within sperm may play a significant role in the origin of the majority of segmental aneuploidies (Babariya *et al.* 2017).

Following appropriate genetic counseling, some couples may wish to consider undertaking ART in conjunction with PGT-A, for indications other than AMA. The use of PGT-A in ART over the past decade has steadily increased in the United States especially for patients of AMA. Couples who are planning to undertake PGT-A with their ART cycle may not benefit from additional sperm aneuploidy testing. However, sperm aneuploidy testing may be useful in at risk patients to assist them in making an informed decision as to whether they wish to consider PGT-A, particularly in the absence of AMA. These at risk patients may not have previously considered, or been offered PGT-A (Fig. 3). Patients with the highest levels of sperm aneuploidy (e.g., structural or numerical chromosome aberrations, and severe teratozoospermia) will likely benefit the most from PGT-A. PGT-A may also be beneficial in patients with unexplained RPL or RIF with accompanying increased levels of sperm aneuploidy, and also perhaps those with sperm aneuploidy levels that are three-fold or higher compared to controls. Whether PGT-A may improve reproductive outcomes in these patients by reducing spontaneous abortions, time to pregnancy and aneuploid offspring and potentially increasing live birth rates remains inconclusive. Given the mounting evidence, this clearly warrants further investigation (Rubio *et al.* 2009, Murugappan *et al.* 2016, Esquerre-Lamare *et al.* 2018).

Conclusions

Male factors contribute to around 50% of infertility; nonetheless, up to 80% of male infertility remains unexplained. Furthermore, evidence is growing to suggest that sperm aneuploidy may contribute to infertility in select patients groups. Despite recent renewed interest in sperm aneuploidy, it remains to be seen whether assessment of sperm aneuploidy will become a more utilized clinical tool in the arsenal of infertility specialists. There are certainly patients who are at risk of having high levels of sperm aneuploidy, including those with structural and numerical chromosome aberrations, azoospermia, severe OAT and teratozoospermia and those who have experienced unexplained RPL and RIF. Numerous studies have suggested that even a modest (two- to three-fold) increase in sperm aneuploidy seemingly translates to a similar increased frequency of aneuploid embryos, ICSI failure, RIF, RPL and aneuploid offspring. Thus, these patients would probably benefit from sperm aneuploidy screening with appropriate genetic counseling that involved discussion of risk to future offspring, availability of PGT-A, appropriate prenatal screening and testing, and the option of donor sperm in rare cases of very high levels of sperm aneuploidy. Recent technological advances allow us to directly examine the

paternal contribution to meiotic aneuploidies through PGT-A testing (e.g., SNP arrays). Studies are needed to address the parental contribution to aneuploidy and should also focus on the aforementioned male patients at a higher risk of producing aneuploid sperm. If these studies reveal a clinically relevant paternal meiotic contribution to segmental and whole chromosome aneuploidy, routine clinical implementation of sperm aneuploidy should follow, at least for those patients at an increased risk. It seems likely that increased access to reliable and accurate automated sperm aneuploidy scoring systems should reduce costs and technical challenges currently associated with sperm aneuploidy screening, which may increase clinical uptake. Future developments may leverage existing technology to further facilitate the rapid detection of more chromosomes simultaneously in a single cell. Examples include the possible development of novel fluorescent dyes to replace traditional organic fluorochromes, such as quantum dots, which has already been applied to FISH with mixed success (Ioannou *et al.* 2009, Yusuf *et al.* 2011, Hwang *et al.* 2015, Wang *et al.* 2018). Quantum dots are inorganic fluorochromes that are resistant to photobleaching and excite at a wide range of wavelengths but emit light in a very narrow band (controlled by particle size) (Ioannou *et al.* 2009). Thus, they have improved photostability and a greater potential for multiplexing experiments compared to traditional organic fluorochromes. Alternative rapid automated systems such as FISH-flow cytometry has emerged for some FISH applications and may be applicable to sperm aneuploidy, and could be potentially combined with quantum dots (Kapoor *et al.* 2009, Arrigucci *et al.* 2017). Quantum dots and FISH-flow cytometry have been around for many years but have not been rapidly adopted in the field of cytogenetics. It remains to be seen if these will be applicable to sperm aneuploidy testing given the unique characteristics of the sperm cell. Another interesting area of future development lies in microfluidics, which is increasingly being applied to various fields of medicine and has been proposed as an additional tool to sort and analyze sperm for ART (Nosrati *et al.* 2017, Quinn *et al.* 2018, Samuel *et al.* 2018). It is plausible that such microfluidic devices may be able to preferentially identify and select certain sperm characteristics and even possibly reduce the chance of selecting aneuploid sperm for use in ART, although this remains to be tested. Perhaps the most transformative developments lie with the rapid advancements in genomics technology, which may minimize the current technical limitations of sperm aneuploidy screening by FISH. For example, microarray technology is now available in microtiter plate formats. This facilitates high volume, low cost genome-wide analysis, and could potentially be used not only to evaluate whole chromosome aneuploidy in sperm but also segmental aneuploidy, which has a relatively high paternal

contribution. The increased uptake in clinical genome sequencing, gene expression assays and epigenetic tests may eventually lead to additional clinically useful and diagnostic test(s) for male infertility. This will likely lead to a better understanding of the paternal contribution to early embryogenesis, and reveal the underlying genetic basis of unexplained male infertility. This will hopefully lead to more appropriate use of ART with better outcomes, and potentially the development of alternative therapies and lifestyle interventions that may reduce current reliance on ART for treatment. In the near future, it seems likely that genome-wide high-throughput technologies will also be utilized to assess the epigenetic potential of sperm, identify rare genomic variants, CNVs and sperm aneuploidy.

Despite the need to develop new tests and approaches for male factor infertility, the field must exercise caution before it adopts new tests. Any new test or therapeutic intervention needs to be carefully validated with close attention paid to patient phenotypes. The large degree of heterogeneity in male infertility phenotypes is often overlooked, but this is critical to determine which patients may benefit from new tests and therapeutic interventions, and which will not. The successful clinical implementation of any tests will also require a shift in attitude amongst many clinicians and embryologists, away from 'you just need any sperm', to a realization that the paternal genome plays a bigger contribution to early embryogenesis and IVF outcomes than is currently appreciated by many. Assessment of sperm aneuploidy may ultimately lead to improved ART outcomes in select patients. It is clear that research has awoken the silent vessel of the sperm cell; we now know it delivers an epigenetically primed genome that is likely crucial for early embryogenesis. Historically, the data from prenatal studies, products of conception and live births suggest that the paternal contribution to aneuploidy is small relative to the maternal contribution. However, many of these studies were performed before the advent of ICSI, which has enabled many infertile men to have biological children. Current evidence suggests that the paternal contribution to aneuploidy in infertile men may be considerably higher than we currently perceive. Furthermore, meiotic segmental aneuploidies in preimplantation embryos are more frequently paternal in origin, which further increases the paternal contribution to aneuploidy. The time is now to carefully reevaluate the paternal contribution to human aneuploidy in infertile patients through well designed research and clinical studies.

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