

The AZFc region of the Y chromosome: at the crossroads between genetic diversity and male infertility

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TABLE OF CONTENTS

- Introduction
 - Methods
 - Genetic organization of the AZFc sequence: the amplicon as building block
 - AZFc gene content
 - Structural complexity of the AZFc sequence
 - Generating genetic diversity: recombination mechanisms in AZFc
 - Evidence for non-homology based recombination
 - Intrachromosomal homologous recombination
 - Genetic and phenotypical aspects of partial AZFc deletions
 - Methodological difficulties in the study of partial AZFc deletions
 - Y chromosome evolutionary lineages and partial AZFc deletions
 - Partial AZFc deletion association studies: an integrated approach
 - Dynamics of partial AZFc duplications
 - Concluding remarks
-

BACKGROUND: The three azoospermia factor (AZF) regions of the Y chromosome represent genomic niches for spermatogenesis genes. Yet, the most distal region, AZFc, is a major generator of large-scale variation in the human genome. Determining to what extent this variability affects spermatogenesis is a highly contentious topic in human reproduction.

METHODS: In this review, an extensive characterization of the molecular mechanisms responsible for AZFc genotypical variation is undertaken. Such data are complemented with the assessment of the clinical consequences for male fertility imputable to the different AZFc variants. For this, a critical re-evaluation of 23 association studies was performed in order to extract unifying conclusions by curtailing methodological heterogeneities.

RESULTS: Intrachromosomal homologous recombination mechanisms, either crossover or non-crossover based, are the main drivers for AZFc genetic diversity. In particular, rearrangements affecting gene dosage are the most likely to introduce phenotypical disruptions in the spermatogenic profile. In the specific cases of partial AZFc deletions, both the actual existence and the severity of the spermatogenic defect are dependent on the evolutionary background of the Y chromosome.

CONCLUSIONS: AZFc is one of the most genetically dynamic regions in the human genome. This property may serve as counter against the genetic degeneracy associated with the lack of a meiotic partner. However, such strategy comes at a price: some rearrangements

represent a risk factor or a de-facto causative agent of spermatogenic disruption. Interestingly, this precarious balance is modulated, among other yet unknown factors, by the evolutionary history of the Y chromosome.

Key words: Y chromosome / AZFc / partial AZFc deletions / spermatogenesis / male infertility

Introduction

The role of the Y chromosome (Y) as a genomic niche for genes involved in male gamete production has been appreciated since the mid 1970s. In their seminal paper, Luciano Tiepolo and Orsetta Zuffardi convincingly argued for the existence of an azoospermia factor (AZF) in the long arm of the Y (Yq) (Tiepolo and Zuffardi, 1976). According to the authors, this factor represented a key genetic determinant for spermatogenesis since its deletion was associated with a lack of sperm in the ejaculate. Owing to the structural complexity of the Y chromosome, a more precise identification of the AZF took ~30 years to be achieved. Indeed, large-scale molecular screening for Y chromosome microdeletions (i.e. those not identifiable via conventional cytogenetic techniques) revealed that such determinants displayed a tripartite organization (Vogt et al., 1996). Thus, AZFa, AZFb and AZFc were established as the Y chromosome regions regulating spermatogenesis. Subsequent DNA sequencing approaches demonstrated that these regions harbour a total of 12 different genes/gene families (Kuroda-Kawaguchi et al., 2001; Tilford et al., 2001; Skaletsky et al., 2003).

In clinical terms, the importance of the AZF regions is paramount as deletions in these domains are one of the most frequent genetic causes of spermatogenic failure (Simoni et al., 2004). Of the three AZF regions, none has been the source of more vigorous debate than AZFc. If the controversy could be summed up in one word, that would be variability. Indeed, AZFc displays significant variation in terms of genomic architecture across the male population. Yet, the phenotypical consequences for spermatogenesis of some of these variants are unclear. In this regard, the aim of the present review is two-fold. First, the drivers for AZFc genetic diversity will be addressed via a comprehensive analysis of the molecular mechanisms shaping this region. Secondly, the impact of this variation on male fertility will be discussed, with particular care being given to contentious topic of partial AZFc deletions. With this strategy, we hope to present a more unified picture of one of the most divisive aspects of reproductive genetics.

Methods

The present manuscript consists of an extensive narrative review coupling a critical discussion of available studies with newly generated data. Previously published reports were collected and analysed by performing multiple searches of the MEDLINE database between September 2004 and December 2009. Generic search terms for the AZFc region were employed, with reports deemed of significant relevance being included in this manuscript.

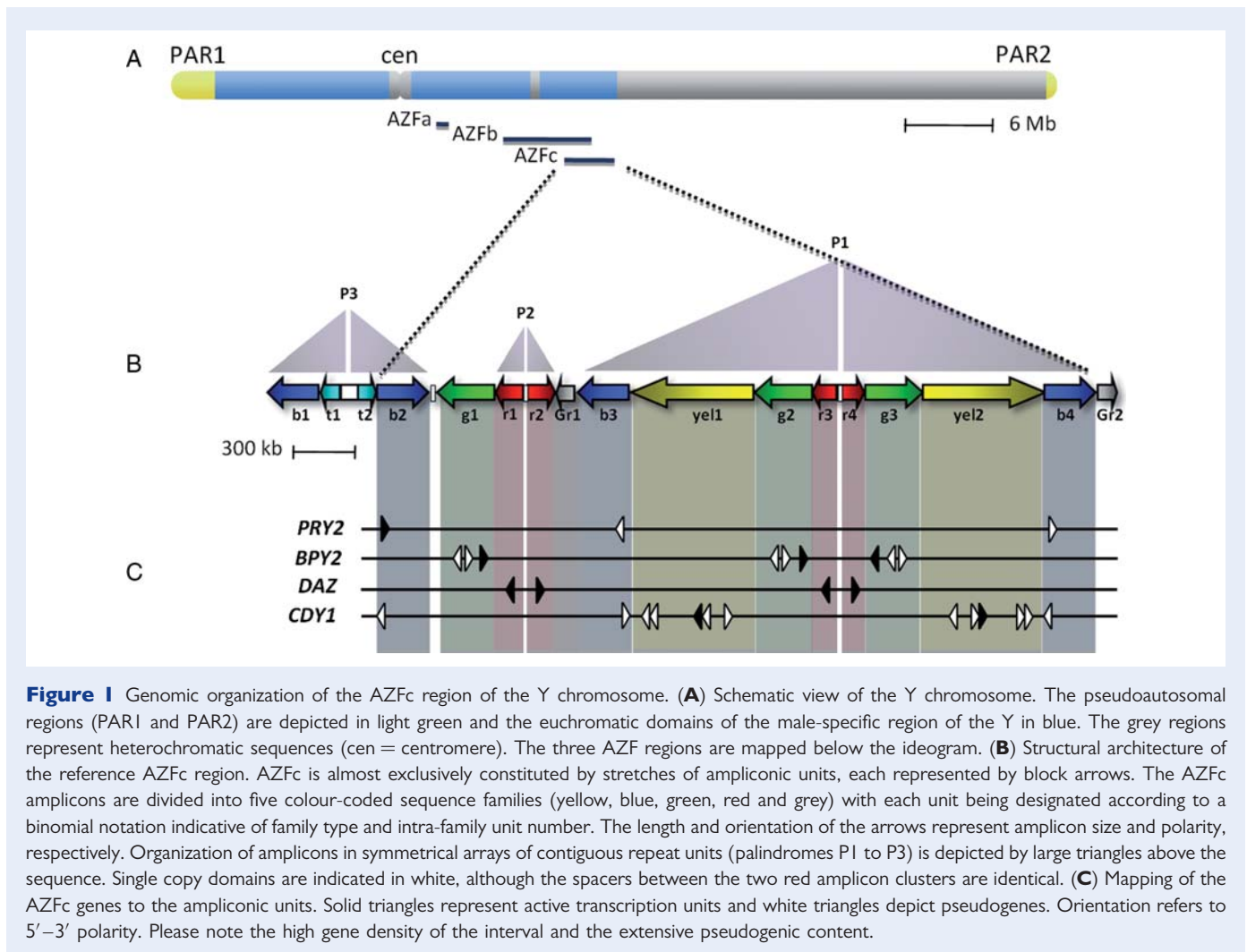
For the integrated evaluation of partial AZFc deletion association studies, 23 reports were selected based on the following criteria: (i) published in international peer-reviewed journals; (ii) listed on National Center for Biotechnology Information databases and accessed using 'AZFc OR gr/gr' as the queried term; (iii) fully written in English; (iv) designed as

association studies selecting men with normal spermatogenic profiles (presumed or verified) as controls and men with spermatogenic impairment as cases; (v) sample size >50 men for either group; (vi) employing sequence tagged site methodology with diagnostic power for partial AZFc deletions; (vii) results sorted by partial deletion pattern; (viii) published between 2003 and 2008. After selection, a careful evaluation of methodological design specificities was performed in order to recalculate, whenever necessary, deletion rates using the most homogenous criteria.

Genetic organization of the AZFc sequence: the amplicon as building block

The three AZF regions were originally defined based on the association of specific spermatogenic disruption phenotypes to precise Y chromosome deletion intervals (Vogt et al., 1996). In the case of AZFc, the observed phenotype was hypospermatogenesis (reduced sperm production). It was later demonstrated that this functional individuality was not reflected at the genomic level, as the proximal end of the AZFc region overlapped with the distal end of AZFb (Repping et al., 2002). Validation for the inherently functional definition of AZFc has been warranted by the development of Y microdeletion screening programmes in the male population. As discussed by several authors, these have produced convincing evidence that AZFc deletions are responsible for marked spermatogenic defects (Vogt, 2005; Krausz and Degl'Innocenti, 2006; Noordam and Repping, 2006; Sadeghi-Nejad and Oates, 2008; and references therein). In fact, men with such deletions display sperm concentrations largely under 1 million sperm/ml (normal value: >20 million sperm/ml; Vogt et al., 1996; Oates et al., 2002; Simoni et al., 2008). Extended clinical experience has also revealed that AZFc deletions account for ~60% of all recorded AZF deletions, further accentuating their significance (Ferlin et al., 2007b; Simoni et al., 2008). Suitably, the genetic disruption of AZFc is generally perceived as a key concern for male reproductive health.

The assembly of the full AZFc sequence represented an undisputed milestone in the study of the Y chromosome. In retrospect, sequencing this 3.5 Mb stretch of Yq euchromatin epitomized the proverbial opening of Pandora's box: AZFc was shown to be constituted by arrays of repeating DNA blocks that made the sequence particularly prone to structural variation in the male population (Fig. 1; Kuroda-Kawaguchi et al., 2001). These blocks were termed amplicons and they correspond to large DNA sequences (range: 115–678 kb) present in multiple copies in AZFc. Appropriately, the closely packed arrangement of the amplicons forms a genetic lattice of unprecedented complexity in the human genome. The AZFc amplicons are organized in sequence families, each displaying a particular genetic signature. Most significantly, intra-family sequence identity levels between amplicon copies exceed 99.9%, making them prime substrates for structural rearrangements. Five different families (colour-coded as



blue, green, red, grey and yellow) map to the reference AZFc sequence, harbouring a total of 13 different ampliconic units (Kuroda-Kawaguchi *et al.*, 2001). The functional relevance of this organization stems from the fact that amplicons accommodate genes required for spermatogenesis. Therefore, changes in amplicon copy number, by implying gene dosage variation, can ultimately resound in phenotypic modifications in the spermatogenic profile.

AZFc gene content

The structural organization of the AZFc transcription units mimics the complexities of the ampliconic architecture. Active copies of four protein-coding gene families map to the AZFc interval: *PRY2*, *BPY2*, *DAZ* and *CDY1* (Fig. 1; Kuroda-Kawaguchi *et al.*, 2001). These genes locate to the blue, green, red and yellow-coded amplicons, respectively, with one transcription unit per amplicon copy. As previously stated, AZFc genes correspond to functional determinants of spermatogenesis, as evidenced by their germline-specific expression and by the fact that their deletion conveys phenotypical consequences only for the gametogenic tissue (Lahn and Page, 1999, 2002; Tse *et al.*, 2003; Stouffs *et al.*, 2004; Huang *et al.*, 2008; Kim *et al.*, 2009). Still, they cannot be considered essential for either spermatogenesis or fertility, as illustrated by the frequent detection of mature sperm in

men with complete AZFc deletions and by the extremely rare cases of natural transmission of AZFc deletions to the progeny (Vogt *et al.*, 1996; Chang *et al.*, 1999; Cram *et al.*, 2000; Saut *et al.*, 2000; Calogero *et al.*, 2002; Gatta *et al.*, 2002; Kuhnert *et al.*, 2004; Xia *et al.*, 2006). However, the dramatic reduction in spermatogenic output associated with complete AZFc deletions is a clear indicator of their biological relevance. Since there are several reviews authoritatively characterizing the AZF genes (for selected reading: Yen, 2004; Reynolds and Cooke, 2005; Vogt *et al.*, 2008), such topic will fall outside the scope of the present manuscript. Succinctly, available evidence suggests that AZFc genes encode for germline-specific functions in: (i) germ cell apoptosis (Stouffs *et al.*, 2001, 2004), (ii) protein ubiquitination (Wong *et al.*, 2002, 2004), (iii) transcriptional regulation coupled to chromatin remodelling (Lahn *et al.*, 2002; Caron *et al.*, 2003) and (iv) transport, storage and translational activation of developmentally regulated transcripts (Collier *et al.*, 2005; Lee *et al.*, 2006; Kee *et al.*, 2009). These have been ascribed to the *PRY2*, *BPY2*, *CDY1* and *DAZ* genes, respectively. It should be noted that the last two families also display autosomal homologues (Dorus *et al.*, 2003; Reynolds and Cooke, 2005). Therefore, some degree of functional redundancy between the Y-borne and the autosomal copies may partially account for the production of mature sperm in AZFc-deleted men.

Structural complexity of the AZFc sequence

The structural complexity of AZFc transcends that of a random assortment of ampliconic units. In fact, amplicons can display a higher-order level of architectural organization: they can be organized in symmetrical arrays of contiguous units. These arrays are designated as palindromes and are defined by a symmetry axis separating two largely identical arms constituted by single or multiple amplicons (Fig. 1; Kuroda-Kawaguchi et al., 2001; Skaletsky et al., 2003). AZFc contains two full palindromes (P1 and P2), as well as the distal end of P3, accounting in total for ~90% of the sequence (Kuroda-Kawaguchi et al., 2001). The exact functional role played the ampliconic/palindromic organization is not fully understood. The current view is that they are an evolutionarily conserved strategy of the Y chromosome striving for maintenance of genetic integrity in the coding domains (Skaletsky et al., 2003; Lange et al., 2009). Considering that AZFc maps to the male-specific region of the Y chromosome (and is therefore bereft of homologous recombination with a chromosome partner), the functional relevance of this model as a counter to the accumulation of deleterious mutations is evident. Appropriately, the multiple gene copies that map to the ampliconic domains may serve both as buffer (via a copy number effect) and correction mechanism (via gene conversion) against the build-up of such events. The establishment of higher-order chromatin configurations arising from the palindromic architecture has also led to some speculation on their involvement in lineage-specific modulation of transcriptional availability and in meiotic sex chromosome inactivation (Vogt and Fernandes, 2003; Yogev et al., 2004; Geoffroy-Siraudin et al., 2007). Yet, experimental evidence to validate either is still scarce.

It can be argued that the most prominent function imputable to the ampliconic organization is the generation of genetic diversity. Seeing that intra-family amplicon copies represent genomic domains of extremely high sequence identity, they potentiate the occurrence of intrachromosomal homologous recombination (Repping et al., 2006; Jobling, 2008; Lange et al., 2009). Available evidence indicates that the scale of this diversity is considerable, as it will be discussed in this review. In fact, AZFc should be regarded as a genetically dynamic chromosomal niche home to frequent large-scale structural rearrangements. These include not only shuffling of existing amplicons (resulting in positional variants), but also changes to the overall number and qualitative state of the sequences. Thus, by acknowledging the extent of this diversity it becomes clear that the structural organization of the reference AZFc sequence corresponds to one out of a multitude of possible genomic states. In the following section, the molecular mechanisms underlying AZFc genetic diversity will be addressed. These will serve as a conceptual premise for a clinically oriented analysis of the phenotypical effects associated with the AZFc variants.

Generating genetic diversity: recombination mechanisms in AZFc

Since the mid 1980s, that polymorphisms in the Yq region later identified as AZFc have been appreciated (Lucotte and Ngo, 1985; Disteche et al., 1986). Yet, a more extensive measure of AZFc

genetic diversity in the Y chromosome population was only established in 2006 (Repping et al., 2006). The latter study demonstrated that AZFc rearrangements were one of the major motifs for large-scale Y structural variation. Such variation stems from a high mutation rate in the interval ($\geq 3.8 \times 10^{-4}$, lower bound) and is illustrated by the detection of 11 different AZFc architectures in 47 chromosomes representing the major evolutionary branches of the Y genealogy (Repping et al., 2006). This rate is particularly striking when compared with the 2.3×10^{-8} single nucleotide substitution rate in the Y chromosome (for 25 year generations). It should be noted that the recorded AZFc mutation rate represents a conservative estimate since it refers solely to changes in the number or order of the reference sequence amplicons. In this regard, more minute differences or even the existence of entirely different amplicon families in the Y genealogy may greatly enhance the scale of diversity. Thus, sequencing AZFc across different Y evolutionary lineages, although a daunting task, may shed new light on the genetic regulation of spermatogenesis.

Given the lack of conventional recombination with a chromosome partner, the use of amplicons as intrachromosomal recombination substrates represents the major adaptive strategy adopted by AZFc to ensure genetic variability (Yen, 2001; Repping et al., 2006; Lange et al., 2009). Appropriately, the high sequence identity recorded between intra-family amplicon copies provides a favourable context for the activation of non-allelic homologous recombination pathways (Sebat et al., 2004; Shaw and Lupski, 2004; Jobling, 2008). Nonetheless, the contribution of non-homology based mechanisms should not be overlooked: AZFc retains evidence at the sequence level for the actions of both homology and non-homology dependent pathways.

Evidence for non-homology based recombination

Even though the molecular origin of the majority of AZFc rearrangements can be imputable to homology-based recombination, the analysis of some variants suggests otherwise (Repping et al., 2004; Ferlin et al., 2005; Hucklenbroich et al., 2005; Lynch et al., 2005; Premi et al., 2007; Balaesque et al., 2008). In these cases, identifying the exact mechanism is a complex task owing to a still limited knowledge on non-homology based pathways and to difficulties in characterizing the variants at the sequence level. Even so, indirect evidence supporting the activation of the non-homologous DNA end joining (NHEJ) pathway can be identified in certain AZFc rearrangements (Repping et al., 2002; Costa et al., 2008; Yang et al., 2008b). Briefly, NHEJ corresponds to an enzyme-based DNA double strand break (DSB) repair pathway promoting the rejoining of two DNA ends via untemplated nucleotide gain or loss at the ligation site (for a review: Lieber, 2008). Since NHEJ is a non-homology based mechanism, it does not require DNA pairing for successful ligation and, consequently, is more frequent in non-duplicated regions (Argueso et al., 2008; Robert et al., 2008). Yet, some repeat-heavy genomic domains seem to potentiate the activation of NHEJ, as observed in the subtelomeric regions (Ribes-Zamora et al., 2007). Furthermore, the processing of the DNA ends can be influenced, in some cases, by the establishment of short terminal homology domains (<25 nucleotides; McVey and Lee, 2008; Pawelczak and Turchi, 2008). The involvement

of NHEJ in AZFc deletions can be inferred from the presence of deletion breakpoints displaying the addition of untemplated nucleotides and flanked by non-homologous sequences. Although the confirmation warranted by breakpoint sequencing is still restricted to a few cases, the analysis of previously published deletion patterns suggests the contribution of NHEJ for some AZFb/AZFb + c deletions (Repping *et al.*, 2002; Costa *et al.*, 2008; Yang *et al.*, 2008b). This is particularly evident in a deletion breakpoint flanked by non-homologous blue and yellow ampliconic sequences (Repping *et al.*, 2002). In this sample, both the fact that the deletion product could not have arisen from homologous recombination and the addition of untemplated nucleotides at the deletion junction are clear hallmarks of the process.

On more general terms, given the extensive opportunity for homology-based recombination provided by the amplicons, it can be envisaged that the contribution of NHEJ (or other non-homologous mechanisms) for AZFc variability may be marginal. Nonetheless, the established practice of interpreting AZFc rearrangements under the auspices of homology-based recombination (usually the most parsimonious model) might further accentuate this bias.

Intrachromosomal homologous recombination

Of the molecular repertoire available to AZFc, intrachromosomal homologous recombination represents the key generator of genetic variability. Recently, an encompassing model for homologous recombination in the palindromic domains has been proposed (Lange *et al.*, 2009). According to this model, recombination is triggered by the generation of a DSB within an amplicon. The occurrence of such lesions are particularly frequent in the male germline, owing to the fact that spermatogenesis requires multiple cell divisions in an oxidative environment depleted of DNA repair enzymes (Crow, 2000; Aitken and Graves, 2002). How this DSB is resolved is the crucial parameter in determining the genetic outcome of the process. If the crossover-dependent pathway is activated, the sequence is modified by the occurrence of a structural rearrangement. Yet, if the non-crossover pathway is selected, modification is restricted to a short sequence that undergoes gene conversion. The balance between the two pathways largely dictates the type and degree of genetic heterogeneity in AZFc.

Gene conversion

Gene conversion corresponds to the unidirectional transfer of genetic material from a donor sequence to a homologous acceptor target (for a review: Chen *et al.*, 2007). The Y chromosome palindromes seem particularly prone to this type of events, as available data points to an estimate of 600 Y–Y converted nucleotides per generation, reflecting a conversion rate of 2.8×10^{-4} per duplicated base per 25-year generation (Rozen *et al.*, 2003). Using the upper limits of mean conversion length (the latest figures point to averages of 300 converted nucleotides per event), this corresponds to at least two distinct Y–Y conversions per generation (Jeffreys and May, 2004; Benovoy and Drouin, 2009). Although the exact molecular mechanisms for gene conversion in the Y chromosome remain elusive, there is little doubt of its relevance as a major modulator of AZFc genetic plasticity. The analysis of amplicon-specific genetic tags (generally designated as

sequence family variants) has revealed not only that these events are extremely frequent, but also that the majority of them are selectively neutral in terms of reproductive fitness (for selected reading: de Vries *et al.*, 2002; Repping *et al.*, 2003a; Machev *et al.*, 2004; Zhang *et al.*, 2006; Lin *et al.*, 2007; Navarro-Costa *et al.*, 2007; Giachini *et al.*, 2008; Stouffs *et al.*, 2008). Interestingly, our results indicate that conversion patterns reflect to some extent differences between Y chromosome evolutionary lineages (Navarro-Costa *et al.*, 2007). This suggests that gene conversion may have played a role in the establishment of lineage-specific AZFc genotypes. The consequences, if any, of these evolutionary variants for the functional regulation of the AZFc genes remain to be assessed.

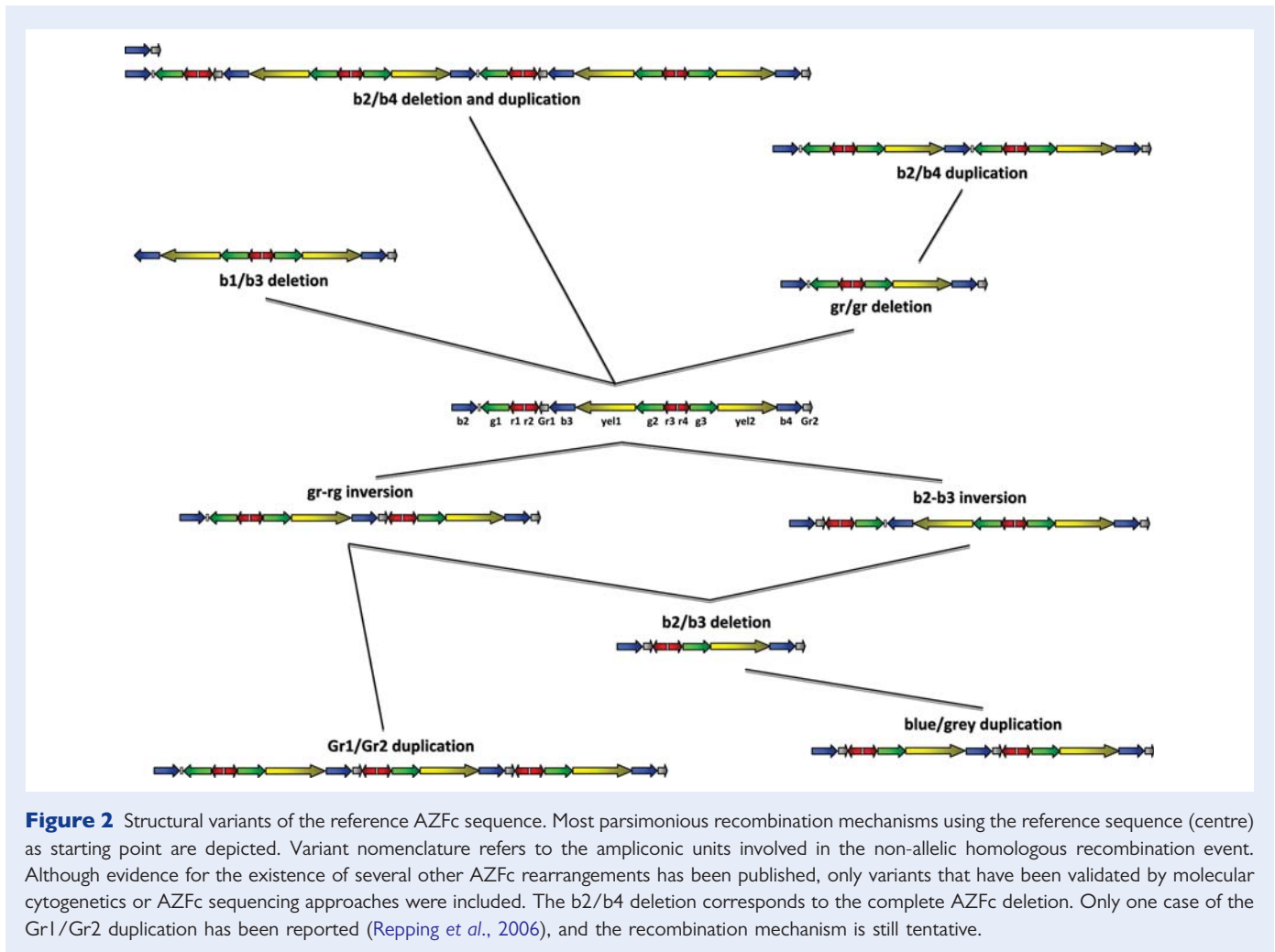
Gene conversion has been proposed to serve as a genetic correction mechanism for ampliconic genes (Skaletsky *et al.*, 2003; Lange *et al.*, 2009). Under this model, conversion displays a directional bias favouring the replacement of defective coding sequences with unaffected templates. Recent studies have revealed the existence of highly directional conversion, arguing against the classic view of a sequence having equal probability of serving as donor or acceptor (Szostak *et al.*, 1983; Bosch *et al.*, 2004; Webster *et al.*, 2005; Dreszer *et al.*, 2007). Seeing that the triggers for gene conversion have only recently begun to be unravelled (Chuzhanova *et al.*, 2009), the drivers for such directionality remain elusive. DNA sequence motifs are bound to play a crucial role in the process, yet how the functional status of the sequence could influence one direction over the other is unclear. Thus, for the time being, proposing a functional directionality for AZFc conversions corresponds to a speculative effort. With or without bias, AZFc conversions are a clearly bi-directional process as demonstrated in the *CDY1* sequence (Rozen *et al.*, 2003). This means that the occurrence of diseases associated with the replacement of active gene copies with defective sequences have to be regarded as a trade-off in the gene correction model (Bischof *et al.*, 2006). Certainly, the extensive array of AZFc pseudogene sequences can serve as fertile ground for such. It can be argued that the loss of specific *CDY1* sequence variants being significantly more frequent in infertile males than in controls partially reflects this effect (Machev *et al.*, 2004).

In light of these considerations, gene conversion should be regarded as a significant driver of AZFc variability, playing until clearly demonstrated otherwise, a largely unpredictable role in the functional regulation of the interval.

Deletions and other structural rearrangements

The activation of the crossover-dependent pathway to resolve a DSB in the ampliconic domains results in a structurally rearranged chromosome. The type of the rearrangement depends on whether the sequences display direct or inverted polarity and on the genetic identity of the recombination intermediates (sister or non-sister chromatids).

If the process involves intrachromatid intermediates displaying inverted sequence polarity, it results in inversions (Lupski, 1998). AZFc seems particularly prone to these rearrangements: an evident bias for inversions is detected both in the main evolutionary branches of the Y genealogy and in the minimum-mutation history of AZFc (Repping *et al.*, 2006). Even though inversions do not seem to convey any specific functional consequences *per se*, they can serve as substrates for the occurrence of AZFc deletion/duplications



(Fig. 2). Fittingly, the role of inversions in AZFc is generally perceived as more of a generator of architectural diversity than of a phenotype modifier. It has been demonstrated that inversions to the reference AZFc sequence are required as substrates for some deletions (Fernandes et al., 2004; Repping et al., 2004), still it is currently unknown whether they can actually increase the likelihood of subsequent rearrangements. Seeing that in some populations partial AZFc deletions have been shown to favour the occurrence of complete deletions (Zhang et al., 2007; Lu et al., 2009), the existence of an analogous effect for inversions is a tangible possibility. More recently, the functional consequences of an interchromatid recombination between inverted Y chromosome sequences have been discussed (Lange et al., 2009). This mechanism results in the formation of an isodicentric Y chromosome, a chromosomal variant associated with severe clinical consequences ranging from spermatogenic failure to sex reversal. The involvement of the AZFc palindromes in the generation of isodicentric Y chromosomes was not only confirmed but also identified as a risk factor for the development of female sexual features due to increased mitotic instability.

Deletions represent one of the most functionally relevant structural rearrangements in AZFc (Fig. 2). Under normal conditions, these are essentially the product of homologous recombination between sequences sharing identical polarity (Lupski and Stankiewicz,

2005; Turner et al., 2008). Nevertheless, in populations subjected to high levels of natural background radiation, less extensive AZFc deletions (arising from the frequent occurrence of DNA lesions) are relatively commonplace (Premi et al., 2009). The clinical significance of AZFc deletions becomes evident when considering that they not only represent one of the most frequent copy-number variants in the human genome, but also that they can decisively impact male fertility (Vogt, 2004; Krausz and Degl'Innocenti, 2006; Noordam and Repping, 2006; Tyler-Smith, 2008). As previously discussed, AZFc was first defined based on the phenotype associated with its complete deletion (Vogt et al., 1996). The latter arises from recombination between the terminal b2 and b4 amplicons (b2/b4 deletions) and leads to a dramatic reduction of spermatogenic output (Fig. 2; Kuroda-Kawaguchi et al., 2001). Nevertheless, as a result of the intricate sequence organization of the interval, AZFc deletions may be less extensive and involve the internal amplicon units; such rearrangements are referred to as partial deletions. Owing to their high prevalence in the male population, these are the focus of particular interest among the andrological community. Interestingly, despite a barrage of published reports, the debate still rages as to whether or not they represent a risk of male infertility. Accordingly, the following section will deal in detail with the divisive topic of partial AZFc deletions.

Genetic and phenotypical aspects of partial AZFc deletions

Unlike complete AZFc deletions, which are consensually regarded as a causative agent of spermatogenic disruption, the clinical consequences of partial deletions are far more difficult to ascertain. This difficulty arises from significant phenotypical heterogeneity, as illustrated by the frequent cases of normal sperm counts and/or deletion transmission to the progeny in affected individuals (for illustrative examples: Hucklenbroich *et al.*, 2005; Lynch *et al.*, 2005; de Carvalho *et al.*, 2006; Zhang *et al.*, 2006; Navarro-Costa *et al.*, 2007; Giachini *et al.*, 2008). The heterogeneities recorded for partial AZFc deletions reflect on two different levels: (i) when comparing between different partial deletion patterns and (ii) when analysing the phenotypical diversity associated with a single pattern. Regarding the first, it is acknowledged that although some deletion types may represent a male infertility risk, others do not (Fernandes *et al.*, 2002; Repping *et al.*, 2003b; Fernandes *et al.*, 2004; Repping *et al.*, 2004; Balaesque *et al.*, 2008). As for the second, a striking variation in spermatogenic profiles has been documented even for partial deletion patterns presumably conveying an infertility risk (Lynch *et al.*, 2005; Giachini *et al.*, 2008; Yang *et al.*, 2008c; Krausz *et al.*, 2009). Variation in these cases corresponds to the full range of male gametogenic phenotypes as defined in terms of sperm number/concentration, morphology and motility. In light of this tremendous plasticity, identifying robust genotype–phenotype correlations is particularly complex. This is evidenced by the multitude of conflicting reports on the effects of partial AZFc deletions on spermatogenesis (for an extensive list, please consult Table 1).

In this section, a critical view on previously published partial AZFc deletion association studies will be undertaken. The objective of this analysis is to present a unified picture based on a pondered evaluation of the specificities of the selected data sets. By carefully curtailing some aspects of inter-study heterogeneity, this approach may accentuate biological similarities and ultimately resound in novel insight on the clinical consequences of partial AZFc deletions.

Methodological difficulties in the study of partial AZFc deletions

As extensively discussed in several reviews, three main partial AZFc deletion patterns have been identified: the b1/b3, b2/b3 and gr/gr deletions (for selected reviews: Vogt, 2004; Krausz and Degl'Innocenti, 2006; Noordam and Repping, 2006; Sadeghi-Nejad and Farrokhi, 2007). These remove between 1.6 and 1.8 Mb of the region and reduce the overall dosage of the AZFc gene families (Fig. 2). Besides these three, several other deletion types have been reported. Yet, restricted numbers of identified cases still limit their characterization (Ferlin *et al.*, 2005; Hucklenbroich *et al.*, 2005; Lynch *et al.*, 2005; Premi *et al.*, 2007; Balaesque *et al.*, 2008). Such limitation is equally extensible to b1/b3 deletions, as attested by their low frequency in the sampled populations (Repping *et al.*, 2003b; Hucklenbroich *et al.*, 2005; Lynch *et al.*, 2005; Stouffs *et al.*, 2008). Similar to that observed in complete AZFc deletions, men with partial deletions do not seem to display any distinctive clinical or hormonal features besides those associated with spermatogenic deregulation. Nonetheless, although a previous association between

gr/gr deletions and increased risk of testicular germ cell tumour has not been replicated in a geographically well-defined population, it is still too premature to draw definitive conclusions on this topic (Nathanson *et al.*, 2005; Ferlin *et al.*, 2007a).

Non-allelic homologous recombination between internal AZFc amplicons is considered the main mechanism responsible for the generation of partial AZFc deletions. Accordingly, partial deletion types are defined based on the identity of the amplicon units involved in the recombination event. Since the gr/gr deletion displays a large recombination target (the green–red–red amplicon blocks), genetic heterogeneity in this deletion type is further accentuated by variation in breakpoint localization. This heterogeneity may express itself in functional terms if, as some authors suggest, the different AZFc gene copies may vary slightly in regulation (Fernandes *et al.*, 2002; Machev *et al.*, 2004; Ferlin *et al.*, 2005; Giachini *et al.*, 2005). Nevertheless, even though an amplicon-specific characterization of gr/gr deletion products (a process designated as partial deletion sub-typing) confirms genotypical diversity, we have been unable to establish definitive genotype–phenotype correlations for these sub-types (Navarro-Costa *et al.*, 2007). Indeed, the similar distribution of deletion sub-types between men with normal and abnormal sperm parameters suggests this degree of variation is insufficient to explain the considerable phenotypical heterogeneity attributed to partial deletions (Krausz *et al.*, 2009). In addition, although it has been hypothesized that the autosomal copies of the *DAZ* and *CDY1* genes can exert some degree of modulation on the phenotypical outcome of such deletions, no evidence to substantiate this claim has yet been recorded (Giachini *et al.*, 2005; Chen *et al.*, 2009). However, a recent indication that the germline DNA demethylation pattern of the *DAZ* gene is under stringent control (Navarro-Costa *et al.*, unpublished results) suggests that epigenetic disturbances in AZFc may be a likely phenotype modulator.

Regarding the functional consequences of partial AZFc deletions, published reports veer between those detecting an association with spermatogenic failure (albeit in the form of a risk factor and not as causative agent) and those that do not. Several authors have already discussed in general terms the theoretical bases for inter-study inconsistencies in the outcome of genetic association tests. Most point out that initial associations tend not to be replicated by subsequent reports (Ioannidis *et al.*, 2001; Vieland, 2001; Trikalinos *et al.*, 2004). In the majority of cases, heterogeneity increases in parallel with the publication of additional studies, until an adequately powered meta-analysis ultimately rebukes the initial association. It has been argued that such heterogeneities stem more from methodological issues than from actual biological differences between the queried populations (Colhoun *et al.*, 2003). This matter is particularly pressing in the context of partial AZFc deletions since sample inclusion criteria vary considerably between reports. Several studies use normozoospermic men as controls, others use fertile men with unknown spermatogenic status and some even use a mix of both. Seeing that partial AZFc deletions have been linked to spermatogenic impairment, the use of normozoospermic men as controls is a more appropriate option. Yet, it should be noted that some authors have identified an association between partial deletions and male infertility despite normal spermogram parameters (Lynch *et al.*, 2005; Wu *et al.*, 2007). Variations in selection criteria are also patent in the cases group. Although most of the published reports use reduced sperm concentration (irrespective of other spermogram data) as the key

Table 1 Main study parameters of the selected case-control reports on the association of *gr/gr* deletions with spermatogenic disruption/male infertility.

Pub. date	Authors	Population	Controls			Cases				Del. confirmation ^c	Gene dosage	Y haplogroup matching ^d	Other remarks
			Total n	Tested for NZ ^a	<i>gr/gr</i> del.	Stated exclusion criteria	Total n	Sperm conc. distrib. ^b	<i>gr/gr</i> del.				
Association													
2003	Repping <i>et al.</i>	Dutch	148	100%	0.0%	– Abnormal karyotype – AZF deletions – Other causes for spermatogenic failure	246	n/a	3.7%	FISH	Yes	Yes	None
2004	de Llanos <i>et al.</i>	Spanish	232	15%	0.0%	– Abnormal karyotype – AZF deletions	283	Azo: 23%; <5 M: 77%	4.2%	None	No	No	Exclusion criteria not tested for all samples
2005	Ferlin <i>et al.</i>	North Italian	263	100%	0.4%	– Abnormal karyotype – AZF deletions – Obstructive azoospermia	337	Azo: 22%; <20 M: 78%	4.7%	None	No	No	Cases display an over-estimation of del. rates due to inclusion of <i>gr/gr</i> deleted siblings
2005	Giachini <i>et al.</i>	Italian	189	100%	0.5%	– Abnormal karyotype – AZF deletions – Severe andrological abnormalities	150	n/a	5.3%	Gene dosage PCR	Yes	No	41% of cases with mild abnormal andrological findings (varicocele, monolateral cryptorchidism and/or recurrent infections)
2005	Lynch <i>et al.</i>	Australian	234	57%	0.4%	Not stated Abnormal karyotype	546 (ART) ^e 607 (databank) ^f	Azo: 21%; <20 M: 79%	4.8% 3.8%	None	No	No	– Recalculated rates (to remove normozoospermic infertile males and men with complete AZF deletions) – Idiopathic infertility not confirmed in 25% of the ART cases
2007	Navarro-Costa <i>et al.</i>	Portuguese	300	0%	1.0%	– Abnormal karyotype – AZF deletions – Other causes for spermatogenic failure	300	Azo: 30%; <10 M: 70%	5.0%	DNA blotting	No	No	None
2007	Yang <i>et al.</i>	Han Chinese	262	100%	5.3%	– Abnormal karyotype – AZF deletions – Obstructive azoospermia – Other causes for spermatogenic failure	414	Azo: 59%; <10 M: 41%	10.6%	None	No	Yes	None
2008	Yang <i>et al.</i>	Han Chinese	634	100%	5.1%	Same as above	1286	n/a	10.0%	None	No	Yes	Possible inclusion of samples from a previous study (Yang <i>et al.</i> , 2007)
2008	Giachini <i>et al.</i>	Central Italian	487	100%	0.4%	– Abnormal karyotype – AZF deletions – Non-central Italian origin – Varicocele grade ≥ 2 – Other causes for spermatogenic failure	556	Azo: 13%; <20 M: 87%	3.2%	Gene dosage PCR	Yes	Yes	– 30% of samples had been included in a previous study (Giachini <i>et al.</i> , 2005) – 49% of cases with mild abnormal andrological findings (unilateral varicocele grade <2 and/or previous infections)

No association

2004	Machev <i>et al.</i>	French	189	2%	2.1%	<ul style="list-style-type: none"> Abnormal karyotype AZF deletions Obstructive azoospermia 	254	<0.1 M: 37%; <5 M: 63%	3.9%	<ul style="list-style-type: none"> Gene dosage PCR FISH 	Yes	No	<ul style="list-style-type: none"> Recalculated rates (to remove normozoospermic infertile males) Admixed population
2004	Hucklenbroich <i>et al.</i>	German	170	100%	1.8%	<ul style="list-style-type: none"> Abnormal karyotype AZF deletions Non-idiopathic oligo/azoospermia 	348	Azo: 18%; <20 M: 82%	4.0%	None	No	No	Statistical difference only for b2/b3 del
2006	Ravel <i>et al.</i>	Admixed	181	9%	3.3%	<ul style="list-style-type: none"> Cytogenetic abnormalities AZF deletions Known causes of infertility 	192	All <2 M	2.1%	None	No	No	Cases with mixed ethnic background
2006	Fernando <i>et al.</i>	Sri Lankan	87	100%	4.6%	Not stated	89	Azo: 82%; <20 M: 18%	4.5%	None	No	No	Recalculated rates (to remove men with complete AZF dels)
2006	Zhang <i>et al.</i>	Han Chinese	89	100%	10.1%	Not stated	87	Azo: 56%; <20 M: 44%	10.3%	Gene dosage PCR	Yes	Yes	None
2006	Carvalho <i>et al.</i>	Brazilian	122	0%	2.5%	<ul style="list-style-type: none"> Abnormal karyotype AZF deletions Obstructive azoospermia 	110	All Azo.	4.5%	None	No	No	Recalculated rates (to select fertile men as controls)
2006	de Carvalho <i>et al.</i>	Japanese	56	0%	33.9%	Not stated	78	Azo: 63%	23.9%	None	No	No	<ul style="list-style-type: none"> Population effect: all dels. in haplogroup D2 Controls with unknown fertility status 37% of cases with no biopsy data Recalculated rates (to remove infertile males with complete AZF dels. and no biopsy data)
2007	Zhang <i>et al.</i>	Han Chinese	280	32%	7.1%	Not stated	296	Azo: 73%; <20 M: 27%	8.1%	Gene dosage PCR	Yes	Yes	Possible inclusion of samples from a previous study (Zhang <i>et al.</i> , 2006)
2007	Wu <i>et al.</i>	Han Chinese	248	35%	7.7%	<ul style="list-style-type: none"> Abnormal karyotype Obstructive azoospermia Cryptorchidism Secondary sterility 	218	Azo: 66%; <20 M: 34%	6.9%	None	No	No	<ul style="list-style-type: none"> Statistical difference only for b2/b3 del. Recalculated rates (to remove men with complete AZF dels. and cases without spermatogenic impairment)
2007	Lin <i>et al.</i>	Han Chinese (in Taiwan)	107	0%	2.8%	Not stated	142	All >0 and <20 M	7.0%	<ul style="list-style-type: none"> Gene dosage PCR DNA blotting 	Yes	Yes	Despite the very distinct gr/gr del. rates, statistical difference was only recorded for the gr/gr duplic.
2007	Lardone <i>et al.</i>	Chilean	77	40%	2.6%	<ul style="list-style-type: none"> AZFc deletions Hypogonadotropic hypogonadism Hypoandrogenism Chronic diseases 	93	Azo: 67%; <5 M: 33%	2.2%	None	No	No	<ul style="list-style-type: none"> Recalculated rates (to remove men with complete AZF dels) Confounding factors in controls

Continued

Table I Continued

Pub. date	Authors	Population	Controls			Cases Stated exclusion criteria	Total n	Sperm conc. distrib. ^b	gr/gr del.	Del. confirmation ^c	Gene dosage	Y haplogroup matching ^d	Other remarks
			Total n	Tested for NZ ^a	gr/gr del.								
2007	Imken et al.	Moroccan	176	43%	4.0%	Not stated	123	Azo: 36%; OAT: 64%	5.7%	None	No	No	Recalculated rates (to remove men with complete AZF dels. and cases with normal sperm counts)
2008	Stouffs et al.	Admixed	394	71%	3.0%	– Abnormal karyotype – AZF deletions – Obstructive azoospermia	187	Azo: 24%; <20 M: 76%	4.3%	None	No	No	Population: Caucasian males living either in Belgium or in the Netherlands
2008	Ravel et al.	Admixed	193	57%	6.7%	– Abnormal karyotype – AZF deletions – Obstructive azoospermia – Other causes for spermatogenic failure	364	Azo: 32%; <20 M: 68%	4.1%	– Gene dosage PCR – DNA blotting	Yes	No	– Cases with mixed ethnic background – Includes samples from a previous study (Ravel et al., 2006)

Values in bold highlight gr/gr deletion rates in cases and controls.

^aPercentage of normozoospermic controls (NZ), as confirmed by sperm parameters. In the remaining individuals no semen analysis was performed.

^bSperm concentration distribution. Azo, Azoospermia; OAT, oligoasthenoteratozoospermia; M, million sperm/ml.

^cDeletion confirmation (of the PCR result) via complementary molecular cytogenetics, DNA blotting or gene dosage methods.

^dAs confirmed by haplogroup typing in cases and controls.

^eSample set corresponds to consecutive infertile males with idiopathic spermatogenic failure enrolled in an ART programme.

^fSample set corresponds to a selection of infertile men with spermatogenic failure retrieved from the Monash Male Infertility database.

n/a, not available.

parameter, the exact cut-off value varies. Additionally, the inclusion of variable fractions of azoospermic men and the presence of secondary clinical features related (or not) to the infertility phenotype also favour the observed heterogeneities.

On a more general note, it is obviously advantageous in terms of association strength to enforce stringent selection criteria in order to ensure a greater homogeneity of the tested population. Nevertheless, even if factors directly attributable to study design are largely controlled, the outcome of partial AZFc deletion association studies may vary greatly, as illustrated by two reports published on Han Chinese men.

Illustrating heterogeneity: the Han Chinese results

In 2007, two association studies on the effects of partial AZFc deletions on spermatogenesis were published weeks apart in two well-renowned journals (Wu *et al.*, 2007; Yang *et al.*, 2008c). What made these reports particularly interesting was that both tested the same population (Han Chinese men) using similar analytical tools. Seeing that both were published almost simultaneously, possible influencing effects were unlikely. In this regard, they present an ideal opportunity to assess the intrinsic variability associated with this type of study, even more so since their conclusions could not have been more disparate: although Yang *et al.* identified an association with spermatogenic failure in *gr/gr* but not in *b2/b3* deletions, an opposite association was recorded by Wu *et al.* What could explain such dramatic differences in two similar reports?

Although both studies analysed the Han Chinese ethnic group, subjects were recruited from two different areas (Eastern and

South-western China) distancing ~ 1500 km. Whereas this may seem trivial, geographical differences can exert a significant effect on the outcome of Y chromosome association tests. The notion of Y haplogroup is central to this discussion. A chromosomal haplogroup refers to a group of chromosomes sharing a similar combination of binary allelic states at multiple loci (for a review: Jobling and Tyler-Smith, 2003). Such a combination is designated as a haplotype and serves as a genetic identifier. The lack of a recombination partner in the male-specific region of the Y implies that these allelic states are passed largely intact from generation to generation, thus representing an ideal tool for the construction of a Y phylogeny (Y Chromosome Consortium, 2002). In fact, thanks both to social and evolutionary factors, the Y haplogroups display very specific patterns of geographical clustering, allowing the definition of male populations based on haplogroup composition (Fig. 3; Seielstad *et al.*, 1998; Tyler-Smith, 2008).

It has been demonstrated that *gr/gr* deletions are fixed in haplogroups D2 and Q1, and *b2/b3* deletions in haplogroup N, although a proximal AZFc deletion is presumably fixed in haplogroup C3* (Repping *et al.*, 2003b; Fernandes *et al.*, 2004; Repping *et al.*, 2004; de Carvalho *et al.*, 2006; Zhang *et al.*, 2007; Balaesque *et al.*, 2008). Since these Y lineages are significantly prevalent in several populations, it is considered that the deletion's deleterious effect on fertility has been countered by lineage-specific compensatory factors. Thus, the overall detection of an association between partial AZFc deletions and spermatogenic impairment for a given population depends on its haplogroup composition, particularly on the frequency of these deletion-fixed lineages, as they will dilute a theoretical risk present in other

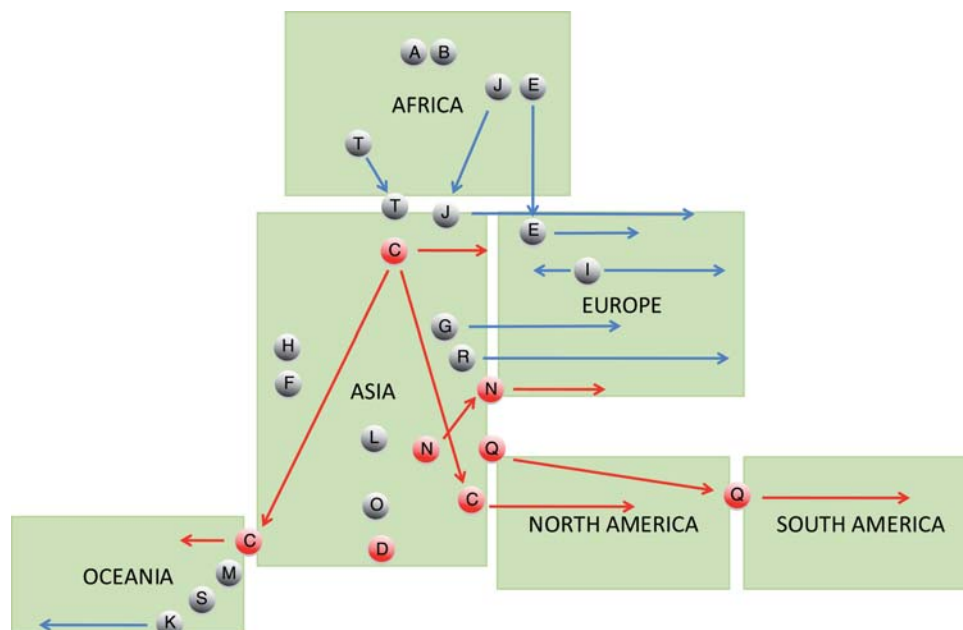


Figure 3 World distribution of Y chromosome haplogroups. The major evolutionary lineages are depicted by the A to T nomenclature of the Y Chromosome Consortium, with the position of the identifiers corresponding to the geographic region where the haplogroup originated or where prevalence is high. Haplogroup P* due to its minimal distribution is not shown. Arrows represent an abridged outlook into the major Y haplogroup migration events. Red identifiers indicate the fixation of partial AZFc deletions in some of the lineages constituting the haplogroup. Their extensive dissemination suggests that partial AZFc deletions in these evolutionary backgrounds do not convey phenotypical consequences for male fertility.

haplogroups. In the specific case of the two Han Chinese studies, although Yang *et al.* recorded statistically similar b2/b3 deletion rates of ~6 and 3% in controls and cases, respectively; such rates were of ~3 and 9% in the Wu *et al.* report. Seeing that b2/b3 deletions are fixed in haplogroup N with no apparent effects on fertility, a variable frequency of this haplogroup in the two populations may partly account for the recorded differences. Indeed, Yang *et al.* demonstrated that ~80% of their recorded b2/b3 deletions belonged to haplogroup N. Given such a high contribution of this haplogroup (where the deletion conveys no functional meaning) to the b2/b3 deletion pool, the lack of association with spermatogenic failure reported in this study is not surprising. On the other hand, the study of Wu *et al.* did not include full haplogroup typing. It can be speculated that their studied population exhibited lower frequencies of haplogroup N and a concomitant increase of other Y lineages where the effects of the b2/b3 deletion could indeed be associated with spermatogenic failure. Actually, this geographic effect may not only explain the divergent results observed for b2/b3 deletions but also account for discrepancies in gr/gr deletion rates. These were similar between controls and cases in the Wu *et al.* study (~8 and 7%, respectively), but varied significantly in that of Yang *et al.* (~5 and 11%). In the study reporting an association, 31% of gr/gr deletions occurred in haplogroup Q1, where this deletion is fixed. Since for this particular haplogroup, no significant differences in deletion rates were recorded between cases and controls, as expected, the detected association must have stemmed from the contribution of other haplogroups. A hypothetical increase in haplogroup Q1 prevalence in East China, by masking the actual association present in the other haplogroups, could thus explain the lack of association reported by Wu *et al.*

The Han Chinese reports serve as a prime example of how slight differences in study settings can empathically alter the end result of an association test. Even though some minor heterogeneities in selection criteria were patent, the contrasting results are most likely the consequence of geographical specificities of the tested populations. In this regard, population dynamics have to be considered a prominent driver for the recorded variability in AZFc association studies. Under such an assumption, the impact of partial AZFc deletions on male (in)fertility will vary depending on the evolutionary lineage of the chromosome where they occur.

Y chromosome evolutionary lineages and partial AZFc deletions

The influence exerted by Y evolutionary lineages on partial AZFc deletion dynamics may transcend that recorded in the deletion-fixed haplogroups. In fact, other haplogroups seem to be able to modulate the degree of spermatogenic disruption associated with partial deletions. Appropriately, haplogroups C and DE* are significantly more represented in gr/gr deleted Chinese men with spermatogenic failure than in equally deleted normozoospermic males (Yang *et al.*, 2008c). It should be noted that this effect was not detected for b2/b3 deletions nor it was replicated in a different regional context. In fact, Krausz *et al.* (2009) found no evidence for distinct haplogroup distributions when analysing gr/gr deleted men with varying spermogram parameters recruited from European centres. Nonetheless, our previous results suggest that Y lineages, as defined at the more detailed haplotype level, might influence the phenotypical expression of

identical gr/gr deletion genotypes (Navarro-Costa *et al.*, 2007). If suitably validated, this observation may shed new light on possible functional specificities of the different Y lineages.

Additionally, susceptibility to the occurrence of partial AZFc deletions seems to vary between Y haplogroups. Haplogroups C and DE* have been associated with increased rates of partial deletions in the Chinese population (Yang *et al.*, 2008a). Thus, these haplogroups convey an increased propensity for the occurrence of partial AZFc deletions, as well as for spermatogenic failure whenever they are present. Furthermore, haplogroups C and G are also particularly prone to partial deletions affecting the proximal AZFc domain (Balaresque *et al.*, 2008). An analogous effect but regarding the complete AZFc deletion has been observed on a precisely defined European population (North Italy): a significant over-representation of haplogroup E was identified in deleted men (Arredi *et al.*, 2007). Inversely, some suggestions for a protective effect have been proposed for haplogroups O3*, J and R (Arredi *et al.*, 2007; Balaresque *et al.*, 2008; Yang *et al.*, 2008a).

Globally, the published results illustrate the complex relationship between Y evolutionary lineages, AZFc rearrangements and spermatogenic phenotype. It seems safe to assume that the evolutionary history of the Y chromosome may modulate the phenotypical expression of partial AZFc deletions via the lineage-specific acquisition of genetic factors. Yet, until the nature of such auxiliary factors and their actual contribution to the spermatogenic network are fully understood, our knowledge of the intersection between Y evolution and male infertility is restricted to the less than unified picture presented by the currently available association studies.

Partial AZFc deletion association studies: an integrated approach

Different strategies have previously been employed in order to gain a more cohesive outlook on partial AZFc deletion association studies. Using a meta-analytic approach, two independent groups have identified a significant association between gr/gr deletions and male infertility/spermatogenic disruption (Tuttelmann *et al.*, 2007; Visser *et al.*, 2009). Yet, seeing that the available association studies display remarkable variation in methods, sample selection criteria and geographical origin (Giachini *et al.*, 2008), a comparative analysis of the published reports is required if the bases for the inter-study outcome differences are to be identified. To characterize this aspect, we selected 23 association studies based on the criteria stated in the methods section. Studies were divided between those reporting an association between gr/gr deletions and spermatogenic disruption/male infertility (9 studies) and those that did not (14 studies), followed by a pondered analysis of methodological design specificities. The latter ensured that deletion rates were recalculated, whenever necessary, so as to ensure greater inter-study homogeneity. For a summary of the main parameters of the selected reports, please consult Table 1.

gr/gr deletions

Globally, ~7500 men (2560 controls and 4961 patients) were analysed in the studies reporting an association compared with ~5000 (2188 controls and 2867 patients) in those that did not. An interesting observation pertains the publication year of the reports: for the first

3 years starting from initial identification of the *gr/gr* deletion, five independent studies reported an association (Repping *et al.*, 2003b; de Llanos *et al.*, 2005; Ferlin *et al.*, 2005; Giachini *et al.*, 2005; Lynch *et al.*, 2005) against only two that did not (Machev *et al.*, 2004; Hucklenbroich *et al.*, 2005). Nevertheless, from 2006 onwards this balance shifted significantly to 4 (Navarro-Costa *et al.*, 2007; Giachini *et al.*, 2008; Yang *et al.*, 2008a, c) against 12 (Carvalho *et al.*, 2006; de Carvalho *et al.*, 2006; Fernando *et al.*, 2006; Ravel *et al.*, 2006; Zhang *et al.*, 2006; Imken *et al.*, 2007; Lardone *et al.*, 2007; Lin *et al.*, 2007; Wu *et al.*, 2007; Zhang *et al.*, 2007; Stouffs *et al.*, 2008; Ravel *et al.*, 2009). The main difference between pre-2006 and post-2006 studies was the geographic origin of the sampled populations. The first wave of studies was mostly conducted in European populations while the latter was more Asian-centric. In fact, when analysing sample geographic origin, six of the nine studies reporting an association were conducted on non-admixed European populations, whereas this figure drops to 2 out of 14 in reports indicating no association. Fittingly, the regional origin of the tested population is a major factor modulating deletion frequency: in studies where an association was reported, *gr/gr* deletion rates in controls were under 1% except in the two non-European populations (5.1 and 5.3% for Asian sample sets). This was equally reflected in the patient group, as deletion frequencies were of ~4.5% for Europeans against 10.0 and 10.6% for studies conducted on Asian populations. Interestingly, the deletion frequency in the control group largely dictated the detection of the association. The range for this parameter was 0–5% in studies reporting an association compared with 1.8–33.9% in those that did not. As before, a population geographical origin effect was clearly observable: deletion rates in controls were similar to those recorded in the patient group for the African and Asian populations (bar the Taiwanese), while the South American and European populations had lower rates that were nevertheless insufficient for the establishment of an association.

The observed regional variability can be explained by population-specific genetic differences in Y chromosome constitution that need to be taken into consideration when performing AZFc deletion diagnosis. Indeed, despite recent developments on microarray-based diagnostic platforms (Osborne *et al.*, 2007), the PCR amplification of a genomic marker initially identified in the reference AZFc sequence remains the most reliable and cost-effective genetic test for *gr/gr* deletions (Repping *et al.*, 2003b). This sequence belongs to haplogroup R, a predominant lineage in European populations (Brion *et al.*, 2005). In other lineages the absence of this marker may not be synonymous with deletion, as previously observed in haplogroup J (Machev *et al.*, 2004), or the deletion may not convey a deleterious effect due to presence of compensatory factors, as in the case of haplogroups Q1 and D2 (de Carvalho *et al.*, 2006; Zhang *et al.*, 2007; Lu *et al.*, 2009). Since the latter are under-represented in European populations but predominant in others (Fig. 3), this can largely explain the apparent regional clustering of the positive association studies. Consequently, we can assume that the relevance of these deletions for male fertility will vary geographically. This is suitably illustrated in the meta-analysis of Visser *et al.* that identified a higher likelihood of spermatogenic impairment in *gr/gr* deleted European populations when compared with Asian counterparts (average OR 6.17 versus 1.84). It should also be noted that the recommended diagnostic test for *gr/gr* deletions does not preclude confirmation via adequate gene dosage

and/or copy type identification assays, as a 5% misdiagnosis rate has been recorded in a large multicenter study (Krausz *et al.*, 2009).

Another controversial aspect is the exact definition of the clinical risk associated with *gr/gr* deletions. In the vast majority of studies reporting an association, *gr/gr* deletions are presented as a risk factor for spermatogenic failure. Yet, one particular report has suggested that infertility, not decreased sperm production, is the main associated phenotype (Lynch *et al.*, 2005). Lynch *et al.* base their assumption on the analysis of normozoospermic infertile men, arguing that the disruption introduced by *gr/gr* deletions may correspond to a fine-scale defect not assessable in a routine spermogram. Given the role in chromatin remodelling and cytoskeletal regulation attributed to the *CDY1* and *BPY2* genes (Lahn *et al.*, 2002; Wong *et al.*, 2004), it is tempting to speculate that either could contribute to this hypothetical deregulation. Indirect evidence supporting these claims has been recorded for another AZFc rearrangement. Accordingly, a link to male infertility and not to low semen quality has been identified for the b2/b3 deletion (Wu *et al.*, 2007). Yet, a recent cross-sectional cohort study reported significantly lower sperm concentration, total sperm count and total motile sperm count in *gr/gr* deleted men when compared with non-deleted individuals (Visser *et al.*, 2009). Even though this result indicates that *gr/gr* deletions affect sperm numbers, the median concentration scores in both groups were still within the normozoospermia range. Such an observation does not formally exclude the possibility that the actual impact of *gr/gr* deletions may surpass that of a quantitative reduction in spermatogenic output.

b2/b3 deletions

Unlike that observed for *gr/gr* deletions, the initial studies on b2/b3 deletions failed to identify any link to spermatogenic failure (Fernandes *et al.*, 2004; Repping *et al.*, 2004). This was largely the consequence of a significant prevalence of the deletion-fixed haplogroup N in the sampled European populations. However, a subsequent study conducted on Asian men identified an association between b2/b3 deletions and male infertility, irrespective of sperm counts (Wu *et al.*, 2007). More recently and after increasing sample numbers, the same team has restricted the risk to spermatogenic impairment (Lu *et al.*, 2009). These results suggest that the occurrence of b2/b3 deletions outside of haplogroup N may represent a risk factor for spermatogenic impairment. Indeed, some indirect observations partially corroborate the proposition: the most recent results of Lu *et al.* (2009) demonstrate that while 80% of the b2/b3 deletions occurring in fertile normozoospermic men belong to the deletion-fixed haplogroup N, this fraction decreases to ~65% when considering infertile men. Furthermore, another study has identified a clustering (although statistically non-significant) of b2/b3 deletions to infertile men enrolled in ART programmes (Mau Kai *et al.*, 2008). Despite this tentative evidence, an unambiguous link between b2/b3 deletions in specific Y lineages and spermatogenic failure remains to be validated.

A propensity for the complete AZFc deletion has been identified in the b2/b3 deletion-fixed haplogroup N (Zhang *et al.*, 2007; Lu *et al.*, 2009). Since a similar observation had been recorded for the *gr/gr* deletion in haplogroup Q1 (Zhang *et al.*, 2007), this supports the theory that specific AZFc architectures may predispose to subsequent rearrangements. It has been suggested this propensity is higher in b2/b3-deleted than in *gr/gr*-deleted backgrounds, although the exact

molecular mechanisms driving this effect remain elusive (Lu et al., 2009). The occurrence of duplications also seems to be potentiated both by b2/b3 and gr/gr deletions, as illustrated by the increased frequency of DAZ gene copy duplications in deletion-fixed lineages (Lin et al., 2007). Taking into account these observations, the existence of structural AZFc triggers potentiating subsequent rearrangements have to be considered. It can be envisaged that the chromatin configurations associated with some architectures may alter the free energy state of the DNA molecule. These may favour the occurrence of more thermodynamically stable configurations via subsequent rearrangements, as identified for autosomal palindromes (Gotter et al., 2007). In this context, b2/b3 deletions may represent not only a presumable risk factor for spermatogenic impairment when present in specific Y lineages, but also a driver for genetic variability in the interval.

Dynamics of partial AZFc duplications

The genotypical and phenotypical characterization of partial AZFc duplications is limited by a significant under-reporting of these cases. The prevailing view is that both partial and complete AZFc duplications do not represent any particular risk for spermatogenic failure since the homeostatic mechanisms regulating spermatogenesis can compensate, to some extent, imbalances associated with gene dosage increases (Bosch and Jobling, 2003; Sebat et al., 2004; Giachini et al., 2008). Appropriately, an early report identified a similar distribution of partial duplication products between fertile and infertile men (Writzl et al., 2005). Furthermore, no evidence of qualitative and quantitatively increased spermatogenic output has been recorded in men with AZFc duplications (Giachini et al., 2008; Krausz et al., 2009). Interestingly, duplications restoring original gene copy number after a partial deletion event fail to exert any noticeable rescue effect on spermatogenic parameters (Krausz et al., 2009). This supports the view that gene dosage imbalances alone are insufficient to account for the spermatogenic disruption associated with partial AZFc deletions.

Nonetheless, a fairly recent study conducted in Taiwanese men identified an association between partial AZFc duplications and spermatogenic failure (Lin et al., 2007). This fits with previous data suggesting that AZFc structural variability is rather conservative regarding gene copy number (Repping et al., 2006). The link between partial AZFc duplications and spermatogenic impairment was later replicated in Han Chinese men from East China, but not in Italian men (Zhang et al., 2007; Giachini et al., 2008). These results imply that if increased AZFc gene content is to play a role in spermatogenic impairment, the effect, much like that of the corresponding partial deletions, will probably be modulated by population-specific factors. In this regard, the Y haplogroup composition of the studied population may be once again pivotal for the outcome of the association test.

Concluding remarks

Genetic diversity is a cornerstone of life. Given the lack of canonical recombination with a chromosome partner, such diversity is a difficult proposition in the male-specific region of the Y chromosome. Nevertheless, the ampliconic organization, by providing an ample substrate for homology-based intrachromosomal recombination, represents

the major driver for genotypical heterogeneity in AZFc. In this regard, two major evolutionary roles may be ascribed to the AZFc amplicons: (i) the generation of novel genetic variants that may confer some degree of selective advantage (even though selection is particularly weak and diffuse in the Y chromosome); and (ii) the maintenance of the functional integrity of AZFc genes via conversion and/or dosage mechanisms. Paradoxically, this strategy threads a precarious equilibrium since the generation of dosage-imbalanced chromosomes, as in the case of AZFc deletions, and the conversion-mediated replacement of functional alleles with affected copies are inevitable by-products. Since both may decisively impact the functional regulation of spermatogenesis as well as promote the genetic degeneracy of the Y, the consequences of this strategy for the evolutionary fate of the chromosome remains an open question.

Owing both to their considerable frequency in the infertile population and ambiguous clinical significance, partial AZFc deletions are the most intensely debated AZFc variants. It has been argued that routine diagnosis for such deletions is not recommended due to its limited diagnostic value and the largely unknown consequences these deletions convey to the well being of the progeny (Stouffs et al., 2008). However, as thoroughly discussed in this review, these deletions are clearly associated with spermatogenic impairment in several geographically defined populations. More specifically, although in some European and Oceania populations the risk is considerable, the deletion conveys no obvious clinical significance in most Asian and some Northern European populations. Since Y haplogroup composition is the main factor dictating the population-dependent response, a thorough analysis of their distribution in our increasingly globalized society may offer an ever more gentrified outlook on the impact of partial AZFc deletions for male fertility. Taking into consideration all available data, we postulate that both gr/gr and b2/b3 partial deletions when occurring outside of Y haplogroups where either deletion has become fixed, represent an infertility risk. In that context, their identification is advantageous both for the elucidation of the molecular aetiology of the infertility phenotype and for genetic counselling in terms of deletion transmission to the progeny. This latter issue is of particular importance since the severity of the spermatogenic impairment defect in the male progeny is impossible to predict, and most significantly, partial deletions have been linked to an increased propensity for the more deleterious complete AZFc deletion. Therefore, partial AZFc deletion screening should be regarded as an advantageous tool in the work-up of infertile couples, provided it is performed in clinically informative populations as defined by their Y haplogroup composition.

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