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human reproduction update

Teratozoospermia: spotlight on the main genetic actors in the human

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BACKGROUND: Male infertility affects >20 million men worldwide and represents a major health concern. Although multifactorial, male infertility has a strong genetic basis which has so far not been extensively studied. Recent studies of consanguineous families and of small cohorts of phenotypically homogeneous patients have however allowed the identification of a number of autosomal recessive causes of teratozoospermia. Homozygous mutations of aurora kinase C (*AURKC*) were first described to be responsible for most cases of macrozoospermia. Other genes defects have later been identified in spermatogenesis associated 16 (*SPATA16*) and dpy-19-like 2 (*DPY19L2*) in patients with globozoospermia and more recently in dynein, axonemal, heavy chain 1 (*DNAH1*) in a heterogeneous group of patients presenting with flagellar abnormalities previously described as dysplasia of the fibrous sheath or short/stump tail syndromes, which we propose to call multiple morphological abnormalities of the flagella (MMAF).

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METHODS: A comprehensive review of the scientific literature available in PubMed/Medline was conducted for studies on human genetics, experimental models and physiopathology related to teratozoospermia in particular globozoospermia, large headed spermatozoa and flagellar abnormalities. The search included all articles with an English abstract available online before September 2014.

RESULTS: Molecular studies of numerous unrelated patients with globozoospermia and large-headed spermatozoa confirmed that mutations in *DPY19L2* and *AURKC* are mainly responsible for their respective pathological phenotype. In globozoospermia, the deletion of the totality of the *DPY19L2* gene represents ~81% of the pathological alleles but point mutations affecting the protein function have also been described. In macrozoospermia only two recurrent mutations were identified in *AURKC*, accounting for almost all the pathological alleles, raising the possibility of a putative positive selection of heterozygous individuals. The recent identification of *DNAH1* mutations in a proportion of patients with MMAF is promising but emphasizes that this phenotype is genetically heterogeneous. Moreover, the identification of mutations in a dynein strengthens the emerging point of view that MMAF may be a phenotypic variation of the classical forms of primary ciliary dyskinesia. Based on data from human and animal models, the MMAF phenotype seems to be favored by defects directly or indirectly affecting the central pair of axonemal microtubules of the sperm flagella.

CONCLUSIONS: The studies described here provide valuable information regarding the genetic and molecular defects causing infertility, to improve our understanding of the physiopathology of teratozoospermia while giving a detailed characterization of specific features of spermatogenesis. Furthermore, these findings have a significant influence on the diagnostic strategy for teratozoospermic patients allowing the clinician to provide the patient with informed genetic counseling, to adopt the best course of treatment and to develop personalized medicine directly targeting the defective gene products.

Key words: male infertility / teratozoospermia / genetic diagnosis / sperm morphology / gene mutations

Introduction

Infertility, observed after 12 months of regular sexual intercourse, is a major health issue with at least 9% of couples requiring medical assistance to conceive a child (Boivin *et al.*, 2007). It is therefore estimated to concern 72.4 million couples worldwide with 40.5 million currently seeking medical care (Boivin *et al.*, 2007). In half of these cases, spermatograms identify a reduced sperm quantity or quality, suggesting that a male factor is present, alone, or in conjunction with a female cause, in half of the concerned couples (Thonneau *et al.*, 1991; Poongothai *et al.*, 2009; Krausz, 2011). The diagnosis of male infertility is often merely descriptive, the etiology of the sperm defect remaining idiopathic in 30–50% of cases (Krausz, 2011; Tüttelmann *et al.*, 2011b). One of the reasons for this lack of fundamental understanding is the heterogeneity of causal factors, as male infertility is a typical multifactorial disorder (Brugo-Olmedo *et al.*, 2001). Genetic factors are however likely to be very frequent as it has been estimated that a genetic origin of male infertility could be found in nearly 1 in 40 men (Tüttelmann *et al.*, 2011a). Chromosomal aberrations (mainly 47,XXY—Klinefelter syndrome), microdeletions of the Y chromosome and cystic fibrosis transmembrane conductance regulator (*CTFR*) mutations have been unambiguously demonstrated to be recurrent genetic causes of male infertility (Popli and Stewart, 2007; Vogt *et al.*, 2008; Jungwirth *et al.*, 2012). Despite this, routine screening for these well-established genetic causes (in principle justified only for patients presenting with certain specific phenotypes) results in a diagnosis in <5% of all phenotypes (Nieschlag *et al.*, 2010). We can therefore safely assume that the bulk of the genetic causes of male infertility is still uncharacterized, likely due to the large number of genes involved (Matzuk and Lamb, 2002) and the lack of ambitious studies carried out on large cohorts of affected men.

Over the past years the genetic investigations of severe teratozoospermias has been one of the most productive areas within the topic of male infertility and recurrent mutations have been identified in three specific phenotypes, which are macrozoospermia, globozoospermia and multiple morphological abnormalities of the flagella (MMAF) (Fig. 1). Teratozoospermia is defined as a percentage of morphologically normal spermatozoa below the lower reference limit. Cut-off values for normality varied greatly in recent decades from 50% in the first World Health Organization (WHO) classification (WHO, 1980) to 4% in the last version published in 2010 (Ombelet *et al.*, 1995; World Health Organization 2010). This traditional definition of teratozoospermia is based on the identification of atypical sperm shapes in sperm smears thereby excluding ultrastructural defects invisible under light microscope (Kruger *et al.*, 1986). Chemes and collaborators proposed to revisit this concept taking into consideration ultrastructural sperm anomalies underlying their functional incompetence. This new approach pushes back the limits of the definition of teratozoospermia beyond the mere description of spermatozoa morphology (Chemes and Rawe, 2003; Chemes and Alvarez Sedo, 2012). Such examination is however not compatible with the routine semen examination carried out in reproductive clinics. Teratozoospermia represents a heterogeneous group including a wide range of abnormal sperm phenotypes affecting, solely or simultaneously, the head, neck, midpiece and tail. Distinctions should also be made between specific forms with a homogenous monomorphic phenotype shared between several patients and forms with heterogeneous sperm anomalies (non-specific) randomly distributed in each individual and among different patients (Chemes and Rawe, 2003).

Oligoasthenoteratozoospermia (OAT) is one of the most common phenotypes of male infertility. It is defined by a combination of qualitative and quantitative sperm defects (Jungwirth *et al.*, 2012). Due to a high phenotypic variability, genetic causes of OAT have not been clearly identified and remain so far largely unknown. Many genetic association studies reported numerous variants associated with OAT, the validity of which remains to be established. Many knockout mice have also been described to present with OAT (Matzuk and Lamb, 2008). No mutations have however been identified in their human orthologs with the exception of CAMK4 which was mutated in two patients with OAT (Khattri *et al.*, 2012). A candidate gene approach also allowed the identification of a double amino-substitution in NANOS1, a gene that is apparently not required for spermatogenesis in the mouse

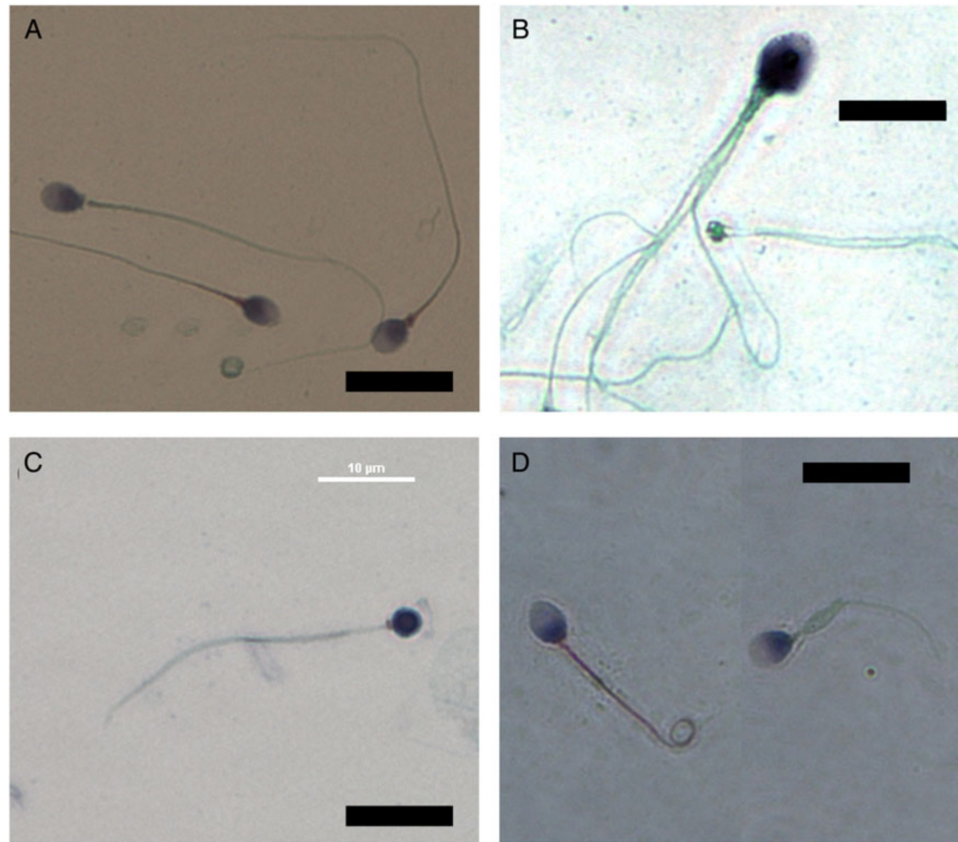

Figure 1 Light microscopy analysis of spermatozoa from different types of genetic infertility. Sperm morphology was assessed with Harris-Shorr staining: nuclei are blue and mitochondria red. (**A**) Control spermatozoa present a normal-shaped head, a typical short midpiece followed by a long principal piece. (**B**) A typical macrospermic spermatozoon from a patient with a homozygous aurora kinase C (*AURKC*) c.144del mutation presents a large head and several flagella. (**C**) A typical globospermic spermatozoon from a dpy-19-like 2 (*DPY19L2*) deleted patient presents a round-shaped head and an absence of mitochondria in the midpiece. (**D**) Spermatozoa from a patient with a homozygous dynein, axonemal, heavy chain 1 (*DNAH1*) mutation with multiple morphological abnormalities of the flagella (MMAF) phenotype present various defects of the flagellum. Scale bars 10 μm.

(Haraguchi *et al.*, 2003), in one man with OAT (Kusz-Zamelczyk *et al.*, 2013). These mutations however, were not formally validated and no further studies confirmed their implication in OAT.

Abnormalities of the sperm flagella always induce asthenozoospermia. They present highly variable morphological defects which can also affect the head and are sometimes associated with oligozoospermia. As such, some patients can be classified as a subgroup of OAT. A mosaic of different flagellar abnormalities is usually observed within the same spermogram including absent, short, bent, angulated or irregular flagella with also a high incidence of head defects. This phenotype has previously been reported as 'short tails' or 'stump tails' (Neugebauer *et al.*, 1990; Baccetti *et al.*, 1993; Stalf *et al.*, 1995; Dávila Garza and Patrizio, 2013) or dysplasia of the fibrous sheath (David *et al.*, 1993; Olmedo *et al.*, 1997, 2000; Chemes *et al.*, 1998; Rawe *et al.*, 2001; Moretti *et al.*, 2011; Ghedir *et al.*, 2014). As none of the previous terms seem to accurately define this phenotype because these patients' flagella present with a mosaic of ultrastructural flagellar defects as well as of morphological abnormalities including short tails but also, bent, curled, thick and missing flagella we previously proposed to call this heterogeneous group of subjects 'MMAF' for multiple morphological anomalies of the flagella (Ben Khelifa *et al.*, 2014). We will use here this descriptive definition for this heterogeneous phenotype. These flagellum morphological defects are however unevenly reported or are sometimes only observed in a small fraction of patient's spermatozoa. Some gene defects have been identified which seem to induce such phenotypes including mutations in solute carrier family 26 (*SLC26A8*) or septin 12 (*SEPT12*) identified in a subset of patients with asthenozoospermia and teratozoospermia including a bent tail and/or a round head with an abnormal acrosome (Kuo *et al.*, 2012; Lin *et al.*, 2012; Dirami *et al.*, 2013). These genes were described as being involved in the organization of the septin rings forming the annulus. Other patients have been studied presenting a more severe phenotype with close to 100% abnormalities and mutations in *DNAH1*, a gene coding for an inner dynein arm protein, identified in ~28% of the patients analyzed (Ben Khelifa *et al.*, 2014).

Other phenotypes can be considered as pure teratozoospermia with 100% abnormal sperm and with a constant uniform pattern of anomalies, such as globozoospermia, large-headed multiflagellar spermatozoa or acephalic spermatozoa. To date, several genes were identified in most of these specific teratozoospermia in humans. The identification and study of these genes shed a much-needed light on the physiopathology

of teratozoospermia, as a prerequisite to improve the patient management, to provide a basis for the development of therapeutic solutions tailored to the gene defect and to provide the patients with adequate genetic counseling and expected treatment outcome. Moreover, since the investigation of gene function in human is difficult, the elucidation of the function of mutated genes and identification of possible candidate genes in animal models is crucial for the identification of factors affecting male fertility. Numerous animal models, and in particular mutant mouse models related to teratozoospermia, have been extensively described in the literature (for review Yan, 2009; De Boer *et al*., 2015). In this review we focus on specific teratozoospermic phenotypes for which a recurrent and reliable genetic cause has been identified in human. We provide an update of the genetic etiology of these phenotypes, summarize their underlying molecular mechanisms and detail the clinical implications arising from these findings.

Methods

A computerized literature search was conducted for all publications in PubMed/Medline until September 2014. We searched using the following MeSH or key word terms: teratozoospermia (402 records) OR globozoospermia (114 records) OR large-headed spermatozoa (35 records) OR macrozoospermia (3 records) OR macrocephalic sperm head syndrome (8 records) OR short tail sperm (187 records) OR stump tail sperm (22 records) OR dysplasia fibrous sheath (39 records) OR primary flagellar abnormalities (22 records) OR primary ciliary dyskinesia (2135 records) OR flagellum biogenesis (260 records) with a possible combination with the keywords genetics, animal models, knockout, diagnosis and ICSI. Any additional relevant articles identified from the bibliography of the initially retrieved articles and reviews were then included (24 records). Only English-language publications or articles in other languages, but with a sufficiently detailed abstract in English were included. Additionally, we have opted to focus on articles regarding specific teratozoospermia phenotypes for which a gene was formally identified in human in several non-related individuals thereby reducing the risk of erroneous diagnosis. Heterogeneous phenotypes such as OAT (249 records) or pure phenotypes but with no identified genetic cause such as 'decapitated spermatozoa' (46 records) were not included in this review. Last, we did not include studies describing the association between single nucleotide polymorphisms (SNPs) and a particular phenotype (588 records) as we believe that many of these reports may have a low level of evidence. Concerning animal models, we selected a restrictive list of knockout (KO) models among every mutant model described in the literature related to infertility and in particular teratozoospermia (1160 records) (for exhaustive review see Yan, 2009; De Boer *et al*., 2015). Only KO mice with a globozoospermia-like phenotype (14 records), mutant mice with inactivated aurora kinase (*Aurk)c*, *Aurkb* and dynein axonemal heavy chain 1 (*Dnah1)* genes (17 records) and mice with central pair microtubule defects leading to flagellar abnormalities (10 records) are presented in this review.

Large headed multiflagellar spermatozoa

Patients with large-headed multiflagellar spermatozoa (MIM 243060), named also macrozoospermia or macrocephalic sperm head syndrome, present with a primary infertility characterized by the presence in the ejaculate of 100% abnormal spermatozoa with an oversized irregular head, abnormal midpiece and acrosome, and multiple flagella (Fig. 1B). Ultrastructural study of such spermatozoa revealed a 3-fold increase in nuclear volume and on average 3.6 flagella for each sperm head (Escalier, 1983). This teratozoospermia is also generally associated with a severe oligoasthenozoospermia (Escalier, 1983; Benzacken *et al*., 2001; Devillard *et al*., 2002). This spectacular phenotype has been regularly described in the scientific literature since its initial description 37 years ago (Nistal *et al*., 1977; German *et al*., 1981; Escalier, 1983; In't Veld *et al*., 1997; Pieters *et al*., 1998; Kahraman *et al*., 1999; Benzacken *et al*., 2001; Devillard *et al*., 2002; Guthauser *et al*., 2006; Mateu *et al*., 2006; Perrin *et al*., 2008; Chelli *et al*., 2010; Brahem *et al*., 2012).

Aneuploidy and abnormal morphology: AURKC is the key

Several studies using Feulgen-stained preparations (German *et al*., 1981), spermatocyte C-banding (Pieters *et al*., 1998) and mainly fluorescence *in situ* hybridization (FISH) analysis showed a high rate of polyploidy and aneuploidy in spermatozoa from men with macrozoospermia. In these reports, the number of spermatozoa interpreted as haploid varied from 0 to 10.9%, as diploid from 19.8 to 60%, as triploid from 10 to 62.4% and as tetraploid from 5.1 to 36% (Yurov *et al*., 1996; In't Veld *et al*., 1997; Weissenberg *et al*., 1998; Viville *et al*., 2000; Benzacken *et al*., 2001; Devillard *et al*., 2002; Lewis-Jones *et al*., 2003; Vicari *et al*., 2003; Guthauser *et al*., 2006; Mateu *et al*., 2006; Achard *et al*., 2007; Perrin *et al*., 2008; Chelli *et al*., 2010; Brahem *et al*., 2012). Partial forms of this syndrome have been described with various percentages of large-headed spermatozoa. These 'mosaic' forms showed a percentage of euploid spermatozoa roughly correlated with their ratio of normally-sized gametes (Vicari *et al*., 2003; Achard *et al*., 2007; Brahem *et al*., 2012). Taken together, these observations provide evidence indicating that chromosome nondisjunction and/or cytokinesis defects occurring during the first, the second or both meiotic divisions are consistently associated with large-headed spermatozoa (Escalier *et al*., 1992; In't Veld *et al*., 1997; Weissenberg *et al*., 1998; Benzacken *et al*., 2001; Devillard *et al*., 2002).

A description of familial cases with consanguineous parents was suggestive of a genetic cause with an autosomal recessive inheritance. In 2007, a genome-wide low-density microsatellite analysis led to the identification of a common region of homozygosity in 7 out of 10 North African macrozoospermic patients, located in the terminal region of chromosome 19 long arm. The *AURKC* gene, localized in the center of this region, appeared as the ideal candidate because it was described as being expressed preferentially in male germ cells and to be involved in chromosomal segregation and cytokinesis (Fig. 2), two functions that could explain the abnormal sperm morphology and cytogenetic content of large-headed spermatozoa (Dieterich *et al*., 2007). Sequencing of the *AURKC* coding sequence allowed the identification of the same homozygous deletion (c.144delC) in all 14 patients included in the study. This mutation introduces a frameshift resulting in a premature termination of translation and yielding a truncated protein lacking its conserved kinase domain (Dieterich *et al*., 2007). It was later demonstrated that the mutated transcript is in fact degraded by the mechanism of nonsense mediated mRNA decay, thus indicating that these patients do not even produce the truncated protein (Ben Khelifa *et al*., 2011). In another study, using flow cytometry in *AURKC*-mutated patients, Dieterich *et al*. (2009) demonstrated that all spermatozoa had a homogenous tetraploid DNA content indicating that patient' germ cells undergo DNA synthesis but remain blocked without completing either of the two meiotic

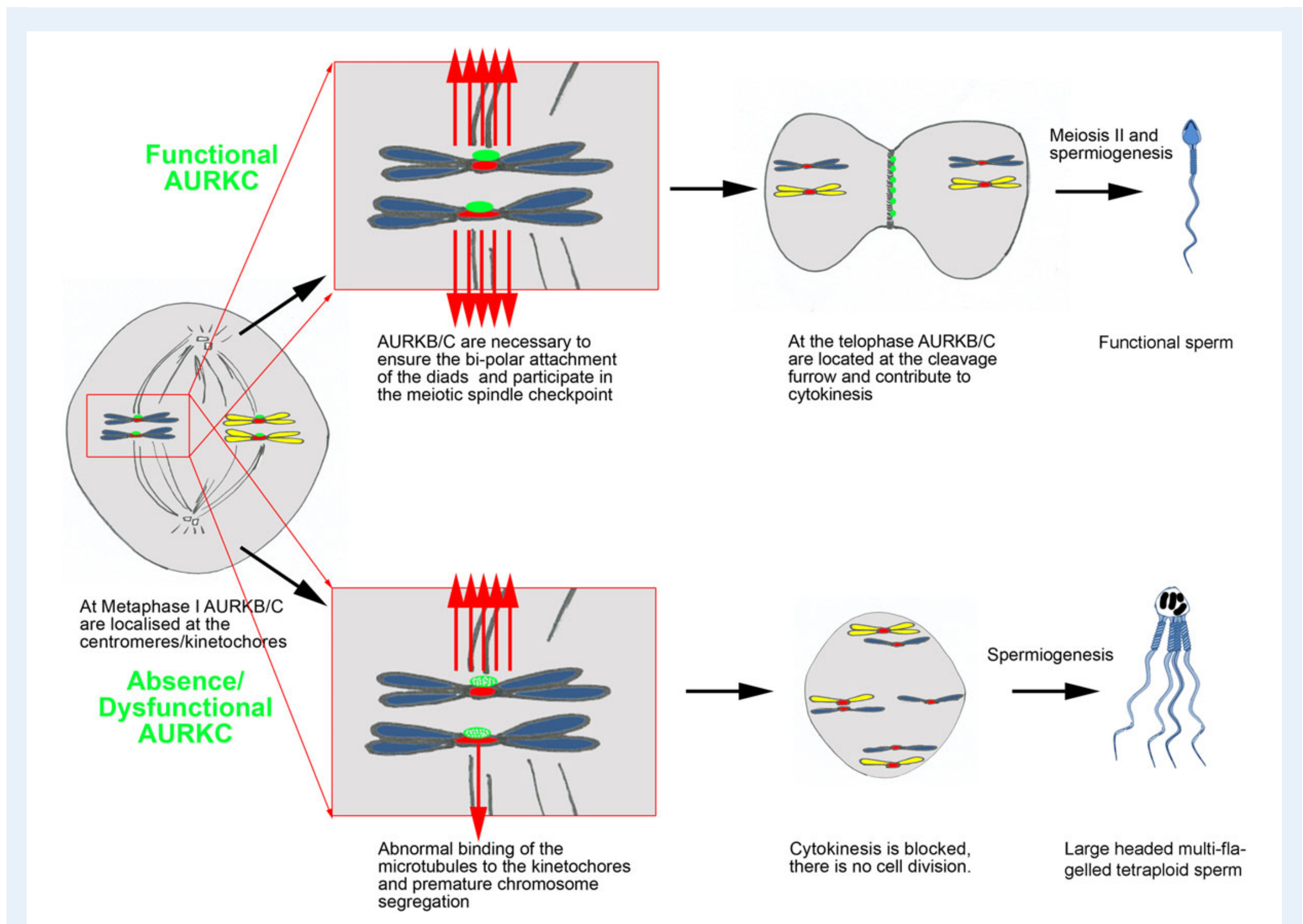

Figure 2 AURKC controls meiosis and spermatocyte cytokinesis.

divisions (Fig. 2). These results were discordant with previous FISH studies, which showed a wide heterogeneity of chromosomal abnormalities. The authors indicated that this difference is mainly due to the limits of the FISH technique and that in these abnormal gametes the superposition of FISH signals led to a great underestimate of the number of chromosomes/chromatids (Dieterich *et al.*, 2009). Surprisingly, in spite of this meiotic arrest, spermiogenesis is not blocked suggesting that meiotic checkpoint controls are abolished or inefficient in these patients. Most macrozoospermic patients however present a severely reduced sperm count concordant with the mathematical reduction in sperm number due to the absence of cell division and a partial effect of the meiotic checkpoints (Ben Khelifa *et al.*, 2011).

AURKC function in meiotic cells: between specificities and complementarities

AURKC belongs to the Aurora kinases family, which are highly evolutionary conserved serine/threonine kinases playing a key role in the control of mitosis and meiosis. In Mammals, aurora kinases also comprise two other family members, aurora kinase (AURKA) and aurora kinase B (AURKB). The three kinases share a high sequence homology in their central kinase domain and have a common ortholog in *Drosophila* and yeast (Sunkel and Glover, 1988; Chan and Botstein, 1993; Glover *et al.*, 1995). Despite their similarities, the three Aurora kinases have distinct localizations and functions. Divergences in the N-terminal domains of these proteins confer specific protein–protein interactions abilities (Carmena and Earnshaw, 2003). AURKA and B play distinct roles during mitosis: AURKA controls centrosome maturation, bipolar assembly and chromosome separation while AURKB regulates chromosome segregation, kinetochore–microtubule interaction and cytokinesis (Goldenson and Crispino, 2014). Much less is known about AURKC which was described more recently. AURKC recently evolved from AURKB after gene duplication (Brown *et al.*, 2004), and therefore shares a high degree of homology with AURKB (73%). AURKC is expressed predominantly in testis, in particular in dividing spermatocytes, and in oocytes whereas AURKA and B are expressed ubiquitously (Bernard *et al.*, 1998; Tseng *et al.*, 1998; Assou *et al.*, 2006; Tang *et al.*, 2006; Yang *et al.*, 2010). AURKC has also been described as highly expressed in early human preimplantation embryos (Avo Santos *et al.*, 2011). There are several lines of evidence that strongly suggest that AURKC can function in place of AURKB in meiotic cells. First, AURKC function can overlap with AURKB as evidenced by the fact that AURKC could rescue AURKB-depleted somatic cells (Li *et al.*, 2004; Sasai *et al.*, 2004; Yan *et al.*, 2005). This was also supported by the fact

that AURKC is sufficient to drive mitotic progression during early embryonic divisions in preimplantation mouse embryos lacking AURKB (Fernández-Miranda et al., 2011). The study of KO mice also demonstrated a respective functional compensation of both kinases during meiosis and embryonic development (Fernández-Miranda et al., 2011; Schindler et al., 2012). Moreover, it has been reported that AURKC, like AURKB, is a component of the chromosomal passenger complex (CPC) with a similar localization and interaction with other subunits such as inner centromere protein antigens 135/155 kDa (INCENP), survivin (BIRC5) and Borealin (CDCA8) (Chen et al., 2005; Vader et al., 2008). CPC is a pivotal regulator of mitotic events including chromosome segregation and cytokinesis (Vagnarelli and Earnshaw, 2004). During chromosome alignment, CPC corrects chromosome-microtubule attachment errors generating temporarily unattached kinetochores, a condition sensed by the spindle assembly checkpoint (SAC) (Vader et al., 2008; Carmena et al., 2012; van der Waal et al., 2012; van der Horst and Lens, 2014). The SAC represents one of the main mechanisms of cell division control ensuring fidelity in chromosome segregation. It verifies whether prerequisites for chromosome segregation have been met and thereby determines whether to execute or to delay chromosome segregation (Musacchio and Hardwick, 2002; Zhou et al., 2002). In addition it has been demonstrated that Aurora kinases could contribute to recruiting different mitotic checkpoint components such as BUB1 mitotic checkpoint serine/threonine kinase (BUB1) to the kinetochore (Becker et al., 2010; van der Waal et al., 2012). Impairment of CPC functions results in chromosome segregation defects, altered SAC and cytokinesis failure. Such perturbations in mitosis can lead to the production of aneuploid/polyploid cells, which then often become malignant. By comparison, abnormalities in CPC during meiosis are expected to lead to the production of chromosomally unbalanced gametes (Sharif et al., 2010; Yang et al., 2010; van der Waal et al., 2012; van der Horst and Lens, 2014).

Many experimental studies tried to address AURKB and AURKC specific functions during meiosis. They have so far been largely hampered by the difficulty to selectively inhibit one of the two kinases (Schindler et al., 2012; Balboula and Schindler, 2014). Only one study supports a preponderant role of AURKB in mouse spermatogenesis based on the study of AURKB transgenic mice, which are shown to be subfertile due to abnormal spermatocytes, increased testicular apoptosis and spermatogenic arrest. The authors however acknowledged that the function of AURKC might also be altered in their AURKB transgenic mutants (Kimmins et al., 2007). KO strategies did not prove more useful because homozygous *Aurkc* KO mice do not perfectly mimic the typical human macrozoospermia phenotype. Unlike infertile *AURKC*-mutated men, KO *Aurkc* male mice are fertile but with reduced litter size. They have normal testis weight and sperm count but do produce abnormal spermatozoa. Sperm abnormalities include abnormal chromatin condensation, acrosome loss and blunted heads (Kimmins et al., 2007). It is possible that the milder phenotype observed in mouse compared with human may be due to a greater overlap of AURKB and C functions in mouse than in human spermatogenesis but this remains to be demonstrated.

The function of AURKC has been extensively studied in oocytes. In female mice, the overexpression of a dominant negative allele inhibiting the AURKC activity (AURKC-DN) in oocytes was shown to cause cytokinesis failure in meiosis I resulting in the production of large polyploid oocytes, a pattern similar to that observed in AURKC-deficient human spermatozoa (Yang et al., 2010). These results are however questionable as AURKB might also be down-regulated in this mouse model (Schindler et al., 2012). Mice oocytes express both AURKB and C and this double expression might provide a backup ensuring the maintaining of critical functions (i.e. SAC and cytokinesis) in a transcriptionally quiescent cell (Schindler et al., 2012). *Aurkc* KO female mice however are subfertile with fewer pups per litter compared with wild type (5.5 versus 7.5 pups, on average per liter), and furthermore their oocytes show a higher incidence of chromosome misalignment and often arrest at meiosis I or later on at the 1-cell stage (Schindler et al., 2012). Balboula and Schindler (2014), using a specific catalytically inactive Aurkc mutant (AURKC-LA), show that most AURKC-LA oocytes arrest at metaphase I suggesting that AURKC-CPC is not the sole CPC complex that regulates the SAC in female meiosis. A small percentage of oocytes proceeded through meiosis and induced cytokinesis normally but were aneuploid, indicating that AURKC-CPC is the critical CPC complex necessary to correct improper chromosome-microtubule attachments during meiosis, a central role for preventing aneuploidy (Balboula and Schindler, 2014). These data indicate that the absence of AURKC is compatible with reproduction but might have an impact during meiosis and/or embryonic development. In human the absence of AURKC is compatible with female reproduction as women with homozygous *AURKC* mutations were shown to be fertile (Dieterich et al., 2009). Women with homozygous *AURKC* mutations could however be subfertile and present an increased risk of miscarriages in relation to the production of aneuploid oocytes. Further studies on the fertility of women with homozygous *AURKC* mutations should be carried out to conclude on this point.

Lastly, AURKC is expressed at a very low level in other somatic cells types such as brain glial cells, lung, placenta (Yan et al., 2005; Fernández-Miranda et al., 2011) or the pineal gland where it has been implicated in circadian clock function in the rat (Price et al., 2009). Apart from infertility, homozygous AURKC-mutated patients display no other clinical features suggesting that AURKC is dispensable in somatic cells (Dieterich et al., 2007, 2009). AURKC is also aberrantly expressed in some cancer cells but its contribution to oncogenesis is not well explored and understood (Khan et al., 2011; Tsou et al., 2011; Zekri et al., 2012; Goldenson and Crispino, 2014).

AURKC mutations: the c.144delC reigns supreme

The genotype of macrozoospermic patients described in the literature is summarized in (Table I). The c.144delC deletion accounts for ~85% of the mutated alleles (Ben Khelifa et al., 2012). Other mutations have been identified: p.C229Y, a novel missense mutation in exon 6 and c.144delC (Dieterich et al., 2009), p.Y248*, a new recurrent non-sense mutation was found in 10 unrelated individuals of European and North African origin (Ben Khelifa et al., 2012) and c436-2A>G, leading to a shortened transcript devoid of exon 5 (Ben Khelifa et al., 2011). Overall, and excluding the study of Eloualid (2014) based on an unselected population of infertile men, a positive *AURKC* mutation diagnosis is found in between 50.8 and 100% of analyzed macrozoospermic patients (Table I).

Nearly all positive mutated *AURKC* patients have a typical phenotype, with close to 100% large-head spermatozoa, whereas negative patients have a lower percentage of large-headed spermatozoa (<75%) (Table II) (Dieterich et al., 2009). Positive hits are strongly correlated with the percentage of large-headed spermatozoa in the ejaculate (Table II). No

Table I **Mutation status for the aurora kinase C (*AURKC*) gene in macrozoospermic men.**

References	Number of patients studied (*n*)				Geographical origin (*n*)	AURKC mutations[¤] (%; *n*)
	Included	Analyzed	Unrelated[?]	Newly tested		
Dieterich *et al.* (2007)	14	14	13	14	North African (14)	c.144delC +/+ 100% (13/13) p.Y248* +/+ 0% (0/13) Other 0% (0/13) No mutation 0% (0/13)
Dieterich *et al.* (2009)	62	62	61	48[&]	North African (43), Middle East (19)	c.144delC +/+ 49.2% (30/61) p.Y248* +/+ 0% (0/61) Other[(1)] 1.6% (1/61) No mutation 49.2% (30/61)
Ben Khelifa *et al.* (2011)	2	2	1	2	North African (2)	c.144delC +/+ 0% (0/1) p.Y248* +/+ 0% (0/1) Other[(2)] 100% (1) No mutation 0 (0%)
El Kerch *et al.* (2011)	18	18	18	18	North African (18)	c.144delC +/+ 61.1% (11/18) p.Y248* +/+ n.d Other n.d No mutation 7 (only not mutated for c.144delC)
Ben Khelifa *et al.* (2012)	87	87	83	44[£]	North African (73), European (14)	c.144delC +/+ 67.5% (56/83) p.Y248* +/+ 9.6% (8/83) Other[(3)] 4.8% (4/83) No mutation 18.1% (15/83)
Eloualid *et al.* (2014)[$]	326[$]	326[$]	326[$]	326[$]	North African (326)	c.144delC +/+ 1.2% (4/326)[$] p.Y248* +/+ n.d Other n.d No mutation 98.8% (322/326)[$]
Ounis *et al.* (2015)	14	14	14	14	North African (14)	c.144delC +/+ 71.4% (10/14) p.Y248* +/+ 7.1% (1/14)

Continued

Table I *Continued*

References	Number of patients studied (*n*)				Geographical origin (*n*)	AURKC mutations[¤] (%; *n*)
	Included	Analyzed	Unrelated[ʔ]	Newly tested		
						Other 0 (0%) No mutation 21.5% (3/14)

n.d: not determined; *n*: number of patients.
[ʔ]Indicated the number of unrelated patients among all analyzed patients.
[¤]Mutation rate (%) is calculated only from the number of unrelated patients.
[$]This study is based on unselected population of 326 idiopathic infertile patients with various sperm parameters and phenotypes. The proportion of patients with macrozoospermia is not available.
[&]The study included 14 patients previously described in Dieterich *et al.* (2007).
[£]The study included 41 patients previously described in Dieterich *et al.* (2009) and 2 patients previously described in Ben Khelifa *et al.* (2011).
[(1)]Patient was compound heterozygote carrying the c.144delC and p.C229Y mutations.
[(2)]Two brothers were compound heterozygotes carrying the c.144delC and c.436-2A>G mutations.
[(3)]One patient was compound heterozygote carrying the c.144delC and p.C229Y mutations, two were compound heterozygotes carrying the c.144delC and c.436-2A>G mutations and two were compound heterozygotes carrying the c.144delC and p.Y248* mutations.

mutation was generally found in patients with <70% of macrocephalic spermatozoa (Dieterich *et al.*, 2009; Ben Khelifa *et al.*, 2011, 2012; El Kerch *et al.*, 2011; Eloualid *et al.*, 2014). Furthermore, although the large majority of men with macrozoospermia present a reduced sperm count, men with extreme oligozoospermia (<0.5 M/ml) were usually not carriers of AURKC mutations.

To date Aurora kinase C remains the only gene where mutations were found in patients with a large-headed spermatozoa phenotype. The sequencing of *AURKC* exons 3 and 6 to search for the two recurrent mutations is appropriate as a first-line genetics test in all patients presenting with large-headed spermatozoa. The sequencing of the remaining exons should be discussed in light of the values of semen parameters. In fact, a sperm concentration >1 million, a percentage of large-head spermatozoa beyond 70% and/or a low percentage of normal spermatozoa (<1%) are in favor of a positive outcomes (Table II) (Ben Khelifa *et al.*, 2012). Others parameters could be taken into account like the presence of multiple flagella. As an example, Molinari *et al.* (2013) reported a case of sperm macrocephaly syndrome with 95% of large-headed spermatozoa but without tail abnormalities. *AURKC* gene sequencing did not reveal any mutations in the patient, suggesting that other genes may be involved in determining this atypical syndrome.

The c.144delC mutation accounts for almost all identified *AURKC* mutations, in particular in a Magrebian population where the prevalence of this mutation at a heterozygous state is estimated to be of 1 in 50 (Dieterich *et al.*, 2009; Eloualid *et al.*, 2014). Surprisingly, it makes *AURKC* gene alterations the most frequent defect in infertile Magrebian men before Klinefelter syndrome and Y-microdeletions (Ounis *et al.*, 2015). It is difficult to precisely calculate the p.Y248* prevalence in North African individuals because the c.144delC is often the only mutation studied (Dieterich *et al.*, 2009; El Kerch *et al.*, 2011; Eloualid *et al.*, 2014). No reliable data are currently available for European individuals due to a small number of studied patients. However, the recently identified non-sense mutation p.Y248* appears to be the main mutation in individuals of European origin (Ben Khelifa *et al.*, 2012). Both recurrent mutations c.144delC and p.Y248* are founding mutations and ancestral haplotypes were used to compute the age of the two *AURKC* mutations. It was estimated that c.144delC occurred between 1350 and 1750 AD and p.Y248* between 675 and 1075 AD. The fact that p.Y248* predates c.144delC is confirmed by the observed wider geographical distribution of p.Y248* (Ben Khelifa *et al.*, 2012). It remains surprising that mutations with a negative effect on reproduction should reach such a high prevalence. A possible explanation lies in the assumption that the heterozygous carriers of *AURKC* mutations may have a selective advantage (Dieterich *et al.*, 2007, 2009; Ben Khelifa *et al.*, 2012; Ounis *et al.*, 2015). No clear demonstration of this putative selective advantage has yet been provided and several hypotheses have been advanced. We previously described that AURKC was involved in the SAC. Men with AURKC heterozygous mutations could therefore have a more relaxed meiotic checkpoint permitting a faster meiosis turnover leading to an increased sperm production (Ben Khelifa *et al.*, 2012). The produced gametes would however be expected to present a higher rate of aneuploidies likely to induce a high rate of spontaneous abortions. This hypothesis is supported by several observations of multiple miscarriages or perinatal deaths in the siblings or parents of macrozoospermic patients (Benzacken *et al.*, 2001; Achard *et al.*, 2007; Ounis *et al.*, 2015).

Curiously, only four different *AURKC* mutations have been reported so far, and all are involved in the complete form of the phenotype. Ben Khelifa *et al.* (2012) propose that some mutations could affect only some functions of the AURKC protein: the microtubule–kinetochore attachment and/or cytokinesis, without altering AURKC SAC function. The severity of the defects may depend on the level of kinase inhibition; some functions of the CPC are already disturbed when the complex is only partially inhibited (e.g. correction of microtubule–kinetochore attachment), while others (e.g. its function in the SAC) may require complete inhibition (van der Horst and Lens, 2014). This would therefore lead to severe quantitative defects, and what could be considered as 'milder' *AURKC* mutations could be found in azoospermic rather than in macrozoospermic patients. This hypothesis has however not been confirmed yet.

AURKC mutational status and patient management

Although assisted reproductive technologies (ART) have revolutionized the treatment of infertile patients with severe oligoasthenoteratozoospermia, they are inefficient for patients presenting large-headed

Table II Sperm parameters of macrozoospermic men genotyped for the *AURKC* gene.

References	AURKC genotype (*n*)[§]	Sperm parameters			
		Nb spz × 10^6 per ml	Large-headed spz (%)	Multiflagellar spz (%)	Normal morphology (%)
Dieterich *et al.* (2007)	Deleterious mutations (14) No mutation (0)	3.2 (0.2–7.76)	56 (34–100)	28.5 (16–50)	2 (0–5)
Dieterich *et al.* (2009)	Deleterious mutations (32) No mutation (30)	7.8 (0.4–28) 116.8 (0.01–280)	76 (34–100) 11.7 (5–75)	40.3 (20–100) 8.5 (0–28)	0.6 (0–15) 20 (0–37)
Ben Khelifa *et al.* (2011)	Deleterious mutations (2) No mutation (0)	0.85 (0.8–0.9)	100 (100)	40 (28–52)	n.a
El Kerch *et al.* (2011)	Deleterious mutations (11) No mutation (7 only not mutated for c.144delC)	n.a n.a	94.6 (71–100) 6.7 (0–38)	34.7 (4–94) 0 (0)	n.a n.a
Ben Khelifa *et al.* (2012)	Deleterious mutations (72) No mutation (15)	13.1 (0.01–98.7) 11.3 (0.01–50)	79.6 (34–100) 35.2 (5–75)	37.9 (7–100) 7.8 (0–28)	0.1 (0–1) 5.1 (0–19)
Eloualid *et al.* (2014)[$]	Deleterious mutations (4) No mutation (322) 322 (98.8%)	6 (0–17)[£] n.a	96.7 (90–100)[£] n.a	44.7 (12–90)[£] n.a	0 (0)[£] n.a
Ounis *et al.* (2015)	Deleterious mutations (11) No mutation (3)	7.51 (0.5–30.5) 13 (0.1–22.8)	99.5 (95–100) 71.7 (70–75)	+ +	n.a n.a

Values are expressed as the mean with the lower and the higher values between brackets if available.
n.a: not available; *n*: number of patients; spz: sperm cells; + indicated that the feature was present but no value was reported.
[§]Indicate the total number of patients with a deleterious mutations including all types of reported mutations in *AURKC* (frameshift, splicing mutations, missense) and the total number of patients with no bi-allelic mutation identified.
[$]This study is based on unselected population of 326 idiopathic infertile patients with various sperm parameters and phenotypes. The proportion of patients with macrozoospermia is not available.
[£]Average was calculated from three out of the four patients because one was azoospermic.

spermatozoa due to the high frequency of sperm chromosomal abnormalities (Perrin *et al.*, 2008). Management of patients diagnosed with macrozoospermia should begin with *AURKC* diagnosis. ICSI will then be formally contraindicated for all homozygous mutated patients who can have recourse to donor sperm or adoption (Ben Khelifa *et al.*, 2011). Careful selection of 'normal-looking spermatozoa' by motile sperm organelle morphology examination (MSOME) has previously been evaluated in several *AURKC* c.144delC deleted patients. FISH analyses performed on all selected spermatozoa showed that all were aneuploid, confirming that ICSI should not be attempted for *AURKC*-mutated patients even after a very thorough morphological selection (Chelli *et al.*, 2010). The prognosis is not as categorically unfavorable for patients not carrying *AURKC* mutations or with a partial/atypical phenotype. Although low, the chances of pregnancy are not negligible (Kahraman *et al.*, 1999; Achard *et al.*, 2007). For these men, sperm FISH should be carried out to assess the rate of euploid sperm and evaluate the probability of success. PGD can be proposed for those with an intermediate rate of aneuploid sperm (Kahraman *et al.*, 2004; Dieterich *et al.*, 2009). Careful scrutiny of the pregnancy and of the perinatal period is however recommended in case of success (Achard *et al.*, 2007).

Globozoospermia

First described in human in 1971, globozoospermia (MIM 613958) is a rare (incidence 0.1%) and severe form of teratozoospermia characterized by the presence in the ejaculate of a large majority of round spermatozoa lacking the acrosome (Fig. 1C) (Sen *et al.*, 1971; Holstein *et al.*, 1973; Dam *et al.*, 2007a). Globozoospermic sperm are unable to adhere and penetrate the zona pellucida, causing primary infertility (Dam *et al.*, 2007a). The initial phenotype was divided into two subtypes: the globozoospermia type I characterized by the complete lack of acrosome and acrosomal enzymes and the globozoospermia type II characterized by a round-headed phenotype due to a residual cytoplasmic droplet surrounding the sperm head and acrosome (Anton-Lamprecht *et al.*, 1976; Singh, 1992). However, this nomenclature is confusing and was subsequently often misemployed in the literature referring to patients with a homogeneous phenotype with ~100% round-headed sperm (type I) or patients with a mosaic of normal and round-headed sperm (type II). The terms 'total' or 'partial' globozoospermia have been proposed and should be preferred to report the homogeneity of the 'original' type I phenotype (Lerer-Goldshtein *et al.*, 2010; Dam *et al.*, 2011) while the rarer, type II phenotype, should be referred as pseudo-globozoospermia.

Globozoospermia and experimental models: a relevant strategy?

Several familial cases of globozoospermia suggested a genetic contribution to this disorder (Kullander and Rausing, 1975; Flörke-Gerloff *et al.*, 1984; Dale *et al.*, 1994; Kilani *et al.*, 2004; Dirican *et al.*, 2008). This hypothesis was strengthened by the description in the literature

of KO mice with a globozoospermia-like phenotype lacking different genes (Table III). Almost all these genes are ubiquitously expressed and therefore do not appear as good candidates for the human globozoospermia phenotype, which only causes primary infertility. These genes encode endoplasmic reticulum proteins such as heat shock protein 90 kDa beta 1 (Hsp90b1) and glucosidase, beta (bile acid) 2 (Gba2) or vesicle trafficking-related proteins such as casein kinase 2, alpha prime polypeptide (Csnk2a2), ArfGAP with FG repeats 1 (AGFG1 or Hrb), golgi-associated PDZ and coiled-coil motif containing (Gopc), protein interacting with PRKCA 1 (Pick1), TATA element modulatory factor 1 (Tmf1), vacuolar protein sorting 54 (Vps54), small ArfGAP 2 (Smap2) and autophagy related 7 (Atg7). All regulate proacrosomal vesicle transport from the Golgi to the acrosome (Table III and Fig. 3). These proteins are implicated in a common pathway and contribute to acrosome biogenesis and sperm head organization (Fig. 3). Only zona pellucida binding protein *(Zpbp or Zpbp1)* and sperm acrosome associated 1 (*Spaca1)* have an expression restricted to the testis (Lin *et al*., 2007; Fujihara *et al*., 2012). Zpbp and Spaca1 are integral acrosomal proteins but display different functions (Lin *et al*., 2007; Fujihara *et al*., 2012). Zpbp is localized in the acrosomal matrix and is involved in the binding and penetration of the sperm into the zona pellucida (Yatsenko *et al*., 2012) while Spaca1 is a transmembrane protein located in the inner acrosomal membrane of spermatids and mature spermatozoa (Fig. 3) and plays an unsolved role in acrosomal morphogenesis and in sperm-egg binding and fusion (Hao *et al*., 2002; Fujihara *et al*., 2012). None of these mouse models perfectly mirrors the round-shaped acrosomeless spermatozoa observed in human globozoospermia. To date, >50 genetically modified mice reported in the Mouse Genome Informatics database (http://www.informatics.jax.org) contain in their phenotypic description a term related to globozoospermia. This is not surprising in view of the complexity and the multitude of proteins involved in acrosome biogenesis (Alvarez Sedó *et al*., 2012). In spite of this large list of mouse candidate genes, only a few mutations have been identified in their human orthologs. Heterozygous missense and splicing mutations in *ZPBP* were described in patients presenting with abnormal sperm head morphology, but their involvement in the disease has not been clearly demonstrated (Yatsenko *et al*., 2012). Similarly, a homozygous missense mutation (G198A) in exon 13 of the *PICK1* gene was identified in a Chinese family (Liu *et al*., 2010). Again, the report of the identification of a relatively mild mutation without any functional validation in a single familial case remains somewhat unconvincing.

DPY19L2 mutational spectrum: toward a diagnostic strategy

Gene candidate approaches, even with strong animal model data, are often unsuccessful (Pirrello *et al*., 2005; Christensen *et al*., 2006). Homozygosity mapping using genome-wide scan analysis of a consanguineous Ashkenazi Jewish family with three globozoospermic brothers identified a homozygous mutation (c.848G>A) in *SPATA16* (spermatogenesis-associated protein 16, previously named *NYD-SP12*) (Dam *et al*., 2007b). The SPATA16 protein localizes to the Golgi apparatus and to the proacrosomal vesicles which fuse to form the acrosome during spermiogenesis (Lu *et al*., 2006; Dam *et al*., 2007b). *SPATA16*, is highly expressed in human testis and contains a conserved tetratricopeptide repeat (TPR) domain (Xu *et al*., 2003) which may interact with the GOPC and HRB proteins (Dam *et al*., 2007b). No mutation was subsequently found in 30 other patients with globozoospermia originating from Europe or North Africa (Dam *et al*., 2007b), suggesting that *SPATA16* is not the main cause of globozoospermia, which is likely genetically heterogeneous. Only recently was a new *SPATA16* mutation identified in single man from a large cohort of patients with globozoospermia (Karaca *et al*., 2014). In 2011, from a cohort of 20 Tunisian patients with 100% globozoospermic sperm, SNP-based genetic linkage analysis identified a common region of homozygosity in 12q14.2 including the dpy-19-like 2 *(DPY19L2)* gene strongly expressed in testis. A 200-kb homozygous deletion of the *DPY19L2* gene was found in 15 of the 20 patients analyzed (Harbuz *et al*., 2011). This deletion was also identified in a second study published simultaneously in 19% (4 out of 21) of the recruited globozoospermic patients (Koscinski *et al*., 2011). Although the deletion frequency is very disparate, both studies conclude that the *DPY19L2* deletion is the main cause of globozoospermia. Subsequently, three large studies confirmed the high prevalence of *DPY19L2* gene alterations, ranging from 60 to 83.3% of analyzed patients in cohorts of globozoospermic patients from different geographic regions and with different ethnic backgrounds (Table IV) (Coutton *et al*., 2012; Elinati *et al*., 2012; Zhu *et al*., 2013). A further two publications further strengthen this conclusion, reporting the presence of homozygous *DPY19L2* deletion in patients from Macedonia and Algeria (Noveski *et al*., 2013; Ounis *et al*., 2015). Homozygous deletions represent 26.7 to 73.3% of the reported *DPY19L2* mutations in the three largest studies (Table IV) (Coutton *et al*., 2012; Elinati *et al*., 2012; Zhu *et al*., 2013). This heterogeneous deletion rate could be explained by a possible founder effect in some studies with a geographic-based recruitment accentuating the deletion prevalence (Harbuz *et al*., 2011; Coutton *et al*., 2012; Elinati *et al*., 2012). In addition, inclusion parameters are different and *DPY19L2* mutations are mainly identified in type I globozoospermia with a typical morphology and a high percentage of affected spermatozoa (Table V) and the inclusion of subjects with a low level of round-headed spermatozoa might in some cases decrease diagnosis efficiency. The genotype-phenotype correlation is however not perfect, indicating that (i) some phenotypic variation exists even within *DPY19L2* deleted subjects and (ii) other gene defects likely induce a pure globozoospermia phenotype (Table V). To enable a better genotype/phenotype correlation great care should be taken to respect the international recommendation for the examination of human semen to standardize the results and avoid protocol variations between the different laboratories (World Health Organization 2010).

Homozygous and compound heterozygous point mutations further broaden the spectrum of *DPY19L2*-dependent globozoospermia (Table IV). *DPY19L2* point mutations can be either missense mutations localized mainly in the central part of the DPY19L2 protein or nonsense/frameshift/splice-site mutations resulting in truncated proteins (Coutton *et al*., 2012; Elinati *et al*., 2012; Zhu *et al*., 2013). Small deletions were also reported (Table IV) indicating that exon deletions are part of the mutational spectrum of the *DPY19L2* gene (Zhu *et al*., 2013). A recurrent missense mutation in exon 8, p.Arg290His, was identified in several unrelated patients. This mutation changes a highly conserved arginine into a histidine and is predicted to be deleterious by multiple prediction tools (Coutton *et al*., 2012; Elinati *et al*., 2012; Zhu *et al*., 2013). Moreover, a recent study demonstrates that *DPY-19*, the *DPY19L2* ortholog in *Caenorhabditis elegans*, encodes a C-mannosyltransferase which is able to glycosylate several target proteins using the lipid-linked glycoside dolichol-phosphate-mannose (Dol-P-Man) as the donor substrate

Table III Mouse models with abnormalities of sperm acrosome morphogenesis and nuclear shaping.

KO mouse ID	Full name	Function	Localization and partners	Acrosome In sperm	Nuclear shape	Nuclear membrane and lamina	References
Atg7	Autophagy related 7	Golgi vesicle fusion; Protein trafficking	Cytoplasm	Present, no hook shaped	Poorly elongated <30% Globular or deformed head	Normal	Wang *et al.* (2014)
Csnk2a2	Casein kinase II alpha' catalytic subunit	Kinase/phosphorylation	Ubiquitous Acrosomal matrix Pick1 partner	Partially separated from the nucleus	Poorly elongated	Swelling of nuclear membranes	Xu *et al.* (1999) and Mannowetz *et al.* (2010)
Dpy19l2	Dpy-19-like 2 (*C. elegans*)	Glycosylase? Structural protein of the Inner Nuclear membrane	Inner Nuclear membrane	Absent	Round, rod shaped	Destabilization of nuclear lamina facing the acrosome. Nuclear membrane splitting off	Pierre *et al.* (2012)
Gba2	Glucosidase beta 2	Hydrolyzes glucosylceramide	Endoplasmic reticulum and/or plasma membrane	Partially present, disordered	Not elongated and irregular outline	?	Yildiz *et al.* (2006) and Walden *et al.* (2007)
Gopc	Golgi-associated PDZ- and coiled-coil motif-containing protein)	Golgi vesicle fusion; Vesicle trafficking	Trans-Golgi Pick1 partner	Fragmented	Round or ovoid not hook shape	Normal	Yao *et al.* (2002) and Ito *et al.* (2004)
Hrb *Agfg1 (official)*	HIV-1 Rev binding protein ArfGAP with FG repeats 1 (official symbol)	Golgi vesicle fusion	Cytoplasmic surface of acrosome	Absent Failure of vesicle fusion	Globular	Apparently normal	Kang-Decker *et al.* (2001) and Kierszenbaum *et al.* (2004)
Hsp90b1	Heat shock protein 90b1 heat shock protein 90, beta (Grp94), member 1 (official symbol)	Endoplasmic chaperone	Endoplasmic reticulum	Partially present	Globular or deformed	?	Audouard and Christians (2011)
*Pick1**	Protein interacting with C kinase 1	Golgi vesicle fusion; Protein trafficking	Trans-Golgi Gopc partner Ck2α' partner	Fragmented	Poorly elongated, not hook shape	Normal	Xiao *et al.* (2009)
Smap2	small ArfGAP 2 Arf GTPase-activating small GTPases protein	Golgi vesicles fusion; Protein trafficking	Trans Golgi binds to both clathrin and the clathrin assembly protein	Partially present, fragmented	Poorly elongated, not hook shape	Apparently normal	Funaki *et al.* (2013)
Spaca1	sperm acrosome associated 1	Structural protein of the acrosomal membrane	Inner acrosomal membrane	Partially present	Round or rod shaped	Loss of the nuclear lamina facing the acrosome	Fujihara *et al.* (2012)
Tmf1	TATA element modulatory factor 1	– Trafficking of Golgi-derived vesicles and/or Golgi vesicles fusion – Cytoplasm removal	Golgi-associated protein	Absent	Round or rod shaped. Nuclei are embedded in aberrant cytoplasm rest.	Apparently normal Dense nuclear lamina is present at the apical side	Lerer-Goldshtein *et al.* (2010)
*Vps54***	Vacuolar-vesicular protein sorting 54 homolog	Vesicular sorting protein Retrograde traffic	Acrosome	Absent Failure of vesicle fusion	Poorly elongated and irregular outline	Normal	Paiardi *et al.* (2011)
Zpbp1	Zona pellucida binding protein 1	Binding and penetration into the zona pellucida	Acrosomal matrix	Bulged	Shortened hook	Normal	Lin *et al.* (2007) and Yatsenko *et al.* (2012)

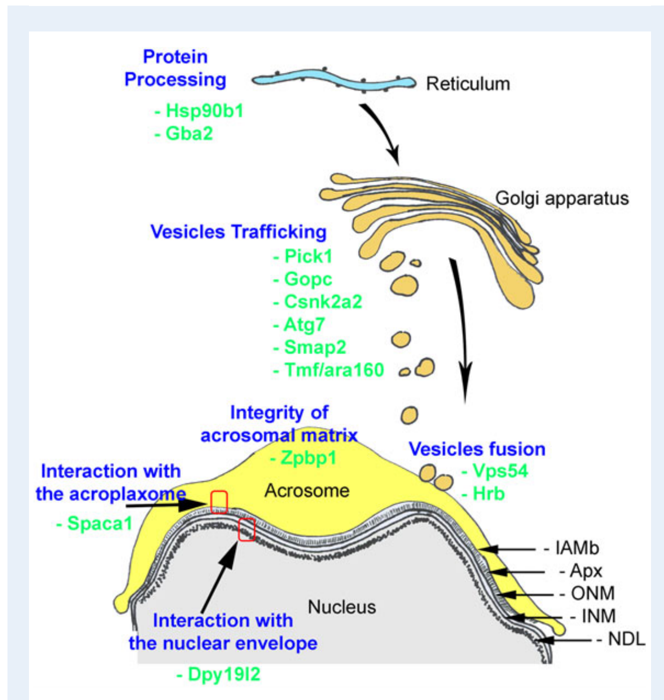

Figure 3 Known proteins involved in acrosome biogenesis whose functional absence leads to globozoospermia or globlozoospermia-like phenotypes in mouse. Acrosome biogenesis includes several steps including protein processing within the reticulum, vesicle trafficking from the Golgi apparatus, vesicle fusion, interaction of the inner acrosomal membrane (IAMb) with the acroplaxome (Apx), interaction of the acroplaxome with the outer and inner nuclear membranes (ONM and INM) of the nuclear envelope and maintaining integrity of the acrosomal matrix. Numerous proteins are involved in these different steps and their absence leads to defective acrosome biogenesis and globozoospermia or globozoospermia-like phenotypes. Only the absence of sperm acrosome associated 1 (*Spaca1)* and Dpy19l2 leads to the disappearance of the nuclear dense lamina (NDL). Genes abbreviations are as follows: heat shock protein 90 kDa beta 1 (Hsp90b1); glucosidase, beta (bile acid) 2 (Gba2); protein interacting with PRKCA 1 (Pick1); golgi-associated PDZ and coiled-coil motif containing (Gopc); casein kinase 2, alpha prime polypeptide (Csnk2a2); autophagy related 7 (Atg7); small ArfGAP 2 (Smap2); TATA element modulatory factor 1; (Tmf1 or ARA160); zona pellucida binding protein *(Zpbp or Zpbp1);* vacuolar protein sorting 54 (Vps54); ArfGAP with FG repeats 1 (Hrb), sperm acrosome associated 1 (*Spaca1*).

(Buettner *et al.*, 2013). Interestingly, the amino acid corresponding to the Arg290 in *C. elegans* has been predicted to be involved in the binding of the Dol-P moiety of the substrate (Buettner *et al.*, 2013). These data suggest that the arginine at position 290 could play a key role in DPY19L2 function. The effect of this potential glycosylation remains to be elucidated. On the whole, the fact that most reported missense mutations occur between exon 8 and 11 could indicate that this central domain of DPY19L2 may support some critical functions.

Overall, the deletion of the totality of the *DPY19L2* gene represents about 81% of the pathological alleles (Table IV). Different approaches have been described to effectively detect these *DPY19L2* deletions. First, a long-range PCR combined with exon-specific amplification is the most widely used strategy to detect both homozygous and heterozygous deletions (Table IV) (Harbuz *et al.*, 2011; Koscinski *et al.*, 2011; Elinati *et al.*, 2012; Noveski *et al.*, 2013; Zhu *et al.*, 2013). This cost effective strategy allows delineating with a good accuracy the breakpoints of the genomic deletion although this information is not, strictly speaking, useful for the diagnosis and clinically relevant. A strong shortcoming of this technique is that it does not detect the deletions with breakpoints falling outside the region covered by the deletion-specific primers therefore presenting a high risk of false-negative results (Coutton *et al.*, 2012; Zhu *et al.*, 2013). An alternative strategy is the use of a multiplex ligation-dependent probe amplification (MLPA) approach to determine the number of *DPY19L2* alleles for each patient, and independently of the localization of the breakpoint (Coutton *et al.*, 2012). Drawbacks of MLPA are the relatively higher cost compared with PCR approaches and the required experience for the design of the MLPA specific probes to avoid cross-hybridization with *DPY19L2* pseudogenes (Zhu *et al.*, 2013). Considering these points, we recommend the research of homozygous and heterozygous *DPY19L2* deletions as the first-line genetic analysis in globozoospermic by long-range PCR or MLPA (Coutton *et al.*, 2012; Elinati *et al.*, 2012; Zhu *et al.*, 2013). Alternatively a quantitative PCR protocol could be used (Chianese *et al.*, 2015). This approach does not rely on breakpoint localization and new robust methods are becoming available which may provide a cheap and fast diagnosis allowing the detection of all recombined alleles. In almost all cases of globozoospermic patients with a heterozygous deletion, an additional point mutation in the non-deleted allele was secondly identified (Coutton *et al.*, 2012; Elinati *et al.*, 2012; Zhu *et al.*, 2013). This strongly encourages researching such a point mutation in globozoospermic patients heterozygous for the genomic deletion (Coutton *et al.*, 2012). In the absence of a deletion (heterozygous or homozygous) the full 22 exon *DPY19L2* sequencing might not be systematically realized as a routine analysis. Similarly, the very low rate of mutations found in others genes, such as *SPATA16* or *PICK1,* questions the clinical relevance of molecular investigations of such genes (Coutton *et al.*, 2012). A ‘hot-spots’ restrictive strategy based on the sequencing of the *DPY19L2* exons 8–11, in which most of the point mutations were identified, could also be envisaged. The genetic diagnosis of globozoospermia does not yet provide any clear prognostic or therapeutic indications. Nonetheless, a molecular diagnosis remains useful to provide adequate genetic counseling and to better understand the physiopathology of globozoospermia, which might help to identify novel therapeutic solutions.

DPY19L2 functions: new insights on the physiopathology of globozoospermia

DPY19L2 belongs to a new family of transmembrane proteins of the nuclear envelope including, in Mammals, fours homologous proteins: DPY19L1 to DPY19L4. Initially, inactivation of DPY-19, the only DPY19L ortholog in *C. elegans*, was shown to induce a shorter (dumpy) than wild-type phenotype (Brenner, 1974). DPY-19 has 10 predicted transmembrane domains and was involved in Q neuroblast polarization during early development (Honigberg and Kenyon, 2000). The four human homologues have 9–11 predicted transmembrane domains, suggesting a similar protein structure to DPY-19 (Carson *et al.*, 2006). Moreover, human DPY19L proteins share a high level of sequence identity with DPY-19, consistent with a conservation of the ancestral protein function (Buettner *et al.*, 2013). Functions of human DPY19L proteins are still poorly understood and in particular for

Table IV **Mutation status for the *DPY19L2* gene in globozoospermic men.**

References	Number of patients				Geographical origin (*n*)	DPY19L2 mutations[¤] (%; *n*)	DPY19L2 deletion detection method	Point mutations identified
	Included	Analyzed	Unrelated[ʔ]	Newly tested				
Harbuz *et al.* (2011)	20	20	19	20	North African (19) and Eastern Europe (1)	Hm deletion 73.7% (14/19) Hm point mutations n.d Compound Htz n.d No mutation 26.3% (5/19)	LR-PCR	None
Koscinski *et al.* (2011)	28	28	21	28	North African (7), Middle East (6), European (12), unknown (3)	Hm deletion 19% (4/21) Hm point mutations n.d Compound Htz n.d No mutation 81% (17/21)	LR-PCR	None
Coutton *et al.* (2012)	34	31	30	14[$]	North African (30), European (4)	Hm deletion 73.3% (22/30) Hm point mutations 3.3% (1/30) Compound Htz 6.7% (2/30) No mutation 16.7% (5/30)	MLPA	p.R290H; p.Q342*; p.M358K
Elinati *et al.* (2012)	54	54	54	33[£]	North African (n.a), Middle East (n.a), European (n.a)	Hm deletion 46.3% (25/54) Hm point mutations 7.4% (4/54) Compound Htz 13% (7/54) No mutation 33.3% (18/54)	LR-PCR	p.R290H; p.R298C; p.Q345*; p.S395LfsX7; p.T493R; p.K680*; c.1218+1G>A; Ex5_6del; Ex5_7del
Zhu *et al.* (2013)	16	15	15	16	Asian (16)	Hm deletion 26.7% (4/15) Hm point mutations 33.3% (5/15) Compound Htz 0% (0/15) No mutation* 33.3% (5/15)	LR-PCR	c.1532delA; c.[1679delT 1681_1682delAC]; p.R290H; p.L330P
Noveski *et al.* (2013)	2	2	2	2	Eastern Europe (2)	Hm deletion 100% (2/2) Hm point mutations 0 (0%) Compound Htz 0 (0%) No mutation 0 (0%)	LR-PCR	None
Ounis *et al.* (2015)	7	5	3	7	North African (7)	Hm deletion 100% (3/3)	MLPA	None

Continued

Table IV *Continued*

References	Number of patients				Geographical origin (*n*)	DPY19L2 mutations[¤] (%; *n*)	DPY19L2 deletion detection method	Point mutations identified
	Included	Analyzed	Unrelated[‽]	Newly tested				
						Hm point mutations 0 (0%) Compound Htz 0 (0%) No mutation 0 (0%)		

n.d: not determined; n.a: not available; *n*: number of patients; spz: sperm cells; MLPA: multiplex ligation-dependent probes amplification; LR-PCR: long-range PCR.
[‽]Indicated the number of unrelated patients among all analyzed patients.
[$]The study included 20 patients previously described in Harbuz *et al*. (2011).
[£]The study included 21 patients previously described in Koscinski *et al*. (2011).
[¤]Mutation rate (%) is calculated only from the number of unrelated patients.
*One additional patient (1/15, 6.7%) had a heterozygous deletion in one allele but with no mutation identified in the non-deleted allele.

Table V **Sperm parameters of globozoospermic men genotyped for the *DPY19L2* gene.**

References	*DPY19L2* genotype (*n*)[$]	Sperm parameters			
		Nb spz × 10^6/ml	Total sperm count (10^6)	Normozoospermic[‽] % (*n*)	Round-headed spz (%)
Harbuz *et al*. (2011)	Deleterious mutations (15)	62.8 (0.02–154)	194.6 (0.09–665)	86.7 (13)	99.5 (94–100)
	No mutation (5)	9.7 (0.04–25.2)	31.2 (0.12–68)	40 (2)	89.8 (64–100)
Koscinski *et al*. (2011)	Deleterious mutations (8)	83.4 (13.2–223)	n.a	100 (8)	100 (100)
	No mutation (20)	38.7 (0.35–109)	n.a	30 (6)	97.6 (84–100)
Coutton *et al*. (2012)	Deleterious mutations (26)	57.3 (0.6–108)	210 (n.a)	n.a	88 (29–100)
	No mutation (5)	20 (14–25)	64 (n.a)	n.a	63 (12–100)
Elinati *et al*. (2012)	Deleterious mutations (36)	n.a	n.a	n.a	n.a
	No mutation (18)	n.a	n.a	n.a	n.a
Zhu *et al*. (2013)	Deleterious mutations (9)	46.7 (0.8–90)	183.6 (n.a)	n.a	100 (100)
	No mutation (5)	20 (14–25)	60 (n.a)	n.a	100 (100)
Noveski *et al*. (2013)	Deleterious mutations (2)	n.a	n.a	n.a	100 (100)
	No mutation (0)				
Ounis *et al*. (2015)	Deleterious mutations (5)	64.6 (16–171)	n.a	100 (5)	99 (95–100)
	No mutation (0)				

Values are expressed as the mean with the lower and the higher values between brackets if available. n.a: not available; *n*: number of patients; spz: sperm cells;
[$]Indicate the number of patients with a deleterious mutations including all types of reported mutations in *DPY19L2* (homozygous deletions, point mutations, compound heterozygotes) and the number of patients with no bi-allelic mutation identified.
[‽]Indicated the total number and percentage of patients normozoospermic with a sperm concentration upper to the 5th centiles of World Health Organization 2010 values (15 × 10^6/ml (CI 12–16)).

DPY19L4 for which nothing is known. It has been demonstrated that down-regulation of Dpy19l1 and Dpy19l3 during neurogenesis of mouse embryos leads to strong neuron migration anomalies (Watanabe *et al*., 2011). *DPY19L3* has also been associated with bipolar disorder (Smith *et al*., 2009). These results strengthen the possibility of a conserved function of the different DPY19L proteins. Interestingly, apart from *DPY19L2*, which presents a predominant expression in the testis, *DPY19L* proteins show a relatively ubiquitous pattern of expression (Carson *et al*., 2006).

After its identification as the main gene involved in globozoospermia, study of *Dpy19l2* KO mice showed that homozygous animals faithfully reproduced the human phenotype with a complete male infertility, 100% of acrosomeless round-headed spermatozoa and manchette abnormalities (Table III). Study of this model led to the conclusion that DPY19L2 is a transmembrane protein located in the inner nuclear membrane (Fig. 3) and that it is necessary to anchor the acrosome to the nucleus. In the absence of Dpy19l2 the forming acrosome slowly separates from the nucleus before being removed from the sperm with the cytoplasm (Pierre *et al*., 2012). In addition to its structural function during acrosome biogenesis, the C-mannosyltransferase function of the ancestral protein DPY-19 has raised the hypothesis that DPY19L2 may have a function in glycosylation of sperm proteins (Buettner *et al*.,

2013). Several potential partner proteins have been proposed to interact with DPY19L2 during spermiogenesis, in particular the SUN proteins belonging to the LINC (linker of nucleoskeleton and cytoskeleton) complexes in association with KASH proteins (Pierre *et al*., 2012). SUN proteins appeared as the best candidate due to their abundance during spermiogenesis and their role in nuclear shaping during sperm head formation (Göb *et al*., 2010; Frohnert *et al*., 2011). This hypothesis has however not been confirmed and the molecular partners of DPY19L2 remain to be characterized (Yassine *et al*., 2015b). Comparative testicular transcriptome studies of wild type and globozoospermic Dpy19l2 KO mice were also realized to attempt to identify Dpy19l2 molecular partners but no conclusive result was obtained (Karaouzène *et al*., 2013).

DPY19L2, low copy repeats and copy number variations: from evolution to mutations

Copy number variations (CNV) refer to gains by duplication or losses by deletion of genetic material greater than 1 kb. They can be pathogenic and cause Mendelian traits or be associated with complex diseases but can also represent benign polymorphic variants. CNVs and in particular gene duplications are involved as a predominant mechanism driving gene and genome evolution (reviewed in Zhang *et al*., 2009). Such a mechanism was involved in the *DPY19L* gene family evolution throughout the vertebrate lineage. The *DPY19L* family genes, encompassing four genes (*DPY19L1*, *2*, *3* and *4*) and six pseudogenes, derived from a common ancestor homologous to the *C. elegans dpy-19* gene owing to multiple gene duplications and pseudogenizations throughout evolution (Carson *et al*., 2006). It is estimated that the duplication that generated *DPY19L2* arose between 173 and 360 million years ago prior to mammalian divergence.

Recurrent CNVs arise mostly by homologous recombination between repetitive DNA sequences. This process is called non-allelic homologous recombination (NAHR) and is well-known in human diseases to be responsible for many recurrent genomic syndromes (Stankiewicz and Lupski, 2010). The DPY19L2 locus is conducive to recurrent deletions and duplications by NAHR due to the presence of two homologous 28-kb low copy repeats (LCRs) located on each side of the gene (Harbuz *et al*., 2011; Koscinski *et al*., 2011). Such a recombination during the meiosis will result in the production of recombinant gametes having either a duplication or a deletion of the *DPY19L2* locus. Sequencing analysis of the NAHR breakpoints at the *DPY19L2* locus demonstrated that the recombination events occurred preferentially in the vicinity of a 13 nucleotide recognition motif for the PRDM9 protein localized in the center of the 28-kb LCRs (Coutton *et al*., 2013). PRDM9, a zinc finger protein that binds to DNA sequences, is a major *trans*-regulator of meiotic recombination hotspots facilitating double strand breaks, a prerequisite for NAHR (Baudat *et al*., 2010; Myers *et al*., 2010; Parvanov *et al*., 2010). An additional putative recombination hotspot within the 28-kb LCRs might constitute a second, less frequent recombination site (Elinati *et al*., 2012).

The NAHR mechanism favors deletions over duplications because the inter-chromatid and inter-chromosome NAHRs create a deleted and a duplicated recombined allele, while intra-chromatid events only generate deletions (Liu *et al*., 2012). More gametes carrying a deletion are thus expected to be produced *de novo* and this was verified for several NAHR hotspots (Turner *et al*., 2008) and in particular for the *DPY19L2* locus (Coutton *et al*., 2013). This is paradoxical regarding the overrepresentation of the duplicated *DPY19L2* allele in the general population. In fact, the frequency of the *DPY19L2* duplication and heterozygous deletion in the general population is estimated to be 1/85 and 1/290, respectively. This can be explained by the loss of deleted alleles occurring during selection against sterile, homozygous deleted men, whereas duplications carriers are not subjected to selection. Also, heterozygous deleted men might also suffer a small fitness penalty, expediting the loss of the deleted allele (Coutton *et al*., 2013).

Clinical management of globozoospermic patients and DPY19L2: what's new?

The relationship between sperm chromosomal abnormalities and sperm morphology has been widely explored. A link has been clearly established in particular for certain types of morphologically abnormal spermatozoa, such as large-headed multiflagellar spermatozoa, but remains controversial for many others (Sun *et al*., 2006). Globozoospermia is no exception and contradictory results were obtained. Some studies report an increased frequency in sperm aneuploidy compared with normal sperm cells while others do not observe any difference (reviewed in Dam *et al*., 2007a; Perrin *et al*., 2013). Finally, even observed, the slightly increased aneuploidy rate in round-headed sperm is comparable to that commonly found in other types of infertility (Perrin *et al*., 2013) and therefore does not counter indicate the treatment of patients by ICSI (Kuentz *et al*., 2013). Despite this, many studies using ICSI for patients with globozoospermia reported low fertilization, pregnancy and live birth rates, overall estimated at 38, 20 and 14%, respectively (reviewed in Dávila Garza and Patrizio, 2013). A first well-established explanation for the low efficiency of ICSI when using globozoospermic sperm is the reduction or absence of the sperm factor, a testis-specific phospholipase (phospholipase C zeta (PLCζ)) involved in the oocyte activation (Yoon *et al*., 2008; Heytens *et al*., 2009; Kashir *et al*., 2010; Amdani *et al*., 2013; Escoffier *et al*., 2015). PLCζ localizes to the inner acrosomal membrane and the nuclear theca in the post-acrosomal region of human sperm which is in accordance with its vanishing in *Dpy19l2*-KO globozoospermic sperm (Escoffier *et al*., 2015). The absence of oocyte activation can be overcome by artificial oocyte activation (AOA) using Ca^{2+} ionophore (Rybouchkin *et al*., 1997; Tejera *et al*., 2008; Kyono *et al*., 2009; Taylor *et al*., 2010). In case of globozoospermia, AOA combined with ICSI improved the efficiency of fertilization, pregnancy and live birth rates compared with conventional ICSI and this, regardless of the genotype (DPY19L2 related or not) of the patients (Kuentz *et al*., 2013). However, the live birth rate per transfer remains lower in globozoospermic patients than in other infertile patients in the same age group (Palermo *et al*., 2009; Kuentz *et al*., 2013). This may be due to sperm DNA damage related to defective chromatin condensation and the DNA fragmentation described in globozoospermic sperm cells (Dam *et al*., 2007a; Brahem *et al*., 2011; Perrin *et al*., 2013; Yassine *et al*., 2015a). Abnormal chromatin condensation in globozoospermia could be linked to an altered replacement of histones by protamines (Blanchard *et al*., 1990; Carrell *et al*., 1999). Yassine *et al*. (2015a) using the *dpy19l2* KO mouse models confirmed that the nuclear invasion by protamines during the last stage of compaction is defective. Moreover, the absence of protamine increases dramatically the susceptibility to DNA breaks. Altogether, epigenetics defects and DNA fragmentation in globozoospermic sperm nuclei impair the developmental potential of embryos generated by ICSI using *Dpy19l2*-dependent spermatozoa (Yassine *et al*., 2015a). In summary,

globozoospermic patients with *DPY19L2* gene alterations combine a low oocyte activation rate and a poor development of embryos generated by ICSI, thus explaining the consistently disappointingly low pregnancy rate obtained with these patients.

Mutational status in globozoospermic patients treated with ICSI was generally unknown or not investigated. Few studies reported successful fertilization or pregnancies in globozoospermic patients with well documented *DPY19L2* or *SPATA16* mutations (Dam *et al.*, 2007b; Harbuz *et al.*, 2011; Kuentz *et al.*, 2013; Karaca *et al.*, 2014). Due to this small number of cases, no evidence of a difference in pregnancy success rate regarding the mutational status of globozoospermic patients has currently been demonstrated. Further studies taking into account genetic mutations may influence the therapeutic strategy and facilitate improving the management of patients diagnosed with globozoospermia. To date, only the type of globozoospermia is known to have an impact on the therapeutic approach as intracytoplasmic morphologically selected sperm injection technique (IMSI) has proven effective only for patients with a partial globozoospermia (Sermondade *et al.*, 2011; Kuentz *et al.*, 2013).

Multiple morphological abnormalities of the flagella

Morphological abnormalities of the sperm flagella leading to asthenozoospermia have been reported regularly since 1984 (Escalier and David, 1984). Chemes and colleagues carried out much of the early work on this phenotype and extensively studied the ultrastructure of the sperm flagella of affected men (Chemes *et al.*, 1987). They observed recurrent abnormalities of the fibrous sheath (FS) which defines the principal piece surrounding the axoneme and the outer dense fibers and consists of two longitudinal columns connected by circumferential ribs (Eddy *et al.*, 2003) (Fig. 4). Escalier had previously identified similar flagellar defects and had described that abnormalities of the peri-axonemal structures (including FS, outer dense fibers, mitochondrial sheath) were always associated with axonemal defects (Escalier and David, 1984). She argued that these peri-axonemal defects might be secondary to the axonemal defects (Escalier and Serres, 1985). Further ultrastructural studies among patients with sperm flagella abnormalities showed a wide range of different peri-axonemal and axonemal defects (Rawe *et al.*, 2001; Chemes and Rawe, 2003).

Cilia and flagellum: a common origin

Although the sperm flagellum and motile cilia (found in the epithelial cells of the airways, the Fallopian tubes, the choroid plexus and the brain ventricles) are specialized for a particular function, they share common structural elements as the central cytoskeletal structure called the axoneme, which is highly conserved throughout evolution. The axoneme contains 9 outer microtubules doublets and 2 central singlets (9+2 structure), several axonemal dyneins, radial spokes, nexin links and many other components that drive and regulate ciliary or flagellar motility. All these microtubule-associated proteins are attached along the axoneme in a regular 96 nm long repeated formation (Inaba, 2003; Satir and Christensen, 2008). The axonemal dyneins are multi-component proteins organized along each microtubule doublet as inner and outer rows (Fig. 4B and C) and comprise motor multiprotein complexes which produce the cilia/flagella beating force (Wickstead and Gull, 2007). Dynein arms are composed of heavy chains (HC), intermediate chains (IC), light-intermediate chains (LIC) and light chains (LC) and numerous regulatory proteins (Bisgrove and Yost, 2006). The structure of the inner dynein arms is more complex than the structure of the outer dynein arms, which, in mammals, contains only two different HCs repeated all along the axoneme. In mammals, the inner arms are organized in seven molecular complexes, viewed in electronic microscopy as globular heads arranged in 3-2-2 groups, thus corresponding to 3 types of inner dynein arms (IDA): IDA1 to IDA3 (Vernon *et al.*, 2005). The mammalian organization was however much less investigated than in *Chlamydomonas* (Bui *et al.*, 2008), and the subunit composition of each inner arm remains to be determined. Although sperm flagella and motile cilia have a similar microtubule structure based on the presence of a 9+2 axoneme, they present several differences. Motile cilia are constituted almost exclusively by the axoneme, whereas mammalian sperm flagella are divided into three principal parts characterized by the presence of additional accessory structures surrounding the axoneme: the outer dense fiber, the mitochondrial sheath, the fibrous sheath (Fig. 4). Recent proteomic analysis identified over 700 proteins that are exclusively localized in the human sperm flagella, of which many are axonemal proteins (Baker *et al.*, 2013). These data highlight the complexity of the flagella structure and biogenesis, suggesting that many genes could be linked to flagellar abnormalities and/or asthenozoospermia.

Primary ciliary dyskinesia

Primary ciliary dyskinesia (PCD) is a multisystemic disorder caused by motility defects of motile cilia and flagella (Afzelius and Eliasson, 1983; Munro *et al.*, 1994). PCD is mainly characterized by recurrent respiratory tract infections with varying symptoms ranging from chronic rhinosinusitis to bronchiectasis and male infertility due to sperm immotility (Ibañez-Tallon *et al.*, 2003). Female subfertility is less common and is caused by dysmotile Fallopian tubes cilia (Lyons *et al.*, 2006). PCD is also associated in 50% of cases with *situs invertus* due to dysfunction of motile embryonic node cilia perturbing organ laterality (Zariwala *et al.*, 1993). More rarely, hydrocephalus arises as a consequence of ependymal cilia dysmotility leading to a blockage of the cerebrospinal fluid flow (Ibañez-Tallon *et al.*, 2002; Kosaki *et al.*, 2004). PCD has an estimated incidence of 1 per 15 000 births (Knowles *et al.*, 2013a) but it may be underestimated due to diagnostic failure (Boon *et al.*, 2013).

A high prevalence of PCD was observed in different genetic isolates and consanguineous populations, a fact consistent with a mainly autosomal recessive mode of inheritance (Jeganathan *et al.*, 2004; O'Callaghan *et al.*, 2010). PCD is genetically heterogeneous (Horani *et al.*, 2014) and research on PCD allowed the characterization of numerous proteins necessary for adequate axonemal molecular structure and assembly. Cilia and flagella are remarkably well conserved throughout evolution. Different experimental models were used to decipher the molecular composition and function of these organelles (Ostrowski *et al.*, 2011). The first model was the green alga *Chlamydomonas* in which *DNAI1*, the first gene to be associated with PCD, was identified (Pennarun *et al.*, 1999). The function of the genes involved in PCD was also efficiently assessed in other multicellular organisms, such as zebrafish, *Xenopus*, *Caenorhabditis elegans* or *Drosophila*, and protists, such as *Paramecium*, *Tetrahymena, Trypanosoma* or *Leishmania,* each bringing specific advantages to the study of cilium biology (review in Vincensini *et al.*, 2011). Thereafter, various studies using a candidate gene approach

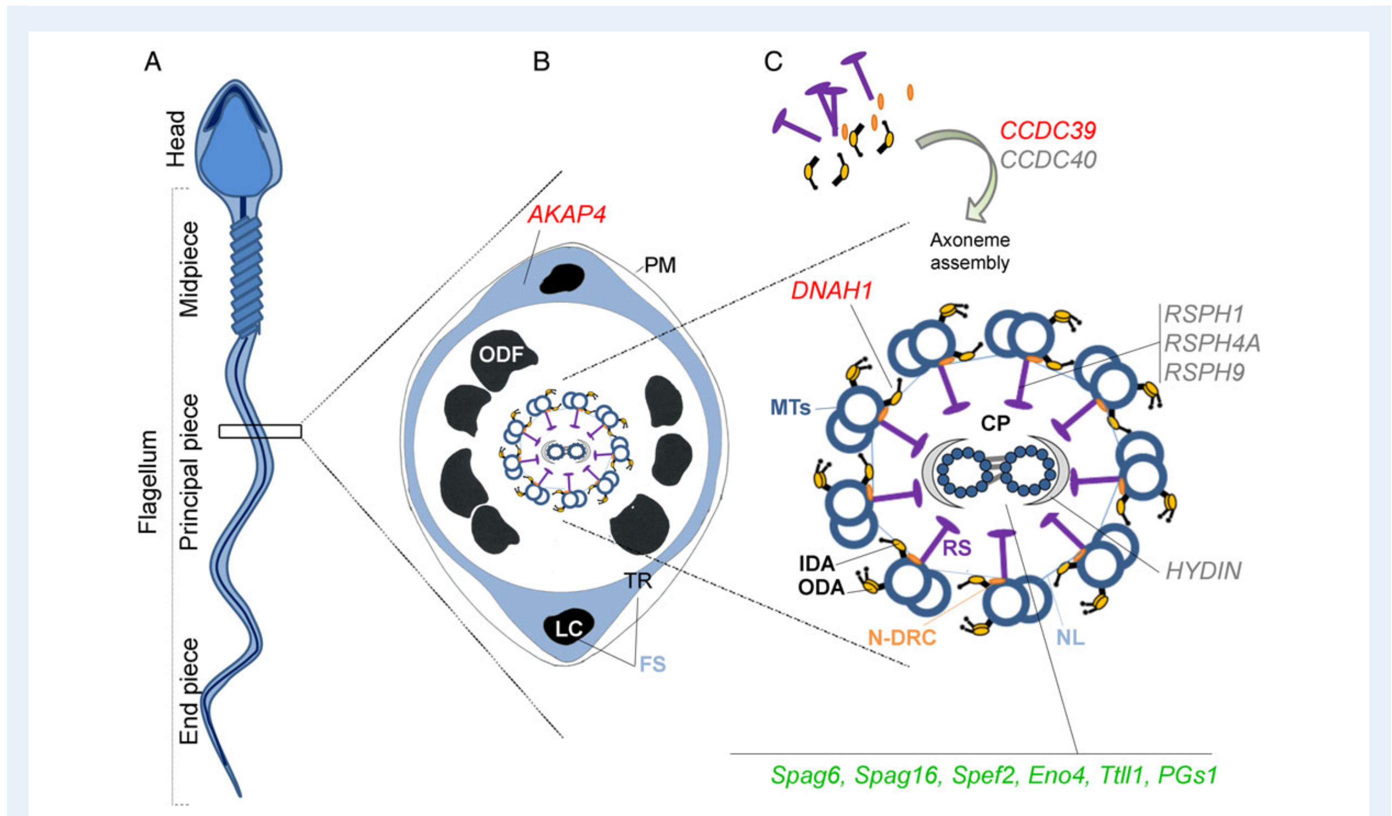

Figure 4 Structure of sperm flagellum and localization of proteins whose functional absence leads to a defective flagellar structure and MMAF-like phenotype. (**A**) Mammalian sperm flagellum is structurally divided into three areas: midpiece, principal piece and end piece. (**B**) Schematic cross section through a representative segment of the principal piece showing the plasma membrane (PM) surrounding 7 outer dense fibers (ODF, there are 9 ODF in the midpiece). The fibrous sheath (FS) is composed of two longitudinal columns (LC) which are connected by transverse ribs (TR). Within the ODF are the components of the axoneme. (**C**) The axoneme is enlarged and the offset shows: the 9 outer microtubule doublets of the axoneme (MTs) with associated inner dynein arms (IDA), outer dynein arms (ODA), radial spokes (RS), nexin-dynein regulator complex (N-DRC), nexin links (NL) and the central pair of microtubule doublets (CP). Projections are represented on the CP (gray bow) but not detailed. Genes formally identified for the MMAF phenotype in human are reported in red. Candidate genes for the MMAF phenotype identified in animal models and in humans are reported in green and gray, respectively. Genes abbreviations are as follows: A kinase (PRKA) anchor protein 4 (*AKAP4;* coiled-coil domain containing 39 (*CCDC39*); coiled-coil domain containing 40 (*CCDC40*); radial spoke head 1 homolog (*RSPH1*), radial spoke head 4 homolog A (*RSPH4A*), radial spoke head 9 homolog *RSPH9*; HYDIN, axonemal central pair apparatus protein *(HYDIN);* sperm associated antigen 6 (Spag6); Sperm associated antigen 16 (Spag16); Sperm flagellar 2 (*Spef2);* Enolase 4 (Eno4); Tubulin tyrosine ligase-like 1 (Ttll1); Tubulin polyglutamylase complex subunit 1 (Psg1).

mainly based on data from *Chlamydomonas* have resulted in the identification of many mutated genes in PCD patients. Different KO mice or inbred dogs deficient in various axonemal components have also been successfully described as mammalian models for PCD (review in Inaba, 2011). Mutations in 28 genes leading to various ultrastructural defects have so far been described to cause PCD (Table VI) and account for the genetic etiology of ~70% of affected individuals (Knowles *et al*., 2014). A male infertility phenotype has often been described as part of the clinical symptoms but this particular aspect of the physiopathology of the PCD is not systematically explored and is often only scarcely described in scientific reports (Table VI). The sperm of infertile male patients with PCD are usually immotile and present various ultrastructural defects of sperm flagella such as missing dynein arms, microtubular translocations, and lack of radial spokes. Nevertheless, in most cases the sperm flagellum appears morphologically normal under direct light microscopy (Table VI) thereby excluding most of these genes as a genetic cause of teratozoospermia or MMAF.

Genetic investigations of MMAF

A genetic origin of MMAF was strongly suspected based on a family clustering reported in at least 20% of patients (Chemes and Alvarez Sedo, 2012). In 2005, Bacetti *et al.* (Baccetti *et al*., 2005a) first reported a partial deletion in the A kinase (PRKA) anchor protein 3 (*AKAP3)* and A kinase (PRKA) anchor protein 4 (*AKAP4)* genes in a patient presenting with short-tail spermatozoa. Ultrastructural sperm evaluation showed MMAF and an altered axonemal structure lacking dynein arms and microtubular doublets including the central pair. A kinase (PRKA) anchor protein 3 *(AKAP3)* and A kinase (PRKA) anchor protein 3 *(AKAP4)* encode two A-kinase anchoring proteins interacting with the regulatory subunits of cAMP-dependent protein kinase A. AKAP3 and AKAP4 are the most abundant structural proteins of the fibrous sheath. AKAP3 is involved in organizing the basic structure of the fibrous sheath while AKAP4 has a major role in completing fibrous sheath assembly (Brown *et al*., 2003). *Akap4* KO mice are infertile with half-length flagella with a

Table VI Primary ciliary dyskinesia gene mutations and their consequences for axonemal ultrastucture and sperm phenotype.

Gene ID	Full name	MIM number	Axonemal localization	Axonemal defects	Infertility phenotype	References
ARMC4	Armadillo repeat containing 4	615408	Assembly of ODA	Reduced outer dynein arms	No data available	Hjeij *et al*. (2013) and Onoufriadis *et al*. (2014a)
C21orf59	Chromosome 21 open reading frame 59	615494	Dynein arm assembly	Absence of both outer and inner dynein arm components	No data available	Austin-Tse *et al*. (2013)
CCDC103	Coiled-coil domain containing 103	614677	Assembly of ODA	Reduced outer dynein arms	No data available	Panizzi *et al*. (2012)
CCDC114	Coiled-coil domain containing 114	615038	DCC2, an ODA microtubule-docking complex	Ciliary outer dynein arm defects	Male are fertile	Knowles *et al*. (2013b) and Onoufriadis *et al*. (2013)
CCDC151	Coiled-coil domain containing 151	615956	ODA docking complex	Outer dynein arm defect	No data available	Hjeij *et al*. (2014) and Jerber *et al*. (2014)
CCDC164 (DRC1)	Coiled-coil domain containing 164	615288	Nexin-dynein regulatory complex	Absence of nexin links, disruption of the nexin-dynein regulatory complex	No data available	Wirschell *et al*. (2013)
CCDC39	Coiled-coil domain containing 39	613798	Assembly of IDA, N-DRC and Radial spokes	– absence of inner dynein arms and nexin links – axonemal disorganization with mislocalized peripheral doublet – displacement or absence of the central pair	Oligoasthenozoospermia midpiece is narrowed flagellum is shortened	Merveille *et al*. (2011), Blanchon *et al*. (2012) and Antony *et al*. (2013)
CCDC40	Coiled-coil domain containing 40	613799	Govern the assembly of N-DRC and inner dynein arm complexes, but not outer dynein arm complexes	– reduction of inner dynein arms – axonemal disorganization with mislocalized peripheral doublet, displacement or absence of the central pair – abnormal radial spokes and nexin links	No data available	Becker-Heck *et al*. 2011, Blanchon *et al*. (2012) and Antony *et al*. (2013)
CCDC65	Coiled-coil domain containing 65	611088	Assembly of the N-DRC	Reduction in inner dynein arms and nexin links	No data available	Austin-Tse *et al*. (2013) and Horani *et al*. (2013b)
DNAAF1 (LRRC50)	Dynein, axonemal, assembly factor 1	613190	Assembly of dynein arm complexes in the cytoplasm	Absence of both outer and inner dynein arms	Male infertility reported but no details were provided	Duquesnoy *et al*. (2009) and Loges *et al*. (2009)
DNAAF2 (KTU)	Dynein, axonemal, assembly factor 2	612517	Assembly of dynein arm complexes in the cytoplasm	Absence of both outer and inner dynein arms	Asthenozoospermia	Omran *et al*. (2008)
DNAAF3	Dynein, axonemal, assembly factor 3	614566	Assembly of dynein arm complexes in the cytoplasm	Absence of both outer and inner dynein arms	Male infertility reported but no details were provided	Mitchison *et al*. (2012)
DNAH11	Dynein, axonemal, heavy chain 11	603339	Outer dynein arm heavy chain	Ultrastructure respiratory cilia is normal and outer dynein arms are intact	Male are fertile	Bartoloni *et al*. (2002), Schwabe *et al*. (2008), Knowles *et al*. (2012) and Lucas *et al*. (2012)
DNAH5	Dynein, axonemal, heavy chain 5	603335	Outer dynein arm heavy chain	Outer dynein arm defect	Asthenozoospermia	Olbrich *et al*. (2002), Fliegauf *et al*. (2005), Hornef *et al*. (2006) and Failly *et al*. (2009)
DNAI1	Dynein, axonemal, intermediate chain 1	604366	Outer dynein arm intermediate chain	The outer dynein arms are shortened or missing.	Asthenozoospermia	Pennarun *et al*. (1999), Guichard *et al*. (2001); Zariwala *et al*. (2001)
DNAI2	Dynein, axonemal, intermediate chain 2	605483	Outer dynein arm intermediate chain	Outer dynein arm defect	Male infertility reported but no details were provided	Loges *et al*. (2008)

DNAL1	Dynein, axonemal, light chain 1	610062	Outer dynein arm light chain	The outer dynein arms are shortened or missing	No data available	Mazor *et al.* (2011)
DYX1C1 (DNAAF4)	Dyslexia susceptibility 1 candidate 1	608706	Assembly of dynein arm complexes in the cytoplasm	Absence of both outer and inner dynein arms	Asthenozoospermia	Tarkar *et al.* (2013)
HEATR2	HEAT repeat containing 2	614864	Assembly or stability of axonemal dynein arms	Absence of outer dynein arms and partial lack of inner dynein arms	Male infertility reported but no details were provided	Horani *et al.* (2012)
HYDIN	HYDIN, axonemal central pair apparatus protein	610812	C2b projection	Lack the C2b projection of the central pair	Asthenozoospermia	Olbrich *et al.* (2012)
LRRC6	Leucine rich repeat containing 6	614930	Assembly of dynein arm complexes in the cytoplasm	Absence of both outer and inner dynein arms	Asthenozoospermia	Kott *et al.* (2012) and Horani *et al.* (2013a)
RGPR	Retinitis pigmentosa GTPase regulator gene	312610	Involved in the transitional zone of motile cilia in airway epithelia/ localized to centrioles	Variable. From normal structure to lack of both dynein arms an abnormal microtubular Disorganization.	No data available	Moore *et al.* (2006) and Bukowy-Bieryłło *et al.* (2013)
RSPH1	Radial spoke head 1 homolog	609314	Radial spoke component	Central pair microtubule complex and radial spoke defects	Male infertility reported but no details were provided	Kott *et al.* (2013), Knowles *et al.* (2014) and Onoufriadis *et al.* (2014b)
RSPH4A	Radial spoke head 4A homolog	612647	Radial spoke component	Central pair microtubule complex and radial spoke defects	Male infertility reported but no details were provided	Castleman *et al.* (2009) and Daniels *et al.* (2013)
RSPH9	Radial spoke head 9 homolog	612648	Radial spoke component	Central pair microtubule complex and radial spoke defects	Male infertility reported but no details were provided	Castleman *et al.* (2009)
SPAG1	Sperm associated antigen 1	603395	Assembly of dynein arm complexes in the cytoplasm	Defects of both outer and inner dynein arms	No data available	Knowles *et al.* (2013c)
TXNDC3	Thioredoxin domain containing 3	607421	Non precisely determined	Shortened or absent outer dynein arms	No data available	Duriez *et al.* (2007)
ZMYND10	Zinc finger, MYND-type containing 10	607070	Assembly of dynein arm complexes in the cytoplasm	Lack of outer and inner dynein arms	Male infertility reported but no details were provided	Moore *et al.* (2013) and Zariwala *et al.* (2013)

thinner principal piece and a tip sometimes curled or splayed into fine filaments. The size and integrity of mutant mitochondrial sheaths are also reduced. However, other cytoskeletal structures, namely the outer dense fibers and the axoneme, remain intact (Miki *et al.*, 2002). Mouse models thus present strong evidence that Akap3 and 4 are involved in MMAF phenotype. Evidence of their implication in the human phenotype is however weaker. A deletion of *AKAP3* and *AKAP4* was described in a MMAF patient but it was only detected using conventional PCR and the genomic breakpoints were not identified (Baccetti *et al.*, 2005a). Moreover, no quantitative analyses of DNA or mRNA were used to confirm the observed deletions and exclude a false negative PCR result. The authors then demonstrated an absence of staining in patient's spermatozoa using immunofluorescence with AKAP4 antibodies (Baccetti *et al.*, 2005a). The absence of the AKAP4 protein in mature sperm may however be secondary to another defect disorganizing the whole sperm flagella structure (Baccetti *et al.*, 2004). Additionally, Turner *et al.* (2001) did not find any mutation in these two genes in 9 MMAF patients.

More recently homozygous mutations in the *DNAH1* gene were identified in several patients with MMAF (Ben Khelifa *et al.*, 2014). *DNAH1* encodes an axonemal inner arm dynein HC and is expressed in various tissues including the testis (Maiti *et al.*, 2000). Homozygosity mapping was carried out on a cohort of 20 North African individuals. Four different homozygous mutations in *DNAH1*, one run-on, one missense and two splice site mutations were identified in seven patients including three brothers. Electron microscopy examination of spermatozoa revealed a general axonemal disorganization including mislocalization of the microtubule doublets, absence of the central pair in about half of the analyzed cross sections, loss of the inner arm dynein as well as severe disorganization of the fibrous sheath, the outer dense fibers and the mitochondrial sheath (Ben Khelifa *et al.*, 2014). All these defects are hallmarks of the MMAF phenotype. Molecular and functional studies on samples from one of the mutated patients carrying the recurrent splicing mutation (c.11788-1G>A) demonstrated that the transcript and the protein were absent, confirming that the observed phenotype is due to the complete absence of DNAH1. This initial study thus indicates that ~70% of DFS patients are expected to bear a genetic alteration in other genes, thus confirming that, like PCD, MMAF is genetically heterogeneous and that many other genes are likely involved in this syndrome. Lastly, the patient with the less severe missense variant presented a milder phenotype with 5% motility and the presence of 6% morphologically normal spermatozoa in contrast to the other *DNAH1*-mutated patients which had 100% abnormal sperm and 0% motility (Ben Khelifa *et al.*, 2014). It could therefore be expected that individuals with intermediate asthenozoospermia and a low level of morphological anomalies could also harbor homozygous or compound heterozygous *DNAH1* mutations of moderate severity.

The phenotype observed in *DNAH1*-mutated patients extends beyond the absence of flagellar motility, as usually observed in other axonemal component defects, and substantiates a role for this protein in the maintenance of the structural integrity of the flagella. In *Dnahc1* KO mice (the mouse ortholog of *DNAH1*, previously called *Mdhc7*), 'rapid-freeze deep-etch electron microscopy' studies indicated that one head of the IDA3 was missing, leading to a 3-2-1 globular head arrangement and suggesting that DNAH1 is one of the components of the IDA3 (Vernon *et al.*, 2005). In *Tetrahymena thermophila*, IDA3 corresponds to the dynein d/a which is directly connected to the radial spoke RS3 through an arc-like structure (King, 2013). In mammals, the three radial spokes (RS1, RS2, and RS3) are multiprotein complexes allowing a connection between the external microtubule doublets and the two central microtubules thus stabilizing the axoneme (Pigino *et al.*, 2011). Therefore, the severe axonemal disorganization observed in *DNAH1*-mutated patients may occur as a result of the abrogation of RS3 anchoring, leading to the absence of the central pair and the mislocalization of the peripheral doublets (Ben Khelifa *et al.*, 2014). KO male mice lacking *Dnah1* are infertile but only present with asthenozoospermia, without the sperm morphological abnormalities observed in human. Aside from the fact that there might be some divergences in flagellar biogenesis between mouse and human, an incomplete disruption of the targeted dynein was reported in the mouse model, which may explain this phenotypic difference (Neesen *et al.*, 2001; Ben Khelifa *et al.*, 2014).

The central pair microtubules: the central key of MMAF phenotype?

The central pair microtubules (CP) are composed of two microtubule singlets, named C1 and C2, which are structurally and biochemically distinct. Structural studies in *Chlamydomonas* showed that the C1 tubule has two long projections (1a and 1b) and two short projections (1c and 1d). The C2 tubule has three short projections termed 2a, 2b and 2c. The CP contains at least 23 distinct proteins and some of these proteins are uniquely associated with either C1 or C2, indicating that the two microtubules may be functionally specialized. Evidence supports the fact that the CP projections interact with the RS heads and modulate dynein activity (reviewed in Wargo and Smith, 2003).

It is worth noting that the lack of the CPs leading to an abnormal '9+0' configuration of the axoneme is the main defect observed in most cases associated with MMAF (Chemes and Rawe, 2003). Such a defect was also observed in patients with *DNAH1* mutations (Ben Khelifa *et al.*, 2014). These observations raise the hypothesis that CP disorganization might be the cornerstone leading to the MMAF phenotype. Several experimental models support the idea that some structural defects impacting the CP give rise to MMAF (Fig. 4C and Table VII). Axonemal abnormalities have been observed in spermatids from testicular biopsies underlining that the onset of the defect occurs during the late stages of spermiogenesis due to a defective assembly of cytoskeletal components of the sperm tail (Chemes and Rawe, 2010). Interestingly, in some cases, the disorganization of the microtubules has been described to increase and the sperm phenotype to worsen during the sperm transit through the epididymal duct (Sapiro *et al.*, 2002; Sironen *et al.*, 2011). This suggests that CP defects precede other more severe structural abnormalities by weakening all the axonemal organization making the flagella vulnerable to mechanical stress during spermiogenesis and sperm transit. This demonstrates that CP plays a major role in maintaining the global flagellum organization throughout spermiogenesis. This could explain why most other axonemal defects, not affecting the CP structure, do not lead to the MMAF phenotype.

As a consequence, all genes encoding a protein interacting directly (CP components) or indirectly with the CP apparatus, like RS, some dynein arms or different partner proteins, are henceforth good candidates for the MMAF phenotype (Fig. 4C). As an example, depletion of mouse Meig1, a protein that interacts directly with Spag16 (Table VII) leads to shorter flagella, absence of the normal '9+2' axoneme arrangement and peri-axonemal disorganization (Zhang *et al.*, 2004; Salzberg *et al.*,

Table VII Animal models with central pair of microtubule doublet defects presenting with a DFS/MMAF-like phenotype.

Inactivated genes ID	Full ID	Localization	Function	Sperm phenotype	Known protein interaction	Associated phenotype	References
Spag6	Sperm associated antigen 6	C1 central pair microtubule	Unknown	Truncated sperm tails. Lack of the central pair microtubules. Alterations in the fibrous sheath and/or outer dense fibers.	SPAG16[£], SPAG17	Hydrocephalus (50%)	Sapiro *et al.* (2000, 2002)
Spag16	Sperm associated antigen 16	Bridges connecting the C1 and C2 microtubules	Unknown	Sperm abnormally shaped. Lack of the central pair microtubules. Marked disorganization of the outer doublet microtubules and outer dense fibers.	SPAG6[£], SPAG17, MEIG1[£]	None	Zhang *et al.* (2004)
Spef2 (Kpl2)	Sperm flagellar 2	C1b (?)	Unknown	Lowered sperm counts, short sperm tails. Absence of central pair and missing of peripheral doublets. Lack of organized mitochondria, outer dense fibers, and fibrous sheath structures.	IFT20	None in pig model; Hydrocephalus and sinusitis in mouse	Sironen *et al.* (2006, 2010, 2011)
Eno4	Enolase 4	C1b (?)	Glycolytic enzymes; ATP production	Sperm shortened, thickened and coiled. Disorganized aggregates of the fibrous sheath components. Disrupted axonemal structures. Displaced outer dense fibers in the principal piece. Defective annulus was also reported	PGAM	None	Nakamura *et al.* (2013)
Ttll1 (PGs3)	Tubulin tyrosine ligase-like 1	Central pair α-tubulin (?)	Axonemal α-tubulin polyglutamylation	Shortened flagella. Various axonemal abnormalities ranged from the absence of central microtubule to complete disorganization of the axonemal and peri-axonemal structures.	Unknown	Rhinosinusitis, otitis media	Ikegami *et al.* (2010) and Vogel *et al.* (2010)
PGs1 (Gtrgeo22)	Tubulin polyglutamylase complex subunit 1	Central pair α-tubulin (?)	Axonemal α-tubulin polyglutamylation	Shortened flagella. Various axonemal abnormalities ranged from the absence of central microtubule to complete disorganization of the axonemal and peri-axonemal structures.	Unknown	Absence of intermale aggression, reduced body fat	Campbell *et al.* (2002)

'?' indicated a probable localization but not formally identified. '£' indicated a protein partner involved in dysplasia of the fibrous sheath/multiple morphological abnormalities of the flagella (DFS/MMAF)-like phenotype. *SPAG17*: Sperm associated antigen 17; *MEIG1*: Meiosis expressed gene 1; *IFT20*: intraflagellar transport 20; *PGAM*: phosphoglycerate mutase.

2010; Teves et al., 2013). In humans, several studies reported mutations in the coiled-coil domain containing 39 (*CCDC39*), coiled-coil domain containing 40 (*CCDC40*), radial spoke head 1 homolog (*RSPH1*), radial spoke head 4 homolog A (*RSPH4A*), radial spoke head 9 homolog *RSPH9* and HYDIN, axonemal central pair apparatus protein *(HYDIN)* genes leading to CP defects. However, few phenotypic descriptions or structural studies have been carried out on sperm samples from mutated patients (Table VI). Only Merveille et al. (2011) reported that the sperm flagellum was shortened in patients with a mutation in the *CCDC39* gene, a feature consistent with the MMAF phenotype and our hypothesis.

PCD to MMAF phenotype: a phenotypic continuum?

DNAH1 is the first inner arm dynein gene involved in a human pathology and the first axonemal gene responsible for a male infertility phenotype without any of the other symptoms usually observed in PCD. These novel mutations in the axonemal dynein *DNAH1* strengthen the emerging point of view that MMAF may be a phenotypic variation of the classical forms of PCD with a continuum of clinical manifestations ranging from infertile PCD patients to MMAF patients with no or low noise PCD manifestations. Indeed, many genes are likely to have a specific function in the sperm flagellum with potentially several paralogs that could carry out a similar function in other ciliated tissues, thus explaining the absence of a more severe PCD phenotype. For instance, absence of respiratory symptoms in *DNAH1*-mutated patients may be explained by other dyneins phylogenetically close to *DNAH1* which may compensate the absence of *DNAH1* in other motile cilia (Ben Khelifa et al., 2014). This hypothesis has also been mentioned in a reverse situation where PCD patients with mutations in the *CCDC114*, a gene encoding an outer dynein arm docking complex, had no fertility problems. The authors proposed that CCDC114 function could be partially replaced by CCDC63, a homologous protein of CCDC114 with an expression restricted to the testis (Onoufriadis et al., 2013). Unfortunately, no nasal brushings or biopsies from *DNAH1*-mutated individuals could be obtained to search for infra-clinical manifestations by exploring the patient's ciliary function in respiratory epithelium. In addition, alternative splicing is widespread in mammals and splice variants often exhibit tissue-specific expression patterns (Yu et al., 2014). Therefore some mutations may affect alternative variants specifically expressed in testis while having no effect in other tissues.

Overall, these observations support the fact that important differences exist between the axonemal assembly/function of respiratory cilia and sperm flagella that explain why PCDs are not always associated with primary flagellar defects and vice versa. This was supported by mutations in *DNAH5*, an outer dynein arm, which are associated with the absence of outer dynein arms in respiratory cilia while a normal distribution of ODA heavy chain was reported in the sperm flagellum of mutated patients (Fliegauf et al., 2005). Mutations in *DNAH11,* another outer dynein arm, although associated with a normal axonemal ultrastructure, led to a PCD phenotype also with normal male fertility (Bartoloni et al., 2002; Schwabe et al., 2008). In many mouse models with a MMAF-like phenotype, the structural disorder appears to be specific to sperm tail development, because no effects on the architecture of cilia in the respiratory or female reproductive tract have been observed (Pilder et al., 1997; Fossella et al., 2000; Campbell et al., 2002; Miki et al., 2002; Sapiro et al., 2002; Sironen et al., 2006, 2011; Zhang et al., 2006; Lessard et al., 2007; Lee et al., 2008; Salzberg et al., 2010; Nakamura et al., 2013). Despite this normal axonemal organization, respiratory symptoms or lower tracheal ciliary beat frequency and/or hydrocephalus were described (Sapiro et al., 2002; Lee et al., 2008; Sironen et al., 2011). These findings highlight the duality of function of various proteins, involved in sperm flagellum biogenesis and in the motility of tracheal and ependymal cilia. Further studies are needed to elucidate the mechanisms underlying differences between cilia and flagella and to decipher the specific proteome of these two closely related organelles.

Clinical implications of flagellar defects: return to base

In dividing cells, the centrioles form the core of the centrosome which is the primary microtubule-organizing center in animal cells and it is involved in numerous functions such as the organization of the mitotic/meiotic spindle (reviewed in Bettencourt-Dias and Glover, 2007). During flagellum biogenesis, the sperm centrioles migrate to the spermatid periphery and form the basal bodies from which the flagellar axoneme originates (Chemes, 2012). Centrioles are embedded in a matrix of proteins known as the pericentriolar material that supports motor proteins such as dyneins or kinesins (Schatten and Sun, 2009). Moreover, during fertilization in human, the sperm centrosome organizes the sperm aster which is essential to unite the sperm and the oocyte pronuclei (Sathananthan et al., 1991). The sperm centrosome also controls the syngamy and the first mitotic divisions after fertilization (Schatten and Sun, 2009).

Primary flagellar abnormalities have been associated with an elevated frequency of gonosomal disomies and diploidies (Lewis-Jones et al., 2003; Baccetti et al., 2005b; Rives et al., 2005; Collodel and Moretti, 2006; Ghedir et al., 2014). In the light of the above, chromosomal abnormalities observed in MMAF patients may be linked to the common components shared between the sperm centrosome and the flagella. Therefore, it could be speculated that defects in some of these centrosome-associated proteins may disturb both flagellum formation and spindle assembly during sperm meiosis, resulting in nondisjunction errors and spermatozoa aneuploidy (Rives et al., 2005). No chromosomal abnormalities were however detected in one patient with short-tailed spermatozoa (Viville et al., 2000) pointing out that all flagellar defects do not impact the centrosomal function.

Sperm aneuploidy could impede ICSI outcomes in patients with flagellar defects. Nevertheless several studies described the successful application of ICSI to treat male infertility due to MMAF or PCD (for review Chemes and Rawe, 2003, Chemes and Alvarez Sedo, 2012; Dávila Garza and Patrizio, 2013). The most recent review estimated the mean overall fertilization, pregnancy and live birth rates in MMAF patients at 63, 57 and 43%, respectively (Dávila Garza and Patrizio, 2013). In PCD patients, this review found fertilization and pregnancy rates ranging from 55 to 65% and from 35 to 45%, respectively, depending with the sperm source (ejaculated or testicular). The overall live birth rate was estimated at 39%.

Interestingly although the results compare favorably with the overall results obtained after ICSI, the rate of ICSI success seems to be influenced by the type of ultrastructural flagellar defects carried by the patients (Mitchell et al., 2006; Fauque et al., 2009). Mitchell et al. (2006) reported a lower implantation and clinical pregnancy rate in patients without axonemal central structures (i.e. '9+0' axoneme).

Likewise, Fauque *et al.* (2009) reported a slower kinetics of early embryo cleavage and a lower implantation rate when a central pair of singlet microtubules was missing. These data question the possibility of a link between some specific axonemal structural defects and abnormal embryonic development in human. Since axonemal structures and sperm asters come from the sperm centrosome, it is possible that some of the described cases of fertilization failure and abnormal embryonic development reported in MMAF patients might be caused by defects in centrosomal or pericentrosomal proteins (Sathananthan, 1994; Van Blerkom, 1996; Chemes, 2012). The use of heterologous ICSI systems (human-bovine, human-rabbit) with sperm from infertile men with MMAF supported this hypothesis, showing a lower rate of sperm aster formation (Rawe *et al.*, 2002; Terada *et al.*, 2004). Interestingly, IVF experiments in *Dnah1* KO mice demonstrated a retarded rate of early embryo development (Neesen *et al.*, 2001). Unfortunately, in patients with *DNAH1* mutations no data relative to ICSI attempts were available (Ben Khelifa *et al.*, 2014). Lastly, although there has been no report of an abnormal birth from patients with MMAF or PCD, the risk of genetic defects other than infertility linked to the sperm structural defects should be discussed during genetic counseling (Sha *et al.*, 2014). We believe that genetics data combined with careful analysis using electron microscopy will, in time, permit us to optimize the course of treatment for MMAF patients.

Translation of the genetic information to the clinic: the way forward

ART can circumvent infertility, and IVF with ISCI is possible for most patients regardless of the etiology of the defects. Unfortunately the availability of these technologies does not guarantee success and almost half the couples who seek reproductive assistance fail to achieve a pregnancy. The improvement of ART procedures is therefore particularly relevant and this strongly relies on a better understanding of spermatogenesis and of the molecular physiopathology of infertility. This mission involves the identification of genes responsible for male infertility. To date, a very short list of genes was identified which is in sharp contrast with the fact that several hundreds of genes (probably well in excess of a thousand) are estimated to be involved in spermatogenesis and male reproduction. Although genetic causes of human male fertility remain largely unknown, genetics investigations of some specific teratozoospermic phenotypes have yielded fruitful results. The key to success in the identification of infertility genes was largely based on the study of clinically and genetically homogenous phenotypes in small cohorts with consanguineous subjects and large families. This led in particular to the identification of *AURKC* and *DPY19L2* and to the development of diagnoses for both genes, which are now recommended for patients presenting macrozoospermia and globozoospermia, respectively. Additional efforts are needed to identify genes in others syndromes and more generally in all phenotypes of male infertility in which many genes remain to be identified in human. The recent identification of *DNAH1* in patients with MMAF is promising although a positive diagnosis is achieved for only ~28% of the patients. This indicates that MMAF is genetically heterogeneous and that many other genes are likely involved in this syndrome.

Homozygosity mapping has so far permitted the identification of the three main genes discussed in this review. This strategy can only be successful when studying large families or consanguineous patients presenting with a very homogeneous phenotypes. Due to the great genetic heterogeneity of infertility, this technique has now reached its limits and microarray analyses are now replaced by the fast evolving technology of next generation sequencing. Sequencing of all the coding sequences, or exome sequencing, can now be realized in a few weeks at a cost of 400–1000 €. Whole exome sequencing will be available on the next day (in a few years time) for probably less than that cost. This technical revolution is what was needed to unlock the secrets of male infertility. Exomic/genomic sequencing of large cohorts of patients is now necessary to identify the thousands of mutations involved in infertility and the hundreds of genes necessary to achieve efficient spermatogenesis. The identification of variants in candidate genes will be fast, but long and complex analyses will be necessary to confirm the pathogenic effect of the identified variants and more so to understand the function of the identified proteins. We can be confident that the number of genes and mutations identified in the pathological context of infertility will increase exponentially over the next few years.

The next step will be to exploit these data to the benefit of the patients. Genotype/phenotype correlation will allow us to provide the best advice and care to patients and to answer many questions: is the risk of aneuploidy increased and is it therefore advisable to do ICSI? Or do we recommend performing PGD? Can we expect to find some sperm in this patient's testis and is it worth doing a biopsy? Such prognoses are already available for a handful of patients: ICSI is contraindicated to patients with a homozygous AURKC mutation and a testicular biopsy should not be attempted for men with an AZFa or b deletion (Krausz *et al.*, 2014). The discovery of *DPY19L2* provided a better insight of the molecular pathogeny of globozoospermia. A recent work showed that testicular sperm present similar DNA defects to epididymal sperm thus suggesting that testicular biopsy is not relevant in this case (Yassine *et al.*, 2015a). Moreover, the demonstration that the sperm factor (PLCzeta) is absent in globozoospermic sperm might justify the use of AOA in this pathology (Escoffier *et al.*, 2015). With an increased list of genes associated with infertility we can expect that a better prognosis and improved advice and treatments will soon be available to many more patients.

We are also convinced that another revolution is brewing. We are currently measuring the limitation of the whole IVF and ICSI strategy that is proposed to most infertile patients, especially those bearing the most severe defects. Alternative treatment strategies will only be possible with an in-depth comprehension of all aspects of spermatogenesis and of the physiopathology of sperm defects. The basis of this comprehension stems from the identification of the genes involved and of their function. We believe that male infertility might be among the pathologies that are best suited for targeted protein therapy and we are convinced that restoration of a functional spermatogenesis will be possible by reintroducing a deficient or missing protein as: (i) treatment success can be measured easily and objectively by a mere spermogram, (ii) in man spermatogenesis lasts ~70 days. This corresponds to the necessary treatment duration to obtain functional gametes, which can then be cryopreserved and used at a later date to initiate any number of pregnancies. This relatively short treatment is in sharp contrast with life-long supplementations needed for most other genetic diseases. We thus believe the identification of the genetic causes of infertility will permit us to develop therapies tailored to restore specifically the identified defects. Different options are possible, ranging from direct protein or mRNA injection into

the rete testis (Ogawa et al., 1997) to injection of expression vectors via lipid shuttles or a virus vector such as adeno-associated viruses (AAV). As the targeted cells produce the gametes that will be used to achieve a pregnancy it is crucial that no genetic material is inserted into the genome, to preclude the transmission of potential deleterious effects to the next generations. At the moment even AAV vectors, which are considered as non-integrating and have been used successfully on post mitotic tissues in many clinical trials, have been found integrating the genome of the target cells at a frequency of 10^{-4}–10^{-5} (Kaufmann et al., 2013). For the time being, this low level of insertion precludes the utilization of viral vectors in the context of infertility, but we can hope that fully non-integrative vector will soon be available. Protein therapy is also possible *ex vivo* in testis culture systems but it is labor intensive and therefore less amenable to transposition to many different specific genetic defects. KO mice that have so far been used to study the physiopathology of abnormal spermatogenesis should now be used to evaluate the feasibility of different treatment options. The efficient delivery of the replacement protein will be subordinate to the nature and the localization of the defective native protein and the mode of delivery will have to be adjusted to ensure specificity. We however firmly believe that the restricted localization of the targeted cells (in the testis) and the limited duration of treatment clearly favor the development of innovative therapies applied to male infertility.

In conclusion, now is a very exciting time in the field of the genetics of infertility. We have so far seen only a small part of the tip of the iceberg but we are confident that the rest will come to light in the foreseeable future. We believe that this work will be the cornerstone of the next revolution in ART allowing the clinician not just to bypass the abnormalities by forceful ICSI but to propose new therapeutic strategies allowing the selective repair of the broken parts.

Authors' roles

Conception and organization of manuscript: C.C., C.A. and P.F.R.; literature search and analysis of evidence: C.C., J.E, and G.M.; writing and editing: C.C., J.E., G.M., C.A. and P.F.R.

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Conflict of interest

None declared.

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