Comprehensive investigation in patients affected by sperm macrocephaly and globozoospermia

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SUMMARY

The aim of this study was to provide a comprehensive genetic/phenotypic characterization of subjects suffering infertility owing to sperm macrocephaly (n = 3) or globozoospermia (n = 9) and to investigate whether the patients’ genetic status was correlated with the alteration of various sperm parameters. AURKC was sequenced in case of sperm macrocephaly while the DPY19L2 status has been analyzed by multiple approaches including a novel qPCR-based copy number assay in case of globozoospermia. Globozoospermic patients were also analyzed for SPACA1, a novel candidate gene herein tested for the first time in humans. The effect of the patients’ genetic status was interrogated by implementing the molecular screening with the characterization of several sperm parameters: (i) routine sperm analysis, integrated with transmission electron microscopy; (ii) sperm fluorescent in situ hybridization (FISH) analysis; (iii) sperm DNA fragmentation (DF) analysis. Moreover, for the first time, we performed microsatellite instability analysis as a marker of genome instability in men with sperm macrocephaly and globozoospermia. Finally, artificial reproductive technology (ART) history has been reported for those patients who underwent the treatment. Macrocephalic patients had an AURKC mutation and >89% tetraploid, highly fragmented spermatozoa. DPY19L2 was mutated in all patients with >80% globozoospermia: the two homozygous deleted men and the compound heterozygous showed the severest phenotype (90–100%). The newly developed qPCR method was fully validated and has the potential of detecting also yet undiscovered deletions. DPY19L2 status is unlikely related to FISH anomalies and DF, although globozoospermic men showed a higher disomy rate and DF compared with internal reference values. No patient was mutated for SPACA1. Our data support the general agreement on the negative correlation between macro/globozoospermia and conventional intracytoplasmic sperm injection outcomes. Microsatellites were stable in all patients analyzed. The comprehensive picture provided on these severe phenotypes causing infertility is of relevance in the management of patients undergoing ART.

INTRODUCTION

It is estimated that infertility affects about 7% of men in their reproductive age (Krausz, 2011). The etiology of male infertility also includes two monomorphic forms of teratozoospermia, sperm macrocephaly, and globozoospermia. Sperm macrocephaly is described as a rare condition with a <1% prevalence in the subfertile population (Nistal et al., 1977) and is characterized by large-headed and multi-flagellated spermatozoa. Globozoospermia (incidence of 0.1%) is characterized by the production of round-headed acrosomeless spermatozoa that are unable to fertilize the oocyte, as no acrosome reaction can occur (Sen et al., 2009).

Literature offers a number of studies dealing with sperm macrocephaly or globozoospermia in relation to artificial reproductive technology (ART) outcomes (Koscinski et al., 2011; Dam et al., 2012; Shimizu et al., 2012; Molinari et al., 2013). These studies demonstrate that such sperm morphological defects are related to impairment of spontaneous conception and that a
better option should be intracytoplasmic sperm injection (ICSI), although the fertilization rate is relatively low or even absent in pure forms of macro/globozoospermia. Assisted oocyte activation (AOA) has been proposed as treatment for globozoospermic patients (Kuentz et al., 2013).

A number of studies focused on the relationship between teratozoospermia and sperm DNA fragmentation (DF), and the majority reports that teratozoospermic males had a sperm DF significantly higher than fertile men (Vicari et al., 2002; Egashira et al., 2009; Brahem et al., 2011; Perrin et al., 2011; Mangiarini et al., 2013). As for sperm aneuploidies, a different picture is portrayed according to whether we refer to sperm macrocephaly or globozoospermia: regarding the former, a high incidence of sperm chromosomal abnormalities is observed in patients with large-headed spermatozoa, typically displaying a >95% of polyploid/aneuploid genetic content (Benzecken et al., 2001; Perrin et al., 2008; Brahem et al., 2011); concerning the latter, a comprehensive review of 16 studies dealing with globozoospermia (Perrin et al., 2013) reports that, on the contrary, this form of teratozoospermia shows a much lower rate of aneuploidy and on average does not appear to be responsible for higher rates of sperm chromosomal anomalies.

Concerning the genetic background, sperm macrocephaly can be caused by the occurrence in homozygosity of a 1-bp deletion (c.144delC) in the AURKC gene, which is essential for correct meiotic chromosomal segregation and cytokinesis (Dieterich et al., 2007). This mutation results in a truncated protein lacking the kinase domain, which leads to a blockage of both meiotic divisions finally causing the presence of tetraploidy and numerous flagella. The c.144delC has been reported to occur exclusively in the North African ancestry, suggesting the possibility of a founder effect (Dieterich et al., 2009; Ben Khelifa et al., 2011, 2012; El Kerch et al., 2011). Dieterich et al. (2009) reported one patient with a pure phenotype displaying the c.144delC mutation in compound heterozygosis with a newly found missense mutation, p.C229Y (c.686G>A). A heterozygous splicing mutation in exon 5 (c.436-2A>G) was also identified in two affected brothers who also carried the c.144delC mutation (Ben Khelifa et al., 2011). Later, the same authors (Ben Khelifa et al., 2012), identified the p.Y248* (c.744C>G; rs55658999) variant in homozygosis in six men of North African origin and in four Europeans, of which two were homozygous and two were compound heterozygous for the c.144delC. This nonsense mutation was always associated with another variant located in AURKC 3′UTR, c.930+38G>A. In addition, a single case of sperm macrocephaly has been recently reported without mutations in the AURKC gene (Moliniari et al., 2013).

Regarding globozoospermia, a genetic basis was suggested by the familial distribution of the syndrome (Kilani et al., 2004), and different patterns of inheritance (polygenic, X-linked, autosomal dominant, autosomal recessive) have been proposed (Trokoudes et al., 1995; Stone et al., 2000). Presently, the most prevalent genetic defect observed in human globozoospermia is a ~200 Kb homoygous deletion of DPY19L2 (12q14.2), firstly identified by a genome-wide scan analysis using a 10K SNP array (Koscinski et al., 2011). It has been proved that DPY19L2 deletion leads to the blockage of sperm head elongation and acrosome formation. This might be explained by the fact that the absence of the protein leads to the destabilization of both the nuclear dense lamina and the junction between the acrosplaxome and the nuclear envelope. Consequently, the acrosome and the manchette fail to be linked to the nucleus leading to the disruption of vesicular trafficking, failure of sperm nuclear shaping and eventually to the elimination of the unbound acrosomal vesicle. Finally, two further genes have been associated with globozoospermia in humans, SPATA16 and PICK1 (Perrin et al., 2013). The former was firstly proposed as possibly implicated in globozoospermia by Dam et al. (2007), who identified a homozygous mutation in the spermato-genesis-specific gene SPATA16. The localization in the Golgi apparatus and the shift with Golgi vesicles to the acrosome observed in round and elongated spermatids suggested a role for the SPATA16 protein in acrosome formation during spermiogenesis (Lu et al., 2006). As for the PICK1 gene, it encodes a peripheral membrane protein involved in protein trafficking, a function that has been well characterized in neurons. Apart from being expressed in the brain, the PICK1 protein shows relatively high levels also in the testes and the pancreas. The first association with globozoospermia was reported by Xiao et al. (2009), who showed that Pick1-knockout mice displayed similar sperm anomalies to those found in human globozoospermia. Then, in another Chinese study, PICK1 was screened for the first time in humans and a homozygous missense mutation (G198A) was reported as the cause of the globozoospermic phenotype. Studies on mice models showed that disruption of other genes, that is, Csnk2a2 (Xu et al., 1999), Hrb (Kang-Decker et al., 2001), Gopc (Yao et al., 2002) and the most recently reported Spacial (Fujihara et al., 2012), results in a phenotype resembling that of globozoospermia in humans. Mutational screening has been performed in humans for CSNK2A2, HRB, and GOPC (Pirrello et al., 2005; Christensen et al., n.d.), but no mutations potentially linked to the pathology were found. Instead, no genetic studies are available on human SPACA1, making this gene an interesting genetic target of investigation. SPACA1 (6q15) encodes a membrane protein localized in the equatorial segment of spermatozoa. Immunohistochemistry of human testicular cells (Hao et al., 2002) demonstrated that SPACA1 distribution coincided with acrosome development and that rat anti-SPACA1 antibodies blocked the binding and fusion of capacitated human spermatozoa with zona-free hamster eggs.

Present literature offers the description of different aspects of these two forms of teratozoospermia, but the picture provided remains partial as available studies focus on specific issues separately. The major aim of this study was to provide a genetic screening of the two known causative genes, AURKC in case of sperm macrocephaly and DPY19L2 in case of globozoospermia. In addition, our patients were also tested for SPACA1, a novel candidate gene herein tested for the first time in humans. To provide a comprehensive phenotypic description, we implemented the genetic investigation with the characterization of both previously analyzed and novel sperm parameters. Hence, our patients were also subjected to routine sperm analysis, integrated with transmission electron microscopy (TEM) to finely characterize sperm morphology, sperm fluorescent in situ hybridization (FISH) analysis and sperm DF analysis. Moreover, for the first time, we performed microsatellite instability (MSI) analysis as a marker of genome instability in teratozoospermic men. Studies in the literature reported that in these two types of monomorphic teratozoospermia the observed pregnancy rate is

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rather low (Viville et al., 2000; Dam et al., 2007; Molinari et al., 2013); therefore, by performing the MSI analysis we aimed at understanding whether this phenomenon might be because of a higher instability of these patients’ genome. Finally, ART history has been reported for those patients who underwent the treatment. This is the first comprehensive study that cumulatively collects a relevant load of information on two types of morphological defects of human spermatozoa with potential benefit for future medical practice.

MATERIALS AND METHODS

Subjects
A total of twelve unrelated patients displaying a >90% teratozoospermic phenotype of sperm macrocephaly (n = 3) and globozoospermia (n = 9) were selected for this study. All patients consulted for primary infertility to the Fundació Puigvert, Spain (n = 4) and to the Division of Sexual Medicine and Andrology Unit, University Hospital Careggi, Italy (n = 8). Two patients, one referring to the Spanish clinic and the other referring to the Italian one, had North African origins, whereas the remaining 10 patients had no known ascendants from North Africa. None of the patients had karyotype anomalies or Y-chromosome microdeletions. The brother of one of the Spanish macrocephalic patients was also recruited. Genetic and sperm analyses were performed in the frame of the diagnostic work-up. All participants and family members gave written, informed consent for the analyses.

Routine semen analysis
Semen parameters were assessed according to the WHO guidelines (WHO, 2010; Data S1).

Transmission electron microscopy
TEM analyses were requested as a service at the University Hospital of Siena and performed as described elsewhere (Baccetti et al., n.d.) (Data S1).

Fluorescent in situ hybridization
For patients attending the Italian clinic, FISH was provided by the University Hospital of Siena according to the protocol described by Baccetti et al. (2003). For patients referring to the Spanish clinic, the analysis was performed at Reprogenetics (Barcelona, Spain) according to the protocol described by Sánchez-Castro et al. (2009) (Data S1).

Terminal deoxynucleotidyl transferase dUTP nick end labeling/prodium iodide assay
Sperm DF was determined by TUNEL/PI assay as described elsewhere (Muratori et al., 2008; Data S1).

MSI analysis
Seven microsatellite loci located on different chromosomes were investigated using genomic DNA from both peripheral blood and sperm samples belonging to the same subject. In this study, selected loci consisted of two mononucleotide tandem repeats (BAT-25 and BAT-26), three dinucleotide tandem repeats (D2S123, D17S250, D5S346), one dinucleotide (TA)\text{n} repeat locus [within the promoter of the estrogen receptor (ESR1)] and one trinucleotide (CAG)\text{n} repeat locus [within exon 1 of the androgen receptor (AR)] (Table S1). MSI was defined as the presence of discordant alleles between blood and sperm DNA belonging to the same subject. Details are provided in the Data S1.

Candidate genes analysis
Sanger sequencing was performed using the BigDye Terminator v3.1 sequencing kit and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). As for macrocephalic patients, the seven AURK\text{C} exons and intron/exon boundaries were analyzed (primers reported in Table S2). Concerning globozoospermic patients, the DPY19L2 and SPACA1 genes were analyzed. As for DPY19L2, screening was performed according to the flow chart represented in Figure S1. Standard ± PCR of exons 10, 14, and 19 served to detect the complete homozygous deletion. Deletion junction fragment analysis (DJFA) was performed to detect the heterozygous DPY19L2 deletion in STS (Sequence-Tagged Sites)-positive patients and to define the type of breakpoint (Elinati et al., 2012) in heterozygous and homozygous deletions. Primers used are reported in Table S3. Mutational screening by direct sequencing was also performed and pathogenic predictions for missense variations were realized using Polyphen (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org) and MutationTaster (http://www.mutationtaster.org). Intronic variants predictions were performed using Human Splice Finder (www.umd.be/HSF1/) and BDGP (www.fruitfly.org/) websites. Multiple sequence alignment of the human DPY19L2 protein among species was performed with MultAlin (http://multalin.toulouse.inra.fr/multalin/). The effect of the missense variants on the properties of the involved extramembrane loops of the DPY19L2 protein was predicted using TMHMM server v.2 (http://www.cbs.dtu.dk/services/TMHMM/). As for SPACA1, primers were designed to amplify and sequence all seven exons and intron/exon boundaries (Table S4).

Quantitative-PCR
Exploration on the DPY19L2 locus in the Database of Genomic Variants (DGV) showed a threefold increase in duplications compared with deletions, and that several deletions might have a different breakpoint possibly undetected by standard DJFA. Therefore, we applied qPCR to identify both novel deletions and duplications involving the DPY19L2 gene by designing a TaqMan assay based on the amplification of the 5’UTR-Exon1 region (Data S1 and Table S5).

Statistical analysis
The statistical software SPSS 20.0 (Chicago, IL, USA) was employed. Comparison of DF mean values was performed with the parametric independent t-test, according to the fact that sperm DF was normally distributed among samples. A p-value <0.05 was considered statistically significant.

RESULTS

Sperm analysis

Routine semen analysis
As for macrocephalic patients, two (11-527 and 12-550) presented a moderate reduction in total sperm count (17.5 and
16 million/ejaculate, respectively), whereas CT154 sperm count was normal (64.3 million/ejaculate). Sperm morphology analysis showed in all patients 100% combined anomalies including the typical features of sperm macrocephaly (Fig. 1A). As for globozoospermic patients, total sperm count varied from very low to normal with variable percentages of round-headed acrosomeless spermatozoa (50–100%).

**TEM analysis**

TEM evaluation was performed in three patients with globozoospermia, A1869, CT158, and CT196. The analysis revealed in all cases the presence of typical sperm head morphology alterations with most of the analyzed nuclei presenting a high level of chromatin decondensation; sperm flagella appeared disorganized and coiled around the nucleus (Fig. 1B). Patient CT158 also presented rarely elliptic or elongated nuclei and some perinuclear structures possibly representing a developmental failure of the acrosome.

**Sperm FISH**

FISH analysis was performed in all patients affected by macrocephaly and in four of nine patients with globozoospermia. Although the number of chromosomes analyzed was different in the Spanish and Italian patients (5 and 3, respectively) the results were very similar. As expected, the most frequent sperm chromosomal anomaly in patients with sperm macrocephaly was tetraploidy (mean value ± standard deviation: 84.27 ± 9.66%), but also disomies/diploidies were higher than reference values (disomies: 4.29 ± 3.40%; diploidies: 7.15 ± 2.55%). Spermatozoa of globozoospermic patients mostly presented with a normal sperm chromosomal content, although average disomies/diploidies levels were higher than reference values (disomies: 0.61 ± 0.26%; diploidies: 0.53 ± 0.31%) (Table S6).

**Sperm DF analysis**

TUNEL/PI assay was performed for two macrocephalic and eight globozoospermic patients. Macrocephalic patients’ (CT154 and 12-550) sperm DF was 54.29 and 52%, respectively, although it did not reach statistical significance compared with the internal reference values calculated on a population of 90 fertile controls, probably because of the small number (n = 2). Among globozoospermic patients, the proportion of fragmented spermatozoa varied from 32.61% (patient CT190) to 64.9% (patient CT196) with a mean value that was significantly higher compared with the internal reference values (mean value ± standard error: 46.92 ± 4.20% vs. 34.04 ± 1.53%; p = 0.017; Table 1).

**Microsatellite instability**

MSI analysis was performed for one of three macrocephalic patients (CT154) and for eight globozoospermic patients. In each patient, all seven markers analyzed resulted stable between spermatozoa and blood DNA samples (Table 1).

**Molecular genetics**

**AURKC screening**

The two macrocephalic patients with North African origin (CT154 and 11-527) carried the c.144delC mutation. The other patient, as well as his brother, were homozygous for the p.Tyr248* (c.744C>G) mutation in exon 6 (Table S7); both brothers also carried the c.930+38C>G mutation in the 3’UTR, reported to be in linkage with the p.Tyr248* mutation (Ben Khelifa et al., 2012).

**DPY19L2 screening**

Three patients carried the DPY19L2 deletion: A1869 and CT190, who were homozygous, and CT158, who was heterozygous. DJFA resulted in the amplification of the expected 1.7 Kb product in all of them, revealing the presence of breakpoint type ‘a’. The rest of patients showed amplification of all exons, thus qPCR analysis was performed to check whether they harbored novel deletions undetectable by DJFA. Q-PCR confirmed the presence of the heterozygous deletion in CT158 as well as the homozygous deletion in CT190 and A1869, but no other novel CNVs were found in the remaining samples. Finally, sequencing was performed to check for the presence of point mutations in
Sperm macrocephaly and globozoospermia are rare forms of teratozoospermia causing male infertility. The literature the heterozygous deletion carrier (CT158) and the non-deleted patients (n = 6).

The heterozygous patient did not show any amplification of exon 7, suggesting a deletion of this exon. The consequence at the protein level is the loss of 20 amino acids, predicted by TMHMM to substantially change the protein conformation owing to hydrophobic changes. As for point mutations, we found a total of nine variants (4 missense, 3 intronic, and 2 synonymous) in five patients (Table S8). Three reported missense variants were found in exon 1: rs10878075, rs10878074, and rs10878073. These variants are seemingly in linkage disequilibrium and in our cohort three non-deleted patients (CT157, CT196, 11-387) carried them in heterozygosis and one (CT176) in homozygosis. The minor allele frequency (MAF) denotes a high frequency of these variants in the general population; for instance, the bioinformatic predictions indicate a non-pathogenic effect of the amino acidic changes. The fourth missense variant, also described in the databases (rs371693431), was detected in heterozygosis in exon 4 of one non-deleted patient only (11-387). This mutation is a serine to leucine substitution (p.Ser165Leu) in a highly conserved region. No MAF is reported for this variant and all prediction tools employed in this study predicted it as potentially pathogenic; for instance, using TMHMM it is predicted to be located in one of the extramembrane loops of the protein and to increase its hydrophobic properties leading to a consistent change of the protein conformation (Fig. 1). Of the three intronic variants found in three different patients, two are reported with a MAF >5% and one is novel, but all seemingly have no effect on splicing. Interestingly patient CT175, displaying the lowest value of globozoospermic spermatozoa (50%), had no variants at all.

**SPACA1 screening**

Sequencing of the whole SPACA1 gene was performed in all globozoospermic patients. No mutations were found in any of them.

**ART outcome**

ART treatment was an option for nine of the patients included in the study (Table 2). In patients with milder forms of globozoospermia oocyte fertilization occurred, although this was not always followed by embryo formation. Patients with severe forms of globozoospermia – who also displayed either the homozygous DPY19L2 deletion or were compound heterozygous – could not even obtain oocyte fertilization. In the case of patient CT154 (macrocephalic), although 5 MII oocytes were injected, none of them was fertilized. Of the other two macrocephalic patients (11-527 and 12-550), the former did not undergo ART, whereas the latter underwent an ICSI cycle reaching embryo transfer, but achieved no pregnancy. We also obtained data on patient 12-550’s family investigating on his brother’s ART history (sample 13-039), who displayed a mild form of sperm macrocephaly (39%); in this case, 2 ICSI cycles were performed and both ended in embryo transfer, but both times no pregnancy was achieved. Pregnancy was achieved only for one couple (patient 11-387), who went for IVF with a donor’s sample.

**DISCUSSION**

Sperm macrocephaly and globozoospermia are rare forms of teratozoospermia causing male infertility. The literature
Figure 2 Bioinformatic analysis of the missense mutation p.Ser165Leu (rs371693431) in DPY19L2. (A) Prediction of the mutation on the properties of the involved extramembrane loop of the DPY19L2 protein using the TMHMM server. Plot of the posterior probabilities for wild type (upper panel) and mutant (lower panel) sequences of the DPY19L2 proteins are shown, based on a hidden Markov model approach. The hydrophobicity, one of the most important parameters incorporated into this model, can be revealed by the plot. The dashed box highlights the significant increase in the protein hydrophobic properties caused by the Serine into Leucine change, which leads to a consistent change in the protein conformation. (B) Amino acid alignment of partial exon 4 of DPY19L2 selected orthologs, performed by MultAlin. The box highlights the Serine in position 165.

![Amino acid alignment](image)

provides well-defined data concerning sperm macrocephaly, for which a consensus exists about the genetic etiology and its consequences on sperm genome and ART outcome. Contrastingly, the picture is rather complex when it comes to globozoospermia, for which more candidate genes have been proposed and data on aneuploidy, sperm DF as well as ART outcomes are largely heterogeneous.

Literature is especially poor of studies where mutational screening of candidate genes for these two conditions is combined with sperm genomic analysis and ART history. In fact, such a comprehensive study has been published only for two patients with sperm macrocephaly (Guthauser et al., 2011; Molinari et al., 2013). Concerning globozoospermia, ours is the first study providing information about the mutational status of DPY19L2 and a novel candidate gene (SPACA1) concurrently to sperm FISH, DF, MSI, and ART outcome.

As expected all patients with macrocephaly had a mutation in the AURKC gene, directly associated with the pathology. Consistent with the literature, the two North African patients in our cohort were homozygous for the c.144delC mutation. The two Spanish brothers, instead, were homozygous for the p.Tyr248* mutation; as they came from a small town in Spain, their parents’ consanguinity cannot be excluded. Interestingly, patient 12-550’s spermatozoa were all macrocephalic, whereas his brother displayed a 39% of macrocephalic spermatozoa, suggesting that the p.Tyr248* mutation has a variable penetrance. Unfortunately, sperm morphology data of distinct p.Tyr248* mutation carriers are not available in the current literature (Ben Kheïla et al., 2012). In accordance with previous studies (Benzacken et al., 2001; Devillard et al., 2002; Guthauser et al., 2006; Perrin et al., 2011; Brahem et al., 2012; Molinari et al., 2013; Achard et al., n.d.), FISH analysis in spermatozoa revealed a high rate of tetraploidy in all three tested patients. Interestingly, the two Moroccan patients carrying the c.1144delC showed >90% polyplody, whereas the Spanish patient 12-550 presented a lower value (68.56%) indicating a milder effect of this mutation on sperm chromosomal constitution. In contrast, the two patients for whom TUNEL/PI assay was performed (CT154 and 12-550) displayed a similarly high level of sperm DF (54.29 and 52%, respectively), regardless of the type of mutation.

As for globozoospermic patients, the genetic analysis included not only the screening of the DPY19L2 gene, the major genetic factor causing globozoospermia, but also that of a novel candidate gene, SPACA1, here studied for the first time in humans. The importance of DPY19L2 mutations in the etiology of globozoospermia has been emphasized by Elinati et al. (2012), who found an involvement of this gene (deletion and/or point mutations on both alleles) in 56% of cases (36/64). We report a frequency of DPY19L2 homozygous deletions of 22.2% (2/9), which seems to be lower compared with the overall frequency reported in the literature. Unfortunately, articles available not always provide data on the consanguinity of homozygous carriers; however, when considering patients from non-consanguineous families and those with unknown family history, we estimated that the frequency of homozygous deletion carriers was of 34.7%.

In our cohort, patients with the severest phenotype had either a homozygous or a heterozygous DPY19L2 deletion together with a deletion of exon 7 on the other allele. When it comes to point mutations, one patient was heterozygous for a deleterious missense variant, but no other potentially deleterious variants could be identified. This finding suggests that in men with the heterozygous mutations the phenotype could either be caused by a second mutation on another candidate gene (digenic etiology) or that the heterozygous mutation is already sufficient to induce a partial globozoospermia. We propose this later scenario as the severity of globozoospermia was milder in the heterozygous patient (83%) compared with the two homozygous deletion carriers (100% globozoospermic) and the compound heterozygote (91%). Moreover, the five patients with wild type DPY19L2 showed the lowest percentage of globozoospermia (50–75%). Our data therefore confirm that DPY19L2 mutations are important contributors to severe forms of globozoospermia suggesting that its screening should not be restricted to the complete forms. The diagnosis of both heterozygous and homozygous mutation is relevant for genetic counseling as loss in DPY19L2 is a frequent cause of inheritance (20–75% of cases).

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proposed to be involved in globozoospermia, data are very scarce and only single mutation carriers with complete globozoospermia are reported for the SPATA16 and PICK1 gene from consanguineous families (Dam et al., 2007; Liu et al., 2010). Later, only SPATA16 was screened in an overall relatively large group of globozoospermic subjects (n = 65) and no mutation was found (Dam et al., 2007; Kuentz et al., 2013; Noveski et al., 2013). Given that research focused on these genes was relatively unsuccessful, we searched for a not yet tested candidate gene. As mentioned above, this is the first time that SPACA1 is considered a candidate gene for human globozoospermia, based on data by Fujihara et al. (2012), reporting a globozoospermia-like phenotype in knockout mice. We found no mutations in SPACA1 in our globozoospermic patients but, given the small cohort tested, it cannot be excluded that this gene might still be involved in this phenotype in humans.

The existence of a clear-cut correlation between globozoospermia and a higher rate of abnormal chromosomal content as well as higher DF is questioned by the fact that only a proportion of patients show abnormal values (Machev et al., 2004; Perrin et al., 2011). In our cohort of globozoospermic patients, we did find an increase in disomies and diploides compared with the reference values; however, inter-individual differences were evident. For the first time, we tested whether the presence of the DPY19L2 deletion might confer a higher rate of both sperm aneuploidies and DF. According to our data, no correlation exists between the presence of the DPY19L2 deletion and the rate of abnormal chromosomal content, as patients carrying a DPY19L2 either homozygous (A1869) or heterozygous (CT158) deletion did not have higher aneuploidy rate compared with non-carrier globozoospermic patients. Considering sperm DF, also this parameter was highly variable between patients (32.6–64.9%) showing on average a significantly higher DF rate (46.9 ± 4.2%) compared with the 90 controls (34.04 ± 1.5%). In relation to the presence of the DPY19L2 deletion, both homozygous carriers displayed a fragmentation rate within the normal range, whereas DF in the heterozygous carrier was definitely above normality (61%). Therefore, a clear relationship between the DPY19L2 deletion and a consistently higher DF cannot be established.

ART history was followed for 8/12 patients, of whom none achieved pregnancy (Table 2). The two patients with 100% large-headed spermatozoa in the ejaculate showed different fertilization rate at ICSI: as for the patient carrying the c.144delC (CT154) and his 28-year-old partner, despite having recovered 5MII oocytes during the ICSI attempt, no oocyte fertilization occurred; instead, patient 12-550, with the p.Tyr248* mutation and a 54-year-old partner, managed to fertilize one of two recovered MII oocytes after ICSI, achieving embryo transfer. Embryo transfer was successful, although not resulting in a pregnancy, only in patient 12-550’s brother (sample 13-039), who carried the same mutation but a lower percentage of macrocephalic spermatozoa.

Concerning the globozoospermic subjects, we observed a correlation between DPY19L2 status and the oocyte fertilization rate; for instance, for the two patients carrying the DPY19L2 deletion that underwent ART treatment (A1869 in homozygosis and CT158 in heterozygosis) oocyte fertilization did not occur, even when the female partner was young (CT158’s case) or had a perfect ovarian response to stimulation (A1869). In non-deleted patients, instead, the ART procedure was carried out until embryo transfer, which in no cases, although, developed in a pregnancy. Given our assumptions above, this correlation is explained by the DPY19L2-dependent severity of the globozoospermic phenotype, for which the fertilization rate is reduced in the presence of the genotype leading to a higher percentage of abnormal spermatozoa that will fail at ICSI. Our data support the general agreement on a negative correlation existing between macro/globozoospermia and conventional ICSI outcome (Viville et al., 2000; Dam et al., 2007; Dirican et al., 2008; Banker et al., 2009; Kuentz et al., 2013). Unsuccessful fertilization derives from the missing PLCδ-dependent induction of calcium increase in the oocyte. AOA has been proposed as an option for patients with complete globozoospermia, although its safety has been questioned: it is, in fact, advisable to restrict its use to selected cases and to avoid it when there is a chance of finding normal spermatozoa, as the case of partial globozoospermia (Kuentz et al., 2013). In these cases, intracytoplasmic morphologically selected sperm injection (IMSI), have been proposed (Kuentz et al., 2013).

In our cohort, neither AOA nor IMSI have been performed.

Given the very low pregnancy rate observed in these two types of monomorphic teratozoospermia (Viville et al., 2000; Dam et al., 2007; Molinari et al., 2013), we aimed to evaluate whether a higher genomic instability would concur to this phenomena. Consequently, another novelty of our study is the analysis of

<table>
<thead>
<tr>
<th>Patient</th>
<th>Phenotype</th>
<th>No. abortions</th>
<th>Partner’s age</th>
<th>ART treatment</th>
<th>No. recovered oocytes</th>
<th>No. fertilized oocytes</th>
<th>No. embryo transfer</th>
<th>Pregnancy</th>
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</tr>
<tr>
<td>13-039*</td>
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</tr>
<tr>
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<tr>
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<td>Globo</td>
<td>0</td>
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</tr>
<tr>
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<td>Globo</td>
<td>0</td>
<td>35</td>
<td>1° ICSI cycle</td>
<td>3 MII</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>Globo</td>
<td>2</td>
<td>39</td>
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<td>2</td>
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<tr>
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<td>4 MII</td>
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</table>

IUI, intra-uterine insemination; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; MII, maturation phase I; MI, maturation phase II; ART, artificial reproductive technology; NA, not available. *13-039 is patient 12-550’s brother.
MSI, which may originate from alteration in the DNA mismatch repair system and is considered a marker of genomic instability (Maduro et al., 2003). All patients resulted stable to MSI analysis, excluding the contribution of genomic instability to the poor ICSI outcomes.

In summary, this study represents the first comprehensive clinical characterization of patients suffering infertility because of two forms of monomorphic teratozoospermia. Our data about sperm macrocephaly basically confirm previous findings on the role of AURKC and the exceptionally high aneuploidy rate in this pathological condition. As for globozoospermia, we observed no direct relationship between the DPY19L2 status and sperm anomalies in terms of FISH, DF but a correlation was detected between the type of DPY19L2 mutations and severity of the phenotype and oocyte fertilization. In the light of our data, we agree that in case of 100% globozoospermia, AOA should be recommended, as there is biological evidence that spermatozoa will not be able to activate the oocyte alone. DPY19L2 genetic screening would help to characterize both complete and partial cases with >80% of globozoospermia, whereas the DPY19L2 status of the female partner of mutation carriers will provide additional information for an appropriate genetic counseling. Apart from offering a comprehensive spermatozoa and clinical characterization, our study presents a number of novel aspects. We aimed to provide an alternative technical approach for the detection of DPY19L2 deletions that might be missed by the previously proposed DJFA analysis. Therefore, we developed the qPCR method herein presented for a rapid and highly reliable analysis of the presence of both common and still undiscovered DPY19L2 deletions. Importantly, for the first time, we provide evidence that neither macrocephaly nor globozoospermia are associated with genomic instability. Finally, the novel candidate gene herein proposed (SPAC1A1) and tested for the first time in humans, does not appear a frequent cause of this phenotype in humans, although studies on a larger study population should be performed to confirm this conclusion.

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DISCLOSURE

The authors have nothing to disclose.

AUTHORS’ CONTRIBUTION

All authors are justifiably credited with authorship, according to the authorship criteria. In detail, CC: sequencing, analysis, and interpretation of data, drafting and revision of the manuscript; MF: sperm analysis; AR and FD: sequencing and qPCR analysis; OL: semen analysis; SV and EG: MSI analysis; MM and LT: sperm DNA fragmentation analysis; DL: sequencing; EA and ER: DNA samples providing; LB: patient recruitment; MC and VP: performance of ART; IN and EC: patient recruitment; SG and AP: sequencing; PP: FISH and TEM analysis; CK: coordination, patient recruitment, drafting, and revision of the manuscript.

REFERENCES


**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Flowchart indicates the molecular investigation performed in globozoospermic patients and potential outcome.

**Figure S2.** qPCR amplification plots. (A) Amplification plot of the normal control carrying two copies of the *DPY19L2*: no difference is observed between the Ct values of the *DPY19L2* gene and the reference gene HAL; (B) Amplification plot of the patient carrying the *DPY19L2* heterozygous deletion: a difference of one Ct is observed between the *DPY19L2* and the reference gene HAL; (C) Amplification plot of the patient carrying the *DPY19L2* homozygous deletion: no amplification is observed for the *DPY19L2*.

**Table S1.** Sequences of primers used for MSI analysis.

**Table S2.** Sequences of primers used for *AURKC* analysis.

**Table S3.** List of primers used for *DPY19L2* analysis.

**Table S4.** List of primers used for *SPACA1* analysis.

**Table S5.** Primers used for qPCR analysis.

**Table S6.** Frequency of chromosomal anomalies found in patients with teratozoospermia.

**Table S7.** *AURKC* mutations identified in macrocephalic patients.

**Table S8.** *DPY19L2* mutations identified in globozoospermic patients.

**Data S1.** Supplemental Materials and Methods.