Genome-wide compound heterozygote analysis highlights *DPY19L2* alleles in a non-consanguineous Spanish family with total globozoospermia

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ABSTRACT

Research question: Would the use of genome-wide genotyping be an advantageous strategy to identify the molecular aetiology of two brothers from a non-consanguineous family, clinically diagnosed with total globozoospermia?

Design: Two related Spanish globozoospermic patients were studied. Eight first- and second-degree family members were also included in the study. The clinical procedure included anamnesis, physical examination, and semen analyses. Acrosome visualization was performed by FITC-PSA labelling and ultrastructural TEM and SEM sperm analysis. Sperm DNA fragmentation was determined by TUNEL and SCD. Molecular analysis included: the detection of deletion of *DPY19L2* gene by a BPa gap-PCR; and genotyping by using a high-throughput genome-wide genotyping platform and a genotype imputation strategy.

Results: The biological characteristics of the two globozoospermic siblings included round-shaped sperm head without acrosome; ultrastructural defects in sperm; increased sperm fragmentation and aneuploidies, inability of sperm to activate oocytes (correctable with artificial activation) and good developmental potential of IVF-ICSI generated embryos. Our genetic study focused on a genome-wide compound heterozygote analysis which identified two deleterious rare coding-variants in *DPY19L2* gene [rs771726551 (c.431T>A exon3) and rs147579680 (c.869G>A exon8)].

Conclusion: Genome-wide compound heterozygote analysis strategy should be considered for molecular screening in globozoospermia and other rare congenital diseases, particularly in cases from non-consanguineous families.

INTRODUCTION

While most cases of male infertility are associated with quantitative defects leading to the absence of sperm or a reduction in sperm number, a significant number of cases of male infertility are caused by morphological or qualitative defects of sperm affecting its fertilizing ability. Morphological characterization of sperm defects during routine light microscopic examination of semen samples permits the definition of specific teratozoospermic phenotypes. Globozoospermia (OMIM 613958) is a severe form of monomorphic teratozoospermia characterized by a round-shaped sperm head without acrosome (best visualized by transmission electron microscopy*)* which negatively impacts the function of the male gamete, leading to the sperm being unable to fertilize an oocyte (Dam *et al.* , 2007a). Other reported defects involve the sperm cell cytoskeleton such as: a round nucleus; absence of the post-acrosomal sheath and their associated proteins; separation of the nuclear membranes; residual cytoplasmic body/droplet surrounding the nucleus or the midpiece; and frequently coiled tails. It is a very rare form of infertility, with an estimated incidence of 0.1% of all cases of male infertility (Dam *et al.*, 2007a); being 0.03% the globozoospermia prevalence in Spanish population (from a cohort of 12094 studied infertile patients in our Center, unpublished results).

Assisted reproduction technology (ART) allows many infertile couples to parent their biological children. A precise diagnosis of infertile couples is essential for the correct clinical management of these cases, and to choose the better ART option. The morphological defects shown in globozoospermic sperm, which are unable to adhere and penetrate the zona pellucida, are related to a worsening of spontaneous conception, accordingly, intracytoplasmic sperm microinjection (ICSI) has been proposed as the best ART option for these patients, however, fertilization, pregnancy and live-birth success rates remain low. The first explanation for the low rate of fertilization is the reduction or the absence of the phospholipase C zeta (PLCζ), a sperm phospholipase responsible for oocyte activation (Escoffier *et al.* , 2015), thus ICSI associated with artificial oocyte activation (AOA) has been implemented in ART treatment for these patients. A second explanation is that it may be due to sperm DNA damages related to defective chromatin condensation and DNA fragmentation described in globozoospermic sperm cells (Dam *et al.*, 2007a, Yassine *et al.* , 2015).

Most publications describing this abnormal sperm morphology disease deal with patients who produced ejaculates showing 100% round sperm heads lacking an acrosome. Nevertheless, having a variable proportion of round headed spermatozoa in ejaculate has been described in a

significant number of patients. Whether this 'partial' globozoospermia is an incomplete form of the same syndrome or a separate disorder remains to be elucidated (Dam *et al.*, 2007a).

A recessive genetic basis has been proposed at least for total and for >50% globozoospermia (Celse *et al.* , 2021). It has been shown that *DPY19L2* (Dpy-19 Like 2) deletion accounts for 80% of total globozoospermia, although mutations in Golgi related genes associated with acrosome biogenesis such as *DPY19L2*, *SPATA16* (spermatogenesis associated 16) (Dam *et al.* , 2007b), *PICK1* (protein interacting PRKCA1) (Liu *et al.* , 2010), *ZPBP1* (Zona Pellucida Binding Protein 1) (Yatsenko *et al.* , 2012) have been also described, supporting a monogenic contribution to globozoospermia. Based on published data, research of homozygous *DPY19L2* deletions is widely proposed as the first-line genetic analysis in globozoospermic patients. Conversely, in the absence of this alteration, full *DPY19L2* sequencing or the investigation of other genes such as *SPATA16* are questionable due to the very low number of reported mutations, thus whole exome sequencing (WES) or whole genome sequencing (WGS) analysis has been proposed as a logical next step of study (Ray *et al.* , 2017).

In the present study, we investigate if the use of a genome-wide genotyping strategy would identify the molecular aetiology of a non-consanguineous Spanish family with two affected brothers who both present a homogeneous phenotype with ≈100% round-headed sperm; and we provide the description of related sperm ultrastructure and the ART treatment outcome.

MATERIALS AND METHODS

Subjects

Two related globozoospermic patients were recruited from the Andrology Service of the Fundació Puigvert (Barcelona, Spain) and they gave their informed consent for the study, which was approved by the institutional ethical committee (June 2018; PR224/18). Eight first- and second-degree family members were also included in the study (Figure 1). They were Spaniards with European ancestry.

The clinical procedure for infertile patients included anamnesis, physical examination and semen analyses, performed in accordance with World Health Organization Guidelines (2010). Routine genetic study included karyotype for chromosomal aberration detection.

Pisum sativum agglutinin sperm labelling for evaluation of acrosome morphology

Spermatozoa from 100-200µl of semen were washed and diluted with PBS to adjust the sperm concentration in the slide. Slide cells were fixed in ethanol for 20 min, allowed to dry and subsequently incubated with fluorescein isothiocyanate conjugated with Pisum sativum agglutinin (FITC-PSA) (50µg/ml in PBS) for 30 min. Slides were examined on a fluorescence microscope (Zeiss, Axioskop 40, Germany) at 100x.

Sperm DNA fragmentation assessment

DNA fragmentation was evaluated using the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay (Cell Death Detection Kit; Roche Diagnostics; Switzerland). Fluorescein isothiocyanate (FITC)-dUTP was used as the label according to the manufacturer's instructions and counterstained with ethidium bromide (10µg/µl). Sperm DNA fragmentation was also evaluated, as an alternative method, by using Sperm Chromatin Dispersion test (Halosperm, Halotech®; Spain). The upper reference limits of DFI for proven fertile donors tested in our laboratory were 30% (SCD) and 26% (TUNEL) respectively.

Fluorescent in situ *hybridization (FISH)*

Sperm aneuploidy study analysis was performed at Reprogenetics (Barcelona, Spain) according to the previously described protocol (Sánchez-Castro *et al.* , 2009).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

TEM analysis was performed as described elsewhere. Briefly a semen sample was diluted 1:5 with 0.1 M phosphate buffer (pH 7.4) and fixed with 2.5% glutaraldehyde. Then, the samples were treated with OsO_4 (1%) and potassium ferrocyanide (0.8%), washed and dehydrated. After inclusion in epoxy resin, ultrafine sections were examined with electron microscope (JEOL J1010 TEM) at 80kV. SEM was also performed in similarly treated samples, with the exception that samples were dried on a SEM support and then examined with a JEOL JSM7001 scanning electron microscope at 15kV.

In vitro fertilization (IVF)

IVF-ICSI (in vitro fertilization/intracytoplasmic sperm injection) was selected as ART treatment for these patients.

Controlled ovarian stimulation was performed according to the standard procedures of the centre, using the GnRH antagonist protocol. Initial daily doses of 150–300 IU of gonadotropins (human menopausal gonadotropin, or recombinant human follicle stimulating hormone) were started on day 2 or 3 of the menstrual cycle. The initial dose of gonadotropins was decided based on female characteristics such as age, body mass index, baseline serum FSH, number of pre-antral follicles and the levels of anti-Müllerian hormone. Gonadotropin-releasing hormone antagonist (Centrotide® 0.25 mg/day) was added starting on the sixth day of the menstrual cycle and maintained until the day of human chorionic gonadotropin administration.

Oocyte retrieval was performed under sedation through transvaginal ultrasonography 34–36 hours after administration of 250 µg of HCG. Cumulus-corona-oocytes complexes were treated with hyaluronidase, denuded, classified according to nuclear maturity and maintained in culture until ICSI. Ejaculated spermatozoa were selected by discontinuous gradient centrifugation (PureSperm®, Nidacon Int. AB, Gothenburg, Sweden) and resuspended in washing medium. Motile sperm were selected, immobilized, and microinjected with an Eppendorf micromanipulator under a 400X magnification. In some cycles, artificial oocyte activation was done immediately after ICSI by incubation with 10 µmol/L of 4-bromo-calcium ionophore A23187 (Sigma-Aldrich; USA) for 30 minutes, followed by extensive washing.

The injected oocytes were incubated individually for 16–18 hours at 37°C and 5.5% CO_2, until confirmation of fertilization. Embryo quality was scored daily, and transfer of one or two

157 embryos was done at day 3 after oocyte retrieval with the help of ultrasound guidance. Luteal
158 phase support was initiated in the same day of oocyte retrieval with 200 mg progesterone
159 vaginal capsules and maintained daily. Embryos, that were not transferred, were cryopreserved
160 with vitrification media (Kitazato; Japan) according to the manufacturer's instructions.

161

162 ***Detection of* DPY19L2 *deletion***

163 DNA was isolated from blood samples according to routine procedures. For detection of deletion
164 of the entire *DPY19L2* gene, a BPa gap-PCR was performed using primers flanking the *DPY19L2*
165 deletion (Figure 2A), generating a fragment of 1700 bp in deletion carriers and primers for exon
166 10 as control for the presence or absence of *DPY19L2*, as previously described (Koscinski *et al.* ,
167 2011). A positive control (a fertile man) and negative controls [homozygous carriers of *DPY19L2*
168 deletion, kindly provided by Dr. Pierre Ray (Institute for Advanced Biosciences, University of
169 Grenoble, and CHU Grenoble Alpes; France)] were also used.

170

171 ***Genome-wide genotyping, imputation and detection of rare coding variants***

172 DNA samples were genotyped using the Infinium™ Global Screening Array-24 v3.0 (GSA,
173 Illumina; USA), following the manufacturer's protocol. All the individuals reached a genotyping
174 call rate over 98% and genotype information was obtained for 660,906 single nucleotide
175 polymorphisms (SNPs). After quality control (QC) analyses, we extended the number of genetic
176 variants analysed through a genotype imputation process, evidencing association at genetic
177 markers that are not directly genotyped. This imputation step was carried out in the TOPMed
178 Imputation Server (https://imputation.biodatacatalyst.nhlbi.nih.gov/) and using the 'NHLBI
179 Trans-OMICs for Precision Medicine' (TOPMed), comprising 97,256 individuals for a total of
180 308,107,085 genetic variants, as a reference panel (Das *et al.* , 2016, Kowalski *et al.* , 2019).

181 After the corresponding QC, we obtained the genotype information of rare variants (minor allele
182 frequency < 0.01 in a population of 1,073 Iberian fertile men), which were polymorphic in the
183 family (minor allele present in at least 1 individual). Finally, we focused on the genetic variation
184 located in coding sequences as defined in GENCODE V36 (GRCh38/hg38). These analyses were
185 performed by the means of a Genome-Wide Association Study gold-standard software, Plink 1.9
186 (Chang *et al.* , 2015) and the bedtools v2.27.1 toolset (Quinlan and Hall, 2010).

187

Compound heterozygosity analysis

We implemented a bio-computational analysis pipeline to identify genetic variants found in homozygosity exclusively in the globozoospermic men. No variants fulfilling these criteria were found in the X chromosome or in spermatogenesis-related autosomal loci. Consequently, we then proceeded to identify gene loci which harboured rare genetic variants that might lead to compound heterozygosity, i.e., the presence of two different mutant alleles (one per chromosome) at a particular gene, only in the affected individuals but not in the rest of the family members (or the fertile population).

Sanger sequencing of the selected variants

The imputed genotype information for each family member for two genetic variants located in the *DPY19L2* locus, was confirmed by direct automated sequencing. *DPY19L2* exons 3 and 8 were amplified using previously described PCR primers (Chianese *et al.* , 2015). Sequences analyses were carried out on a 3130 Genetic Analyzer sequencer (Applied Biosystems, Hitachi, Japan).

In silico analysis of sequence variants

Multiple alignments were performed as implemented in Clustal Omega (Sievers *et al.* , 2011). We addressed the impact of the selected variants on protein function based on the predictions reported by the Polyphen and SIFT algorithms, which evaluate the effect of non-synonymous single amino acid substitutions. PolyPhen-2 calculates naïve Bayes posterior probabilities that the mutation is damaging and classifies the variant as benign, possibly damaging, or probably damaging based on the false positive rate thresholds (Adzhubei *et al.* , 2010). SIFT scores range from 0 to 1 and the amino acid substitution is predicted to be damaging (score <= 0.05) or tolerated (score > 0.05) (Sim *et al.* , 2012).

RESULTS

Sperm analysis

Patients were two brothers (28 and 29 years old) from a non-consanguineous Spanish family who consulted for primary infertility for at least 18 months. No previous history of male infertility was diagnosed in their family although semen analysis identified mild teratozoospermia in one of the two cousins of the maternal side of the family (III.7, III.8; Table 1) and asthenozoospermia in one maternal uncle (II.9, Table 1).

Detection of 100% round-shaped sperm heads of both infertile siblings was determined during routine light microscopic examination of the semen sample (Figure 3B, 3C; semen sample from a fertile individual was also assessed as control Figure 3A). The absence of acrosome was visualized by FITC-PSA labelling (Figure 3E, 3F compared with control Figure 3D) and thus, the two brothers were diagnosed with total globozoospermia. Subsequent ultrastructural sperm analysis confirmed the absence of acrosome and revealed the presence of redundant nuclear membrane and intermediate piece defects such as disorganization or absence of the mitochondrial sheath (Figure 3H, 3I; control Figure 3G). Other ultrastructural defects are associated with sperm flagellum, and an increased frequency of short, coiled and irregular flagella was determined (Figure 3K, 3L; control Figure 3J).

An increased sperm DNA fragmentation was observed, by using both TUNEL [values 34% (P1) and 43% (P2)] and SCD [values 37% (P1) and 28% (P2)] (Table 1). There were 0.68% (P1) and 2.26% (P2) total sperm aneuploidies. Karyotype analysis showed no chromosomal aberration.

Molecular analysis

DNA samples of the affected brothers (III.4, III.5) were used for genetic analysis. DNA from the parents (II.3, II.4) was also available as well as from other members of the maternal side of the family [grandparents (I.3, I.4), aunt (II.5), uncle (II.9) and cousins (III.7, III.8)] (Figure 1; Table 1).

First, patients were tested but were negative for recurrent *DPY19L2* deletion, not in homozygosis or heterozygosis (data not shown), thus we used a high-throughput genome-wide genotyping platform and a genotype imputation strategy to determine potential pathogenic genetic variants at a genome-wide scale (Figure 4). Primary analysis of GWAS allowed us to obtain the genotype information of 277,483 rare variants (minor allele frequency < 0.01 in a population of 1,073 Iberian fertile men), which were polymorphic in the family (minor allele

present in at least 1 individual). Then, we focused on the 2651 genetic variants located in coding sequences. After genome-wide analysis of these polymorphisms, 887 instances were identified as possibly involved in compound heterozygosity (at least two mutant alleles, one per chromosome, linked to one gene). But only two SNPs in the *DPY19L2* locus were both present in the globozoospermic brothers and not in the rest of the members of the family (Figure 4). These *DPY19L2* SNPs, which result in amino acid substitutions, were later confirmed by direct automatic sequencing: c.869G>A in exon 8 (p.R290H; rs147579680) and c.431T>A in exon 3 (p.M144K; rs771726551) (Figure 2B, 2C, 2D), the latter is described as associated with globozoospermia for the first time in this report. The i*n silico* mutant model predicts c.431T>A SNP as a clinically damaging missense mutation, similarly as c.869G>A (Figure 2D). Sanger sequencing was used to confirm the variants obtained from the imputed genotype information for the available family members (Figure 1; Table 1). Both uncle (II.9) and cousins (III.7, III.8) were carriers of *DPY19L2* SNP c.431T>A and neither of them presented a globozoospermic phenotype (Table 1). Subsequent mutation screening by GWAS in a cohort of 1037 Spanish fertile control individuals identified no carrier for either of these *DPY19L2* mutations.

SNP c.431T>A in *DPY19L2* was absent from the 1000 Genomes Project (1000G) (Auton *et al.* , 2015) and the Exome Aggregation Consortium (ExAC) databases (Karczewski *et al.* , 2017). However, it was identified at a very low frequency in European-related cohorts (Latino/Admixed American 7/31984 allele frequency; and European non-Finnish 5/126408 allele frequency) included in the Genome Aggregation Database (gnomAD) project (Karczewski *et al.* , 2020), compared to a higher allele frequency for the c.869G>A *DPY19L2* SNP (European non-Finnish 41/128530 allele frequency). These variants are both found in males and females in a similar frequency. They are very rare SNPs in overall population.

Thus, the pathogenicity classification of these rare variants in an acrosome related gene make these heterozygous *DPY19L2* alleles the most likely cause of globozoospermia in this family.

ART reproductive results

A total of 4 IVF-ICSI cycles were performed using ejaculated spermatozoa from the two globozoospermic brothers (Table 2). In the first cycle (P1) of IVF-ICSI, 11 metaphases II (MII) were obtained and injected with motile sperm without achieving fertilization. In the second cycle, 12 MII were injected, and 25% normal fertilization was obtained with artificial oocyte activation (AOA) using Ca^{2+} ionophore A23187, transfer of 2 quality A embryos and pregnancy and delivery of a girl. In the third cycle, 6 MII, 16.7% fertilization (with AOA), pregnancy of one transferred

279 embryo and miscarriage at 8 weeks. In P2, a cycle of IVF-ICSI, 18 MII, fertilization 22% (with
280 AOA), transfer of 2 embryos of qualities B and C, pregnancy and delivery of a girl was achieved.

281 To summarize, the sperm of globozoospermic siblings were unable to activate oocytes, which
282 could be partially corrected with AOA. Generated embryos showed good developmental
283 potential.

DISCUSSION

The first studies of consanguineous families allowed the identification of different genetic factors involved in the pathogenesis of globozoospermia, most of them related to proteins involved in the biogenesis of the acrosome (Coutton *et al.* , 2015, Dam *et al.*, 2007b, Koscinski *et al.*, 2011). To date, globozoospermia-associated mutations in *C2CD6*, *C7orf61, CCDC62*, *CCIN*, *DNAH17*, *GGN*, *PICK1*, *SPATA16*, *ZPBP1* have been identified, but alterations in the testis-specific *DPY19L2* gene, which encodes an eleven-domain transmembrane protein necessary for sperm head elongation and acrosome formation (Harbuz *et al.* , 2011, Pierre *et al.* , 2012) are by far the most frequent molecular etiologic factors for this condition. The described prevalence rate and distribution pattern of *DPY19L2* alterations is highly heterogeneous in the literature, probably due to context-dependency such as the geographical origin of affected patients, the severity of globozoospermia and the variable degrees of consanguinity of patients studied. In globozoospermic patients of European origin, *DPY19L2* gene defects have been identified in 74% of patients with >50% of round-headed spermatozoa, while *DPY19L2* diagnosis efficiency rose to 80% for cases with >90% of globozoospermia (Celse *et al.*, 2021). *DPY19L2* deletion account for 45-50% of alleles, thus the majority of globozoospermic patients are homozygous or compound heterozygous for this alteration: *DPY19L2* homozygous deletions were identified in 36% of globozoospermic patients or compound heterozygous of *DPY19L2* deletion on one allele and *DPY19L2* mutation in the other (18.2%) (Celse *et al.*, 2021). Thirty deleterious *DPY19L2* variants have been described, accounting for approximately 20% of the pathological alleles (Ray *et al.*, 2017) (Celse *et al.*, 2021).

The results of the present study revealed that total globozoospermia can result from the transmission of heterozygous mutations in *DPY19L2* from non-consanguineous parents. In fact, in patients with rare recessive diseases, compound heterozygosity of pathogenic mutations is the most likely inheritance model if the parents are non-consanguineous. To our knowledge, we describe the first case of compound heterozygosis for 2 different *DPY19L2* mutations in globozoospermic patients of European ancestry. The first change, in exon 3, p.M144K implies the exchange of a hydrophobic, nonpolar, aliphatic amino acid to a positively charged hydrophobic one in position 144. Variant in exon 8 p.R290H is a recurrent missense mutation, that affects a highly conserved arginine which was described as essential for the C-mannosyltransferase activity of DPY-19, the DPY19L2 ortholog in *Caenorhabditis elegans* (Buettner *et al.* , 2013, Ray *et al.*, 2017). Moreover, these changes affect the extramembrane domains (extramembrane 1 and 3 in the perinuclear space) of the protein and are predicted to greatly affect the properties of DPY19L2.

The *DPY19L2* gene, located on 12q14.2, has 22 exons encoding for a 11-transmembrane domain protein and is flanked by two low-copy repeats (LCRs) sharing 96.5% identity. A non-allelic homologous recombination process between those LCRs underlies the complete *DPY19L2* deletion related to globozoospermia (Elinati *et al.* , 2012, Stankiewicz and Lupski, 2002). On the other hand, these LCR or segmental duplications have been shown to significantly contribute to evolution by duplication of the functional gene. In fact, another functional gene, *DPY19L1*, and six pseudogenes have been described within those LCRs (Carson *et al.* , 2006).

The emergence of Next generation sequencing technologies (NGS) has made it possible to analyse many genes in a single procedure, but all sequencing technologies have limitations. WES and WGS allow the analysis of the genetic variation in every position of either the coding regions or the complete genome sequence. Despite the technical advances and the decreasing economic cost of sequencing, WES and WGS currently involve long and still expensive procedures that present some difficulties in identifying pathogenic variants related to segmental duplications and pseudogenes. Remarkably, in this report, instead of NGS we have applied an innovative approach to make the most out of the genotyping data obtained by a high-throughput genotyping assay, which is used in genome-wide association studies (GWAS). This genotyping platform is fast, has very low cost per sample and contains probes for hundreds of thousands of polymorphic positions in the genome. Additionally, we expanded the number of interrogated loci to millions using a huge reference panel and an imputation algorithm, a common procedure in GWAS. Contrary to standard GWAS approach, we did not analyse common genetic variation, but we focused only on rare polymorphic coding variants and, among them, on those loci which showed compound heterozygous inheritance patterns. By these means, we were able to identify the two *DPY19L2* mutations associated with globozoospermia and we propose that this strategy could be applied in similar contexts.

It is worth noting, however, that our GWAS-based approach relies on the availability of DNA samples and genome-wide genotype information from several family members. Moreover, the causal variants should be present either in the genotyping arrays or in the haplotypes included in the imputation panel. Thus, the presented strategy would not be able to identify *de novo* mutations in affected individuals and WES or WGS would be necessary in these cases.

We confirm the negative correlation between *DPY19L2* mutation-related-100% globozoospermia and conventional intra-cytoplasmic spermatozoa injection outcomes, and the necessity of artificial oocyte activation using Ca^{+2} ionophores for a successful ART outcome. A higher rate of DNA fragmentation, probably associated with the absence of protamines and poor

sperm chromatin compaction (Yassine *et al.*, 2015), observed in these globozoospermic patients could lead to an impaired development potential of embryos (Yassine *et al.*, 2015), which could be a contributing factor to the absence of PLCζ, for low-IVF success rate, as previously suggested for *DPY19L2* homozygous-deleted patients (Coutton *et al.*, 2015).

As expected, heterozygous *DPY19L2* mutations do not affect female reproduction in this family. In fact, DPY19L2 is mainly expressed in the spermatids and in no female reproductive organs. To our knowledge, no woman with homozygous *DPY19L2* mutations has been described, suggesting the absence of any phenotype in those women.

Overall, we describe the first case of compound heterozygosis for 2 different *DPY19L2* mutations in globozoospermic patients of European origin. The biological and molecular characteristics of two siblings with globozoospermia are described, of which the following stand out: increased sperm fragmentation and sperm aneuploidies; inability of sperm to activate oocytes (which could be partially corrected with artificial oocyte activation similarly as described in globozoospermic patients with different *DPY19L2* mutational status); and good developmental potential of the generated embryos. All these data highlight the fact that compound heterozygous mutations in *DPY19L2* should be considered for molecular screening in globozoospermia, particularly in cases from families without consanguineous relationships, and currently unexplained cases of globozoospermia in non-consanguineous families may be due to *DPY19L2* mutations.

The identification of genetic factors in infertile couples is clinically relevant not only to improve ART treatment but also to determine the risk for the future offspring since these genetic defects, which would not have been inherited otherwise, can be transmitted to the progeny through assisted reproductive techniques.

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FIGURE LEGENDS

Figure 1. Pedigree of the Spanish family and segregation analysis of *DPY19L2* alleles

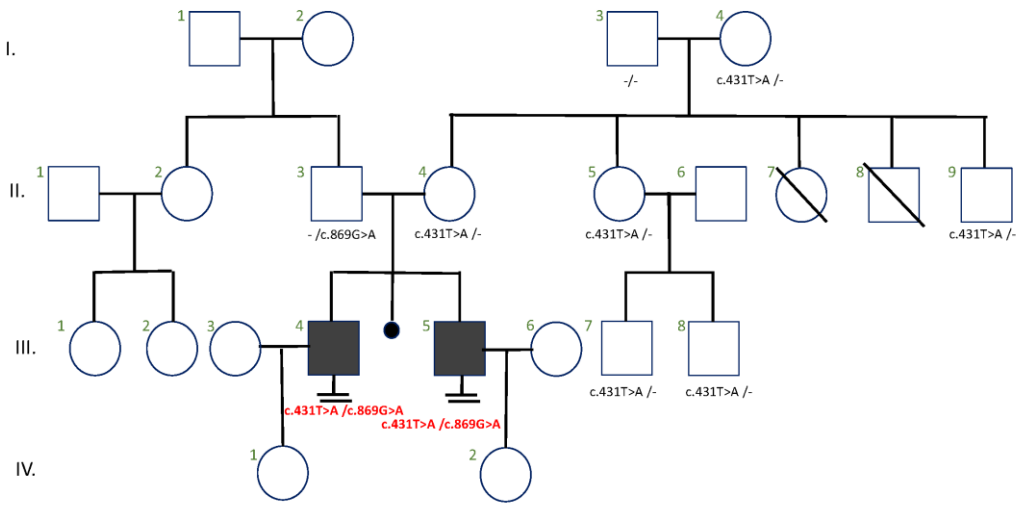
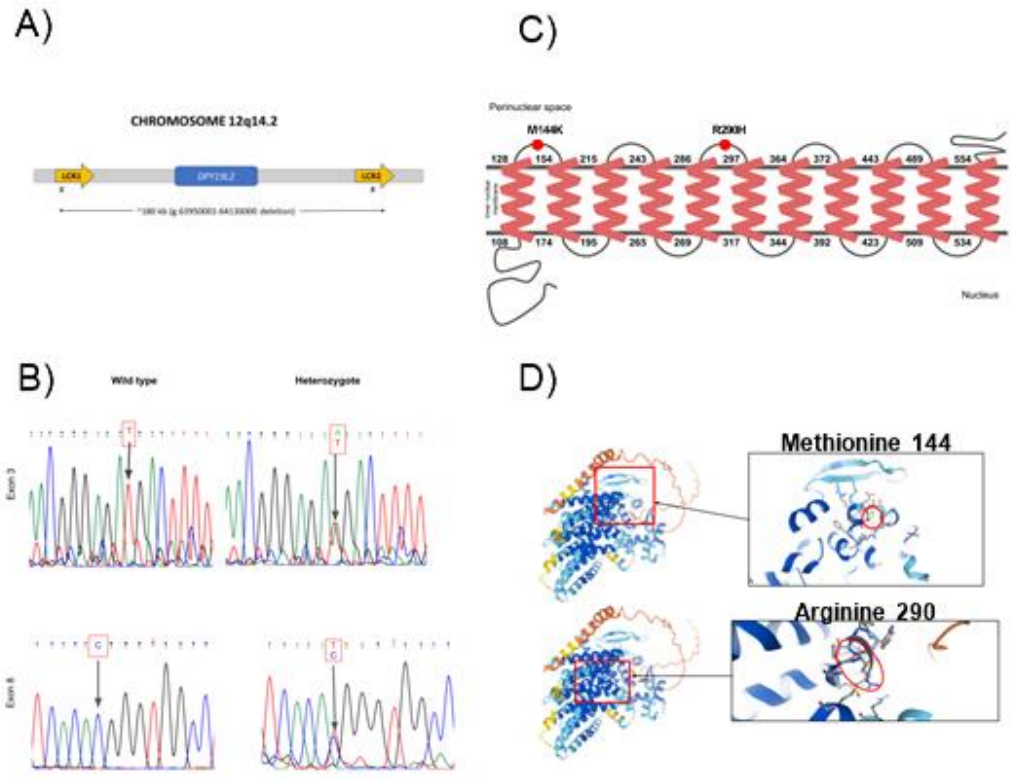


Figure 2. *DPY19L2* mutations in globozoospermic siblings. A) Schematic representation of the *DPY19L2* gene and the surrounding LCR regions. # symbol indicates the location of PCR oligonucleotide sequences to amplify BPa fragment B) Electropherograms of *DPY19L2* exon 3, and 8 showing the control and mutated sequences. C) Schematic representation of the 11-transmembrane domain DPY19L2 protein. The *DPY19L2* SNPs [c.431T>A in exon 3 (p.M144K; rs771726551) and c.869G>A in exon 8 (p.R290H; rs147579680)] in globozoospermic brothers affect amino acids in the extramembrane domains (extramembrane 1 and 3 in the perinuclear space) of the protein. D) Structural model of DPY19L2 obtained by the EBI tool (Jumper *et al.* , 2021) showing the wild-type amino acids affected by the two sequence variants. These missense mutations are predicted to greatly affect the properties of DPY19L2.


A)
CHROMOSOME 12q14.2
C)
Perinuclear space
M144K
R290H
Nucleus
B)
Wild type
Heterozygote
Exon 3
Exon 8
D)
Methionine 144
Arginine 290

405

Figure 3. Round-shaped sperm head without acrosome in globozoospermic siblings

Panels (A, D, G, J) show control spermatozoa, (B, E, H, K) P1 globozoospermic sperm and (C, F, I, L) P2 globozoospermic sperm.

(A-C) Light microscopy. Conventional sperm analysis evidence round-shaped sperm head in P1 and P2 infertile patients

(D-F) Fluorescence microscopy. Fluorescein- pisum sativum agglutinin (FITC-PSA) labelling of sperm evidence the absence of acrosome in globozoospermic sperm.

(G-I) Transmission electronic microscopy. Acrosome of control sperm is clearly identified (G). In globozoospermic sperm, a round shape nucleus without acrosome is evidenced and mitochondria disorganization at the midpiece of sperm

(J- L) Scanning electronic microscopy evidence additional morphological alterations at the middle piece and tail of globozoospermic sperm

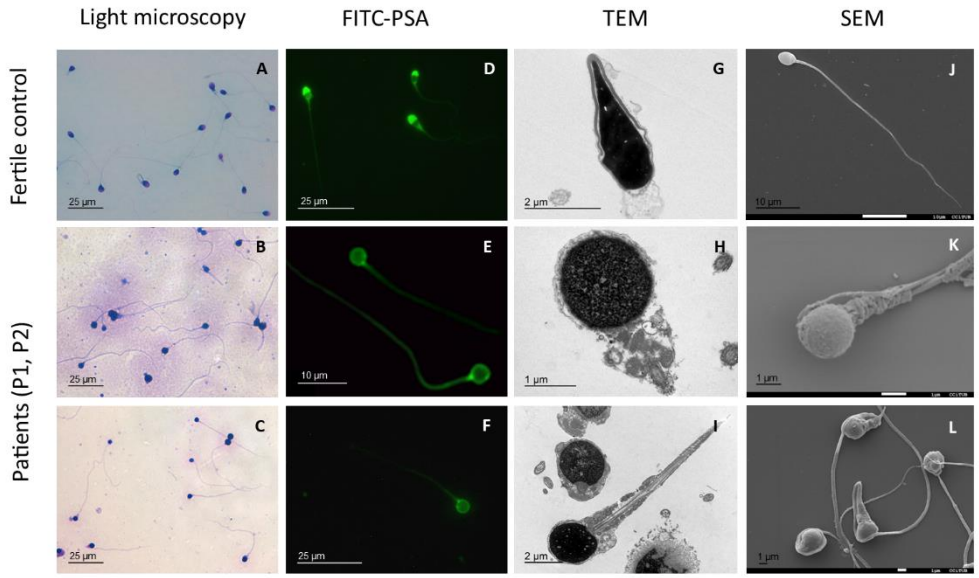
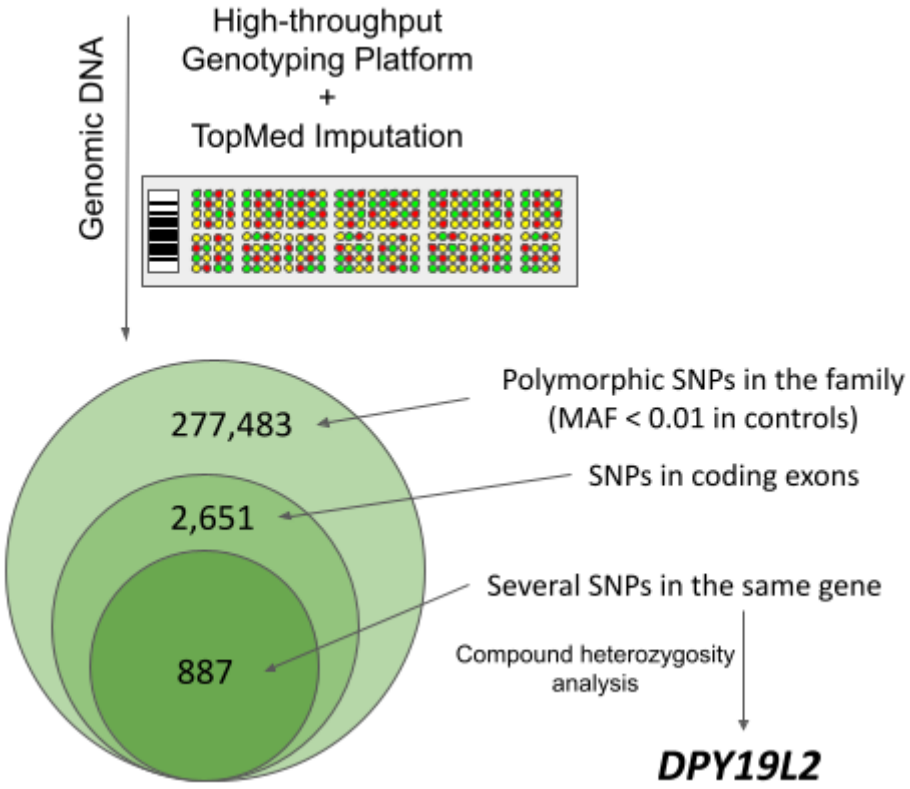


Figure 4. Summary of results from GWAS bioinformatic analysis of globozoospermic brothers


Genomic DNA
High-throughput
Genotyping Platform
+
TopMed Imputation
277,483
2,651
887
Polymorphic SNPs in the family
(MAF < 0.01 in controls)
SNPs in coding exons
Several SNPs in the same gene
Compound heterozygosity
analysis
DPY19L2

420

421

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512