Ayed *et al. Middle East Fertility Society Journal* (2026) 31:3
https://doi.org/10.1186/s43043-025-00295-w

Middle East Fertility Society Journal

RESEARCH Open Access

CFAP251 deletion and ICSI outcome in Tunisian infertile men with multiple morphological abnormalities of the sperm flagellum

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Abstract

Background Multiple Morphological Abnormalities of the Sperm Flagella syndrome (MMAF) is a specific asthéno –tératozoospermia leading to male infertility and characterized by severe defects in the sperm's tail or flagellum. These abnormalities lead to greatly reduced or absent sperm motility, making it difficult for sperm to reach and fertilize an egg. MMAF is considered a genetic disorder, often inherited in an autosomal recessive manner. Significant advancements in high-throughput DNA sequencing have led to the identification of numerous genes linked to MMAF. While research is ongoing and the exact mechanisms are still being fully elucidated, over 40 genes have been associated with MMAF, accounting for about 60–70% of cases.

Patients and methods Two infertile MMAF brothers undertaking ICSI were included. Whole exome sequencing was carried out for one of them.

Results We identified a homozygous framshift variant c.[3007_3337del] in *CFAP251* gene in the patient and in his affected brother. The deletion encompasses exons 20 and 21, transmitted from heterozygous parents. Consequently, 12.8% of the protein (147 amino acids) is predicted to be lost. The analysis of their ICSI attempts showed that no pregnancy was achieved for their couples.

Conclusion Our findings highlight the importance of whole exome sequencing in identifying genetic causes of MMAF and may provide valuable insights for clinical genetic counseling and assisted reproductive treatments for affected individuals.

Keywords Male infertility, MMAF, Whole exome sequencing, *CFAP251*, *ICSI*

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Introduction

Asthenozoospermia, characterized by reduced sperm motility, and teratozoospermia, marked by abnormal sperm morphology, are common causes of male infertility [1]. A specific subset of these disorders, introduced in 2014, and called Multiple Morphological Abnormalities of the Sperm Flagellum (MMAF). It is characterized by a spectrum of flagellar defects: absent, short, coiled, bent, and irregular flagella due to various ultrastructural defects of the axoneme without association with respiratory cilia symptoms [2, 3].Genetic factors play a crucial role in MMAF etiology. With advances in high-throughput sequencing technology, more than one hundred genes have been implicated in sperm flagella anomalies in humans and mammals. To date, over 40 candidate genes have been validated in infertile men with MMAF syndrome [2]. *DNAH1* was the first gene described in this phenotype and has been reported in patients of North African origin [3]. Subsequent studies have identified DNAH1 mutations in other cohorts from different ethnic backgrounds [2]. Several genes, including those encoding components of the axoneme and peri-axonemal structures, have been implicated. Among these, genes involved in the inner and outer dynein arms (*DNAH2, DNAH6, DNAH17*), members of the Cilia and Flagella Associated Protein family (*CFAP43, CFAP44, CFAP69, CFAP251*) [2] or peri-axonemal structures (*FSIP2*). However, genetic studies can explain only around 60% of MMAF cases. Therefore, further research is needed to discover new genetic factors that contribute to MMAF.

The aim of this study is to identify the genetic factor of familial case with MMAF using whole exome sequencing and to analyze the result of their ICSI attempts.

Materials and methods

Patients

In this study, we initially included two infertile brothers referred to the Genetic Department at the Pasteur Institute of Tunis from Department of Reproduction Biology, of Aziza Othmana Hospital in Tunis. Each individual presented with a typical MMAF phenotype characterized by severe asthenoteratozoospermia resulting from a combination of sperm flagellar defects. Symptoms associated with primary ciliary dyskinesia (PCD), such as sinusitis, bronchitis, and pneumonia, were carefully reviewed and excluded from the MMAF cohort. Peripheral whole blood samples were collected for genetic analyses. Signed informed consent was obtained from the participants included in the study, and approval was granted by the local ethics committee of the Pasteur Institute of Tunis.

Semen analysis and DNA fragmentation test

Several sperm analyses were conducted for both patients. Semen samples were collected by masturbation after two to seven days of sexual abstinence whether in the andrology unit of Reproductive Biology Department of Aziza Othmana Hospital or in a city andrology lab. The semen samples were incubated at 37 °C for 30 min for liquefaction and the ejaculate volume, sperm concentration, vitality, motility and morphology were evaluated. Sperm vitality was assessed by eosin staining, and sperm morphology was analyzed based on Schorr-stained semen smears according to the modified Kruger classification.

Semen analysis was conducted according to the World Health Organization (WHO) guidelines 2010 as they took place between 2019 and 2022.

Sperm images were captured with Microscope Clever C320® equipped with OPTOSCAN S40 camera.

A DNA fragmentation test was conducted twice for patient II.2 (P0733) using the GoldCyto sperm® kit (Microptic®), which evaluates on the evaluation of sperm chromatin dispersion (SCD). A threshold of 15% indicates a high DNA fragmentation rate.

Whole-exome sequencing and bioinformatics analysis

Genomic DNA was extracted from blood lymphocytes collected on EDTA, using a commercially available kit (FlexiGene Kit; Qiagen), according to the manufacturer's instructions. DNA quantity and purity were evaluated using a NanoDrop™ spectrophotometer.

Whole exome sequencing was performed only for P0733, using the Human Core Exome + Human RefSeq panel exome capture kit (Twist Bioscience, CA, USA). Sequencing was performed on Illumina NovaSeq 6000 (S4 flow cell) with an expected throughput of 5 GB of paired-end reads (2 x 150 bp). 96% of all Ensembl v111 canonical exons were covered at least 10x and 82% were covered at least 20x. Reads were analyzed using an updated version of the previously described bioinformatics pipeline developed in-house [4]. It consists of two modules, both distributed under the GNU General Public License v3.0.

Briefly, using the first module (https://github.com/ntm/grexome-TIMC-Primary), adapters were trimmed and low-quality reads filtered with fastp 0.23.2, reads were aligned on GRCh38 with bwa- mem2 v2.2.1 [https://doi.org/10.1109/IPDPS.2019.00041], duplicates were marked using samblaster v0.1.26 [https://doi.org/10.1093/bioinformatics/btu314], and the BAM file was sorted and indexed with samtools 1.18 [https://doi.org/10.1093/gigascience/giab008]. SNVs and short indels were called using strelka 2.9.10 [https://doi.org/10.1038/s41592-018-0051-x] and GATK 4.4 to produce individual GVCF files. These were finally merged with similar GVCFs corresponding to 1038 patients or controls available in-house, to obtain a single multi-sample GVCF per caller. This cumulative approach, where new exomes are integrated with previous samples before analysis, greatly increases

the power to identify potentially causal variants. Furthermore, using two variant callers independently provides a simple initial cross- validation procedure.

The second module (https://github.com/ntm/grexome-TIMC-Secondary) processed each merged GVCF and produced annotated analysis-ready TSV files. This was achieved by performing 17 streamlined tasks, including the following. Low-quality variant calls (DP<10, GQX<20, or less than 15% of reads supporting the ALT allele) were discarded. Variant Effect Predictor v111 [https://doi.org/10.1186/s13059-016-0974-4] was used to annotate the variants and predict their impact and tier. Gene expression data from the Genotype-Tissue Expression project (GTEx v7) were added. Variants with a minor allele frequency greater than 1% in gnomAD v2.0 or 3% in 1000 Genomes Project phase 3 were filtered.

Results

Characteristics of the patients and semen analysis

Our two patients, II.1 and II.2 (P0733), are aged 44 and 40 years, respectively, and were born from a consanguineous marriage (Figure 1). All semen analyses conducted showed that both progressive and total motility were severely reduced, with values ranging from 8.8% to akinetopsermia. In Table 1, we provide details on the various parameters from one of the analyses for each patient, particularly focusing on sperm morphology. Multiple morphological abnormalities of the flagella were observed in 97% to 100% of the spermatozoa, with short and coiled flagella being predominant, as illustrated in Figure 2. Since the semen analyses were performed by multiple operators across two different laboratories, there were no doubt regarding the validity of the findings.

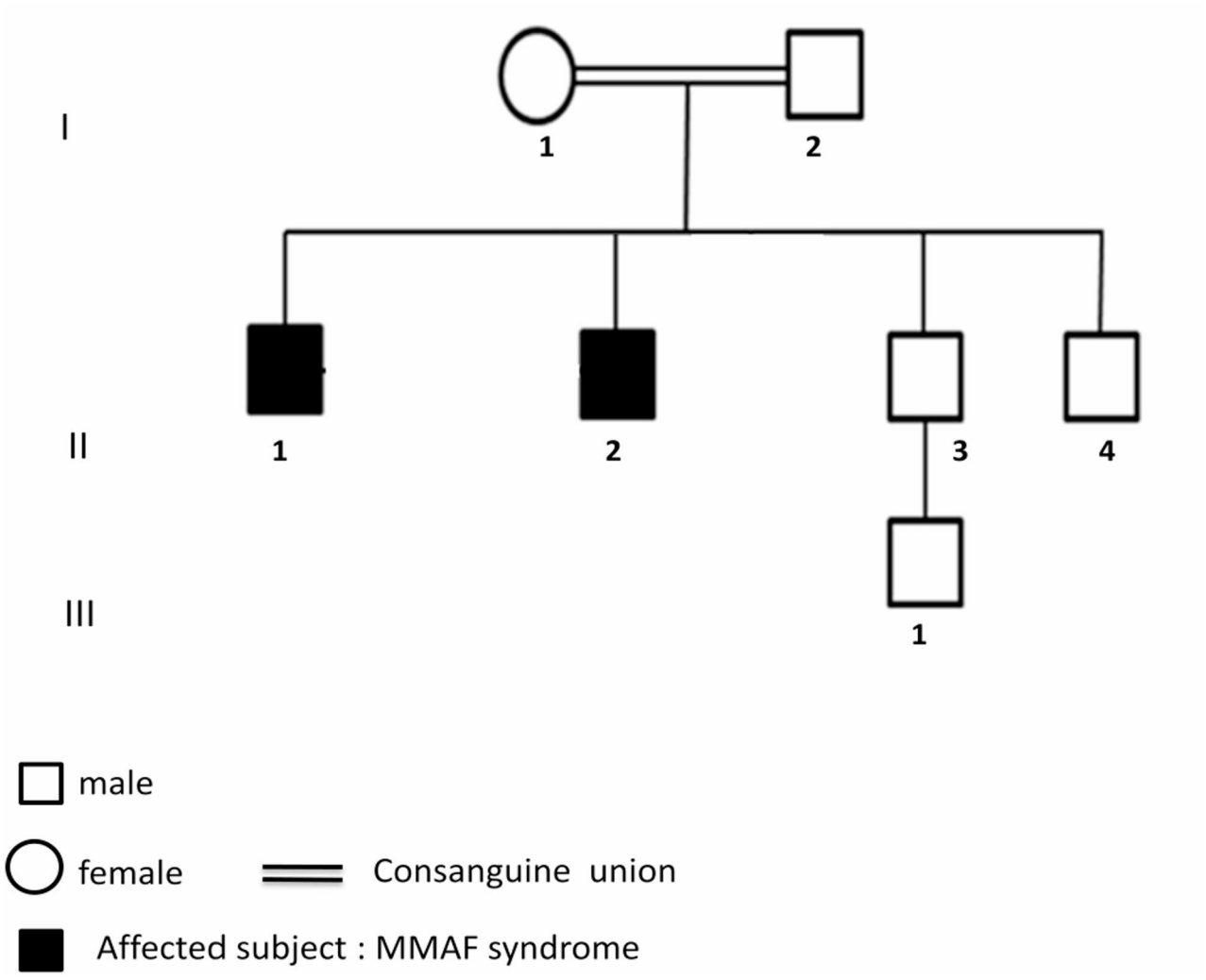

Fig. 1 Pedigree of familial cases with MMAF syndrome II.1 and II.2 (P0733)

Table 1 Semen parameters of the two MMAF patients (*)

	Patient II.1	Patient II.2 = P0733
Volume (mL)	3.7	2.9
Sperm concentration (10^6/mL)	9.7	1.7
Total motility (%)	0	8
Sperm vitality (%)	17	39
Normal spermatozoa (%)	0	3
Abnormal spermatozoa	100	97
Short flagella	58	32
Coiled flagella	10	27
Absent flagella	0	16
Angulated flagella	0	4
Flagella with irregular caliber	0	0
Double flagella	0	0

Here we present an example of two of the several sperm analyses taken by the two brothers in the andrology unit of Reproductive Biology Department of Aziza Othmana Hospital or in a city andrology lab

(*) All semen analyses were conducted between 2019 and 2022 based on the World Health Organization (WHO) guidelines 2010 for sperm analysis

Data analysis of whole exome sequencing

Due to limit financial resources, WES was performed only in one brother II.2=P0733 with better DNA quality.

Since this study was limited to a single exome, CNV calling was not possible and our systematic analysis was therefore limited to SNVs and short indels, preventing the direct discovery of any causal CNV.

An initial analysis of the bioinformatics pipeline results identified a homozygous missense variant predicted to strongly impact the protein encoded by a testis-enriched gene (*DNAH6*). However, Sanger validation showed that the variant is heterozygous in the affected brother, refuting this variant as causal since the father is presumably heterozygous.

A deeper re-analysis identified three intriguing variants, all homozygous in P0733. Although unremarkable in terms of impact - two were synonymous and one was a predicted-benign missense variant, all three were homozygous in seven other MMAF exomes marked as "diagnosed" in our database, and they were in the same region of chromosome 12.

Further investigation revealed that the identified causal variant for the seven other MMAF patients was a single variant: a homozygous deletion of exons 20–21 in *CFAP251*, also located on chromosome 12 in close proximity with the three benign SNVs.

Finally, an examination of the BAM of P0733 using IGV showed that P0733 was very likely affected by the same homozygous deletion c.[3007_3337del] of *CFAP251* (Figure 3).

Confirmation of the CFAP251 deletion

Polymerase Chain Reaction was performed according to the routine protocol of the laboratory. Two couples of primers were used: F1=Int19F: GAATAGAAAGGCGG GAAAGG and R1=Int22R: AACTCTGGGTGGAAATT GCTTA; Ex20F: AGCTTTGAGGTGCTCGGTTA and Int20R: CCTCATGGCTCAAATCTGGT as described previously by Kherraf et al, [4]. F1 (upstream of exon 20) and R1 (downstream of exon 21) amplified a 950 pb fragment in affected individual. Ex20F and Int20R amplified a 220pb fragment in normal individual. Duplexe PCR confirmed the c.[3007_3337del] in P0733 (II.2) and his brother II.1 (Figure 4b). Segregation study in parents showed that they were heterozygous for this variant (Figure 4c). In 13 other Tunisian MMAF patients, no deletion of *CFAP251* was found (data not shown).

ICSI outcomes

Between November 2020 and November 2023, patient II.2 (P0733) and his wife underwent four ICSI cycles. In their first two attempts (2020 and 2021), the wife had an antagonist ovarian stimulation (using r-hFSH) and after ovum pickup and ICSI, the couple achieved fertilization rates of 55% and 60%, respectively. However, they encountered fertilization abnormalities, with zygotes showing either one pronucleus or three pronuclei in 11% and 20% of the two attempts, respectively. They were able to benefit from embryo transfers of good to average quality embryos in both attempts—specifically, one blastocyst on day 5 during the first attempt and two embryos on day 3 during the second. Unfortunately, neither attempt resulted in a pregnancy. The last two attempts, the ovarian stimulation protocol was switched to an agonist protocol (with different gonadotrophins : either menotropins or an association of r-hFSH and r-hLH), but unfortunately, fewer oocytes were collected (4 and 5 respectively) with only lesser mature oocytes than the first attempts. These two cycles resulted in fertilization failures, with no embryos available for transfer.

It should be noted that during the four ICSI attempts, sperm vitality tests without vital dyes (such as Sperm-Mobil® Vitrolife) were required. Indeed, the application of substances such as theophylline can stimulate flagellar movement in sperm cells, allowing for the selection of viable sperm for microinjection.

After these unsuccessful attempts, additional tests were proposed to the couple, including a Sperm DNA fragmentation test. The first test done for II.2 (P0733) found a rate of 22.5%, a fairly high index. Following a four-month antioxidant treatment—primarily consisting of a combination of L-carnitine, L-arginine, Coenzyme Q10, vitamin E, zinc, and selenium—the same DNA fragmentation test was repeated, yielding a normal rate of 5%.

Discussion

Disruption of spermatogenesis is a major cause of infertility, and genetic abnormalities affecting spermatogenesis can be the cause of many unknown male infertility

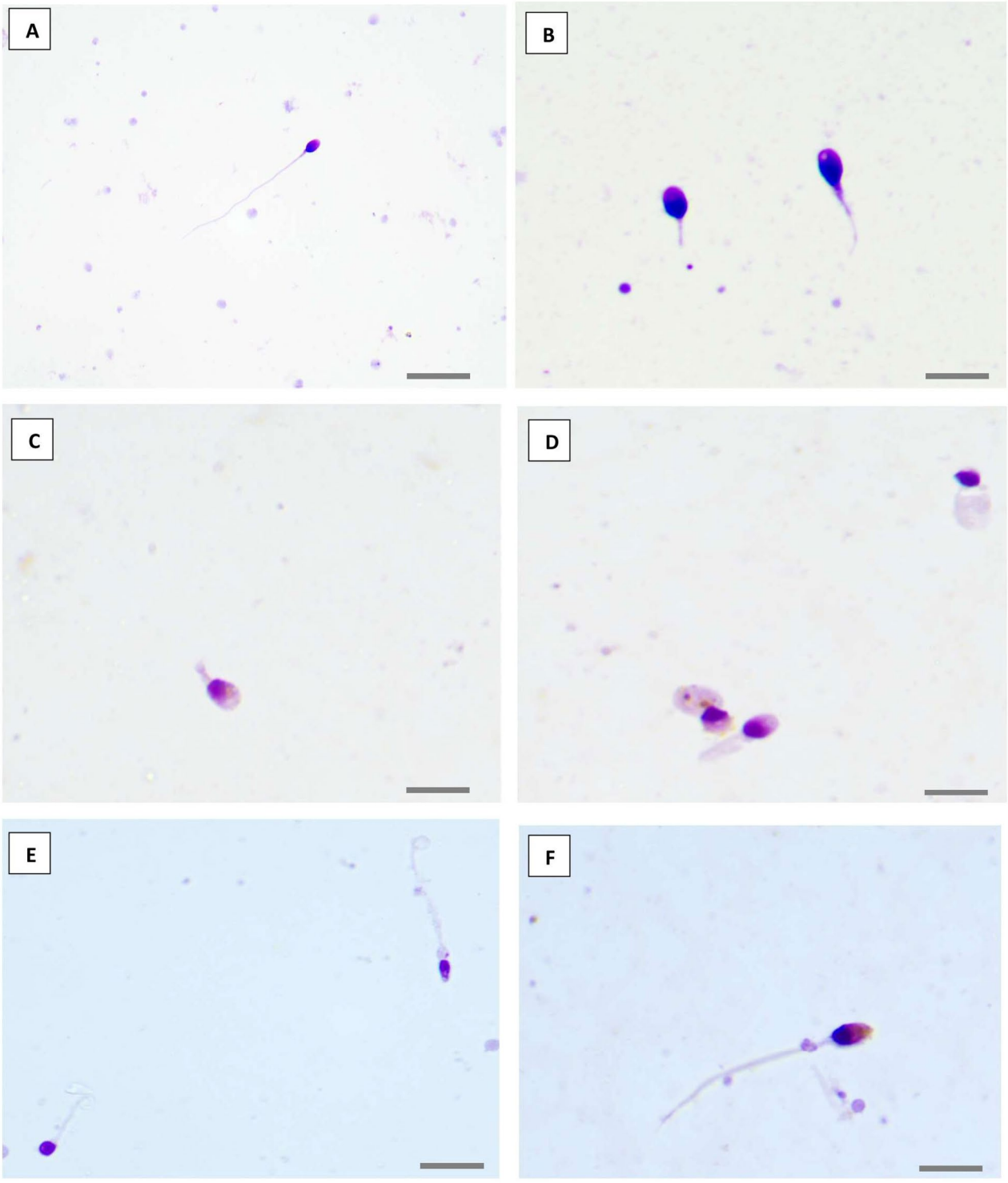

Fig. 2 Light microscopy imaging of MMAF sperm of patient II.2 (P0733) (x1000) Photos taken with a Clever C320 Microscope equipped with an OPTO-SCAM S40 camera, at x1000. Scale bars 10 µm; Testsimplets® slides for **A**-**B**-**C**-**D** and Diff-Quick TM staining for **E** and **F**; **A**- Picture of a normal spermatozoa; **B**- Spermatozoas with short flagella; **C**- Spermatozoo with absent flagellum; **D**- Spermatozoa with coiled flagella; **E**- Spermatozoa with longer flagella but coiled terminal piece; **F**- Spermatozoa with longer flagellum but missing terminal piece

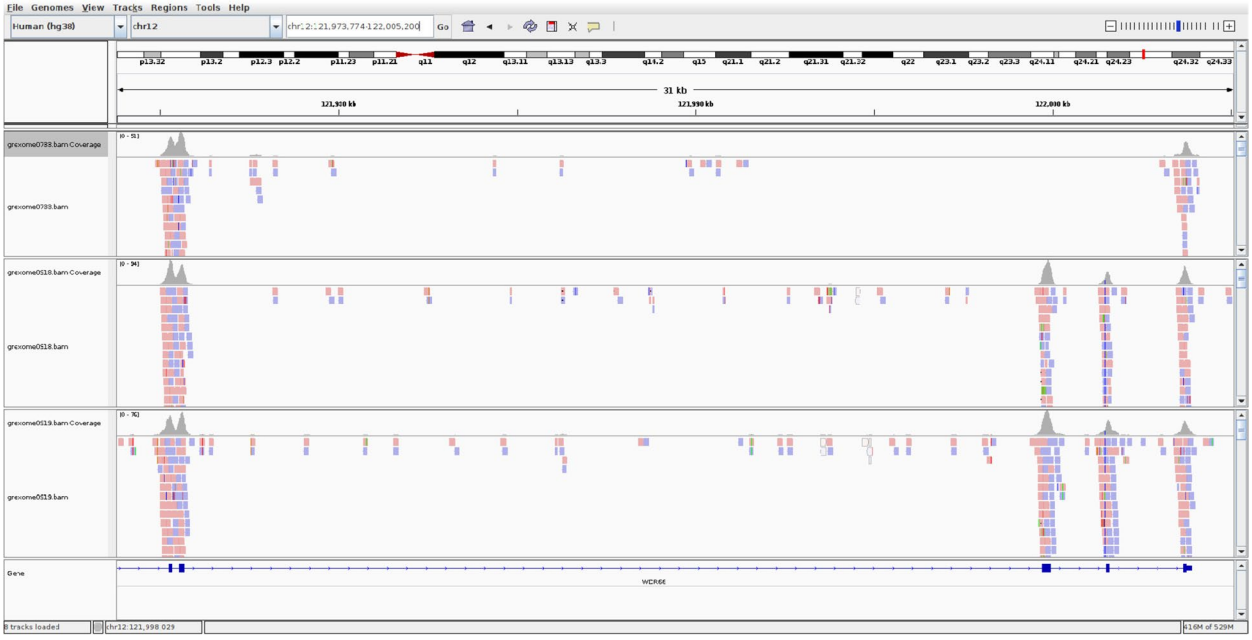

Fig. 3 Deletion of Exons 20–21 of *CFAP251* gene identified in P0733 which is absent in two other patients P0918 and P0919 with no MMAF. For each patient, red and blue rectangles represent aligned forward and reverse reads (bottom) and the grey histogram represents the total sequencing depth at each position. P0733 has no aligned reads overlapping exons 20 and 21 of CFAP251, contrary to the two controls

cases [5]. Spermatozoon is a highly specialized cell well adapted for motion and the delivery of paternal DNA material to the egg. It consists of the following: a head, containing the nucleus and acrosome; a midpiece containing the centrioles, the proximal part of the flagellum, and the mitochondrial helix; and the tail, consisting of a highly specialized flagellum [6].

Motile cilia and eukaryotic flagella are evolutionarily conserved organelles. Their dysfunction can cause many human diseases known as ciliopathies. The tail or flagellum of sperm is a specialized form of cilium having a complex structure with more than 1000 proteins involved in its function [7]. It is divided into four parts; a connecting piece which is attached to the head, mid-piece, principal piece, and the end piece [8]. The sperm tail has a complex anatomy, with the surrounding axoneme having 9 + 2 microtubule arrangements along its entire length and peri-axonemal structures that contribute to sperm motility and fertilization [7]. The axoneme is surrounded by: outer dense fiber (ODF), Fibrous sheath (FS), and the mitochondrial sheath (MS) [8] and serving as the core structure along the length of the sperm tail and extends from the remnant of the centriole attached to the implantation fossa of the nucleus [8].

Due to the conserved axonemal structure between motile cilia and sperm flagella and within the common spectrum of sperm immotility disorders, a range of pathological sub-types exist with overlapping phenotypes: Dysplasia of the fibrous sheath (DFS), primary ciliary dyskinesia (PCD) and multiple morphological abnormalities of the sperm flagella (MMAF) [9].

MMAF syndrome is a human reproduction disorder due to the dysplastic development of sperm flagella. The spermatozoa of MMAF patients manifest an: absent, short, coiled, bent, and/or irregular-caliber flagella [10].

Different approaches (candidate gene and whole genome sequencing) have been used to identify the causal genes of MMAF syndrome in Humans. To date, over 40 genes have been demonstrated to be associated with the MMAF phenotype [11].

DNAH1 encoding an Inner Arm Heavy Chain Dynein is the first gene reported in humans with MMAF by Ben Khelifa et al, [3] in North African patients. Subsequently, cilia and Flagella-associated proteins (CFAPs), including *CFAP43, CFAP44* [12, 13], *CFAP69* [14], *CFAP65* [15], and *CFAP251* [4, 16] were demonstrated to be a recurrent cause of MMAF.

Some other genes have also been reported to be causal of MMAF in different ethnic groups including *QRICH2* [17], *FSIP2* [18], *ARMC2* [19], *SPEF2* [20], and *TTC21A* [21]. Other rarer homozygous missense mutations have been reported in single familial cases like *CEP135* and *AK7* [4] *ARMC3* [22],*TTC12* [23] *and SPAG17* [24]. However, 50% of cases of MMAF can be attributed to these identified genes. The genetic causes and pathogenic mechanisms in the remaining unresolved MMAF cases still need to be further illustrated. In this context, due to the limit financial resources, we conducted WES for one familial case with MMAF syndrome

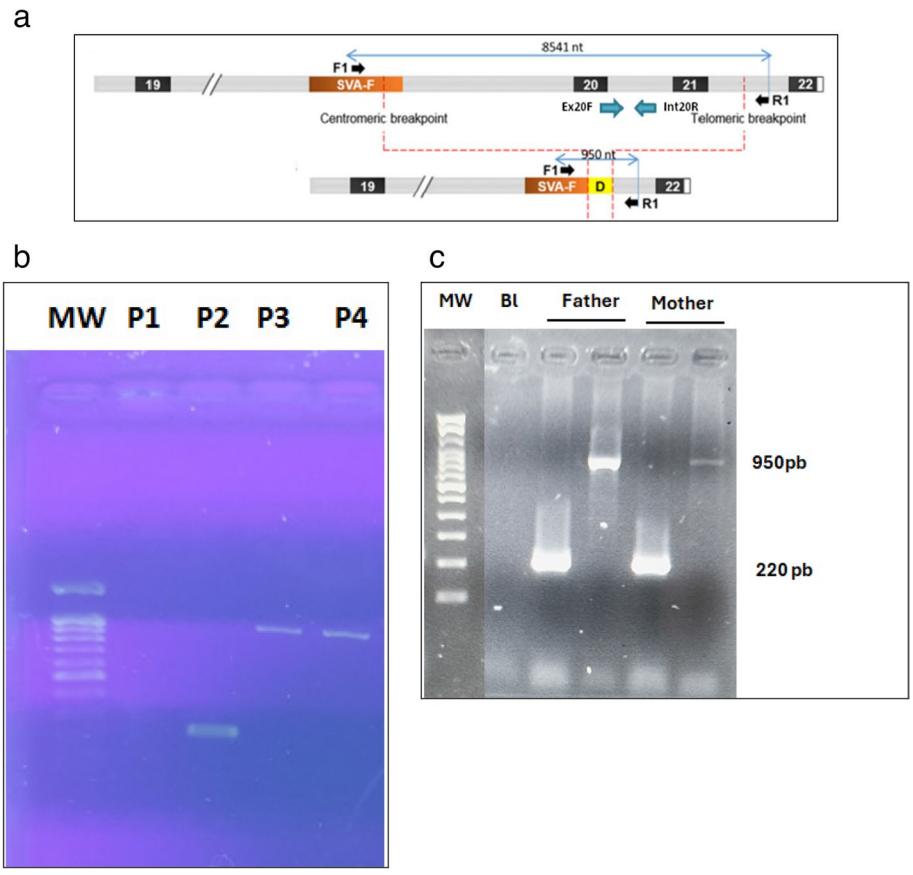

Fig. 4 **a** Localization of primes F1, R1, Ex20F and Int20R used to confirm the deletion of exon 20 and exon 21 of *CFAP251* gene [Kharraf et al. 2018 modified]. **b** Duplex PCR confirming the deletion of exon 20 and 21 of *CFAP251 in patient and his affected brother.* The primer used permit to amplify a 220 nucleotide sequence located within the *CFAP251* deletion present in non-deleted alleles (Ex20F and Int20R) and a 950 nucleotide sequence encompassing the deletion breakpoint (Int19F = F1and Ex22R = R1) only present in the deleted alleles. In lane P2 the amplified DNA was extracted from a non-deleted control, in P3 from the homozygous deleted patient P0733 and P4 from his affected brother, in P1: Blank and MW = molecular weight. **c** Segregation study of c.[3007_3337del] of CFAP251 in parents MW: molecular weight; Bl: Blank PCRs showed that parents presented two fragments 950 and 220 pb confirming that they heterozygous for this variant

and we indentified a homozygous ancestral funder deletion of exon 20–21 of *CFAP251*.

CFAP251 gene is one of Cilia and Flagella Associated Protein family (Previously known as *WDR66*) which encodes a 14 WD-repeat protein (Q8TBY9) with prominent expression in the testis [4]. The CFAP251 protein plays a crucial role in the assembly and stability of the axoneme, the fundamental structure of the flagellum. In humans, mutations in CFAP251 have been linked to MMAF, contributing to defects in sperm motility and morphology [4, 10, 16]. In our study, c.[3007_3337del] of *CFAP251* was found in two affected brothers among 15 MMAF patients (13%)one patient. His affected brother shared the same variant. Previously, this variant was reported in 15% of Tunisian Infertile men and was demonstrated that has been an ancestral variant... this deletion has been [4].

The loss of exons 20 and 21 results in a frameshift mutation that creates a premature stop codon at the beginning of exon 22. Consequently, 12.8% of the protein (147 amino acids) is predicted to be lost in a previous study [4].

Calling copy number variants (CNVs) in WES data remains a difficult task, and most software tools typically require at least ten to twenty technically homogeneous exomes - i.e. produced with the same or similar capture kits, sequencers and protocols. We identified firstly a homozygous pathogencic *DNAH6 variant*. However, Sanger validation in affected brother (heterozygous state) rejected the implication of this variant. Then, the analysis of data from our patient showed that he shared with 7 other Tunisian MMAF patients explored previously, three benign SNVs.located on chromosome 12 and closely to a homozygous deletion of exons 20–21 in *CFAP251.* The examination of BAM revealed the c.[3007_3337del] of *CFAP251in our patient.*

The c.[3007_3337del] is the first CNV reported by WES in MMAF, which is a cost-effective technique permitting the detection of pathogenic variants (SNV and small indels). CNVs, a frequent cause of disease, have

Table 2 Summary of variants and candidate genes reported in MMAF Tunisian patients from different studies

Gene	Variant coordinates	c.DNA variant	Protein variation	Variant classification	Study
DNAH1	chr3:52,348,707	c.11788 – 1G > A	p.Gly3930Alafs*12	pathogenic	*Khelifa* et al., *2014* [3]
	Chr3:52,414,073	c.7531delC	p.Gln2511SerfsTer27	troncnated protein	*Coutton el al, 2018* [13]
	Chr3:52,382,924	c.2127dupC	p.Ile710HisfsTer4	troncated protein	
	Chr3:52,395,227	c.4744_4752delCCAGCTGGC	p.Pro1582_Gly1584del	pathogenic	
	Chr3:52,394,055	c.4531G > A	p.Val1511Met	pathogenic	
	chr3:52,362,390	c.4983G > A	p.Val1661%3D	VUS/likely Benign	*Coutton* et al., *2019* [19]
	chr3:52,389,587	c.9621 + 1G > C	splice_donor_variant	pathogenic	
CFAP43	Chr10:105,912,486	c.3541-2A > C	p.Ser1181Lysfs*4	pathogenic	*Coutton* et al., *2018* [13]
	Chr10:105,905,296	c.3882delA	p.Glu1294AspfsTer47	pathogenic	
	Chr10:105,921,781	c.3352C > T	p.Arg1118Ter	pathogenic	
	chr10:104,230,787	c.120_121delTT +	p.Ile40MetfsTer12	pathogenic	*Coutton* et al., *2019* [19]
	chr10:104,230,791	c.114_117delCACC	p.Asn38LysfsTer10	pathogenic	
CFAP44	Chr3:113,114,596	c.1890 + 1G > A	p.Pro631Ile*22	pathogenic	*Coutton* et al., *2018* [13]
	Chr3:113,063,450	c.3175C > T	p.Arg1059Ter	pathogenic	
CFAP69	chr7:90,271,861	c.763C > T	p.Gln255Ter	pathogenic	*Coutton* et al., *2019* [19]
FSIP2	chr2:186,654,145	c.2282dupA	p.Asn761LysfsTer4	pathogenic	*Martinez e atl, 2018* [18]
	chr2:186,618,487	c.910delC	p.Gln304LysfsTer13	pathogenic	
	chr2:186653470_186653479	c.[1606_1607insTGT; 1607_1616delAAAGATTGCA],	p.Lys536MetfsTer1	pathogenic	
	chr2:186670422_186670425	c.[16389_16392delAATA	p.Glu5463GlufsTer7	pathogenic	
	chr2:185,795,139	c.8003C > A	p.Ser2668Ter	pathogenic	*Coutton* et al., *2019* [19]
	chr2:185,790,382	c.3252delA	p.Lys1084AsnfsTer19	pathogenic	
ARMC2	chr6:108,904,406	c.1023 + 1G > A	splice_donor_variant	pathogenic	*Coutton* et al., *2019* [19]
	chr6:108,964,306	c.2279 T > A	p.Ile760Asn	pathogenic	
	chr6:108,965,045	c.2353_2354delTT	p.Leu785MetfsTer5	pathogenic	
TTC21A	chr3:39,106,225–39108964	c.3116 + 5G > T	p.Ile240*	pathogenic	*Liu* et al., *2019* [21]
CFAP65	chr2:219,002,846–219,041,527	c.3047 T > G	p.Leu1016Arg	pathogenic	*Li* et al., *2019* [10]
CFAP251	chr12:122355768_122441833	c.[3007_3337del]	p.Ile1003Lysfs26	pathogenic	*Kherraf* et al., *2018* [4] *and Our study*

not been studied in the context MMAF phenotype using whole-exome sequencing (WES) data. High-throughput sequencing technology has significantly advanced the development of novel methodologies and approaches for investigating genomic structural variation (whole genome sequencing, long read sequencing) but they are still expensive Intragenic deletion isnot the only pathogenic mechanisms of *CFAP251* associated with MMAF. In a Chinese study, 3 biallelic mutations were identified in three patients: a homozygous nonsense mutation, c.799 C > T; a nonsense mutation c.415 C > T (p.Gln139*) and a frameshift mutation, c.1718delT (p.Phe574Leu*3). Both mRNA and protein levels of CFAP251, decreased or even disappeared, suggesting the detrimental effects of loss-of-function mutations in *CFAP251* [10].

The genetic basis of MMAF syndrome is heterogeneous, involving various candidate genes across individuals and populations. For instance, in Tunisian men with MMAF-related infertility, around ten genes have been reported in various studies, including: *DNAH1*, *CFAP43, CFAP44, CFAP65, CFAP69, FSIP2, TTC21A, ARMC2* and more frequently, *CFAP251* (Table2). Consequently, it has been suggested to incorporate *CFAP251* into a panel of genes for further investigation into male infertility [25] in Tunisian population [Greither 2023]. No empirical medical treatment has been reported to improve the semen parameters of MMAF men, and intracytoplasmic sperm injection (ICSI) is the only way to conceive with a female partner.

Few studies to date have provided detailed analyses regarding the flagellar ultrastructural defects underlying this phenotype, its genetic aetiologies, and the results of ICSI in such cases of male infertility. Success rates after ICSI may be influenced by the type of ultrastructural flagellar defects and/or by the gene defects carried by the patients. Several studies regarding the ICSI outcome have reported satisfactory fertilization rates for MMAF patients with some genes like *DNAH1*, *DNAH2, CFAP43*, and *CFAP44* [2]. In the study of Wambergue et al, the fertilization rate was 70.8% for 9 MMAF/DNAH1+ patients versus 76.5% for 13 MMAF/DNAH1- patients and 69.8% for patients with comparable characteristics but without MMAF [26]. *CFAP251* has been also correlated with favorable ICSI outcomes. In the study of Wang

et al, successful pregnancy was achieved after ICSI who the male partner carried out splicing mutations (c.1192-3C>G) in *CFAP251* [27].

In our study, the fertilization rate of our two patients MMAF/del CFAP251+ was 55 % vs 49 % respectively. For patient (II.2), the ICSI outcome differed between the attempts. In the first and second attempts, the couple was able to obtain good-quality embryos for transfer (a blastocyst in the first attempt and 2 day 3 embryos in the second), but in the last two attempts, total fertilization failure was observed.

This could potentially be explained by poor sperm nuclear quality in patients with MMAF. Numerous studies have reported a strong link between abnormalities in sperm flagella and both decreased sperm nuclear quality and an increased rate of aneuploidy [28] and [29], which could impede ICSI outcomes as shown in our patient who has a DNA fragmentation rate of 22.5%.

Additionally, it's worth noticing that a female factor may also be involved, as the patient's II.2 (P0733) partner reported symptoms indicative of developing endometriosis during recent consultations. She was then diagnosed with a stage four endometriosis with a right ovarian cyst.

Furthermore, the average number of mature oocytes collected was significantly lower in the later ICSI attempts (3.5) compared to the first attempts (7), which could contribute to the impaired fertilization rates.

The small number of cases with the CFAP251 deletion (n=2) and the lack of functional validation may have introduced bias into the unblinded ICSI outcome assessment. More genetic investigation could be achieved. We suggest starting with the search for this ancestral deletion by duplex PCR before performing WES.

To conclude, MMAF syndrome is a heterogeneous syndrome with multiple genetic factors. WES allowed us to identify a new familial case with Tunisian founder *CFA251* deletion in two brothers and to provide a novel marker for genetic counseling and diagnosis of male infertility in Tunisian population with MMAF syndrome. A larger study of ICSI outcome in couples with CFAP251 deletion should be taken to conclude about their prognosis.

Acknowledgements
We thank all the individuals for their cooperation, as well as all the referring physicians and biologists

Authors' contributions
W.A and N.T analyzed the data and wrote the manuscripts; W.A performed genetic consultation and collected clinical data of patients; W.F and W.F performed DNA extraction; W.A performed PCR; K.K.B, S.C,N;C and M.B referred patients, performed semen analysis and ICSI; A.T, P.R and A.A supervised all molecular laboratory work. All authors have read and agreed to the published version of the manuscript.

Funding
This work was supported by the « Ministère de l'Enseignement Supérieur et de la Recherche Scientifique », the « Université Tunis El Manar » and the « Laboratoire de Génomique Biomédicale et Oncogénétique lR16IPT05 à l'Institut Pasteur de Tunis ».

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethic approval and consent to participate
The study was approved by the Bio-Medical Ethics committee of the Institute Pasteur of Tunis. The committee's reference number is 2022/23/I/V1. All patients included in this study, were consent to participate and signed a written informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 8 July 2025 / Accepted: 29 December 2025
Published online: 13 January 2026

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