Human Reproduction, Vol.34, No.10, pp. 2071–2079, 2019
Advance Access Publication on October 17, 2019 doi:10.1093/humrep/dez166

human reproduction

CASE REPORT *Andrology*

CFAP70 mutations lead to male infertility due to severe astheno-teratozoospermia. A case report

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Submitted on April 9, 2019; resubmitted on July 14, 2019; editorial decision on July 18, 2019

ABSTRACT: The use of high-throughput sequencing techniques has allowed the identification of numerous mutations in genes responsible for severe astheno-teratozoospermia due to multiple morphological abnormalities of the sperm flagella (MMAF). However, more than half of the analysed cases remain unresolved suggesting that many yet uncharacterised gene defects account for this phenotype. Based on whole-exome sequencing data from a large cohort of 167 MMAF-affected subjects, we identified two unrelated affected individuals carrying a homozygous deleterious mutation in *CFAP70*, a gene not previously linked to the MMAF phenotype. One patient had a homozygous splice variant c.1723-1G>T, altering a consensus splice acceptor site of *CFAP70* exon 16, and one had a likely deleterious missense variant in exon 3 (p.Phe60Ile). The *CFAP70* gene encodes a regulator protein of the outer dynein arms (ODA) strongly expressed in the human testis. In the sperm cells from the patient carrying the splice variant, immunofluorescence (IF) experiments confirmed the absence of the protein in the sperm flagellum. Moreover, IF analysis showed the absence of markers for the ODAs and the central pair complex of the axoneme. Interestingly, whereas CFAP70 staining was present in sperm cells from patients with mutations in the three other MMAF-related genes *ARMC2*, *FSIP2* and *CFAP43*, we observed an absence of staining in sperm cells from patients mutated in the *WDR66* gene, suggesting a possible interaction between two different axonemal components. In conclusion, this work provides the first evidence that loss of CFAP70 function causes MMAF and that ODA-related proteins may be crucial for the assembly and/or stability of the flagellum axoneme in addition to its motility.

Key words: male infertility / genetics / MMAF / CFAP70 / outer dynein arm / gene mutation / flagellum / whole-exome sequencing

Introduction

The past decade has been marked by the emergence of the next generation sequencing (NGS), a fast-evolving technology which has allowed important advances in human genetics. This technical revolution has proved to be a highly powerful research and diagnostic tool in male infertility and has permitted a major step forward for the identification of genetic causes of male infertility (Krausz and Riera-Escamilla, 2018). In particular, NGS has contributed massively to deciphering the genetic causes of the multiple morphological abnormalities of the flagella

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(MMAF phenotype), which is a subgroup of astheno-teratozoospermia characterised by the presence in the ejaculate of immotile spermatozoa with several flagellar defects including short, coiled or absent flagella and flagella of irregular caliber (Ray *et al.*, 2017). To date, mutations in 14 genes have been found to be associated with MMAF (*AK7*, *AKAP3*, *AKAP4*, *ARMC2*, *CEP135*, *CFAP43*, *CFAP44*, *CFAP65*, *CFAP69*, *DNAH1*, *FSIP2*, *QRICH2*, *TTC21A* and *WDR66*) (Supplementary Fig. S1) (Baccetti *et al.*, 2005; Ben Khelifa *et al.*, 2014; Sha *et al.*, 2017a; Tang *et al.*, 2017; Auguste *et al.*, 2018; Coutton *et al.*, 2018; Dong *et al.*, 2018; Kherraf *et al.*, 2018; Lorès *et al.*, 2018; Martinez *et al.*, 2018; Coutton *et al.*, 2019; Liu *et al.*, 2019; Shen *et al.*, 2019). However, despite these plethoric findings, the etiology of MMAF remains unknown in more than 50% of the cases and the discovery of the remaining genes is a now a major challenge (Coutton *et al.*, 2019).

Using different animal models and in particular flagellated protists such as Chlamydomonas or Trypanosoma, at least five of these known MMAF-related genes (*DNAH1*, *CFAP43*, *CFAP44*, *WDR66* and *CEP135*) have been already reported to encode different components of the axoneme, a central multi-protein complex which is highly conserved throughout evolution and shared by both the sperm flagellum and other motile cilia (Inaba, 2007; Satir and Christensen, 2008). Briefly, the axoneme consists of nine outer doublet microtubules (DMTs) surrounding two central singlets (9 + 2 structure) known as the central pair complex (CPC). DMTs carry several components that drive and regulate ciliary or flagellar motility including axonemal dynein motors subdivided into inner (IDA) and outer arms (ODA), radial spokes, nexin links and many others (Satir and Christensen, 2008). DNAH1 is located in the inner dynein arm which drives axoneme bending and ciliary or flagellar motility (Ben Khelifa *et al.*, 2014); CFAP43 and CFAP44 seem to be related to the tether and tether head (T/TH) complex, which connects dynein motor domains to DMTs (Fu *et al.*, 2018; Urbanska *et al.*, 2018); WDR66 is a component of the spoke-associated complex (CSC) which mediates regulatory signals between the radial spokes and dynein arms (Urbanska *et al.*, 2015); and CEP135 is located in the centrosomal complex (Ohta *et al.*, 2002) (Supplementary Fig. S1).

In this work, we analysed genetic data obtained by whole-exome sequencing (WES) from a large cohort of 167 MMAF patients, and identified two patients carrying a homozygous deleterious mutation in the *CFAP70* gene, which encodes an axonemal protein that is localised at the base of the outer dynein arm (ODA) and regulates ciliary motility (Shamoto *et al.*, 2018). Therefore, this work shows for the first time the involvement of an ODA-linked protein to the MMAF phenotype and confirmed that the ODA complex, in addition to its motility function, plays an important role in the assembly and/or stability of the flagellum axoneme.

Case report

WES was performed on a large cohort of 167 MMAF patients as previously described (Coutton *et al.*, 2019). All individuals presented with a typical MMAF phenotype characterised by severe astheno-zoospermia (total sperm motility below 10%) with at least three of the following flagellar abnormalities present in >5% of the spermatozoa: short, absent, coiled, bent or irregular flagella (for details, see Coutton *et al.*, 2019). Sperm analysis was carried out in the clinical laboratories during routine biological examination of the patient according to World Health Organization (WHO) guidelines (Wang *et al.*, 2014). The morphology of the sperm cells from the *CFAP70*-mutated patients was assessed with Papanicolaou staining (Fig. 1A). Detailed semen parameters of the two mutated patients are presented in Table I. All individuals have a normal somatic karyotype (46,XY) with normal bilateral testicular size, normal hormone levels and secondary sexual characteristics. Informed consent was obtained from all the subjects participating in the study according to local protocols and the principles of the Declaration of Helsinki. The study was approved by local ethics committees, and samples were then stored in the CRB Germethèque (certification under ISO-9001 and NF-S 96-900) following a standardised procedure or were part of the Fertithèque collection declared to the French Ministry of health (DC-2015-2580) and the French Data Protection Authority (DR-2016-392).

Bioinformatics analysis was performed according to our previously described protocol (Coutton *et al.*, 2019). Data analysis of the whole cohort of 167 MMAF individuals permitted the identification of 54 individuals (32.3%) with harmful mutations in known MMAF-related genes (Coutton *et al.*, 2019). In addition, we identified here two patients (CFAP70_1 and CFAP70_2) (accounting for 1.2% of our cohort) with homozygous deleterious variants of *CFAP70*, a gene not previously associated with any pathology. The *CFAP70* mutations were subsequently validated by Sanger sequencing (Fig. 1B). PCR primers are listed in Supplementary Table S1, and a detailed protocol can be found in Coutton *et al.* (2019). The first variant identified in the patient CFAP70_1 is a splice variant c.1723-1G>T, altering a consensus splice acceptor site of *CFAP70* exon 16 (Fig. 1C). The second variant identified in individual CFAP70_2 is a missense variation c.178T>A (p.Phe60Ile) located in exon 3 (Fig. 1C). Both variants were absent from gnomAD, the largest control sequence database regrouping sequence data from over 120 000 individuals. No mRNA analysis or immunostaining could be performed on sperm cells from CFAP70_2 due to the lack of biological samples. However, using prediction software for non-synonymous SNPs, we found that this missense change is predicted to be deleterious by SIFT (score of 0) and probably damaging by PolyPhen (score of 0.996). Moreover, the concerned amino acid (Phe60) was found to be conserved in *CFAP70* orthologues (Fig. 1D). For these two patients, no other candidate variants reported to be associated with cilia, flagella or male fertility were found.

CFAP70 (NM_145170.3, formerly reported as *TTC18*) is located on chromosome 10 and contains 27 exons encoding a predicted 1121-amino acid protein (Q5T0N1). *CFAP70* is predominantly expressed in the testis according to data from GTEx (GTEx Consortium, 2015) and is described to be associated with cilia and flagella (Shamoto *et al.*, 2018). RT-qPCR experiments performed with a panel of 10 human tissues including other ciliated tissues such as trachea confirmed the results showing that *CFAP70* transcripts are most highly expressed in the testis compared with all the other tested tissues (Supplementary Fig. S2). Primer sequences and RT-qPCR conditions are indicated in Supplementary Table SII and in Coutton *et al.* (2019). According to the UniProt server, CFAP70 contains 8 tetratricopeptide repeats (TPR repeats), which are known to form scaffolds which mediate protein–protein interactions (The UniProt Consortium, 2017) (Fig. 1C).

We performed immunofluorescence (IF) assays on sperm cells from a fertile control, a patient CFAP70_1 carrying the splice variant c.1723-1G>T and other MMAF patients mutated in other genes. For each

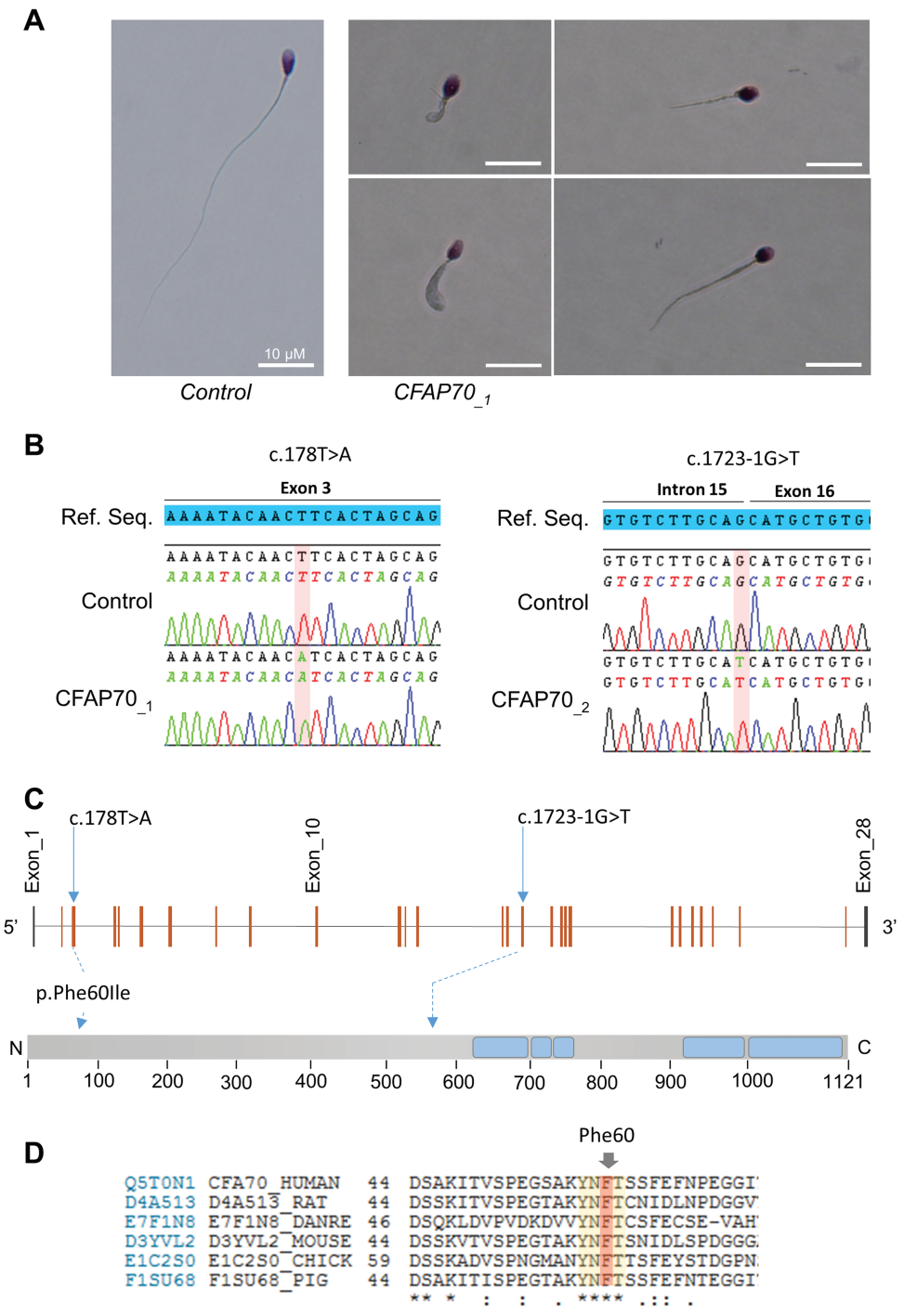


Figure 1 Morphology of normal and CFAP70 mutant spermatozoa, and the mutations identified in the *CFAP70* gene. (**A**) Light microscopy analysis of spermatozoa from fertile control and individual CFAP70_1. All spermatozoa from the patient have shorter flagella than controls. Additional features of morphological abnormalities of the sperm flagella (MMAF) spermatozoa such as thick and coiled flagella are also observed. Scale bars 10 µm. (**B**) Electropherograms of Sanger sequencing showing the two *CFAP70* variants identified in the MMAF cohort. The substituted nucleotides are highlighted in red. (**C**) Location of *CFAP70* mutations in the intron–exon structure and in the protein representation. Blue square stands for tetratricopeptide repeats (TPR) as predicted by the InterPro server (https://www.ebi.ac.uk/interpro/). Mutations are annotated in accordance to the Human Genome Variation Society's recommendations. (**D**) Amino acid sequence alignment of CFAP70 in different vertebrate species showing the conservation of the amino acid (Phe, F) in position 60 (highlighted in red) as well as the surrounding amino acids.

Table I Detailed semen parameters for the two MMAF individuals harbouring a *CFAP70* mutation.

CFAP70 mutated patients		Semen parameters														
Patients	***CFAP70* mutations**	**Sperm volume (ml)**	**Sperm conc. (10^6/ml)**	**Total motility 1 h**	**Vitality**	**Normal spermatozoa**	**Absent flagella**	**Short flagella**	**Coiled flagella**	**Bent flagella**	**Flagella of irregular caliber**	**Tapered head**	**Thin head**	**Micro-cephalic**	**Macro-cephalic**	**Multiple heads**	**Abnormal base**	**Abnormal acrosomal region**
CFAP70_1	c.1723-1G > T	2.5	2	0	75	0	0	90	0	0	0	10	0	1	0	0	0	0
CFAP70_2	c.178 T > A	3	6.9	13	72	0	28	24	10	0	58	10	4	0	2	0	54	80
Reference limits[a]		1.5 (1.4–1.7)	15 (12–16)	40 (38–42)	58 (55–63)	23 (20–26)	5 (4–6)	1 (0–2)	17 (15–19)	13 (11–15)	2 (1–3)	3 (2–4)	14 (12–16)	7 (5–9)	1 (0–2)	2 (1–3)	42 (39–45)	60 (57–63)

Values are percentages unless specified otherwise. MMAF: morphological abnormalities of the sperm flagella
[a]Reference limits (5th centiles and their 95% confidence intervals) according to the World Health Organization

MMAF patient studied, 200 sperm cells were analysed by two different experienced operators and the IF staining intensity and pattern were compared with a fertile control. CFAP70 immunostaining in sperm cells from the control was present all along the flagellum, with a marked signal at the base of the flagellum. In the CFAP70_1 patient, CFAP70 staining was totally absent in all analysed spermatozoa (Fig. 2), confirming the deleterious effect of this mutation. In order to determine if the loss of CFAP70 was specifically linked to the *CFAP70* mutation and was not a hallmark of the MMAF phenotype, secondary to structural defects, we performed IF assays on sperm cells from five other MMAF patients including four patients previously identified with a mutation in *ARMC2*, *FSIP2*, *CFAP43* or *WDR66* and one with no genetic cause identified (Fig. 2). Genotype information of the four additional MMAF patients mutated in *ARMC2*, *FSIP2*, *CFAP43* and *WDR66* is indicated in Supplementary Table SIII. In all but the *WDR66*-mutated patient, CFAP70 immunostaining was comparable with that observed on fertile controls (Fig. 2), indicating that CFAP70 expression and localisation were not altered in the sperm from MMAF patients with mutations in these genes. Interestingly, CFAP70 staining was mostly absent in the *WDR66*-mutated patient irrespective of the sperm morphology (Supplementary Fig. S3), suggesting a possible link, direct or indirect, between, two axonemal components in the sperm flagellum (Fig. 2, Supplementary Fig. S1).

To further characterise the molecular defects induced by *CFAP70* mutations in human sperm, we used an indirect approach studying, by IF, the presence and localisation of some proteins belonging to different substructures of the axoneme. The presence of the only two proteins was investigated: SPAG6 as a marker of the CPC and DNAI2 as markers of the ODA. We observed that in sperm from individual CFAP70_1, staining of SPAG6, an axoneme CPC protein (Sapiro *et al.*, 2000), was totally absent from the flagellum, suggesting defects in the CPC structure (Fig. 3). This finding is in concordance with previous results showing that the absence of CPC is a frequent characteristic of MMAF spermatozoa (Coutton *et al.*, 2015). Similarly, the staining of DNAI2 was totally absent or dramatically reduced in most of the analysed sperm irrespective of the sperm morphology (Fig. 4). This result is consistent with the function of CFAP70 as a regulatory component of the ODA in motile cilia and flagella (Shamoto *et al.*, 2018). To formally conclude, all the potential ultrastructural defects observed using IF should be further confirmed by transmission electron microscopy (TEM). However, TEM could not be performed in this present work due to a very low number of sperm cells available. Moreover, due to limited sample availability, IF analyses could not be repeated on sperm from the other patient with the *CFAP70_2* mutation.

Discussion

In this present work, we identified two infertile patients with a typical MMAF phenotype carrying deleterious homozygous mutations in the *CFAP70* gene. The first patient harbours a deleterious splicing mutation leading to the absence of the protein in the sperm flagellum (Fig. 2). The second patient has a missense mutation at position Phe60 predicted to be deleterious using mutation-prediction software. However, bioinformatics predictions are not sufficient to conclude to the pathogenicity of this mutation and additional supporting functional work (e.g. mRNA or protein studies, IF experiments or CRISPR/Cas9-targeted mutagenesis in different animal models) could be useful to reinforce this

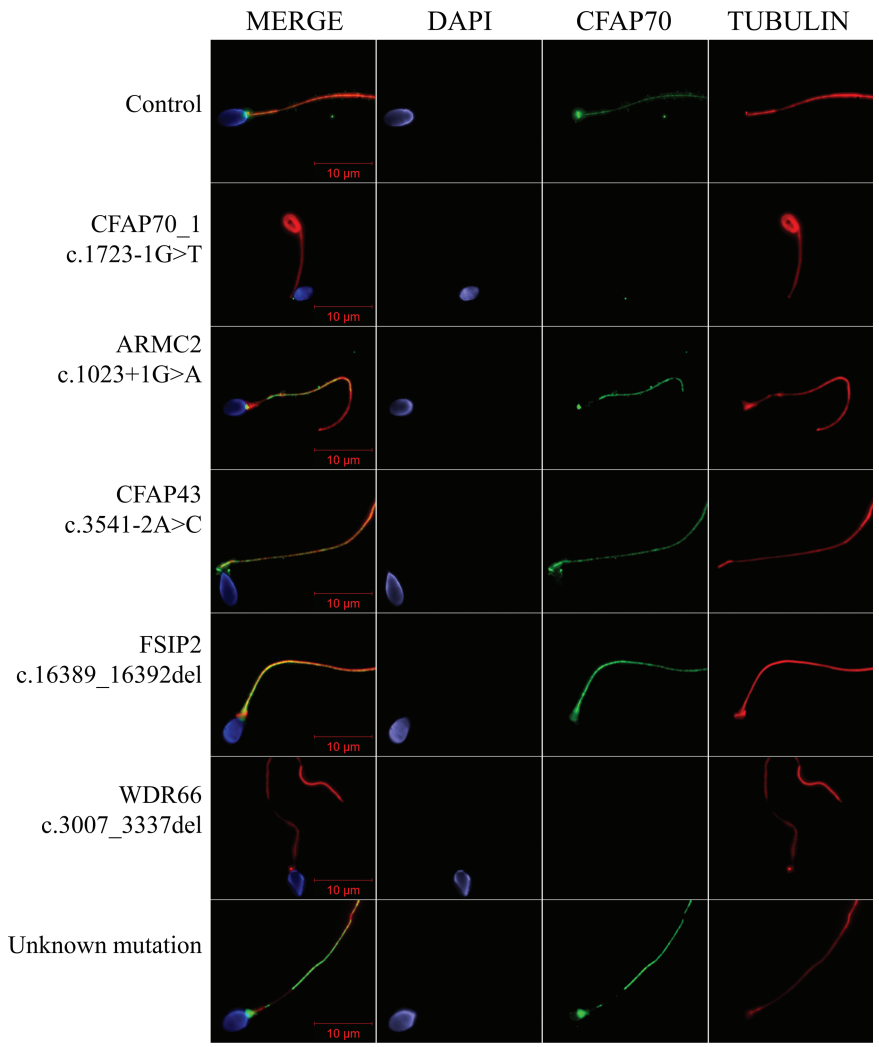


Figure 2 CFAP70 immunostaining in human spermatozoa from control and MMAF patients. Sperm cells from a fertile control, the patient CFAP70_1, four other MMAF patients mutated in *ARMC2*, *FSIP2*, *CFAP43* and *WDR66* and one MMAF patient with unknown genetic cause were stained with anti-CFAP70 (HPA037582, Sigma-Aldrich, rabbit, 1:100, green) and anti-acetylated tubulin (32-2500, Thermo Fisher, mouse, 1: 1000, red) antibodies. DNA was counterstained with DAPI showing sperm nuclear DNA (blue). The CFAP70 immunostaining (green) uniformly stains the full-length flagellum from the fertile control, the three patients mutated in *ARMC2*, *FSIP2* and *CFAP43* and the MMAF patient with unknown genetic cause. Conversely, CFAP70 staining is absent in sperm from the CFAP70_1 patient mutated in *CFAP70* and the patient mutated in *WDR66*. Mutations are specified for each patient. Scale bars 10 µm.

assumption. Mutations in *CFAP70* appeared as the best candidates to explain the MMAF phenotype observed in these two patients based on robust evidence in the literature supporting its critical function for cilia/flagella formation and function. First, transcriptomic studies showed that *Cfap70* was strongly upregulated in differentiating mouse tracheal epithelial cells (Xu *et al.*, 2015). Furthermore, inactivation of *ttc18*, the orthologue of the human *CFAP70* in zebrafish, led to a significant reduced cilia length and number in Kupffer's vesicle (van Dam *et al.*, 2017). Moreover, extensive work on Chlamydomonas FAP70 demonstrated that the protein is a regulator of the ODA (Shamoto *et al.*, 2018). Importantly, the authors demonstrated that FAP70 is not formally a component of the ODA complex but seems to be involved in the coordination of the ODA activity in response to changes in the surrounding mechanical and/or chemical conditions to produce proper ciliary motility (Shamoto *et al.*, 2018). In this work, IF experiments indicated ODA defects in sperm cells from the patient with the CFAP70_1 mutation (Fig. 4). Moreover, previous studies demonstrated that the ODAs were not disorganised in the sperm flagellum from MMAF patients mutated in other axonemal genes suggesting that ODA defects may be specifically linked to *CFAP70* mutations. In Chlamydomonas, the lack of FAP70 did not affect the ODA ultrastructure (Shamoto *et al.*, 2018), in contrast to what was observed in the sperm cells from our patient (Fig. 4). However, such a discrepancy between human and flagellate flagella was already found for two other genes involved in MMAF (*CFAP43* and *CFAP44*) (Coutton *et al.*, 2018). These IF results should nevertheless be confirmed by TEM analysis in sperm

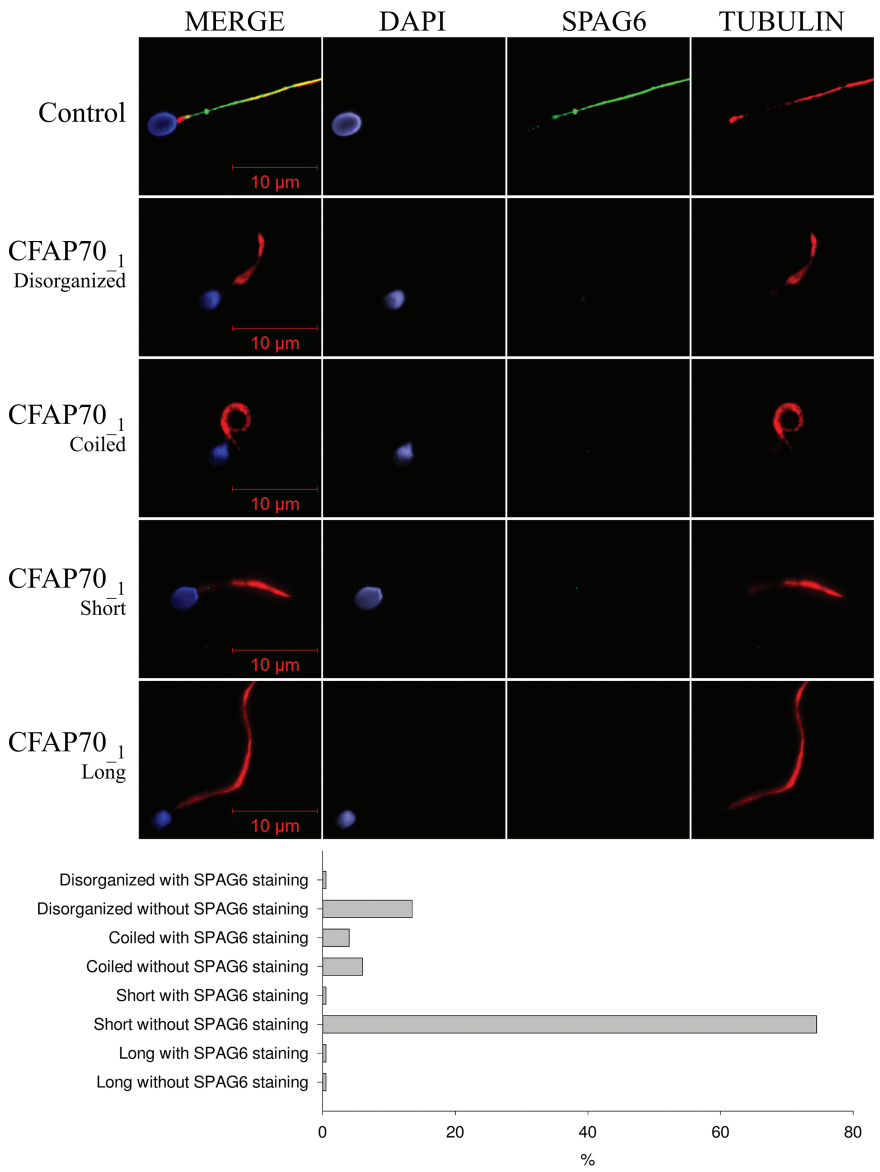


Figure 3 The central pair complex (CPC) is affected by *CFAP70* mutations. Sperm cells from a fertile control and the patient CFAP70_1 were stained with anti-SPAG6 (HPA038440, Sigma-Aldrich, rabbit, 1:500, green), a protein located in the CPC, and anti-acetylated tubulin (32-2500, Thermo Fisher, mouse, 1:1000, red) antibodies. DNA was counterstained with DAPI. SPAG6 staining is mostly absent in sperm from the patient mutated in *WDR66* irrespective of the sperm morphology (short, long, coiled or disorganised). The histogram showing the percentage of analysed spermatozoa with or without SPAG6 staining according to their morphology is presented (number of sperm cells analysed = 200). Scale bars 10 µm.

cells from CFAP70-mutated patients to allow reliable and definitive conclusions. *CFAP70* encodes a TPR protein composed of eight TPR repeats located at the C-terminal domain. TPR domains are known to mediate protein–protein interactions, and it has been proposed that FAP70 localises at the base of ODA through the N-terminal end of the protein and the C-terminal region may define the binding partner that regulates ODA function (Shamoto *et al.*, 2018). The partners of CFAP70 are still unknown, but Shamoto *et al.* (2018) suggested that CFAP70 could interact with various ciliary regulatory proteins, such as protein phosphatases (Elam *et al.*, 2011) and calcium-binding proteins (Mizuno *et al.*, 2012). Interestingly, we showed in the present study that CFAP70 staining is absent in a patient with mutation in the *WDR66* gene. *WDR66* is a MMAF-related gene previously reported to encode an axonemal protein containing a calcium-regulating EF-hand domain in its C-terminal end (Auguste *et al.*, 2018; Kherraf *et al.*, 2018). Interestingly, the WDR66 patient included in this present work harboured the recurrent terminal deletion previously described to remove the C-terminal domain of the protein (Kherraf *et al.*, 2018). Therefore, this observation may suggest an additional role of the C-ter domain of CFAP251 in the recruitment and assembly of the other component of the flagellum, as CFAP70 may be. Moreover, WDR66 has been described to localise to the calmodulin- and spoke-associated (CSC) complex at the base of radial spoke 3 in *Tetrahymena* and *Chlamydomonas* (Urbanska *et al.*, 2015). Interestingly, the CSC is involved

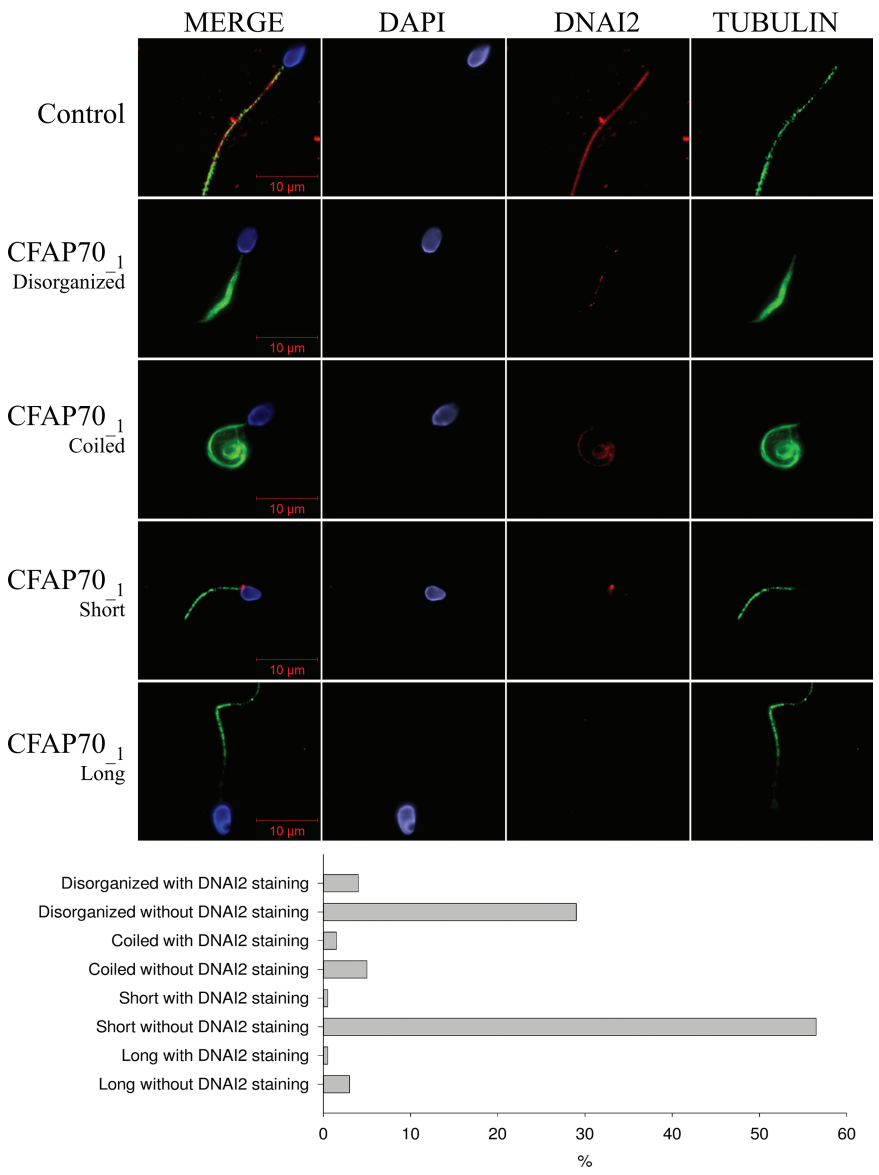


Figure 4 Outer dynein arms (ODAs) are affected by *CFAP70* mutations. Sperm cells from a fertile control and the patient CFAP70_1 were stained with anti-DNAI2 (H00064446-M01, Abnova, mouse, 1:400, red), a protein located in the ODA, and anti-acetylated tubulin (PA5-19489, Thermo Fisher, rabbit, 1:500, green) antibodies. DNA was counterstained with DAPI. The DNAI2 immunostaining is mostly absent or dramatically reduced in sperm from the patient CFAP70_1 compared with the fertile control and irrespective of the sperm morphology (short, long, coiled or disorganised). The histogram showing the percentage of analysed spermatozoa with or without the DNAI2 staining according to their morphology is presented (number of sperm cells analysed = 200). Scale bars 10 µm.

in the modulation of dynein activity and therefore in the regulation of flagellar motility (Heuser *et al.*, 2012). These findings support the fact that WDR66 is a potential partner of CFAP70 and may work in synergy to modulate inner and outer dynein activity. However, further investigations (such as co-immunoprecipitation (co-IP) experiments) are necessary to clarify this hypothesis and will permit elucidation of the mechanisms by which CFAP70 regulates ODA function and flagellum and cilia length in mammals.

It has been demonstrated that *Cfap70* inactivation in mouse ependymal cells led to shortened cilia in addition to motility defects (Shamoto *et al.*, 2018). This observation echoes the sperm phenotype observed in our two MMAF patients presenting sperm with short and immotile flagella. Moreover, this supports our findings that CFAP70 may be involved in axoneme biogenesis or stability beyond its function in ODA regulation.

As observed for the other MMAF individuals of the cohort (Coutton *et al.*, 2019), analysis of sperm morphology from patients CFAP70_1–2 showed a high proportion of spermatozoa with abnormal flagella (Fig. 1, Table I). Interestingly, we also found low sperm concentrations in the ejaculates (oligozoospermia) as was observed for patients mutated in *CFAP69* (Dong *et al.*, 2018). However, the two CFAP70 patients presented a different pattern of morphological defects of the

sperm flagellum with only short flagella for patient 1 while a mosaic of defects (short, absent or irregular caliber) was observed in patient 2. In addition, patient CFAP70_1 with the most severe variant presents spermatozoa with 0% motility in contrast with the patient with the missense variant (p.Phe60Ile), who presents a milder phenotype with 13% motility (Table I). This could suggest that the Phe60Ile-mutated protein may preserve residual activity, as reported for other missense mutations in other MMAF-related genes (Ben Khelifa *et al.*, 2014; Amiri-Yekta *et al.*, 2016). Moreover, this may reinforce the hypothesis of a possible phenotype continuum depending on the severity of mutations in MMAF genes, as shown for *DNAH1* (Ben Khelifa *et al.*, 2014). Concerning the prognosis of intracytoplasmic sperm injection (ICSI) using sperm cells from *CFAP70*-mutated subjects, only ICSI outcomes for patient CFAP70_1 were available, and one clinical pregnancy with one live birth after 2 cycles was reported. This positive outcome is consistent with previous studies demonstrating that MMAF-affected individuals have a good prognosis following ICSI (Coutton *et al.*, 2015; Wambergue *et al.*, 2016; Sha *et al.*, 2017b).

Finally, we know that *CFAP70* is expressed in different ciliated tissues such as trachea or ependymal epithelium. However, the two patients with CFAP70 mutations presented only with primary infertility without any other clinical features, thus excluding a phenotype of primary cilia dyskinesia (PCD), as has been observed for all genes associated with a MMAF phenotype. These recurrent observations confirm once again that flagellum biogenesis requires some proteins and pathways that are at least partly different from those necessary for cilia biogenesis. Interestingly, we observed that many of these MMAF-related genes encode proteins which are linked directly or indirectly with the protein complex close to the radial spoke 3 (RS3) (DNAH1, WDR66, CFAP43, CFAP44 and now CFAP70), suggesting that the RS3 biogenesis and function may be one of the major differences between cilia and sperm flagella.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We thank all patients and control individuals for their participation.

Authors' roles

J.B., C.C., G.M., P.F.R., C.A., S.B. and A.T. analysed the data and wrote the manuscript; Z-E.K., N.T-M., P.F.R. and C.C. performed and analysed the genetic data; J.B., C.C. and G.M. performed the IF experiments. G.P. and M.B. performed the RT-qPCR experiments; A.A-Y. and V.S. provided clinical samples and data; C.C., P.F.R., C.A. and G.M. designed the study, supervised all molecular laboratory work, had full access to all of the data in the study and took responsibility for the integrity of the data and its accuracy. All authors contributed to the report.

Funding

'MAS-Flagella' project financed by Agence Nationale de la Recherche and Direction Générale de l'Offre de Soins for the program PRTS 2014; 'Whole genome sequencing of patients with Flagellar Growth Defects (FGD)' project financed by the Fondation Maladies Rares for the program Séquençage à haut débit 2012.

Conflict of interest

The authors have declared that no conflict of interest exists.

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