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Whole exome sequencing of men with multiple morphological abnormalities of the sperm flagella reveals novel homozygous *QRICH2* mutations

Zine-Eddine Kherraf[1,2] | Caroline Cazin[1,2] | Charles Coutton[1,3] | Amir Amiri-Yekta[1,2,4] | Guillaume Martinez[1,3] | Magalie Boguenet[1] | Selima Fourati Ben Mustapha[5] | Mahmoud Kharouf[5] | Hamid Gourabi[4] | Seyedeh Hanieh Hosseini[6] | Abbas Daneshipour[4] | Aminata Touré[7,8,9] | Nicolas Thierry-Mieg[10] | Raoudha Zouari[5] | Christophe Arnoult[1] | Pierre F. Ray[1,2]

[1]INSERM U1209, CNRS UMR 5309, Institute for Advanced Biosciences, Team Genetics Epigenetics and Therapies of Infertility, Université Grenoble Alpes, Grenoble, France

[2]UM GI-DPI, CHU Grenoble Alpes, Grenoble, France

[3]UM de Génétique Chromosomique, CHU Grenoble Alpes, Grenoble, France

[4]Department of Genetics, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

[5]Polyclinique les Jasmins, Centre d'Aide Médicale à la Procréation, Centre Urbain Nord, Tunis, Tunisia

[6]Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

[7]INSERM U1016, Institut Cochin, Paris, France

[8]UMR8104, Centre National de la Recherche Scientifique, Paris, France

[9]Faculté de Médecine, Université Paris Descartes, Sorbonne Paris Cité, Paris, France

[10]CNRS, TIMC-IMAG, Université Grenoble Alpes, Grenoble, France

Correspondence
Pierre F. Ray, INSERM U1209, CNRS UMR 5309, Institute for Advanced Biosciences, Team Genetics Epigenetics and Therapies of Infertility, Université Grenoble Alpes, and UM GI-DPI, CHUGA, Grenoble 38000, France.
Email: pray@chu-grenoble.fr

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Abstract

Multiple morphological anomalies of the sperm flagella (MMAF syndrome) is a severe male infertility phenotype which has so far been formally linked to the presence of biallelic mutations in nine genes mainly coding for axonemal proteins overexpressed in the sperm flagellum. Homozygous mutations in *QRICH2*, a gene coding for a protein known to be required for stabilizing proteins involved in sperm flagellum biogenesis, have recently been identified in MMAF patients from two Chinese consanguineous families. Here, in order to better assess the contribution of *QRICH2* in the etiology of the MMAF phenotype, we analyzed all *QRICH2* variants from whole exome sequencing data of a cohort of 167 MMAF-affected subjects originating from North Africa, Iran, and Europe. We identified a total of 14 potentially deleterious variants in 18 unrelated individuals. Two unrelated subjects, representing 1% of the cohort, carried a homozygous loss-of-function variant: c.3501C>G [p.Tyr1167Ter] and c.4614C>G [p.Tyr1538Ter], thus confirming the implication of *QRICH2* in the MMAF phenotype and human male infertility. Sixteen MMAF patients (9.6%) carried a heterozygous *QRICH2* potentially deleterious variant. This rate was comparable to

what was observed in a control group (15.5%) suggesting that the presence of *QRICH2* heterozygous variants is not associated with MMAF syndrome.

KEYWORDS
flagella, gene defects, genetic diagnosis, male infertility, spermatogenesis, whole exome sequencing

1 | INTRODUCTION

Teratozoospermia is one of the most severe causes of male infertility and represents a heterogeneous group including a wide range of abnormal sperm subphenotypes affecting, solely or simultaneously, the head, neck, midpiece and tail.[1] Morphological defects affecting the sperm flagellum alter sperm motility and lead to asthenozoospermia and male infertility. Multiple morphological abnormalities of the sperm flagellum (MMAF) is the most frequent severe phenotype affecting sperm tail morphology. The genetic characterization of this phenotype began in 2014 with the identification of *DNAH1* (dynein axonemal heavy chain 1) as a major gene implicated in MMAF.[2] After the development and the introduction of high throughput sequencing (HTS) techniques such as whole exome sequencing (WES), deleterious mutations were formally identified in eight additional MMAF genes (*AK7*, *ARMC2*, *CFAP43*, *CFAP44*, *CFAP69*, *FSIP2*, *TTC21A*, and *WDR66*)[3-15] showing the high genetic heterogeneity of this phenotype. Despite these important advances in gene identification, about half of the MMAF cases remain idiopathic without a genetic diagnosis.

Very recently, the presence of variants in *QRICH2* (Glutamine Rich 2) was shown to induce a MMAF phenotype.[16] The authors studied two Chinese familial cases and reported homozygous nonsense mutations altering the functional structure of the encoded protein. QRICH2 was shown to be involved in stabilizing and enhancing the expression of numerous proteins related to sperm flagellar development. By characterizing a knock-out (KO) mouse model, they showed that homozygous *Qrich2* KO males were infertile and displayed the same sperm defects than those observed in MMAF subjects.[16] However, the prevalence of *QRICH2* mutations in MMAF patients remains unknown because *QRICH2* mutations were identified only in two Chinese familial cases. In addition, the realization of independent replicative studies is important to confirm the clinical relevance of new genetic findings.[17] In this manuscript, we explored our large cohort of 167 MMAF individuals and report novel homozygous mutations in *QRICH2*, in two unrelated subjects originating from different geographical regions: North Africa (Algeria) and the Middle East (Iran), therefore confirming that *QRICH2* mutations are a recurrent but rare cause of the MMAF phenotype. Moreover, we assess if the presence of heterozygous *QRICH2* potentially deleterious variants could be associated with MMAF.

2 | PATIENTS AND METHODS

Here, we reanalyzed our data obtained by WES performed for a total of 167 individuals[4] affected by primary infertility associated with a MMAF syndrome by focusing on variants located in the newly described *QRICH2* gene. All the recruited subjects displayed isolated infertility with no other clinical features; in particular, primary ciliary dyskinesia syndrome was excluded. In this cohort, 83 individuals originated from North Africa (mainly from Algeria, Libya, and Tunisia) and sought consultation for primary infertility at the "Clinique des Jasmins" in Tunis, 52 individuals originated from the Middle East (Iran) and were treated in Tehran at the Royan Institute (Reproductive Biomedicine Research Center) for primary infertility, and 32 subjects were recruited in France. All individuals presented with a typical MMAF phenotype, which is characterized by severe asthenozoospermia (total sperm motility below 10%; normal values 40%) with at least three of the following flagellar abnormalities present in >5% of the spermatozoa: short, absent, coiled, bent, or irregular flagella. All individuals had a normal somatic karyotype (46, XY) with normal bilateral testicular size, hormone levels, and secondary sexual characteristics.

Informed consent was obtained from all the individuals 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) according to a standardized 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).

Whole exome sequencing and bioinformatics analysis were performed according to our previously described protocol using the human genome assembly GRCh38 as a reference sequence. Our initial analysis permitted to identify homozygous variants in the following genes *ARMC2*, *CFAP43*, *CFAP44*, *DNAH1*, *FSIP2*, and *WDR66* (*CFAP251*)[4] in a total of 57 men (34%) from the studied cohort. Here, examining the pooled variants from the 167 MMAF subjects, we extracted and analyzed all the potentially deleterious variants in *QRICH2*.

3 | RESULTS

Here, we extracted all potentially deleterious *QRICH2* variants present in all tested subjects (Table 1). We assessed the effect of all *QRICH2* variants using the variant frequency indicated in gnomAD and their predicted impact using SIFT (sorting intolerant from tolerant), PolyPhen (polymorphism phenotyping), and HSF (human splicing finder). Because the MMAF phenotype is rare and deleterious variants are expected to be negatively selected, we excluded all variants with a frequency greater than 1% in gnomAD. We then considered as

TABLE 1 List of all *QRICH2* potentially deleterious variants present in a total of 167 MMAF subjects

Patients	Genotype	Effect	cDNA	Protein	SIFT	PolyPhen	HSF	gnomAD global frequency	gnomAD maximal Frequency
170	Homo	Stop gained	c.3501C>G	p.Tyr1167Ter				$3.97 \times x10^{-6}$	2.89×10^{-5} (Latino)
216	Homo	Stop gained	c.4614C>G	p.Tyr1538Ter				4.02×10^{-6}	8.84×10^{-6} (European non-Finnish)
167	Hetero	Splice region	c.4855+4_4855+7delAGTG	-			Probably affecting splicing	8.89×10^{-4}	3.88×10^{-3} (unknown origin)
206	Hetero	Missense	c.4119G>A	p.Met1373Ile	Deleterious	Probably damaging		0	0
154	Hetero	Missense	c.3587C>T	p.Pro1196Leu	Deleterious	Probably damaging		1.42×10^{-5}	4.04×10^{-5} (European, Finnish)
415	Hetero	Missense	c.3433G>A	p.Glu1145Lys	Deleterious	Probably damaging		1.76×10^{-5}	1.38×10^{-4} (Latino)
424	Hetero	Missense	c.3278A>G	p.Glu1093Gly	Deleterious	Probably damaging		2.62×10^{-4}	2.65×10^{-3} (Latino)
233, 336, 391, 430	Hetero	Synonymous	c.3081A>G	p.Ala1027Ala			Potential alt. of splicing	5.44×10^{-4}	$2,51 \times 10^{-3}$ (Ashkenazi Jewish)
304	Hetero	Frameshift	c.290_291delTC	p.Ser97TyrfsTer7				0	0
339	Hetero	Missense	c.4618C>T	p.Arg1540Trp	Deleterious	Probably damaging	Probably affecting splicing	1.51×10^{-3}	4.64×10^{-3} (Ashkenazi Jewish)
222, 251, 415, 428	Hetero	Missense	c.3910A>G	p.Lys1304Glu	Tolerated	P1robably damaging		8.99×10^{-3}	2.74×10^{-2} (European Finnish)
296	Hetero	Missense	c.3169G>A	p.Gly1057Ser	Deleterious	Probably damaging	Potential alteration of splicing	1.98×10^{-3}	1.82×10^{-2}
105, 127	Hetero	Inframe deletion	c.2042_2071del	p.Val681_Ala690del				0	0
117	Hetero	Missense	c.1996G>T	p.Val666Phe	Deleterious	Benign		1.13×10^{-3}	9.12×10^{-3}
Nb of subjects = 18	Nb of variants = 14								

Abbreviations: HSF, human splicing finder; MMAF, multiple morphological anomalies of the sperm flagella; SIFT, sorting intolerant from tolerant.

potentially deleterious: all the truncating variants, all variants scored as deleterious or probably damaging by SIFT and/or PolyPhen, respectively, and all exonic or near intronic variants predicted to have a significant impact on splicing by HSF (predicted to create or obliterate a donor or acceptor splice site and scored as probably affecting splicing or potentially altering splicing).

We identified a total of 14 potentially deleterious variants in a total of 18 individuals (Table 1). Interestingly, two unrelated subjects (Figure 1A) each carried a *QRICH2* homozygous loss-of-function variant (c.3501C>G [p.Tyr1167Ter] and c.4614C>G [p.Tyr1538Ter], respectively). Sanger Sequencing was performed using primers listed in Table S1, and confirmed the presence of these variants in the corresponding subjects (Figure 1B). Both presented a typical MMAF phenotype as described in Table 2. Unfortunately, the affected relative of patient P170 could not be studied. *QRICH2* (NM_032134) is located on human chromosome 17 and contains 19 exons encoding a predicted 1663 amino acid protein (NP_115510) with three-principal domains (Figure 1C). Individual P170, originated from North Africa (Algeria), had a homozygous nonsense variant c.3501C>G [p.Tyr1167Ter]. This variant is located in exon 7 and is predicted to cause either the degradation of the mRNA through nonsense-

TABLE 2 Semen parameters and sperm morphology assessment in subjects P170 and P216 carrying biallelic mutations in *QRICH2*

	Subjects	
	P170	P216
Gene	QRICH2	QRICH2
Mutation type	Nonsense	Nonsense
Genotype	Homozygous	Homozygous
Coding DNA sequence change	c.3501C>G	c.4614C>G
Predicted protein impact	p.Tyr1167Ter	p.Tyr1538Ter
Allelic frequency in gnomAD	3.97×10^{-6}	4.02×10^{-6}
Semen parameters		
Semen volume	4.8 mL	6.5 mL
Sperm concentration	17.5×10^{6}/mL	4.0×10^{6}/mL
Total motility (PR + NP)	7%	0%
Progressive motility (PR)	2%	0%
Vitality	35%	70%
Morphology (typical forms)	0%	0%
Abnormal flagella	100%	100%

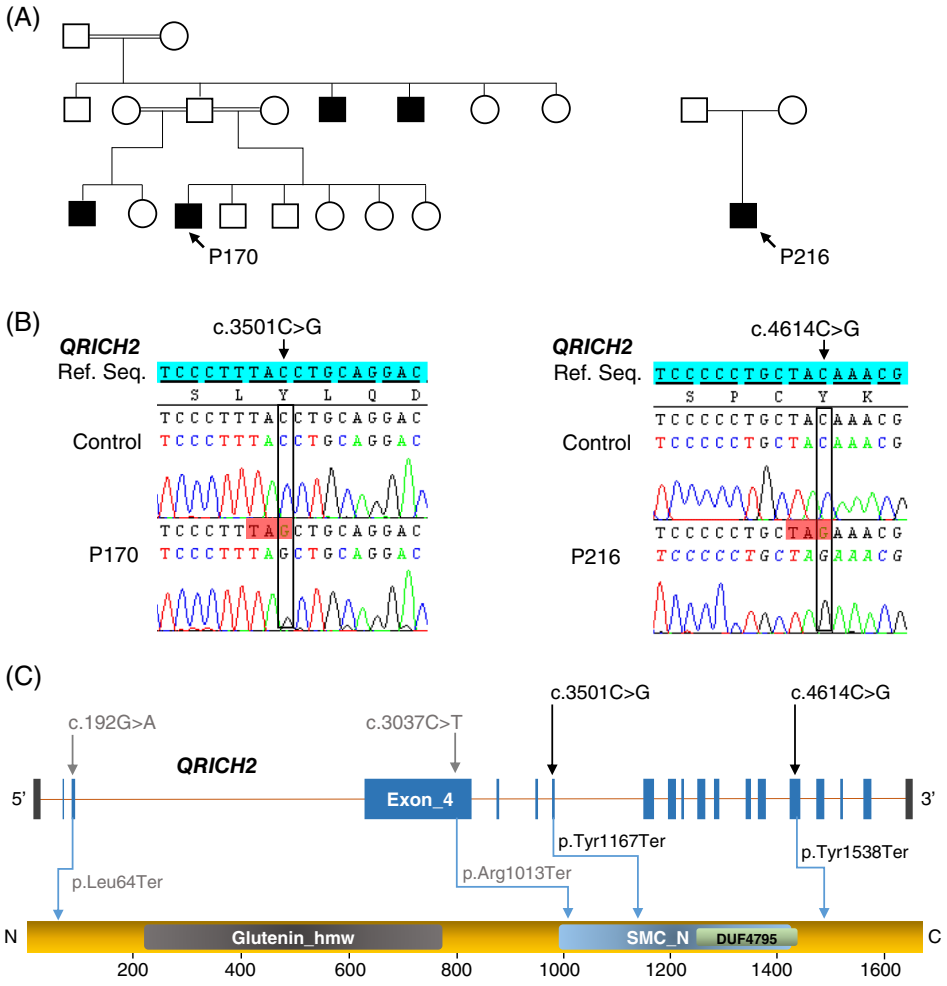

FIGURE 1 Homozygous loss-of-function *QRICH2* mutations in two independent cases of male infertility associated with MMAF. A, Pedigrees of two independent families. Black squares indicate infertile subjects in each family. Black arrows indicate the subjects analyzed by WES. B, Sanger sequencing verification of the identified variants from subjects P170 and P216. Black arrows indicate the position of the identified variants. These two variants induce a premature translational termination highlighted in red. C, *QRICH2* gene structure and functional structure of the encoded protein. Gray arrows show the previously identified variants, whereas black arrow show the newly identified mutations and their respective impact on protein translation. MMAF, multiple morphological anomalies of the sperm flagella; WES, whole exome sequencing [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 List of all *QRICH2* potentially deleterious variants present in a total of 103 azoospermic subjects

Patients	Genotype	Effect	cDNA	Protein	SIFT	PolyPhen	HSF	gnomAD global frequency	gnomAD maximal frequency
203	Hetero	Splice region	c.4617A>G	p.Lys1539Lys			Probably affecting splicing	4.5×10^{-4}	7.47×10^{-4} (European, non-Finnish)
400	Hetero	Synonymous	c.3432C>T	p.Ala1144Ala			Potential alt. of splicing	5.8×10^{-4}	1.4×10^{-3} (European, Finnish)
196	Hetero	Missense	c.3266C>T	p.Thr1089Met	Deleterious	Possibly damaging		6.73×10^{-5}	3.62×10^{-4} (African)
276	Hetero	Missense	c.3230C>G	p.Pro1077Arg	Deleterious	Probably damaging		1.91×10^{-4}	1.57×10^{-3} (African)
405	Hetero	Missense	c.3053G>A	p.Arg1018His	Deleterious	Benign		6.01×10^{-5}	2.39×10^{-4} (European, Finnish)
351	Hetero	Synonymous	c.2292C>T	p.Gly764Gly			Potential alt. of splicing	3.89×10^{-5}	4.00×10^{-4} (African)
351	Hetero	Inframe deletion	c.2042_2071del	p.Val681_Ala690del				0	0
356	Hetero	Stop gained	c.2242_2270dup	p.Gln757HisfsTer4			Potential alt. of splicing	0	0
358	Hetero	Synonymous	c.2070A>G	p.Ala690Ala			Potential alt. of splicing	1.99×10^{-5}	5.45×10^{-5} (East Asian)
277	Hetero	Missense	c.2078G>A	p.Arg693His	Tolerated	Benign	Potential alt. of splicing	3.98×10^{-5}	7.75×10^{-5}
277	Hetero	Missense	c.1499A>G	p.Asp500Gly	Tolerated	Benign	Potential alt. of splicing	0	0
277	Hetero	Missense	c.4370G>A	p.Arg1457His	Deleterious	Probably damaging		2.11×10^{-3}	1.82×10^{-2} (South Asian)
141, 326	Hetero	Inframe deletion	c.1888_1947del	p.Ile630_Gly649del				0	0
80, 87	Hetero	Inframe deletion	c.1345_1404del	p.Ala449_Gly468del			Potential alt. of splicing	4.61×10^{-3}	4.74×10^{-2} (African)
255	Hetero	Missense	c.3910A>G	p.Lys1304Glu	Tolerated	Probably damaging		8.99×10^{-3}	2.74×10^{-2} (European, Finnish)
85, 365	Hetero	Missense	c.791G>A	p.Arg264Gln	Tolerated	Benign	Potential alt. of splicing	7.65×10^{-3}	3.56×10^{-2} (Ashkenazi Jewish)
Nb of subjects = 16	Nb of variants = 16								

Abbreviations: HSF, human splicing finder; SIFT, sorting intolerant from tolerant.

mediated mRNA decay (NMD) or to induce a premature translational termination disrupting the SMC_N super family domain. This last effect results in the loss of the DUF4795 domain implying that this mutation may have a strong impact on QRICH2 function. Moreover, we note that this variant in located in the SMC_N domain (Figure 1C), like one of the variants described in the initial report.[16] The second individual, P216, originated from the Middle East (Iran), was homozygous for the variant c.4614C>G [p.Tyr1538Ter] located in exon 15. The identified variant is predicted to induce a premature stop-codon leading to the production, if the abnormal mRNA is not degraded by the NMD mechanism, of a truncated protein lacking the last 126 amino acids of the C-terminal region. According to the gnomAD database (https://gnomad.broadinstitute.org), the allelic frequencies of these two variants are estimated at 3.97×10^{-6} and 4.02×10^{-6}, respectively, indicating that both variants are very rare in the human population. These results therefore confirm that *QRICH2* is a new "MMAF gene," contributing to approximately 1% of all cases in our cohort.

Because heterozygous *Qrich2* KO mice were described to have an alteration of sperm motility, we wanted to evaluate if MMAF patients from our cohort were more frequently carriers of heterozygous *QRICH2* variants than other non-MMAF subjects and hence if the presence of heterozygous *QRICH2* variants could contribute to the MMAF phenotype in human. Sixteen out of 167 subjects carried a potentially deleterious heterozygous *QRICH2* variant (9.6%).

We then analyzed whole exome data from a cohort of 103 subjects with azoospermia. These individuals can be considered as suitable controls as they were recruited by the same centers, sequenced and analyzed using the same processes. Similarly to what is described above, we extracted all the *QRICH2* potentially deleterious variants from this cohort. We did not identify any homozygous variants but identified a total of 16 *QRICH2* potentially deleterious variants in 16 (15.5%) individuals (Table 3). This result indicates that the frequency of MMAF individuals carrying heterozygous *QRICH2* potentially deleterious variants is not higher than what is observed in patients with another sperm defect unrelated to flagellar defects (azoospermia).

Interestingly, one MMAF subject (P415) carried two heterozygous missense variants scored as probably damaging by PolyPhen. As his parents were not tested, we cannot know if the variants are allelic. Analysis of the rest of the exome data for that patient showed that he is in fact homozygous for a *DNAH1* truncating variant, expected to be at the origin of his defect. It is therefore unlikely that these two *QRICH2* variants play a role in this patient's phenotype.

Additionally, in the cohort of azoospermic subjects analyzed (Table 3), one subject carried two potentially deleterious *QRICH2* variants (P351) and one carried three (P277). As *QRICH2* is very unlikely to be associated with azoospermia, this suggests that the presence of several potentially deleterious variants in these patients has probably no effect.

4 | DISCUSSION

In the last few years, the emergence and availability of HTS technologies and especially of WES allowed the identification of numerous new genetic causes underlying severe phenotypes of human male infertility such as nonobstructive azoospermia and monomorphic teratozoospermia.[18,19] Among these severe phenotypes, the multiple morphological anomalies of the sperm flagella (MMAF) syndrome showed a high genetic heterogeneity highlighting the abundance of genes involving in flagellum biogenesis and implicated solely in this phenotype.[20] Despite the identification of numerous causative genes, half of MMAF cases remain without an accurate diagnosis. These remaining cases could be explained in part by the difficulty to interpret some variants of unknown significance and by the fact that some variants are not detected by WES such as variants located in non-covered exonic regions or deep intronic regions that are not targeted and sequenced by this technique. In addition, bioinformatic pipelines do not yet efficiently detect complex genomic rearrangements.[21]

In their recent manuscript, Shen et al[16] studied two Chinese consanguineous families with MMAF cases and identified two nonsense mutations: c.192G>A [p.Leu64Ter] and c.3037C>T [p.Arg1013Ter]. The discovery of new probands harboring *QRICH2* mutations in MMAF syndrome by independent research teams is an important step in the validation process of genes involved in human infertility.[17,22] Herein, we reinvestigated the data obtained from WES of a large cohort of 167 unrelated MMAF individuals and identified two subjects carrying two novel homozygous nonsense mutations in *QRICH2*: c.3501C>G [p.Tyr1167Ter] identified in a North African individual born from first-degree consanguineous parents and c.4614C>G [p.Tyr1538Ter] identified in an Iranian individual. In addition to these homozygous variants, we also investigated rare heterozygous variants of *QRICH2* predicted to be potentially damaging by SIFT, PolyPhen, and/or HSF. Sixteen patients (9.6%) carried at least a *QRICH2* heterozygous variant, this rate was however comparable to what was observed in a control group (15.5%) suggesting that the presence of *QRICH2* heterozygous variants is not associated with MMAF syndrome.

In their study, Shen et al[16] observed that *Qrich2* heterozygous mice presented with asthenozoospermia. Here, we conclude that the presence of heterozygous *QRICH2* variants is unlikely to be associated with a MMAF phenotype. This was expected as, although not tested, both parents of affected men are expected to be heterozygous and they could conceive spontaneously. In men, we can therefore suppose that if heterozygous *QRICH2* deleterious variants have an effect on spermatogenesis it has to be mild and remain compatible with natural reproduction. We however cannot rule out that *QRICH2* heterozygous men could present with a moderate asthenozoospermia.

Interestingly, one MMAF subject (P415) carried two heterozygous missense variants scored as probably damaging by PolyPhen. As his parents were not tested, we cannot know if the variants are biallelic. Moreover, prediction softwares only give an indication of a potential effect on the protein and it is our belief that a diagnosis cannot be

given on missense variants if a functional test has not been performed confirming the deleterious effect of the variant on the protein function. Here, we would therefore not consider that the MMAF syndrome of P415 is caused by the two *QRICH2* identified variants. This is confirmed by the fact that this subject is homozygous for a *DNAH1* truncating variant which is expected to be at the origin of his defect. Missense variants could however be at the origin of male infertility and biallelic missense variants in *QRICH2* could induce MMAF or a milder phenotype. In the studied cohort, we however did not identify any patients carrying a homozygous missense variant. Moreover, the presence of two or more potentially deleterious variants in one gene is a frequent occurrence and without a segregation analysis we cannot know if the variant are allelic or biallelic. This is exemplified in the cohort of azoospermic subjects analyzed for *QRICH2* variants (Table 3) in which we identified one subject with two potentially deleterious *QRICH2* variants (P351) and one with three (P277). As we have established that *QRICH2* is very unlikely to be associated with azoospermia, we believe that the fact that several variants are found in these individuals is caused by chance and we do not expect the combination of these variants to be pathogenic.

Overall, these results confirm that biallelic loss-of-function mutations in *QRICH2* are a primary cause of MMAF in different ethnic's groups. It is interesting to underline that all MMAF genes identified first in North African and/or Middle East populations were secondarily confirmed in Chinese population and vice versa. This discovery confirms the importance of QRICH2 in spermatogenesis and particularly in flagellum biogenesis and shows its implication in the pathogeny of MMAF and male infertility. By confirming the role of *QRICH2* in male infertility, this work contributes to improving the molecular diagnosis of male infertility, a prerequisite to improve the care of infertile couple.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

DATA ACCESSIBILITY

All described variants and data that support the findings will be available in [ClinVar] at [https://www.ncbi.nlm.nih.gov/clinvar/] following an embargo from the date of publication.

ORCID

Charles Coutton https://orcid.org/0000-0002-8873-8098
Pierre F. Ray https://orcid.org/0000-0003-1544-7449

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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