#### **ORIGINAL RESEARCH ARTICLE**

## Association of the CAG repeat polymorphism in mitochondrial polymerase gamma (POLG1) with male infertility: a case-control study in an Algerian population

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Mohamed Larbi MLR Rezgoune<sup>1,2\*</sup>, Djalila DC Chellat<sup>1,2</sup>, Noureddine NA Abadi<sup>2</sup>, Abdelhamid AS Slama<sup>3</sup>, Dalila DS Satta<sup>1,2</sup>

Laboratoire de biologie moléculaire et cellulaire, Université des frères Mentouri Constantine 1, Constantine, Algérie<sup>1</sup>; Laboratoire de biologie et génétique moléculaire, Centre hospitalo-universitaire Ben-Badis, Université Salah Boubnider Constantine 3, Constantine, Algérie<sup>2</sup>; Laboratoire de biochimie, APHP Kremlin-Bicêtre, Paris, France<sup>3</sup>

\*For Correspondence: Email: rezgoune.mohamed.larbi@umc.edu.dz

#### Abstract

Polymorphisms in the mitochondrial DNA polymerase gamma (POLG) have been speculated to be associated with male infertility. The main objective of our study was to assess the possible association of CAG repeat polymorphism in *POLG*1 gene and male infertility in Algerian population. Genomic DNA from 89 infertile men and 84 controls was extracted using *salting-out method*. CAG repeat polymorphism was analyzed by the automated direct sequencing protocol. Statistical analysis was performed by Epiinfo® (v6.0) software. A significant association with male infertility was found for CAG repeat polymorphism in heterozygous genotypes ( $10/ \neq 10$  vs 10/10: OR = 2.00 [0.99 - 4.05], p = 0.03; "infertile vs control groups";  $10/ \neq 10$  vs 10/10: OR = 3.75 [1.20-11.96], p=0.01 "oligoasthenoteratospermic group"). Also, the results showed a significant association between the morbid allele ( $\neq 10$ ) and male infertility (2.07 [01.07 - 04.02], p = 0.01). Our results showed that *POLG*1 CAG repeat polymorphism might be a risk factor for male infertility in Algerian population. Investigations with larger sample sizes and representative population-based cases and matched controls are needed to validate our results. (*Afr J Reprod Health 2021; 25[1]: 67-75*).

Keywords: Infertility, CAG repeat length, Mitochondria, POLG1 gene, sequencing

#### Résumé

Les polymorphismes de l'ADN polymérase gamma mitochondriale (POLG) ont été supposés être associés à l'infertilité masculine. L'objectif principal de notre étude était d'évaluer l'association possible du polymorphisme de répétition CAG dans le gène *POLG*1 et l'infertilité masculine dans la population algérienne. L'ADN génomique de 89 hommes stériles et 84 témoins a été extrait en utilisant la méthode de *salting-out*. Le polymorphisme de répétition CAG a été analysé par le protocole de séquençage direct automatisé. L'analyse statistique a été réalisée par le logiciel Epi-info® (v6.0). Une association significative avec l'infertilité masculine a été trouvée pour le polymorphisme de répétition CAG dans les génotypes hétérozygotes (10 /  $\neq$  10 vs 10/10: OR = 2,00 [0,99 -4,05], p = 0,03; «infertiles vs groupes témoins»; 10 /  $\neq$  10 vs 10/10: OR = 3,75 [1,20-11,96], p = 0,01 «groupe oligoasthénotératospermique»). De plus, les résultats ont montré une association significative entre l'allèle morbide ( $\neq$  10) et l'infertilité masculine (2,07 [1.07-4.02], p = 0.01). Nos résultats ont montré que le polymorphisme répété de *POLG*1 CAG pourrait être un facteur de risque d'infertilité masculine dans la population algérienne. Des enquêtes avec des échantillons de plus grande taille et des cas représentatifs basés sur la population et des témois appariés sont nécessaires pour valider nos résultats. (*Afr J Reprod Health 2021; 25*[1]:67-75).

Mots-clés: Infertilité, longueur de répétition CAG, mitochondries, gène POLG1, séquençage

## Introduction

Infertility has been recognized as a very common health problem that affects about 15%-20% of couples who want to conceive, and almost 50% cases are because of male factors<sup>1</sup>. Abnormal sperm parameters (oligoasthenoteratozoospermia) or azoospermia are mostly related (75%) to primitive testicular injury, whereas obstructive (post-testicular) and secondary (pre-testicular) forms are relatively rare<sup>2</sup>. Despite important advancements in the male infertility diagnoses, the etiology remains unknown in almost half of the all male infertile cases<sup>3</sup>.

Until now, it has been assumed that genetic aberrations are thought to account for 15%-30% of male factor infertility, which include Y chromosome microdeletions, chromosomal aberrations and single-gene mutations<sup>4,5</sup>. In the last few years, novel approaches such as single nucleotide polymorphism (SNP) array, comparative genomic hybridization-array (array-CGH) and next generation sequencing (NGS) provided important data on rare variants.

Quite recently, considerable attention has been paid to mitochondrial genome<sup>6</sup>. It is usually accepted that mitochondria plays an important role in the energy metabolism as they contain the enzymes of the Oxidative PHOsphorylation System (OXPHOS), which satisfy the energetic needs of the cells. Mitochondrial DNA (mtDNA) codes for only few subunits of the OXPHOS enzymatic complexes<sup>7</sup>. Mutations of mitochondrial or nuclear DNA coding for subunits of mitochondrial machinery have been associated with a variety of human diseases especially in the organs with a high request for respiratory energy, like skeletal muscle, heart, kidney, brain, liver, and germinal tissue<sup>8,9</sup>.

For the reason that spermatozoa contain a large number of mitochondria, which play a vital role in their quality and quantity by providing the energy required to complete their functions, particularly sperm motility, it is generally hypothesized that accumulation of pathogenic mtDNA alterations influences the function of spermatozoa<sup>10</sup>. Several publications have appeared in recent years documenting an association between mtDNA alterations and sperm dysfunction<sup>6,11</sup>.

The human mtDNA polymerase gamma (POLG, POL $\gamma$ ) is an enzyme involved in the replication and repair of mtDNA, believed to be the only polymerase acting in the mitochondria. The holoenzyme POLG is a heterotrimer composed of two subunits: a one catalytic subunit (POLG1) of 140 kDa, with both polymerase and 3' $\rightarrow$ 5' exonuclease activity, and two accessory subunits (POLG2) of 53 kDa, which confers processivity<sup>12</sup>.

Mutations in *POLG1* result in mtDNA deletions and/or depletion, which then lead to decreased energy production in the affected cell via respiratory chain deficiency<sup>13.</sup> To date more than

180 pathogenic mutations have been reported in the *POLG1* gene as well as several SNP (http://tools.niehs.nih.gov/polg/).

The catalytic subunit of POLG is encoded by the POLG1 gene located on 15q25, with 23 exons (the first one is non-coding). The second exon of POLG1 contains a potentially unstable trinucleotide CAG repeat region (CAG) 10CAACAGCAG) that codes for a polyglutamine stretch near the N-terminus of the mature protein downstream of the presumed mitochondrial targeting sequence<sup>14</sup>. This polyglutamine tract can be the site for protein-protein interactions; altering the tract in POLG which may outcome to a suboptimal or indecorous mtDNA replication<sup>15</sup>. CAG repeat length is polymorphic (ranging from 6-15) with a major allele (wild-type), containing 10 repeats, whereas the mutant alleles are said not 10 CAG repeats, not 10 or  $\neq 10^{16}$ .

In 2001, an association between the  $\neq 10/\neq 10$  *POLG*1 genotype and male sub-fertility was claimed, firstly, by Rovio et *al.*, 2001<sup>17</sup>. Meanwhile, this original publication, many studies have assessed the association between the  $\neq 10/\neq 10$  CAG-repeat variant in *POLG*1 and male infertility and/or spermatogenic failure<sup>18</sup>. Few researchers rose that variation in CAG-repeat can affect the male reproductive ability; others did not find the association between CAG-repeat polymorphisms and male infertility<sup>19</sup>.

In this first work carried out in Algeria, we sequenced the exon 2 of *POLG*1 to investigate CAG repeat and associated SNP, in the Algerian infertile and control men, with the aim to assess whether this variant is associated with male infertility.

## Methods

This case-control study comprised a total of 89 infertile Algerian patients, including 59 with idiopathic azoospermia (AZOs), 21 with severe oligoasthenoteratozoospermia (OATs) and 9 with asthenospermia (ASTs) aged from 25 to 50 years, who were recruited from the Ibn-Sina Laboratory and Ibn-Rochd Clinic both located in the area of Constantine (East Algeria). These non-obstructive idiopathic infertile patients were classified by

semen analysis according to the World Health Organization guidelines<sup>20</sup>. The control group consisted of 84 men with normal sperm analysis. All patients and controls were of Algerian ethnic origin and have, in appearance (referring to interrogatories), no associated pathology. A written consent was obtained from each subject to participate in this study. The study was approved by the local Ethics Committee (Centre Hospitalouniversitaire, Constantine).

#### **DNA** extraction

Human genomic DNA was extracted from peripheral blood leukocytes using an inorganic solvent (NaCl method). 5-7 ml of peripheral blood was collected in EDTA tubes. DNA extracted from each patient was prepared at a concentration of 100 ng/ $\mu$ l.

#### Polymerase chain reaction amplification

To perform the (CAG)n repeat polymorphism of the POLG1 gene (OMIM : 174763) and SNP associated, the exon 2 (ENSE00000943530) was amplified by PCR in a thermal cycler (BIORAD®I Cycler) using Taq Polymerase kit (Gold® 250U supplied with dNTP and buffer) and two couples of primers; 2.1 Forward (CCACGTCTTCCAGCCAGTAA) and 2.1 2.2 Reverse (GCTTCTGCAGGTGCTCGAC), Forward (CGAGCAAATCTTCGGGCA) and 2.2 Reverse (CCCGTAACAGGACCTCAGAA). These primers were used to amplify the whole exon 2 of the *POLG1*; two fragments, respectively of 414 and 500-bp were amplified separately. PCR was performed in the following conditions: initial denaturation at 95°C for 10 min, followed by 35 cycles at 94°C for 30 Sec, 60°C for 30 Sec, 72°C for 45 sec and a final extension at 72°C for 7 min. products The PCR were controlled by electrophoresis (100 volt, 30 min) on 2% agarose gel (NuSieve®) stained with ethidium bromide and visualized under ultraviolet light. The PCR products were purified by automat (BIOMEK® NXP, Beckman-Coulter) using AMPure®-XP Agencourt®kit exhausting SPRI® (Solid Phase Reversible Immobilization) technology as described in the manufacturer's protocol.

# Genotyping of SNP (CAG)n in the POLG1 gene

The purified DNA was then sequenced by the automated direct sequencing protocol (Sanger et al., 1997)<sup>21</sup> using an ABI PRISM Big-Dye di-deoxy terminator v1.1 cycle sequencing kit (Applied Biosystems<sup>®</sup>). The sequences were purified using CleanSEQ®-XP Agencourt® kit (Applied Biosystems®) and performed with 3730xl DNA Analyzer (Applied Biosystems<sup>®</sup>). Obtained sequences were read with the software Sequencher®v5.3 (genecodes®corporation). The screening of POLG1 exon 2 SNP was performed using reference sequence extracted from the NCBI database (http://www.ncbi.nlm.nih.gov/). The number of CAG repeats was determined and genotype defined into; homozygote wild-type (10/10), homozygote mutant ( $\neq 10/\neq 10$ ) when they did not have a 10 CAG allele (regardless of the number of CAG repeat in each allele (>10 or <10)) and heterozygote  $(10/\neq 10)$ .

#### Statistical analysis

All samples were genotyped, genotypic and allelic frequencies of patients and controls were calculated by counting (Excel® 2010). To analyze the significance of the POLG1 polymorphism in male infertility, the differences in allelic and genotypic rates of the POLG1 locus between the groups (cases-controls) were evaluated using the Chisquared  $\chi^2$  (*Pearson* and *Yates*) test and Odds Ratio (OR) using Epi-info® (v6.0) software. P-values< 0.05 were deemed as being statistically significant. The departure from Hardy-Weinberg Equilibrium (HWE) for each group was assessed using a Pearson  $\gamma 2$  test for goodness of fit in the HWE online program (http://ihg2.helmholtzmuenchen.de/cgi-

bin/hw/hwa1.pl).

## Results

Our study was performed to assess the possible associations between the locus polymorphism and semen quality and fecundity in Algerian infertile men.

#### Genotypic and allelic distribution of POLG1 CAG repeat in the two study populations

The analysis and simulation indicate that the most frequent *POLG1* genotype in both groups of subjects was 10/10 genotype with a frequency of 55.05% (n = 49) in the infertile patients and 71.42% (n = 60) in the control group. The heterozygous genotype (10/ $\neq$ 10) was significantly more frequent among the patients (40.44%, n = 36) than among the controls (26.19%, n = 22), the *p*-value was 0.03. The frequencies of  $\neq$ 10/ $\neq$ 10 genotype were very low in the two groups (4.51 % in the infertile group and 2.39% in the control group) with no significant difference (*p*= 0.29).

There were no significant differences found between the two groups in the distribution of genotypes of the POLG1 under co-dominant (for homozygous genotype) and recessive models (p= 0.29; p=0.44, respectively). However, under the dominant model ( $10/\neq 10 + \neq 10/\neq 10$ ) the difference between cases and controls was significant (p=0.02). On the other hand, the frequency of the morbid allele ( $\neq 10$  allele) in infertile and controls, 24.72% and 15.48%, respectively, was significant (p=0.01) (Table 1).

#### Genotypic and allelic distribution of POLG1 CAG repeat in the sub-groups of infertile patients

The respective frequencies of the various genotypes in the three sub-groups of infertile men are given in Table 2. The most frequent genotype was the homozygous wild type in ASTs and AZOs subgroups (88.88% and 55.93%, respectively) followed by the heterozygous (40.67 % and 11.12%) and mutant homozygous (3.34% and 0%). According to these results, the frequency of the  $\neq$ 10 allele was calculated for ASTs and AZOs (5.56% and 23.76%) but no statistically significance difference in genotype and allele distribution in theses sub-groups were observed. However, in OATs sub-group, the most frequent genotype was the heterozygous (52.38%) followed by the homozygous wild type (38.09%) and the mutant homozygous (9.53%). A statistically significant difference was observed in heterozygous genotype between OATs infertile sub-group and controls (p= 0.01) under the co-dominant (10/ $\neq$ 10 vs 10/10) and the dominant models (10/ $\neq$ 10 +  $\neq$ 10/ $\neq$ 10) (p= 0.009; p=0.009, respectively). We also found a significant difference in the frequency of the  $\neq$ 10 allele (35.72%; p= 0.002) in OATs vs normal subjects (Table 2). Table 3 summaries the results of several studies in different populations of infertile men patients.

#### Discussion

In this paper we examine the implication of the *POLG1* CAG repeat polymorphism in male infertility, but molecular mechanisms leading to this impairment remain to be elucidated. Our results describe for the first time a significant association between CAG repeat polymorphism and male infertility in Algerian population.

Recently, genetic polymorphisms involved in spermatogenesis have been considered in association with male infertility, but are supposed to be co-factors rather than specific sources of spermatogenic failure, because they are also present in fertile men<sup>22,23</sup>. These sorts of genetic variants by themselves would probably be liable to a relatively minor damage of sperm production and/or function, but the effects of these variants may be worsened by the presence of other co-factors such as environmental factors<sup>24</sup>.

The most debated and controversial in this topic concerns the polymorphic CAG repeat-length variations of *POLG1* gene. Several studies have reported a relationship between the length of the CAG-repeat in *POLG1* gene and male infertility<sup>25-27</sup>. However, other studies have not reproduced this result<sup>24,28-34</sup>.

Our results show a distribution similar to that reported in European and Indian populations

**Table 1:** Frequencies of different POLG1 CAG repeat genotypes and alleles in infertile patients and controls (casecontrol study)

	Controls		Inferti	le patients	OR CI	Р
	%	n	%	'n		
Codominant	71.42	60	55.05	49	Ref	-
10/10	26.19	22	40.44	36	2.00 [0.99 - 4.05]	0.03
<b>10/</b> ≠ <b>10</b>	2.39	2	4.51	4	2.46 [0.36 - 20.21]	0.29
<b>≠10/ ≠10</b>						
Dominant	28.58	24	44.95	40	2.04 [1.04 - 4.03]	0.02
<b>10/≠10</b> + ≠ <b>10/</b> ≠ <b>10</b>						
10/10						
Recessive	97,61	82	95,49	85	0.52 [0.06-3.41]	0.73
<b>10/≠10 + 10/10</b>						
≠10/≠10						
Allele	84.51	142	75.27	134	Ref	-
10	15.48	26	24.73	44	2.07 [01.07 - 04.02]	0.01
≠10						

Ref: reference; CI: confidence interval, OR: odds ratio

**Table 2:** Frequencies of different *POLG1* CAG repeat genotypes and alleles in subgroups of infertile patients (AZOs, OATs and ASTs)

Genotypes	Controls			AZOs			OATs			ASTs		
	n (%)	OR	р	n	OR	р	n	OR	р	n	OR	р
Co-dominant												
10/10	60			33	-	-	8	-	-	8	-	-
	71.42			55.93			38.09			88.88		
	22	-	-	24	1.98	0.08	11	03.75	0.01	1	0.34	0.53
<b>10/</b> ≠ <b>10</b>	46.19			40.67	[0.91- 4.33]		52.38	[01.20-		11.12	[0.02-	
								11.96]			2.99]	
	2	-	-	2	1.82	0.95	2	07.50	0.16	0	0	0.54
<i>≠</i> 10/ <i>≠</i> 10	2.39			3.43	[0.17-19.19]		3.43	[00.63 -		0	[0-7.18]	
								91.56]				
Dominant												
<b>10/≠10</b> +	24	-	-	26	1.97	0.08	13	04.06	0.009	1	0.31	0.46
≠10/≠10 vs	48.58			44.1	[0.92-4.21]		61.91	[1.35-		11.12	[0.01-	
10/10								12.46]			2.72]	
Recessive												
<b>10/≠10 + 10/10</b>	82	-	-	57	0.70 [0.07-	0.87	19	0.23	0.37	9	-	0.45
VS	117.61			96.6	.16]		90.47	[0.02-		100		
<i>≠</i> 10/ <i>≠</i> 10								2.50]				
Allele												
10	142	-	-	90	-	-	27	-	-	17	-	-
<b>≠10</b>	26	-	-	28	1.70 [0.90-	0.10	15	3.03	0.006	1	0.32	0.43
	15.48			23.76	3,21]		35.72	[1.33-		5.56	[0.02-	
								6.91]			2.47]	

with a high frequency of homozygous genotype (10/10) in controls<sup>17,25,26,30,31,34</sup>. However, this frequency seems to be lower than those reported in Chinese population<sup>33</sup>. In spite of the genetic similitude of Algerian and Tunisian population, the frequencies of the 10/10 genotype reported in Tunisian controls were higher than founded in our

study<sup>27</sup>. This difference of distribution could be related to different ethnic and geographical origins and/or statistical bias selection.

Contrary to Rovio *et al.*, 2001, Amaral *et al.*, 2007, Shu-Yuan Liu *et al.*<sup>17,18,26</sup>, but in accord with some other reports<sup>27,28</sup>, the homozygous mutant genotype was not an exclusive feature of

**Table 3:** Review of genotypic and allelic *POLG1* CAG repeat polymorphism in different populations of infertile men patients

		Genotypes				Alleles		
Authors	Studied population	Cohort	10/10	<b>10/≠10</b>	<b>≠10/≠10</b>	10	<b>≠10</b>	
Rovio et al., 2001	European	99	55.56	35.35	9.09	73.23	26.77	
Martin Jensen et al., 2004	Danish	429	71.79	25.41	2.80	84.50	15.50	
Krausz et al., 2004	Italian	195	73.33	24.10	2.56	85.38	14.62	
Aknin-Seifer et al., 2005	French	433	70.90	26.10	3.00	83.95	16.05	
Brusco et al., 2006	Italian	277	70.40	28.16	1.44	84.48	15.52	
Harris et al., 2006	Neo-Zeeland	182	73.63	22.53	3.85	84.89	15.11	
Yao et al., 2006	Chinese	146	93.84	6.16	0.00	96.92	3.08	
Plaseski et al., 2007	Macedonian	225	76.89	20.89	2.22	87.33	12.67	
Amaral et al., 2007	Portuguese	95	72.63	27.37	0.00	86.32	13.68	
Rani et al., 2008	Indian	509	75.05	20.63	4.32	85.36	14.64	
Shu-Yuan Liu et al., 2011	Chinese	150	92.67	6.00	1.33	95.67	4.33	
Poongothai et al., 2013	Indian	124	79.03	18.55	2.42	88.31	11.69	
Baklouti-Gargouri et al., 2013	Tunisian	216	84.26	11.11	4.63	89.81	10.19	
The present study	Algerian	89	55.06	40.45	4.49	75.28	24.72	

the patients group but was also found in the controls group with not significantly different frequencies in our study. The heterozygous genotype  $(10/\neq 10)$  was found in a relatively high frequency in patients with fertility problems (40.45%) in comparison with some previous data (Table 3), the difference was statistically significant in comparison with controls. However, the frequencies of abnormal homozvgous mutant genotype ( $\neq 10/\neq 10$ ) was not significantly higher in infertile than in fertile men. The frequency of this genotype was similar to studies that claimed the no implication of CAG POLG1 in male infertility<sup>25-27,35</sup>. We also observed a statistically significant difference in the distribution of the  $\neq 10$ allele with patients and controls. Our results show that allelic variants of *POLG1* are somehow deleterious to sperm function or differentiation. So far, the significance of this finding is not clear.

Mutated alleles in *POLG1* ( $\neq$ 10) would produce suboptimal mtDNA polymerase leading to the accumulation of mutations in the mtDNA with the consequence of impaired energy metabolism of the spermatogenic cells and finally bring about a disturbance of sperm production and/or differentiation<sup>28</sup>. The identification of this partner protein and the confirmation of the hypothesized increased rate of mtDNA mutations in association with the mutated *POLG1* genotype are awaited<sup>17</sup>.

Thus, spermatozoa are heavily dependent on respiratory energy for motility, and impaired energy

metabolism has long been hypothesized to contribute to infertility. The relatively high frequency of the  $10/\neq 10$  genotype in our fertile controls was reported in other investigations<sup>28-31,33</sup> in accord with the hypothesis formulated by *Jensen* et *al.*, 2004 that the *POLG1* gene polymorphism seems to damage sperm function in a discrete manner, without affecting seriously semen parameters<sup>25</sup>.

The *POLG1* genotype distribution in the different sub-groups of infertile patients showed that  $10/\neq 10$  and  $\neq 10/\neq 10$  genotypes were found with higher frequency in OATs than in AZOs and ASTs infertile men in comparison with controls (*p*=0.002). These data suggest that the presence of the mutant allele disrupts the spermatogenetic process and leads to a reduced semen quality. Yao *et al.*<sup>32</sup>, found that the frequency of  $10/\neq 10$  CAG genotype was slightly higher in asthenospermic patients than in oligospermic and azoospermic patients and in control groups (with no significance); the authors concluded that  $10/\neq 10$  genotype could affect sperm motility<sup>32</sup>.

We observed a low frequency of the heterozygous and homozygous mutant genotype in the ASTs. However, the lower size of this infertile sub-group (n=9) does not allow to consider this result as significant. Xiao *et al.*<sup>36</sup>, conducted a metaanalysis and the results showed statistically significantly longer CAG repeat length among

cases compared with controls. However, shorter repeat length was associated with a lower risk of male infertility compared with a longer repeat length in the overall analysis<sup>36</sup>. The same metaanalysis reported that the CAG repeat length was associated with male infertility in Caucasian populations, but not Asian or Egyptian populations<sup>36</sup>.

Zhang et al.<sup>19</sup>, have also conducted a metaanalysis and demonstrated no apparent association between POLG-CAG-repeat and male infertility<sup>19</sup>. The discrepancy between the first and subsequent studies are frequently observed for case-control genetic association studies, and both bias and real population diversity may explain the overestimation of the disease protection or conferred predisposition by а genetic polymorphism. In the case of POLG1 CAG polymorphism, both conditions may contribute to the observed discrepancy. One possible explanation could be that polymorphisms with mild functional effects would be of pathogenic significance only in the presence of specific environmental factors or in association with a certain genetic background.

At last, we demonstrated in this study that the analyzed polymorphism seems to be implicated in male infertility in Algeria and that the presence of non-common allele appears to introduce male infertility, probably through the perturbation of the energetic metabolism in the cell and decreasing sperm motility.

Some limitations of our study must be addressed. One is that the lifestyles of patients including smoking, or other potentially negative habits are not presented. Furthermore, geneenvironment interaction analysis for male infertility was not performed. As in all case-controlled studies, a selection bias may exist, which may influence the detection of real associations. Finally, the results should be confirmed with a larger more diverse cohort.

In conclusion, it is evident that the biological significance of *POLG*1 CAG-repeat variants in male infertility remains unclear, and the heterozygous genotype seems to be associated with OATs. This work also suggests that *POLG*1,

possibly in conjunction with other factors, may play a role in regulating mtDNA copy in human sperm.

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## **Contribution of Authors**

Mohamed Larbi Rezgoune: study design, sequencing and preparation of draft manuscript Chellat Djalila: patient recruitment, DNA extraction and proof reading of the manuscript Noureddine Abadi: data collection and statistical analysis Abdelhamid Slame: study design

Abdelhamid Slama: study design

Satta Dalila: conception of the research idea All authors mentioned in the paper have read and approved the manuscript.

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