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0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(9):4292–4297

doi: 10.1210/jc.2004-0008

The Clinical Significance of the POLG Gene Polymorphism in Male Infertility

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Based on association studies, an increasing number of gene polymorphisms have been proposed as modulators of spermatogenesis. Interestingly, a clear cause-effect relationship between a polymorphism of the POLG gene and oligo(asthe-no)zoospermia was recently described. The POLG gene contains a polymorphic CAG repeat, and the presence of a homozygous mutant (not10/not10 CAG) genotype was found only in infertile men. In the present study, a large number of infertile patients and normospermic men of Italian origin were studied to define the effect of POLG genotypes on spermatogenic potential and whether the homozygous mutant is specific for spermatogenic disturbances. The mutated genotype was found at the same frequency in both infertile and normospermic men. Mean values of sperm parameters such as sperm count, motility, and morphology did not differ significantly between carriers of the three different genotypes. Our study failed to confirm any influence of the POLG gene polymorphism on the efficiency of the spermatogenesis. More importantly, considering that the homozygous mutant genotype has been found in normospermic fertile men, the analysis of the CAG repeat tract of the POLG gene does not appear to have any clinical diagnostic value. (*J Clin Endocrinol Metab* 89: 4292–4297, 2004)

INFERTILITY/SUBFERTILITY ALREADY affects about 5–7% of the general male population and may further increase in the future, considering an apparent trend of declining sperm count in industrialized countries over the last 40–50 yr (1, 2). Abnormal sperm parameters (oligoasthenoteratozoospermia) or absence of spermatozoa in the ejaculate (azoospermia) are mainly related (75%) to primitive testicular damage, whereas obstructive (posttesticular) and central or secondary (pretesticular) forms are relatively rare (3). Despite our increasing knowledge of the physiology of male reproduction and the availability of new diagnostic tools, the etiopathogenesis of testicular failure remains undefined in about 50% of cases, which are referred to as idiopathic infertility.

These idiopathic cases are likely to be of genetic origin because the number of genes involved in human spermatogenesis is probably over thousands (4), and only a small proportion of them have been identified and screened in infertile men so far.

Chromosomal anomalies (mainly numerical, such as Klinefelter syndrome) and microdeletions of the azoospermia factor (AZF) regions of the Y chromosome (5–7) are the most frequent known genetic causes of spermatogenic failure. The frequency of these two genetic anomalies increases with the severity of the spermatogenic defect, reaching to an overall 30% (15% karyotype abnormalities and 15% of AZF microdeletions) in azoospermic men. Besides these relatively frequent causes, mutations in specific genes such as KALIG-1 (8), KALIG-2 (FGFR-1) (9), GPR54 (10), GnRH receptor (11), gonadotropins (12), gonadotropin receptors (13), and androgen receptor (14) have also been identified in patients affected by syndromic hypogonadism or in patients with a characteristic picture of hormonal abnormalities. Mutation analyses in spermatogenesis candidate genes revealed an extremely low incidence, for instance in the USP9Y gene (15), or no functionally relevant mutations in the DBY (15), DAZLA (16), or FATE genes (17).

More recently, a number of reports have focused upon the role of certain haplogroups, allele variants, and single-nucleotide polymorphisms in male infertility. Based on association studies, an increasing number of gene polymorphisms have been proposed as modulators of the efficiency of the spermatogenic process leading to moderate or severe impairment of sperm production. In many cases, only sporadic data are available: polymorphisms in the genes DAZLA (16), DAZ (18), protamine-2 (19), estrogen receptor α (20), P450–1A1 (21), and mtDNA haplogroups (22); whereas results for CAG repeat-length polymorphism in the androgen receptor gene are discordant among different studies (for review, see Ref. 23). Association studies concerning human histocompatibility leukocyte antigen haplotypes (24, 25) represent an important starting point for the search of major histocompatibility complex genes involved in spermatogenesis. Similarly, a Y chromosome effect on spermatogenesis (other than AZF deletions) can be determined indirectly by the definition of Y chromosome haplogroups predisposing to male infertility (26).

Although Klinefelter sdr, Y microdeletions, and some of the gene-specific mutations are specific for spermatogenic

Abbreviations: AZF, Azoospermia factor; mtDNA, mitochondrial DNA.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

failure and, thus, not found in normozoospermic men, the above-listed polymorphisms must be considered risk factors given their presence (although at a lower frequency) in fertile (normospermic) men.

Interestingly, a clear cause-effect relationship between a polymorphism of the gene of POLG and oligo(astheno)zoospermia has recently been described by Rovio *et al.* (27). The POLG gene contains a polymorphic CAG repeat with a major allele at 10 CAG in the general population. Many repeat-length alleles different from 10 (ranging from 6–15) have been found and are considered mutants. Rovio *et al.* (27) reported the presence of homozygous mutant (not10/not10 CAG) genotype in infertile males (nine of 99) but not in fertile men (zero of 98), suggesting a new genetic cause of male infertility. The authors hypothesized that the presence of two mutated alleles would lead to a suboptimal mtDNA polymerase resulting in the accumulation of mutations in the mtDNA that would cause impaired energy metabolism of the spermatogenic cells and finally a disturbance of sperm production and/or differentiation.

The aim of the present study was to assess whether different CAG allele genotypes are associated with different spermatogenic potential and whether the not10/not10 CAG genotype is specific for spermatogenic disturbances, and, thus, whether the screening for POLG polymorphism can be proposed as new diagnostic test. A large number of infertile patients with different sperm count (ranging from azoospermia to mild forms of oligozoospermia) and a control group of normospermic men of Italian origin have been studied.

Subjects and Methods

Subjects

The study population consisted of 245 patients seeking complete andrological work-up for couple infertility at the Andrology Unit and the Unit of Physiopathology of Reproduction of the University Hospital Careggi (Florence, Italy). All patients underwent comprehensive andrological examination including semen analysis and karyotype and Y chromosome microdeletion screening. A total of 50 patients were excluded because of the presence of karyotype/Y chromosome anomalies (n = 21), hypogonadotrophic hypogonadism (n = 3), iatrogenic causes (n = 6), and patients of non-Italian origin (n = 20).

Of the 195 patients included in this study, sperm count revealed azoospermia (the complete absence of spermatozoa) in 50 patients, cryptozoospermia (<1 million spermatozoa/ml) in 25 patients, severe oligozoospermia (1–5 million spermatozoa/ml) in 72 patients, moderate oligozoospermia (5–20 million spermatozoa/ml) in 33 patients, asthenoteratozoospermia (total progressive motility < 50% with total normal morphology < 30%) in 11 patients, and pure teratozoospermia in four patients.

A total of 190 men with normal sperm parameters have been recruited from the Florence and Ancona areas. Ninety men fathered at least one child or had normal fertilization after *in vitro* fertilization for pure tubal factor infertility. All subjects gave an informed consent for molecular analysis of their blood samples.

Semen analysis

Semen analysis has been performed, according to the 1992 World Health Organization guidelines (28), at the Andrology Laboratory of the University Hospital of Careggi (Florence, Italy).

Molecular analysis

DNA source. DNA was extracted from peripheral lymphocytes in the infertile group and in 130 controls, whereas DNA was isolated from frozen semen in 60 controls.

CAG repeat-length analysis. The CAG-repeat region of the POLG gene was amplified by PCR according to Rovio *et al.* (29). The CAG repeat-length analysis was performed using an autosequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA). The size of the PCR products was determined by GeneScan software (Applied Biosystems). CAG repeat regions with different CAG repeat numbers were subjected to direct sequencing on the autosequencer for the definition of the correct CAG repeat length. The number of CAG repeats was calculated, using GeneScan software, by comparing the detected PCR fragment with the sequenced fragments. CAG repeat number determination was repeated twice on two separate gel runs.

Each homozygous mutant (not10 CAG) sample was confirmed by direct sequencing.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS for Windows (version 11; SPSS Inc. Chicago, IL). All variables were checked for normal distribution by Kolmogorov-Smirnov one-sample test.

For comparisons of means between groups of different genotypes, Student's *t* test for independent samples, when normal distribution was observed, was applied. Logarithmic transformation of data was performed to normalize the distribution when the presence of log normal distribution was checked. Finally, in the case of nonnormalized distribution, the nonparametric Mann-Whitney H test was applied to achieve the same objective.

$P < 0.01$ was considered statistically significant in each situation. The null hypothesis of means (or median) equality was refused.

Differences between frequencies in the subgroups of infertile men were quantified using Fisher's exact test.

Results

POLG allele and genotype distribution in the two study populations

The most frequently observed POLG allele in both groups of subjects was the common allele of 10 CAG repeats with a frequency of 85% in the infertile and 81% in the control group. We found a number of other not10 CAG alleles (mutated alleles) ranging from seven to 12 in the infertile group and from five to 12 in the control group (Table 1).

The combination of these alleles gave origin to three different genotypes: 10/10 CAG (homozygous wild type), 10/not10 CAG (heterozygous), and not10/not10 CAG (homozygous mutant). Some of the allele combinations such as 11/7 CAG were specifically found only in the infertile group, whereas others (10/5, 10/7, 10/9, and 9/12) were found in the control normospermic group (Fig. 1B)

In 10 cases, DNA extracted from both peripheral lymphocytes and spermatozoa was tested. The CAG genotype was the same, excluding *de novo* tissue-specific mutations.

TABLE 1. Allelic frequency of the POLG gene CAG repeats in the two study populations, infertile men and normospermic controls

No. of CAG repeats	Infertile patients (n = 195) Frequency (no. of total alleles − 390)	Normozoospermic controls (n = 190) Frequency (no. of total alleles − 380)
10	0.85	0.81
11	0.11	0.13
12	0.02	0.03
9	0	0.014
8	0.01	0.012
7	0.01	0.002
5	0	0.002

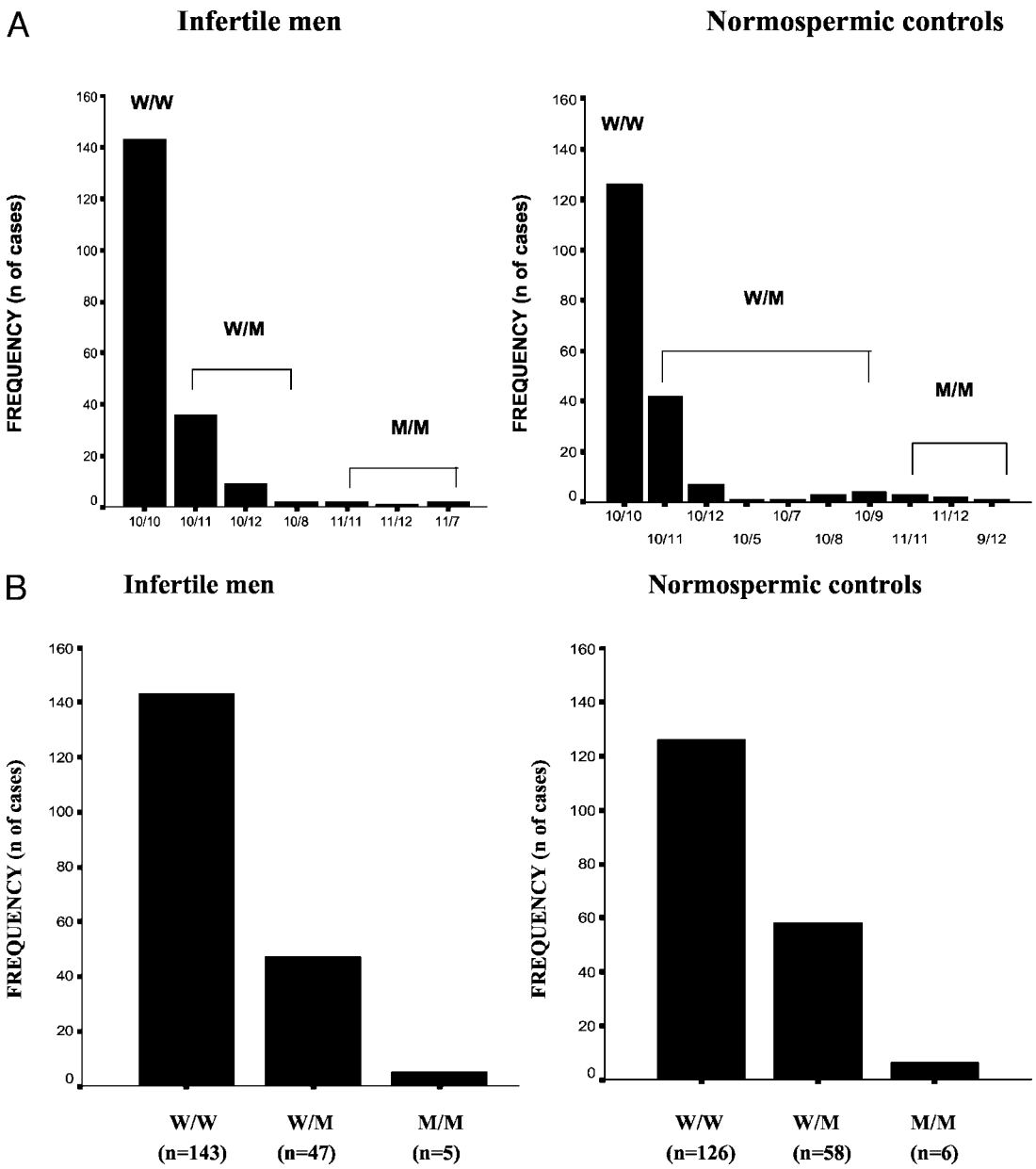

FIG. 1. POLG gene CAG genotype in patients and controls. A, Combination of different CAG alleles in patients and in normospermic controls. B, Genotype frequencies of the POLG gene in infertile and in normospermic men. W/W genotype indicates the presence of two common alleles (10/10 CAG); W/M genotype indicates heterozygosity for the common allele (10 CAG) and for a mutated not10CAG allele (> or <10 CAG); M/M genotype indicates homozygosity for the not10CAG allele (> or <10 CAG). Some of the combinations are specific for the patient group, whereas others are specific for the control group.

A similar distribution of the three different POLG genotypes has been observed in both groups. The most frequent genotype in the infertile and control group was the homozygous wild type (10/10CAG), 73.3 and 66.3%, followed by the heterozygous (10/not10 CAG), 24.1 and 30.5%, and the homozygous mutant genotype (not10/not10 CAG), 2.6 and 3.2%, respectively (Fig. 1A).

In contrast to the paper by Rovio *et al.* (27), the homozygous mutant genotype was not an exclusive feature of the patient group but was found also in the normospermic group, at a similar frequency (2.6 *vs.* 3.2%; P = not significant).

POLG genotype distribution in subgroups of infertile patients

The group of infertile patients included men with different sperm parameters ranging from azoospermia to asthenoteratozoospermia. The frequency of the three POLG genotypes has been calculated for four different subgroups: azoospermic (n = 50), cryptozoospermic (n = 25), severe oligozoospermic (n = 72), and mixed moderate oligospermic and asthenoteratozoospermic men (n = 48). In all subgroups, the most frequent genotype was the homozygous wild type, followed by the heterozygous mutant. No homozygous mu-

tant was found in the group of cryptozoospermic men; however, the relevance of this finding is of limited value due to the size of this subgroup (n = 25). The frequency of the homozygous mutant genotype in the remaining three groups (azoospermic, severe oligospermic, and mixed moderate oligo-asthenoteratospermic men) was 2, 2.8, and 4.2%, respectively. The relatively high frequency in the moderate oligospermic and asthenoteratospermic group (4.2%) was not statistically different from the control group (3.2%).

Genotype-phenotype correlation

A total of 11 homozygous mutants have been found in the two study populations (Table 2). The sperm concentration ranged from azoospermia to 85 million spermatozoa/ml. Therefore, the previously suggested genotype-phenotype correlation (mutant genotype and moderate oligozoospermia) has not been confirmed in our study. Three of the six normospermic men had conceived at least one child, which indicates that spermatozoa bearing the mutant genotype are fully fertile. The remaining three normospermic individuals had not yet attempted conception.

TABLE 2. Seminal parameters observed in subjects with M/M (not10/not10CAG) POLG genotype

	Genotype	Sperm no. × 10^6/ml	Motility (%)	Morphology (%)
Patient A54	11/12	0.8	6	0
Patient A102	11/7	15.5	17	12
Patient A122	11/11	0		
Patient A146	7/11	3.6	9	15
Patient A202	11/11	10	13	20
Control C52	11/11	85	57	34
Control C60	11/11	74.4	55	33
Control mm26	11/11	42	56	45
Control CS14	11/12	75	85	38
Control mm100	11/12	60	52	43
Control mm85	9/12	38	51	50

The "mutated genotype" has been found in both normospermic controls and patients with a variety of sperm parameters ranging from azoospermia (A122) to moderate oligoasthenoteratozoospermia (A102).

Comparison of sperm parameters among patients with different POLG genotype

To assess whether different POLG genotypes are able to influence spermatogenesis or sperm function, we compared the mean values of the three principal sperm parameters (concentration, morphology, and motility) among men bearing the three different genotypes. The mean values of the three parameters were not significantly different among the three groups in both normospermic and infertile men (Table 3).

Discussion

The search for new genetic anomalies represents one of the major tasks in modern andrology because it is expected that the majority of the so-called idiopathic cases of male infertility are of genetic origin. Although the number of spermatogenesis candidate genes is steadily increasing, their pathogenetic role, *i.e.* the frequency and the associated phenotype of their mutations, is largely unknown. To date, only two genetic tests became part of the routine genetic analysis of oligospermic and nonobstructive azoospermic men: karyotyping and Y chromosome microdeletion screening. Although karyotype anomalies and AZF microdeletions are frequently (~30%) found in nonobstructive azoospermic men, their incidence is much lower (~10%) in oligospermic patients. On the other hand, some of the known andrological pathologies causing male infertility such as varicocele, cryptorchidism, or environmental toxins (for example, heavy smoking) are associated with different degrees of spermatogenic damage in different individuals, indicating the importance of interaction between environment and genetic background.

Recently, the role of polymorphisms in genes potentially involved in spermatogenesis became of increasing interest. Polymorphisms are supposed to be cofactors rather than specific causes of spermatogenic failure because they are also present in normospermic men, although at a lower frequency. These types of genetic variants by themselves would probably be responsible for a relatively mild impairment of sperm production and/or function, but the effects of these variants may be worsened by the presence of other cofactors.

TABLE 3. Mean ± SD of the three semen parameters: sperm concentration/ml, percentage of spermatozoa with normal morphology (Morphology %), and percentage of spermatozoa with rapid and slow progressive motility (Motility %) in the group of infertile patients and in the group of normospermic men

	Genotype W/W	Genotype W/M	Genotype M/M
Infertile patients			
Sperm concentration/ml (×10^6/ml)	4.09 ± 7.43	6.95 ± 16.59	5.98 ± 6.61
	n = 143	n = 47	n = 5
Morphology (%)	13.03 ± 9.07	11.68 ± 7.95	11.25 ± 4.78
	n = 85	n = 31	n = 4
Motility (%)	21.56 ± 14.47	20.70 ± 16.61	11.75 ± 4.25
	n = 77	n = 30	n = 4
Normospermic controls			
Sperm concentration/ml (×10^6/ml)	80 ± 48.95	87.59 ± 75.27	62.40 ± 19.13
	n = 126	n = 58	n = 6
Morphology (%)	40.08 ± 7.53	41.17 ± 8.43	40.50 ± 6.65
	n = 126	n = 58	n = 6
Motility (%)	60.94 ± 9.89	59.21 ± 8.25	59.33 ± 12.78
	n = 126	n = 58	n = 6

The comparison of the mean values among the three genotypes (W/W *vs.* W/M and W/W *vs.* M/M and W/M *vs.* M/M) in both groups gives $P > 0.01$ (not significant).

The majority of data are originating from single studies (19–22) that necessitate further confirmation; however, some of the polymorphisms have been extensively studied in different populations (23). The most debated and controversial issue concerns the polymorphic CAG repeat-length in the first exon of the androgen receptor. Different repeat length leads to differences in the androgen receptor's transactivating capacity (30), and it would affect the spermatogenic process given its dependency on androgens. In some studies, an increased length of the CAG repeat (still in the polymorphic range) has been reported to be associated with male infertility and in particular a cutoff value of more than 26 CAG repeats (specific for infertile patients) has been reported (31). These data have not been confirmed in other publications (23, 32).

In the present study, we reported a similar discrepancy in respect to previous data from Rovio *et al.* (27) and from another very recent paper (33). Although Rovio *et al.* (27) indicated a clear cause-effect relationship (and not a simple association) between the presence of a homozygous mutant genotype of the POLG gene and idiopathic oligospermia with a calculated frequency of 5–10%, we did fail to confirm such a finding. The mutated not10/not10 CAG genotype has been found at the same frequency in both infertile and control normospermic men. Moreover, the mean values of the three principal sperm parameters (number, motility, and morphology) did not differ significantly between carriers of the three different genotypes (10/10, 10/not10, and not10/not10). The recent paper on a Danish population (33) reports a significantly higher frequency of the mutated genotype in normospermic men affected by unexplained couple infertility. Although the total number of patients and controls analyzed is large, the main clinical conclusion is based on the comparison of a small subgroup of normospermic patients (n = 49) *vs.* a large subgroup of controls with the same semen profile (total n = 572). Apart from the size of the patient's subgroup, there is also a problem with the selection criteria used to define it because the presence of functional female factors cannot be completely ruled out with the standard diagnostic exams and thus the couple's infertility can be related, with the same likelihood, to a functional female component as well as to a functional male factor. Moreover, six of seven normospermic patients with the mutated POLG genotype are in fact asthenozoospermic (sperm motility $< 50\%$); thus, the true frequency of homozygous mutant genotype would be one of 43 (2%) instead of seven of 49 (14.3%), which is similar to that found in the controls. Therefore, the author's conclusion about the association between reduced sperm fertilizing ability and POLG polymorphism is questionable.

The effect, if any, of the not10/not10 CAG genotype on the polymerase's activity is poorly understood. It is well known that human mtDNA encodes 13 of the polypeptides of the electron transport chain associated with the process of oxidative phosphorylation, the spermatozoa's most important ATP-generating pathway. Although both point mutations (34) and multiple large-scale deletions (35) of mtDNA have been reported in men with poor semen quality, their clinical significance is not yet clearly defined. In addition, an association between asthenozoospermia and certain mtDNA haplogroups that reflect significant differences in sperm oxidative performance has also been observed (22). Rovio *et al.* (27) proposed a reduced efficiency of the polymerase γ in the presence of homozygous mutated polyglutamine tract, which would lead to the accumulation of mtDNA mutations and impaired sperm energy metabolism and spermatogenic dysfunction. Although *in vitro* studies in cultured HEK293T cells failed to reveal a detectable effect of the deletion of the polyglutamine tract on enzymatic activity of the polymerase γ (36), the authors hypothesized that a sperm-specific protein could eventually interact with this region of POLG, and the interaction could be negatively influenced by the not10 CAG repeat length. The identification of this partner protein and the confirmation of the hypothesized increased rate of mtDNA mutations in association with the mutated POLG genotype is awaited.

Discrepancy between the first and subsequent studies are frequently observed for case-control genetic association studies, and both bias and genuine population diversity may explain the overestimation of the disease protection or predisposition conferred by a genetic polymorphism.

In the case of POLG polymorphism, both conditions may contribute to the observed discrepancy. One possible explanation could be that polymorphisms with mild functional effects (and this should still be demonstrated in the case of POLG polymorphism) would be of pathogenetic significance only in the presence of specific environmental factors or in association with a certain genetic background.

Apart from different ethnic and geographic origin, other factors related to the study populations (size and selection criteria) may give another plausible explanation for the discordance. An ideal control group for studies dealing with male infertility should contain normospermic-proven fertile men because around 10% of fertile men are oligospermic, and functional sperm defects rarely coexist with normal sperm parameters. However, because the putative effect of the POLG mutation is oligo/astheno/teratozoospermia, to avoid selection bias we selected our control group on the basis of their normal semen parameters. Although fertility was not assessed in about half of the control cases, three of six control men bearing the homozygous mutation were both normospermic and fertile. This finding clearly shows that spermatozoa from these men are fully fertile.

The most likely explanation for the observed discrepancy between our data and the paper from Rovio *et al.* (27) is the relatively low number of controls in their study (n = 98) *vs.* a much larger group (n = 190) in our study. Ioannidis *et al.* (37) have recently evaluated by meta-analysis 370 studies addressing 36 genetic associations for various outcomes of disease. They demonstrated that the small size (with a critical sample size of <150) of the first publication and a large number of studies were independent predictors of reaching discrepancies.

In conclusion, our study failed to confirm any influence of the CAG repeat length of the POLG gene on the principal sperm parameters. More importantly, considering that the homozygous mutant genotype has been found at the same frequency in normospermic men as in infertile patients, the analysis of the CAG repeat tract of the POLG gene does not appear to have any clinical diagnostic value.

Acknowledgments

We are grateful to Dr. Antonella Bucciantini for her valuable contribution to the statistical analysis of our data, Dr. R. Lazzarini for her technical assistance, and the clinical staff of the Andrology Unit and Reproductive Physiology (Prof. M. Maggi, Dr. G. Corona, Dr. M. Magini, Dr. L. Petrone, Dr. A. Cilotti, Dr. R. Mansani, Prof. E. Coccia, and Prof. I. Noci) for recruitment of patients.

Received May 12, 2004. Accepted May 26, 2004.

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This work was supported by a grant from the University of Florence.

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