

File ID 119740
Filename Chapter 7: CAG repeat length in the polymerase gamma (POLG) gene :
effect on semen quality

SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type Dissertation
Title Unraveling the genetics of spermatogenic failure
Author G.H. Westerveld
Faculty Faculty of Medicine
Year 2008
Pages 128
ISBN 9789064642951

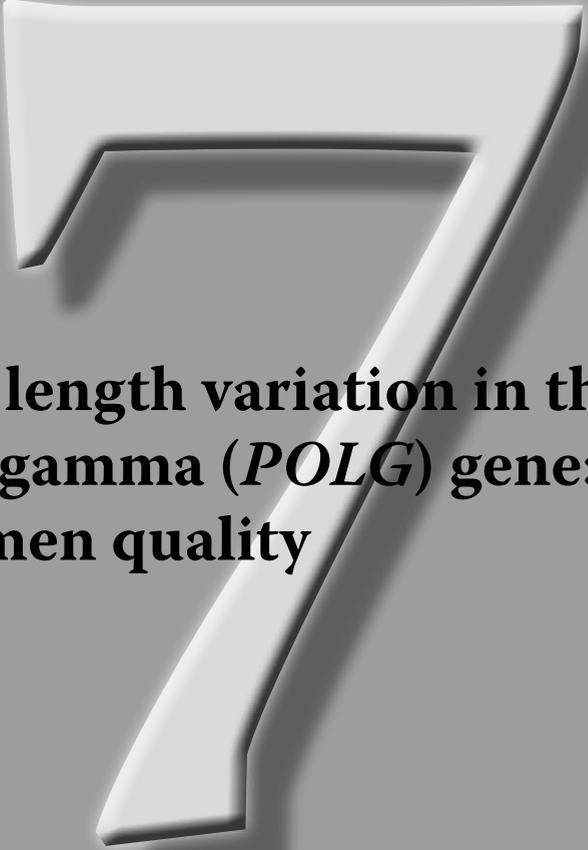
FULL BIBLIOGRAPHIC DETAILS:

<http://dare.uva.nl/record/288572>

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**CAG repeat length variation in the
polymerase gamma (*POLG*) gene:
effect on semen quality**

Molecular Human Reproduction 2008; 14: 245-249

Abstract

Several case-control studies have investigated the effect of CAG-repeat length variation in the *POLG* gene on male fertility and semen quality. Some described an association between the homozygous not10 CAG-repeat genotype and male subfertility and/or reduced semen quality, whereas others did not. The aim of our study was to investigate whether the not10/not10 variant is associated with spermatogenic failure.

By direct sequencing methods, we determined the CAG-repeat length of *POLG* in a cohort of 700 consecutive included men with variable degrees of spermatogenesis to investigate its effect on semen quality.

The frequency of the not10/not10 variant in our cohort was 4.7%. There were no differences in semen quality between groups with various *POLG* genotypes. There was a significant difference in frequency of the three CAG-repeat genotypes between ethnic subgroups. In conclusion, the not10/not10 *POLG* variant is not associated with clinically significant decreases in semen quality, but its frequency is dependent on ethnic background.

Introduction

Subfertility, i.e. the inability to conceive within one year of unprotected intercourse, is an increasing problem in the Western world. It is estimated that one in every eight couples experiences difficulties in conceiving naturally. In about half of these cases, the male partner has spermatogenic failure, defined as one or more semen parameters below the WHO threshold for normal semen quality (1, 2). Spermatogenic failure can result from exogenous factors, such as previous chemotherapy, or endogenous factors, such as Y-chromosome deletions (3, 4). Unfortunately, the cause of spermatogenic failure remains unknown in the majority of cases. Many case-control studies have attempted to determine whether certain genetic variants are associated with spermatogenic failure (5). One of these genetic variants studied is the not10/not10 CAG-repeat variant in the polymerase gamma (*POLG*) gene. The not10/not10 genetic variant describes a genotype in which both alleles in exon 1 of the *POLG* gene have a CAG-repeat length with more or less than 10 repeats.

The human *POLG* gene is located on the long arm of chromosome 15 and has a function in the replication of human mitochondrial DNA (6). The first exon of *POLG* contains a potentially unstable CAG repeat. The length of the CAG repeat is polymorphic with the most frequent allele containing 10 repeats (7). Mouse models showed that knock-in mice that expressed a proofreading-deficient version of PolgA, the mouse homolog of human *POLG*, have increased levels of mtDNA mutations. These mutations are associated with reduced life span and premature onset of aging-related phenotypes such as reduced fertility and alopecia (8, 9).

In 2001, an association between the not10/not10 *POLG* genotype and male subfertility was claimed (10). More precisely, men with reduced semen quality more often had a not10/not10 CAG-repeat genotype as compared to men with normal semen quality and male partners from subfertile couples (regardless of their semen quality) more often had this not10/not10 genotype than males partners from fertile couples.

Since this initial publication, five studies have assessed the association between the not10/not10 CAG-repeat variant in *POLG* and male subfertility and/or spermatogenic failure with conflicting results (Table I)(11-15). These conflicting results may be due to the case-control design used in these studies, which is prone to selection bias. Patients and, especially, controls were defined differently in the various studies and in most studies, controls were recruited from a different population than the patients. Given these conflicting results, the clinical significance of the not10/not10 *POLG* variant is at present unclear.

The aim of the present study was therefore to assess the effect of CAG repeat length variation in *POLG* on semen quality by performing a genotype driven rather than phenotype driven approach. To do so, we studied a large cohort of consecutively included men with variable degrees of spermatogenesis and compared semen quality in men with different *POLG* genotypes.

Table 1. Literature overview

Study	Patients		Controls		Conclusions	
	Number	Definition	not10/not10	Number	Definition	not10/not10
Rovio, 2001 ^a	99	Subfertile	9 (9.1)	98	Fertile	0
Jensen, 2004	405	Subfertile	12 (3.0)	374	Fertile	3 (0.8)
	144	<5x10 ⁶ /ml	1 (0.7)	13	<5x10 ⁶ /ml	0
	157	5-20x10 ⁶ /ml	3 (1.9)	42	5-20x10 ⁶ /ml	0
	104	>20x10 ⁶ /ml	8 (7.7)	319	>20x10 ⁶ /ml	3 (0.9)
				495	Unselected	8 (1.6)
				16	<5x10 ⁶ /ml	0
				106	5-20x10 ⁶ /ml	0
				373	>20x10 ⁶ /ml	8 (2.1)
Krausz, 2004	195	Subfertile ↓ semen quality	5 (2.6)	190	Normospermic	6 (3.2)
	147	<5x10 ⁶ /ml	3 (2.0)			
	33	5-20x10 ⁶ /ml	2 (6.1)			
	15	>20x10 ⁶ /ml	0			
Aknin-Seifer, 2005	433	Subfertile + ↓ semen quality	13 (3.0)	91	Normospermic	1 (1.1)
	372	<5x10 ⁶ /ml	11 (3.0)			
	26	5-20x10 ⁶ /ml	2 (7.7)			
	35	>20x10 ⁶ /ml	0			
Brusco, 2006	277	Subfertile + ↓ semen quality ^c	4 (1.4)	121	Fertile	6 (5.0)
				227	Unselected	9 (4.0)
Harris, 2006	182	Subfertile + ↓ semen quality	7 (3.8)	93	Subfertile, normospermic	3 (3.2)
	73	<5x10 ⁶ /ml	2 (2.7)			
	56	5-20x10 ⁶ /ml	3 (5.4)			
	53	>20x10 ⁶ /ml	2 (3.8)			

^a semen analyses unknown^b women included^c sperm concentration 5-19x10⁶/ml

Values in parentheses are percentages

Material and Methods

Study subjects

All men who attended the Center for Reproductive Medicine in the Academic Medical Center from January 2000 until September 2005 as part of a subfertile couple, defined as a couple failing to conceive within one year of unprotected intercourse, and who gave informed consent were included in our study. During the first visit, a medical history was taken to check for possible exclusion criteria. Primary exclusion criteria were a history of surgery of the vasa deferentia, bilateral orchidectomy, chemo- or radiotherapy and bilateral cryptorchidism. Men were also excluded if the fertility workup had identified obstructive azoospermia, retrograde ejaculation, numerical or structural chromosome abnormalities or Y-chromosome deletions. At least two semen analyses were performed for each patient according to WHO guidelines as part of standard patient care, and retrospectively linked to each included patient (2). Ethnic background was registered for each patient. The Institutional Review Board of the Academic Medical Center approved this study.

Genetic analysis

DNA was extracted from peripheral blood leucocytes according to standard procedures. The CAG repeat in exon 1 of the *POLG* gene was amplified by using sense (5'-ccaagccaggtgtctgac) and antisense (5'-gaagtctgtccagttgt) primers, using the available *POLG* genomic sequence information on the web (NM_002693). PCR was carried out in a total volume of 25 μ l and contained 250 ng DNA, 5.0 μ l 5x betaine buffer (0.25 M Tris (pH 9.2), 70 mM NH_4SO_4 , 10% DMSO and 1.5% Tween), 2.5 μ l 5M betaine, 0.2 mM dNTPs, 30 pmol forward and reverse primer, 2 mM MgCl_2 and 1 U SuperTaq polymerase. We used a touchdown PCR program with a temperature range of 69 – 62 $^\circ\text{C}$ with a 1 $^\circ\text{C}$ decrement per cycle and 1 cycle increment per temperature step and a final amplification for 20 cycles at 94 $^\circ\text{C}$ for 30 s, 50 $^\circ\text{C}$ for 30 s and 72 $^\circ\text{C}$ for 30 s with a final extension at 72 $^\circ\text{C}$ for 5 min.

To determine the CAG repeat length we used direct sequencing methods, using the same primers as those used for PCR, on an automated ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed with the CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA).

Statistic analysis

A chi-square test was used to determine whether the observed frequencies of genotypes conformed to Hardy-Weinberg (H-W) expectations.

The average of all semen analyses for each individual patient was used in all statistical analyses. Semen parameters were tested for normal distribution using the Shapiro-Wilk test. Data are either presented as means \pm standard deviation (SD) in case of normally distributed variables or as median with 25th and 75th percentiles in case of a non-normal distribution. Associations between the not10/not10 *POLG* variant and semen parameters were analyzed

using a Kruskal-Wallis test. To investigate the possible effect of genetic heterogeneity on the association between the not10/not10 *POLG* variant and semen quality, all semen characteristics were analyzed using a (non-parametric) ANOVA model that included CAG repeat length, ethnicity and their interaction as predictors. All statistical analyses were carried out using SPSS (version 14.0). P-values < 0.05 were considered significant.

Results

We included 700 men with variable degrees of spermatogenesis. Cohort characteristics are shown in Table II. Forty-three (6.1%) men were azoospermic, 70 (10.0%) men had severe oligozoospermia (<5.0x10⁶/ml), 87 (12.4%) men moderate oligozoospermia (5.0x10⁶/ml – 20.0x10⁶/ml) and 500 (71.4%) men were normospermic (>20.0x10⁶/ml) according to the WHO criteria for normal sperm concentration. The frequency of the not10/not10 *POLG* variant was 4.7%. Distribution of genotypes was in H-W equilibrium.

Men with the not10/not10 *POLG* variant did not have a significant reduction in semen quality as compared to men with the 10/not10 or 10/10 genotype (Figure 1).

There was a significant difference in frequency of the three *POLG* CAG-repeat genotypes between ethnic subgroups (P=0.015). The wildtype genotype (10/10) varied from 52% in African men to 79% in Turkish men. Similarly, the frequency of the not10/not10 genotype varied from 2.3% in Asian men to 11% in African men (Table III). An ANOVA prediction model that included CAG repeat length, ethnicity and their interaction showed a significant interaction between ethnicity, CAG repeat length and semen concentration (P=0.012), as well as between ethnicity, CAG repeat length and total motile count (TMC) (P=0.042)(Table IV). We therefore performed a subgroup analysis in the two largest ethnic groups, which suggested a different effect of the *POLG* genotypes on concentration and TMC for Dutch and African men (Figure 2). In Dutch men the *POLG* genotype had no influence on semen quality, whereas in men from African origin, the heterozygous genotype (10/not10) had a positive effect on concentration (P=0.015) and TMC (P=0.040).

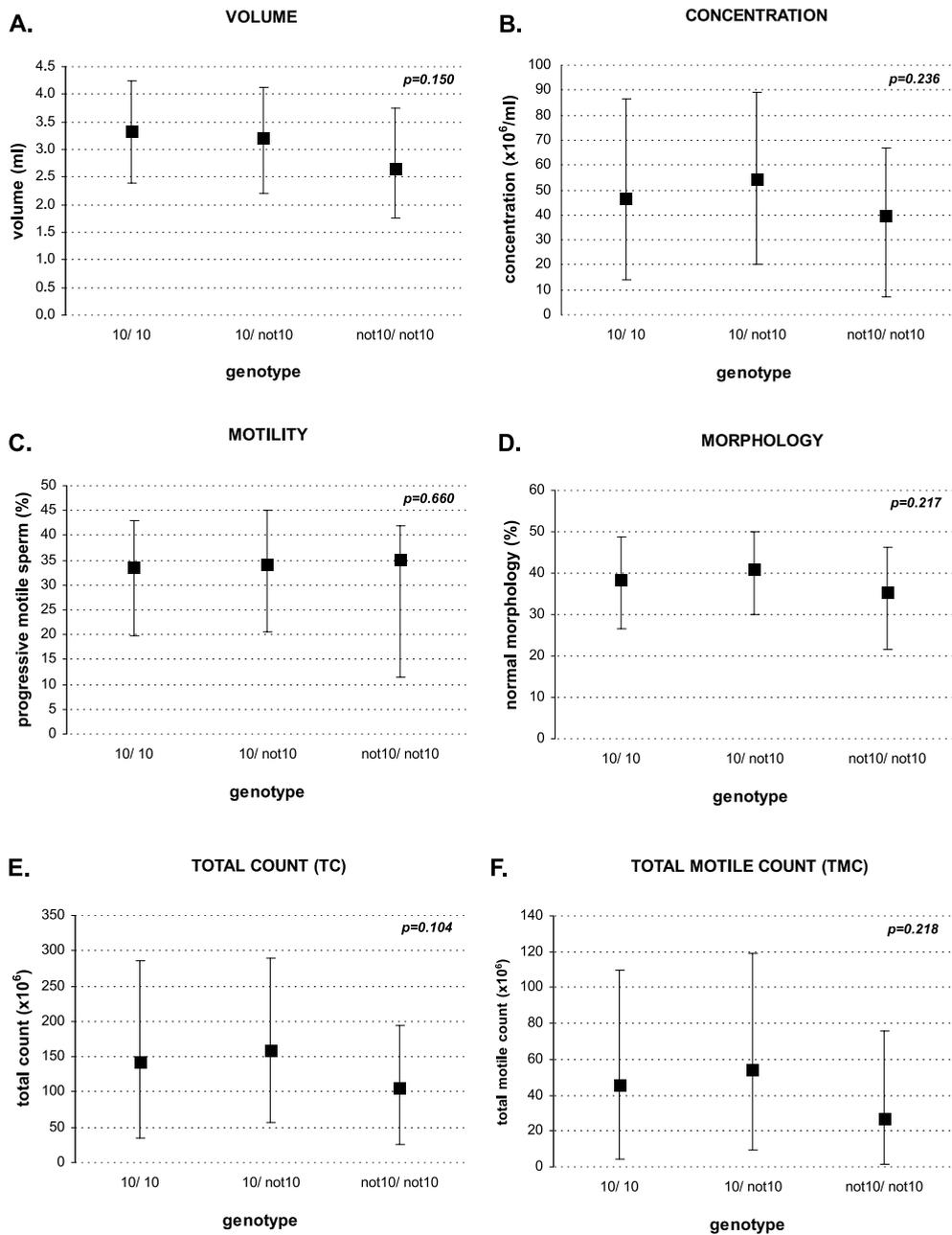


Figure 1. Semen quality in men with different *POLG* genotypes. Graphs illustrate semen quality (median \pm quartiles) for the three different CAG-repeat length genotypes: homozygous wildtype (10/10), heterozygous (10/not10) and homozygous mutant (not10/not10). (A) volume, (B) sperm concentration, (C) percentage progressively motile sperm, (D) percentage morphologically normal sperm, (E) total sperm count and (F) total motile sperm count. In the top right corner of each graph the *P*-value of the Kruskal-Wallis equation is given.

Table II. Cohort characteristics (n=700)

	Mean (SD) or median (25 th -75 th percentile)*
Age (years)	39.2 (6.3)
<i>Semen analysis</i>	
• volume (ml)	3.4 (± 1.5)
• concentration (10 ⁶ /ml)	48.5 (15.0-87.6)*
• motility (% progressive)	33.5 (19.6-43.5)*
• morphology (% normal)	37.2 (± 15.7)
• total count (10 ⁶)	144.2 (42.4-281.3)*
• total motile count (10 ⁶)	46.9 (5.5-108.4)*
	Number
<i>Ethnicity</i>	
• Dutch	458 (65.4)
• African	91 (13.0)
• Asian	44 (6.3)
• Northern African	34 (4.9)
• Turkish	24 (3.4)
• non-Dutch European	17 (2.4)
• Middle Eastern	14 (2.0)
• other	18 (2.6)

Values in parentheses are percentages

Table III. Genotype distribution per ethnic group

	10/10	10/not10	not10/not10
Cohort (n=700)	451 (64.4)	216 (30.9)	33 (4.7)
Dutch (n=458)	294 (64.2)	147 (32.1)	17 (3.7)
African (n=91)	47 (51.6)	34 (37.4)	10 (11.0)
Asian (n=44)	36 (81.8)	7 (15.9)	1 (2.3)
Northern African (n=34)	24 (70.6)	7 (20.6)	3 (8.8)
Turkish (n=24)	19 (79.2)	4 (16.7)	1 (4.2)
non-Dutch European (n=17)	11 (64.7)	6 (35.3)	0
Middle Eastern (n=14)	10 (71.4)	4 (28.6)	0
other (n=18)	10 (55.6)	7 (38.9)	1 (5.6)

Values in parentheses are percentages

Table IV. Interaction between semen parameters, genotype and ethnicity

Effect	Volume <i>P</i> -value	Concentration <i>P</i> -value	Motility <i>P</i> -value	Morphology <i>P</i> -value	Total Count <i>P</i> -value	TMC <i>P</i> -value
POLG	0.441	0.113	0.380	0.120	0.215	0.188
Ethnicity (E)	0.010	0.077	0.704	0.236	0.044	0.081
POLG*E	0.972	0.012	0.138	0.614	0.066	0.042

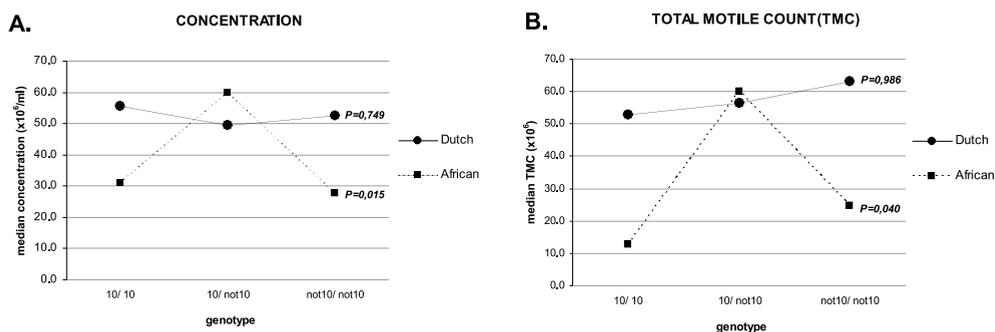


Figure 2. Subgroup analysis into the effect of *POLG* genotypes on semen quality in the two largest ethnic subgroups.

The effect of the three CAG-repeat *POLG* genotypes on sperm concentration (A) and total motile count (B) in the Dutch and African subgroups. On the right in each graph the *P*-values for both subgroups are given.

Note: the other ethnic subgroups were not analyzed since the total number of the not10/not10 *POLG* variant occurred in less than five patients.

Discussion

In this cohort of 700 men, we found no differences in semen quality between men with or without the not10/not10 *POLG* genotype. The frequency of the three *POLG* genotypes differed between ethnic subgroups. Subgroup analysis showed that the *POLG* genotype had no influence on semen quality in Dutch men, whereas in men from African origin, the heterozygous genotype (10/not10) had a positive effect on semen quality.

One of the limitations of our study is that we included only 700 men. In our cohort, the mean concentration of men without the not10/not10 *POLG* variant was 57x10⁶/ml and in men with this homozygous variant 48x10⁶/ml. Given the population size of 700 and an observed *POLG* not10 allele frequency of 0.2, we could detect a minimal difference of 13x10⁶/ml between carriers and non-carriers of the *POLG* not10 allele, or in case of a recessive genetic model, a minimal difference of 27x10⁶/ml between homozygous not10/not10 patients and the other variants.

We included men with variable degrees of spermatogenesis, because of uncertainty about the phenotype associated with the not10/not10 variant. Our cohort design allows for comparison of the average semen quality of men with a certain (genetic) risk factor (such as in this case the not10/not10 *POLG* variant) to the semen quality of men without this risk factor and avoids phenotype based bias.

We found differences in frequency of the three *POLG* genotypes between ethnic subgroups, which has been described earlier in 12 ethnic subgroups from North Eurasia (16). Although subgroup analysis showed a different effect of the *POLG* genotypes on spermatogenesis in Dutch men compared with men from African origin, this is most likely due to small numbers of the three genotypic subgroups in the African subgroup. Moreover, it is biologically not very plausible that only the heterozygous genotype has an effect on the phenotype. These differences in frequency between (ethnic) groups illustrate the importance of proper study design when performing genetic association studies. In contrast to candidate gene screening in which the purpose of study

is to find a direct cause-effect relationship, i.e. finding a gene mutation that causes spermatogenic failure, association studies aim to find an association between a genetic locus and a certain disease. Association studies are thus genetic mapping studies in which the genetic variant in itself is not the direct or only cause of the phenotype. Consequently, these variants (also known as polymorphisms) also occur in controls, but, in case of an existing association, at a significantly lower frequency. Thus, association studies are more sensitive to selection bias than candidate gene screening. In our opinion, a cohort design is the preferred design for association studies, whereas a case-control design is a more useful design for candidate gene screening.

In conclusion, the not10/not10 *POLG* variant is not associated with clinically significant decreases in semen quality. We therefore advise not to screen for *POLG* CAG repeat length in men from subfertile couples.

Acknowledgements

We thank S.K. van Daalen for technical assistance.

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