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What is influencing the phenotype of the common homozygous polymerase-γ mutation p.Ala467Thr?

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Polymerase-γ (*POLG*) is a major human disease gene and may account for up to 25% of all mitochondrial diseases in the UK and in Italy. To date, >150 different pathogenic mutations have been described in *POLG*. Some mutations behave as both dominant and recessive alleles, but an autosomal recessive inheritance pattern is much more common. The most frequently detected pathogenic *POLG* mutation in the Caucasian population is c.1399G>A leading to a p.Ala467Thr missense mutation in the linker domain of the protein. Although many patients are homozygous for this mutation, clinical presentation is highly variable, ranging from childhood-onset Alpers-Huttenlocher syndrome to adult-onset sensory ataxic neuropathy dysarthria and

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ophthalmoparesis. The reasons for this are not clear, but familial clustering of phenotypes suggests that modifying factors may influence the clinical manifestation. In this study, we collected clinical, histological and biochemical data from 68 patients carrying the homozygous p.Ala467Thr mutation from eight diagnostic centres in Europe and the USA. We performed DNA analysis in 44 of these patients to search for a genetic modifier within *POLG* and flanking regions potentially involved in the regulation of gene expression, and extended our analysis to other genes affecting mitochondrial DNA maintenance (*POLG2, PEO1 and ANT1*). The clinical presentation included almost the entire phenotypic spectrum of all known *POLG* mutations. Interestingly, the clinical presentation was similar in siblings, implying a genetic basis for the phenotypic variability amongst homozygotes. However, the p.Ala467Thr allele was present on a shared haplotype in each affected individual, and there was no correlation between the clinical presentation and genetic variants in any of the analysed nuclear genes. Patients with mitochondrial DNA haplogroup U developed epilepsy significantly less frequently than patients with any other mitochondrial DNA haplotype. Epilepsy was reported significantly more frequently in females than in males, and also showed an association with one of the chromosomal markers defining the *POLG* haplotype. In conclusion, our clinical results show that the homozygous p.Ala467Thr *POLG* mutation does not cause discrete phenotypes, as previously suggested, but rather there is a continuum of clinical symptoms. Our results suggest that the mitochondrial DNA background plays an important role in modifying the disease phenotype but nuclear modifiers, epigenetic and environmental factors may also influence the severity of disease.

Keywords: mitochondrial diseases; neuromuscular disorders; genetics; phenotype; molecular biology

Abbreviation: SANDO = sensory ataxic neuropathy, dysarthria and ophthalmoparesis

Introduction

The *POLG* gene codes for the catalytic subunit of polymerase γ, the only DNA polymerase found within mitochondria (Ropp and Copeland, 1996; Chinnery and Zeviani, 2008). During the past 10 years, mutations in *POLG* have been identified in a wide range of mitochondrial diseases, including progressive external ophthalmoparesis (Van Goethem *et al.*, 2001), Alpers/Alpers-Huttenlocher syndrome (Naviaux and Nguyen, 2004), adult-onset spinocerebellar ataxia (Van Goethem *et al.*, 2003, 2004), parkinsonism and premature ovarian failure (Luoma *et al.*, 2004). Although *POLG* is a nuclear gene, clinical symptoms are related to a secondary impairment of mitochondrial DNA (accumulation of multiple mitochondrial DNA deletions, decreased mitochondrial DNA copy numbers or somatic mitochondrial DNA point mutations) in the affected tissues. It is becoming clear that *POLG* is a major human disease gene, possibly accounting for up to 25% of all patients with mitochondrial diseases in the UK and in Italy (Chinnery and Zeviani, 2008). To date, ~150 pathological mutations have been identified in this gene (http://tools.niehs.nih.gov/polg/). *POLG* mutations may behave as both dominant and recessive alleles and multiple (>3 or 4) mutations may occur in single individuals, further complicating the genetic diagnosis of *POLG*-related disease (Horvath *et al.*, 2006). In addition to clearly pathogenic mutations, polymorphic genetic variants may also modulate the phenotype (Luoma *et al.*, 2005).

The two most frequent pathogenic *POLG* mutations in Caucasians are c.1399G>A (p.Ala467Thr) and c.2243G>C (p.Trp748Ser) (Hakonen *et al.*, 2005, 2007). In one study, both mutations appeared to reside on a common ancient European haplotype in patients throughout Europe, Australia, New Zealand and the USA (Hakonen *et al.*, 2007). The associated alleles have spread to populations of European descent with carrier frequencies up to 1% in several of them, therefore representing a common cause of ataxia and Alpers' syndrome in the Western world (Hakonen *et al.*, 2007).

It has been shown that the p.Trp748Ser mutation is one of the most common causes of inherited ataxia in Finland and Norway (Hakonen *et al.*, 2005). Despite the fact that affected individuals had the identical homozygous p.Trp748Ser mutation, the phenotypes in this study were surprisingly heterogeneous and typically included ataxia, peripheral neuropathy, dysarthria, mild cognitive impairment, involuntary movements, psychiatric symptoms and epileptic seizures (Hakonen *et al.*, 2005).

Except for the Scandinavian population discussed above, the p.Ala467Thr mutation seems to be the most common non-synonymous disease-causing *POLG* mutation worldwide (Luoma *et al.*, 2004; Horvath *et al.*, 2006; Chinnery and Zeviani, 2008; Blok *et al.*, 2009). *POLG* containing the p.Ala467Thr mutation has a polymerase activity that is only ~4% of the wild-type enzyme (Chan *et al.*, 2005; Luoma *et al.*, 2005), most likely due to reduced DNA binding and to impaired initiation of DNA strand elongation by modified interaction with the 55 kD accessory subunit (*POLG2*). The p.Ala467Thr mutation is generally considered recessive, but it was suggested that p.Ala467Thr may give rise to a mild dominant phenotype causing late-onset ptosis (Luoma *et al.*, 2005). Patients homozygous for p.Trp748Ser also have variable clinical presentation, although published phenotypes fall into several discrete clinical groups, including childhood-onset severe epilepsy and liver failure (Alpers-Huttenlocher syndrome), and adult-onset sensory ataxic neuropathy, dysarthria and ophthalmoparesis (SANDO). The suggestion of familial clustering of these phenotypes implicates additional genetic or shared environmental factors influencing the expression of the clinical phenotype (Tzoulis *et al.*, 2006; Cohen and Naviaux, 2010). However, there have been no large-scale multi-national studies of patients harbouring p.Ala467Thr or p.Trp748Ser, and our understanding of the phenotypic spectrum is based on small case series and isolated case

reports, with publication bias favouring the description of different phenotypes.

Previously, some European mitochondrial DNA haplogroups have been correlated with clinical conditions caused by mitochondrial DNA mutations (Hudson *et al.*, 2007). The functional consequences of mitochondrial DNA genetic backgrounds (haplotypes, haplogroups) have been demonstrated by both disease association and *in vitro* studies (Wallace, 2005, 2008; Hudson *et al.*, 2007).

We collected clinical data and DNA samples from 68 patients from 58 families homozygous for the p.Ala467Thr *POLG* mutation and provide the first comprehensive phenotypic description of this common genetic disease, with a view to defining the cause of the variable clinical presentation of this single homozygous mutation through the detailed characterization of relevant nuclear genes and the mitochondrial genome.

Patients and methods

We contacted eight national diagnostic centres in Europe and the USA, allowing the identification of 68 patients from 58 families, homozygous for the p.Ala467Thr mutation (Supplementary material and Table 1). These patients were prospectively collected over a 10-year period (2001–11), and follow-up information was available for all patients. The following patients were reported previously: five Dutch patients (Blok *et al.*, 2009), two Belgian siblings (Van Goethem *et al*., 2004), one isolated Belgian patient (Van Goethem *et al*, 2003), two UK patients (Stewart *et al.*, 2009) and one German patient (Horvath *et al.*, 2006).

Using a standardized questionnaire, we collected clinical data on age, gender, case histories, clinical presentation, examination findings including skeletal muscle biopsy and analysed them as a group (Supplementary material).

Genetic analysis

Genomic DNA of 44 patients was available for further studies. The analyses for mitochondrial DNA deletions and depletion and sequencing of the 22 coding exons and flanking intronic regions of *POLG* were performed in the diagnostic laboratories. The predicted promoter region of *POLG* was determined from the Transcriptional Regulatory Element Database (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home), and direct Sanger sequencing of this entire promoter was performed (Supplementary material). Sequencing of the genes *POLG2, PEO1* and *ANT1* was performed by standard methods using intronic primers (Supplementary material). Microsatellite marker analysis of the *POLG* locus was performed using four markers (D15S979, D15S1045, D15S202, D15S127) closely flanking the *POLG* gene on chromosome 15 by standard methods. The rationale behind studying microsatellite markers and not single nucleotide polymorphisms was that microsatellite markers evolve faster, and thus might tag an allele that alters the phenotype, whereas ancient single nucleotide polymorphisms may only show a core haplotype. The mitochondrial DNA haplogroup in the 44 samples were determined by PCR amplification and restriction fragment length polymorphism analysis using a standard algorithm (Supplementary material).

Table 1 Number of patients from the different centres presented with the major phenotypes

Phenotype	UK	G	NL1	NL2	B	N	F	USA	
Alpers	1	6	4	2	1	0	0	0	14
SANDO	4	6	2	7	3	1	2	2	27
Mixed	8	2	3	3	4	5	1	1	27
	13	14	9	12	8	6	3	3	68

B = Belgium; F = France; G = Germany; N = Norway; NL = Netherlands.

Statistical analysis

To assess the relationship between the clinical and genetic findings, logistic regression table and Fisher's exact tests (SPSS Statistics 17.0) were performed. The associations between the microsatellite marker length and phenotype were assessed by *t*-test and by the non-parametric Wilcoxon rank sum test with continuity correction, carried out in using the R statistical package. We compared age of onset with clinical symptoms (e.g. epilepsy, liver failure, ataxia, neuropathy) using Kaplan–Meier survival curves, and the statistical significance was calculated by the Mantel–Cox test.

Results

Clinical presentation

The clinical presentation of the 68 patients is summarized in the Supplementary material. The phenotypes did not fall into discrete groups, but formed a continuous spectrum of disease. Age at onset in our cohort varied from 2–40 years (mean, 18.8 ± 9.6 years) (Table 2). Comparing disease onset and the presence or absence of clinical symptoms, we found that in the presence of epilepsy, the age of disease onset was significantly ($P = 2.6 \times 10^{-4}$) lower (15.9 ± 8.1 years, $n = 41$) than in patients without epilepsy (24.2 ± 9.3, $n = 27$). Ataxia and neuropathy were both associated with a later onset of disease (Tables 1–4). In general, patients presenting at a younger age tended to have epilepsy and hepatopathy, thus resembling patients with compound heterozygous *POLG* mutations, whereas older patients tended to have ataxia and peripheral neuropathy (Fig. 1).

There was a striking association of epilepsy and mortality. Of the 41 patients who had epilepsy, 24 (59%) died either following a severe episode of epileptic encephalopathy ($n = 13$) or because of liver failure ($n = 11$) and most deaths occurred either in the late second and third or in the fifth and sixth decades. In contrast, of the 27 patients without epilepsy, only one patient died (age 46 years), suggesting that these patients have a better prognosis. We also observed a significant difference in the presence or absence of epilepsy between genders (female predominance, $P < 0.025$) (Fig. 2A and B). Because of the low number of patients, we have not subclassified them based on the different types of epilepsy.

Liver failure occurred exclusively in patients with epilepsy. Nineteen out of 41 patients with epilepsy developed liver problems, including abnormal liver enzymes and liver enlargement (46%); this was fatal in 11 cases, and in six cases, it became fatal after sodium valproate therapy (Table 3). These data indicate

Table 2 Clinical presentation, age of onset and death in 68 patients homozygous for p.Ala467Thr

Symptom	Alpers	SANDO	Mixed
Epilepsy	+	−	+
Neuropathy	−	+	+
Ataxia	±	+	+
	14 patients (20%)	**27 patients (40%)**	**14 patients (20%)**
Age of onset	13.2 ± 5.6 years	24.2 ± 9.3 years	17.2 ± 8.9 years
Age at death	18.1 ± 7.5 years ($n = 9$)	46 years ($n = 1$)	40 ± 14.4 years ($n = 15$)
Death rate	9/14 (70%)	1/27 (3.7%)	15/27 (55%)

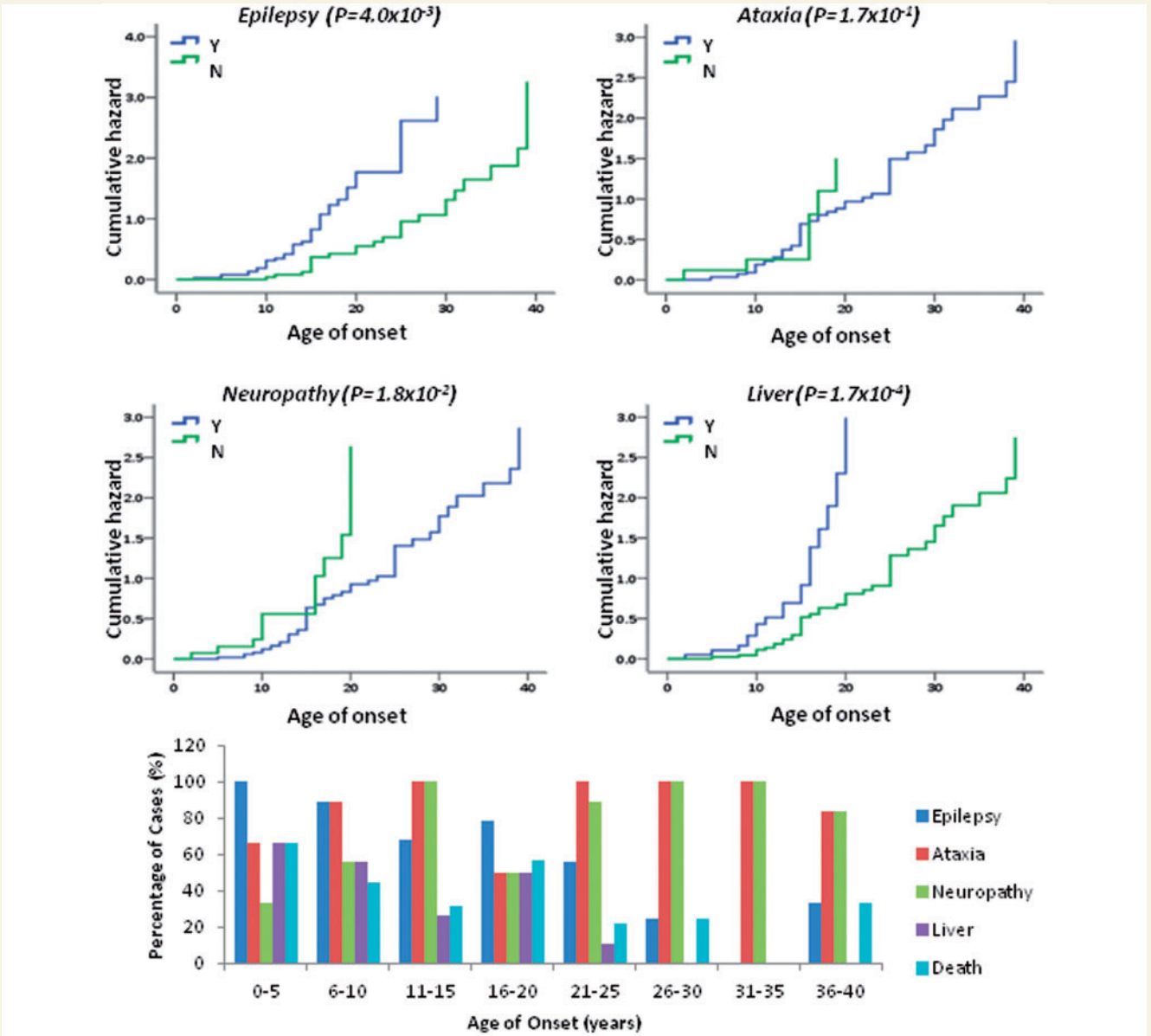

Figure 1 Kaplan–Meier hazard curves of 'age of onset' versus phenotype (i.e. epilepsy Y/N) in our cohort of 68 patients ($n = 68$) analysed by Log-Rank test (Mantel–Cox). Epilepsy and especially liver dysfunction are clearly correlated with younger age (both significant, $P \leqslant 0.05$), but neuropathy and ataxia have 'crossed lines' ($P \geqslant 0.05$). Number of patients in each category are as follows: epilepsy—with (yes) $n = 41$, without $n = 27$; ataxia—with $n = 58$, without $n = 10$; neuropathy— with $n = 54$, without $n = 14$; liver failure—with $n = 19$, without $n = 49$.

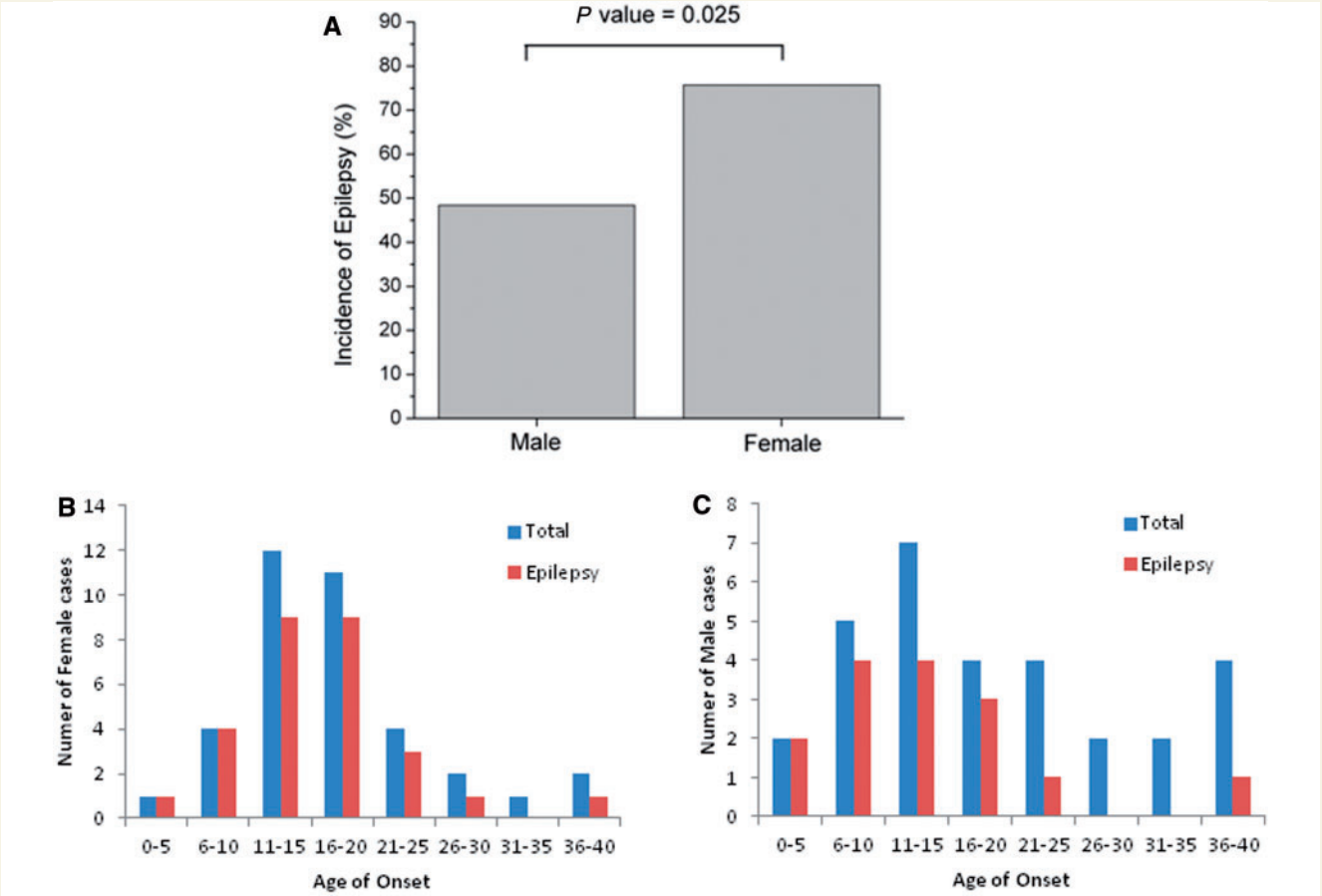

Figure 2 (**A**) Females are more likely to develop epilepsy. (**B**) The presence or absence of epilepsy in males and (**C**) females is shown.

Table 3 Summary of the clinical presentation of the homozygous p.Ala467Thr in our cohort

Clinical presentation	Present	Age	Absent	Age
Family history	35 (51%)	N/A	33 (49%)	N/A
Ataxia	58 (85%)	19.7 ± 9.76	10 (15%)	15 ± 5.0
Epilepsy	41 (62%)	15.9 ± 8.1	27 (38%)	24.2 ± 9.3
Neuropathy	54 (79%)	20.4 ± 9.68	14 (21%)	13.92 ± 6.52
PEO	29 (43%)		Ragged red fibres 39 (57%)	
Myopathy	14 (21%)		54 (79%)	
SLE	16 (24%)		52 (76%)	
Extrapyramidal	6 (9%)		62 (91%)	
Liver involvement	19 (28%)		49 (72%)	
Cardiac symptom	2 (3%)		66 (97%)	

PEO = progressive external ophthalmoparesis; SLE = stroke-like episodes.

that the unfavourable prognosis associated with epilepsy was not always related to valproate toxicity, as 5 out of 11 patients who developed fatal liver failure did not receive valproate therapy.

Muscle histology revealed ragged red fibres or cytochrome *c* oxidase–deficient fibres in 91% of the patients (31/34), a mitochondrial respiratory chain defect in 54% (13/24), multiple mitochondrial DNA deletions in 60% (18/30), whereas mitochondrial DNA depletion was present in only 13% of the patients (2/16) (Table 4). Histological, biochemical and genetic analysis of skeletal muscle did not show a correlation with disease severity. However, histological abnormalities, biochemical respiratory chain deficiency and multiple mitochondrial DNA deletions were more frequent in older patients. All patients with progressive external ophthalmoparesis and/or myopathy had abnormal muscle histology.

Interestingly, the clinical presentation was similar in siblings from seven of eight families. Clinical presentation and age at onset of patients within these families are summarized in Table 5.

Although we observed a continuum of clinical features across the group, we subdivided the patients into 'extreme phenotypes' to facilitate our search for potential genetic modifiers (Supplementary material), and allow for a comparison of allele and haplotype/haplogroup frequencies. These groups were Alpers (-Huttenlocher) syndrome (defined by the presence of an epileptic encephalopathy without evidence of a peripheral neuropathy); SANDO (defined by the presence of sensory-ataxia, neuropathy, dysarthria and ophthalmoparesis without epilepsy); patients presenting with an intermediate phenotype, including epilepsy, ataxia and peripheral neuropathy (intermediate group). Based on these criteria, 14 patients presented with Alpers (-Huttenlocher) syndrome, 27 patients with SANDO and 27 patients showed a combination of epilepsy, ataxia and sensory-axonal neuropathy (intermediate group).

Heterozygous carriers of the p.Ala467Thr mutation were not investigated in this study, but family histories of the homozygous patients did not suggest that heterozygous carriers had clinical symptoms.

Table 4 Summary of the muscle biopsy results of the homozygous p.Ala467Thr in our cohort

Muscle biopsy	Present	Absent	Not analysed
RRF	31 (91%)	3 (9%)	34
RC activity	13 (54%)	11 (46%)	44
mtDNA deletions	18 (60%)	12 (40%)	38
mtDNA depletion	2 (13%)	14 (87%)	52

mtDNA = mitochondrial DNA; RC = respiratory chain; RRF = ragged red fibres.

Genetic variants in *POLG* including the promoter region

No additional polymorphic variants were detected in the *POLG* coding region compared with the reference sequence; this is consistent with a common haplotype and single founder mutation. Many single nucleotide polymorphisms have been reported in *POLG*, some of them with high frequency in Caucasian populations (http://tools.niehs.nih.gov/polg/, Supplementary material). In one of the diagnostic laboratories involved in this study Medical Genetics Center, diagnostic sequencing of the entire coding region of *POLG* with flanking intronic sequences showed that 620 out of 897 (~70%) patients from independent families carried different single nucleotide polymorphisms in *POLG*, indicating that the wild-type sequence is much less frequent than the presence of single nucleotide polymorphisms (data not shown). In addition to the *POLG* allele containing p.Ala467Thr, a few other alleles containing single pathogenic mutations (p.Gly848Ser, p.Thr251Ile/p.Pro587Leu, p.Ala862Thr, p.Arg964Cys, p.Ser764Arg, p.Ser1104Cys, p.Lys1191Asn) lacked any other single nucleotide polymorphisms. Sequencing of the predicted 297 bp promoter region of *POLG* (Transcriptional Regulatory Element Database, Genomatix software) revealed only one reported single homozygous nucleotide polymorphism (g.4650T>A, National Center for Biotechnology Information (NCBI) rs6496572, Minor allele Frequency/minor allele count A = 0.006/14) that was found in all homozygous p.Ala467Thr patient samples, indicating that this single nucleotide polymorphism is part of the original *POLG* haplotype.

Table 5 The clinical presentation of the homozygous p.Ala467Thr *POLG* mutation in siblings

Family	Clinical presentation	Age of onset	Current age/died	Symptoms				
				E	A	N	M	L
Family A	SANDO	10	37	–	+	+	–	–
	SANDO	15	33	–	+	+	–	–
Family B	SANDO	22	37	–	+	+	+	–
	SANDO	20	37	–	+	+	+	–
Family C	SANDO	20	42	–	+	+	+	–
	ALPERS	20	26†	+	–	–	–	+
Family D	Intermediate	9	18†	+	+	+	–	+
	Intermediate	19	35	+	+	+	–	–
Family E	Intermediate	12	44	+	+	+	+	–
	Intermediate	14	20†	+	+	+	–	–
	Intermediate	13	17†	+	+	+	–	+
	Intermediate	16	44	+	+	+	–	–
Family F	Intermediate	15	44†	+	+	+	+	–
	Intermediate	8	47†	+	+	+	+	+
Family G	Intermediate	17	53†	+	+	+	+	+
	Intermediate	5	55†	+	+	+	+	–
Family H	SANDO	15	23	+	+	+	+	–
	SANDO	25	30	+	+	+	+	–

E = epilepsy; A = ataxia; N = neuropathy; M = myopathy; L = liver problem; † = died.

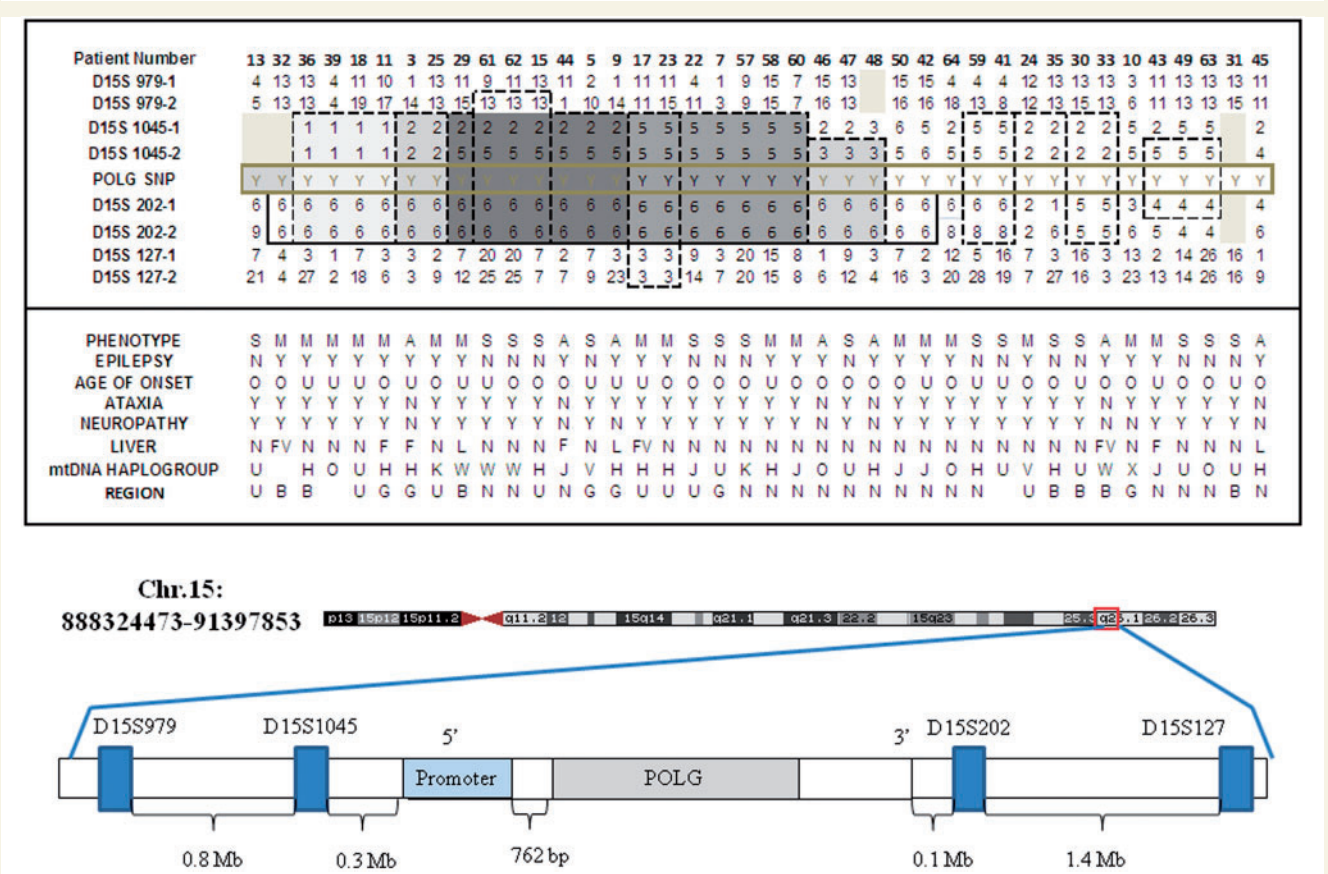

Figure 3 Schematic representation of chromosome 15 showing the position of *POLG* gene in the middle with flanking markers. Below we illustrate the location of the *POLG* promoter region and the tested markers. The table shows the manually reconstructed haplotypes for individual patients. The -1 and -2 variants in each marker mean the two alleles. Patients with matching haplotypes are blocked. The table also demonstrates the clinical aspects of each patient in respect to their haplotype. A = Alpers, S = SANDO, M = intermediate. Age of onset: O = >16 years; U = ⩽15 years. N = none; FV = fatal valproate; F = liver fatal; L = liver non-fatal; O = other; U = UK; G = Germany; B = Belgium; N = The Netherlands.

Sequencing of *POLG2*, *PEO1* and *ANT1*

Two known intronic and two exonic single nucleotide polymorphisms have been found in *POLG2*: c.1292 ± 31T>G (rs9908620) in nine patients, c.1292 ± 146C>T (rs9908927) in seven patients, a non-synonymous single nucleotide polymorphism (c.505G>A, p.Ala169Thr, rs1427463) in eight patients and the synonymous c.1269C>T, p.Ser423Ser (rs61733782) in six patients. The occurrence of these variants in our cohort was not statistically different in patients with different clinical presentations and was a fair representation of the frequency in normal Caucasian controls (Supplementary material). No correlation was detected between the missense variant c.505G>A, p.Ala169Thr and clinical phenotype (chi-square test, $P > 0.05$).

In 30% of patients (12 patients), we detected three known intronic single nucleotide polymorphisms in *PEO1* (c.1593-5C>T: rs3740485, c.1593-3T>C: rs3740486, c.1734 ± 16C>A: rs3740487) and one non-synonymous single nucleotide polymorphism, p.Val368Ile (rs17113613) in a single patient. One intronic single nucleotide polymorphism was found in *ANT1*, c.-25G>A (rs3733652). No significant correlation (χ^2 test, $P > 0.05$) was observed between these single nucleotide polymorphisms and clinical severity, and the representation of these single nucleotide polymorphisms in our cohort was similar to that in normal controls (Supplementary material). There was no correlation between clinical presentation and number of single nucleotide polymorphisms (mutational burden), suggesting no additional effect on the phenotype of more single nucleotide polymorphisms in one gene or in different genes. Detailed statistical evaluation is outlined in the Supplementary material. As the analysis of these three nuclear genes interacting with POLG on mitochondrial DNA maintenance did not show any association with clinical presentation, we did not complete the analysis of the other mitochondrial DNA maintenance genes, which have a more distant interaction with *POLG* on mitochondrial DNA.

Correlation with chromosomal haplotype

Using both genetic and clinical data, we compiled a schematic diagram (Fig. 3) to show the chromosomal haplogroups together

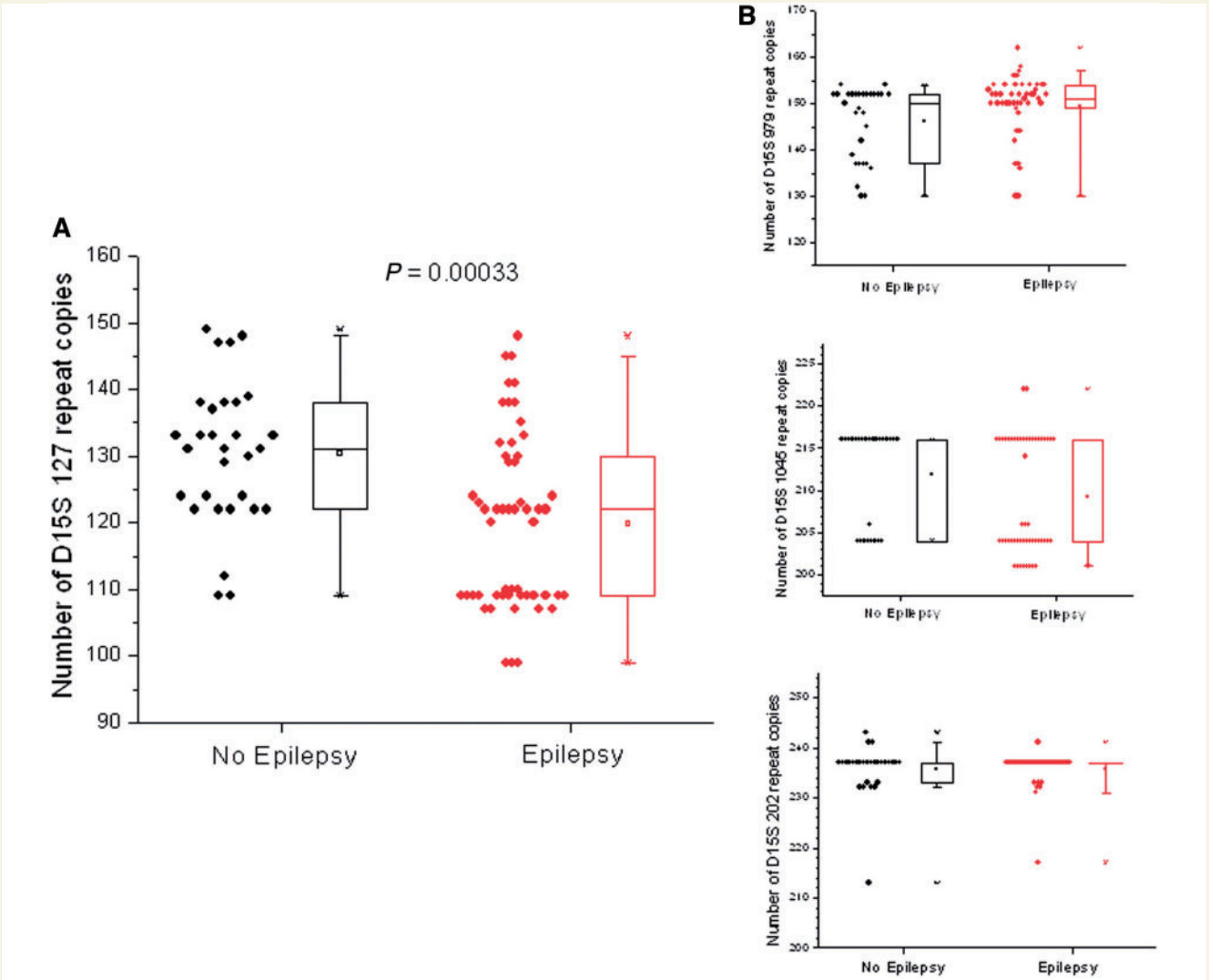

Figure 4 (**A**) The figure shows the analysis for association of the D15S127 marker and epilepsy. (**B**) The boxes to the right give the mean, median and the inter-quartile ranges for each data set. The whiskers on the boxes are the 95% confidence intervals for the data range.

and to compare these data to the phenotype. We also looked for correlation with age at onset of symptoms (<15 years/ >16 years). Analysis of the four markers showed increasing variability, especially for D15S979 and D15S127 further upstream or downstream from *POLG* (Fig. 3). There are no population frequency data available for the microsatellite markers used in this study, but the presence of homogeneous blocks in the proximity of *POLG* in the face of high variability in these markers further downstream or upstream is in keeping with a single common European ancestor for the p.Ala467Thr mutation, as suggested previously (Luoma *et al.*, 2005). Comparison of the chromosomal haplotype-blocks with age at onset and phenotype showed only one possible association involving one of the four polymorphic repeat regions (D15S127) and epilepsy. Comparing the distribution of markers in the epilepsy cases versus the non-epilepsy cases showed that the numbers of the D15S127 repeats was lower in the epilepsy cases than in controls ($P < 0.00033$, Fig. 4), suggesting that closer genealogical relationship may have an effect on the development of epilepsy. There are no population frequency data known for this marker, therefore we cannot predict whether the low repeat number is frequent in normal population or part of the ancient *POLG* haplotype. This marker is also not located within a known gene. None of the other three markers were significant at the 0.05 level (Fig. 4B). We repeated the statistical tests using the non-parametric Wilcoxon test and found the same result with only D15S127 significantly associated with epilepsy ($P = 0.00032$).

Correlation with mitochondrial DNA haplogroups

We used the Fisher's exact test to compare mitochondrial DNA haplogroup with the following major symptoms: epilepsy, ataxia, neuropathy, progressive external ophthalmoparesis, myopathy, stroke, cardiac involvement and liver involvement. Haplogroup

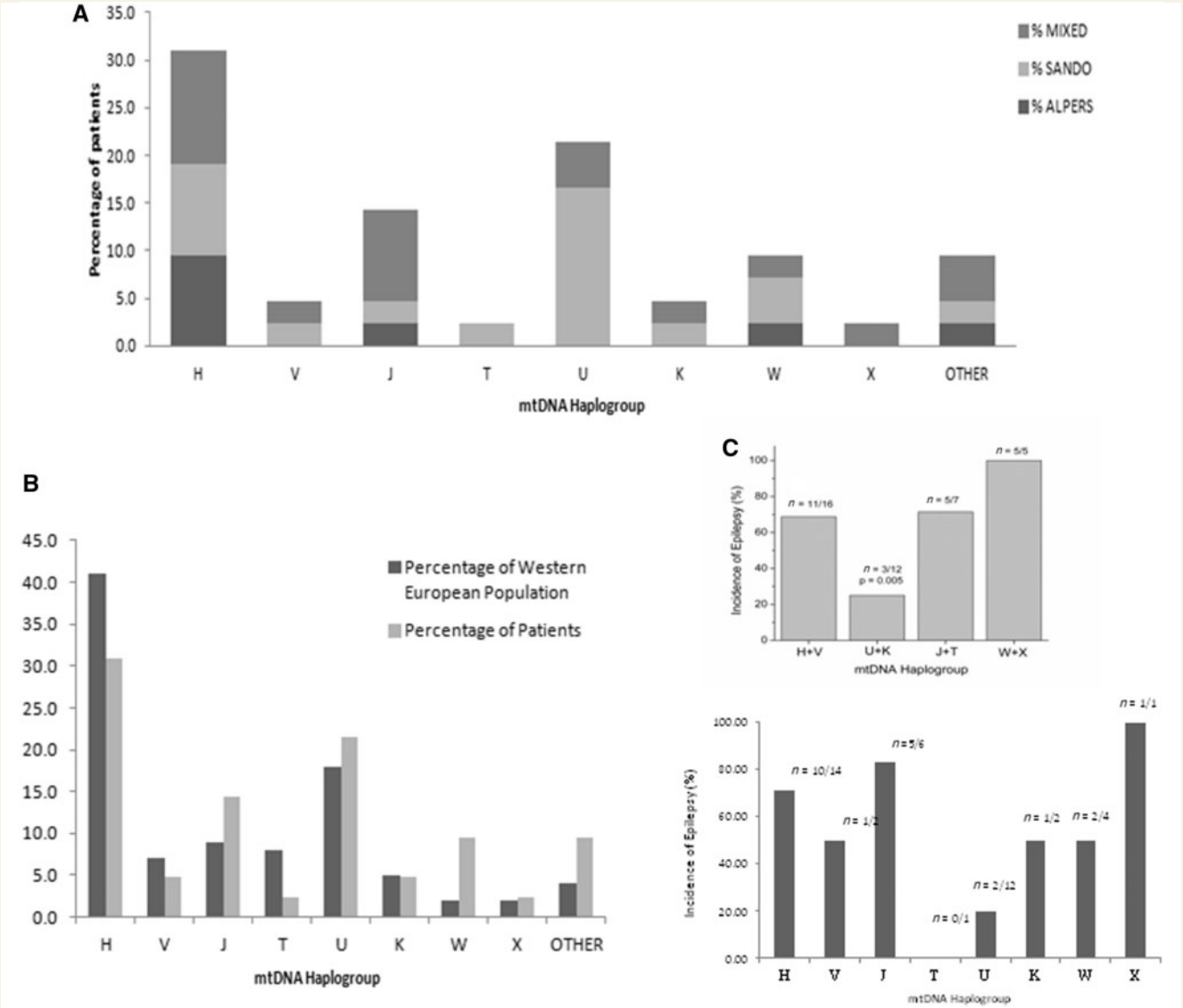

Figure 5 (**A**) Stacked graph is showing the mitochondrial DNA haplogroup as a % of the patient population and the phenotype as a % of individual mitochondrial DNA haplogroups. (**B**) Population frequency data for mitochondrial DNA haplogroups from Mitomap. (**C**) Incidence of epilepsy in our patient cohort in association with the combined and individual mtDNA haplogroups.

frequencies were similar to the European population controls (Fig. 5). Mitochondrial DNA haplogroup U alone demonstrated a significant negative association with epilepsy ($P < 0.025$), implying that mitochondrial DNA haplogroup U may protect against the development of epilepsy. For analysis, we combined the haplogroups into reasonable categories based on the phylogenetic tree. We chose to use 'H ± V' ($n = 16$), 'U ± K' ($n = 12$), 'J ± T' ($n = 7$) and 'W ± X' ($n = 5$). Our data suggest that the 'U ± K' haplogroup is protective against epilepsy (3/12, $P = 0.005$ by Fishers exact test). The effect size here is large, giving a rather good *P*-value despite the small sample size (Fig. 5). This *P*-value is low enough to remain significant even with a multiple testing correction for the four haplogroup sets. We note that all three 'U ± K' individuals with epilepsy were female, and gender is, in fact, a significant factor.

Discussion

The high clinical variability in patients carrying different *POLG* mutations is well known and has been illustrated in numerous publications during the past 10 years (Van Goethem *et al.*, 2001, 2003, 2004; Luoma *et al.*, 2004; Naviaux and Nguyen, 2004; Horvath *et al.*, 2006; Chinnery and Zeviani, 2008). Although it was originally suggested that the location of the mutation in different protein domains (exonuclease, linker, polymerase) may influence the clinical presentation (Horvath *et al.*, 2006; Stumpf and Copeland, 2011), recent studies in numerous patients do not support this hypothesis (Milone *et al.*, 2011; Tang *et al.*, 2011). However, a recent study modelled patient mutations onto POLG crystal structure and noted that in 3D, the mutations fall into specific functional domains, and specific combinations of

mutations led to specific phenotypes (Euro *et al.*, 2011). Evaluation of 92 patients with two mutant *POLG* alleles showed that these exhibited a wide spectrum of clinical symptoms (Tang *et al.*, 2011). Seizures, hepatopathy and lactic acidosis were predominant in younger patients, whereas patients with adult-onset disease had a higher percentage of myopathy, sensory-ataxia and ophthalmoparesis (Tang *et al.*, 2011). Our data show similar age-related trends and indicate that clinical presentations can be almost as diverse in patients carrying the homozygous p.Ala467Thr mutation, as in compound heterozygous cases, suggesting that the major determinant of clinical severity is not the location or type of mutation, but other genetic or epigenetic factors. Our aim in this study was to search for these factors.

Patients that were homozygous for the p.Ala467Thr presented with epilepsy and liver involvement in young age, supporting the clinical observation made earlier that epilepsy is usually the first clinical sign of POLG deficiency when the disease manifests in childhood (Isohanni *et al.*, 2011). Those who developed epilepsy in their teens also had poor prognosis because of progressive epileptic encephalopathy leading to stepwise decline in motor and cognitive functions. There was another peak of epileptic encephalopathy in adult patients in their forties or fifties, which determined clinical course and prognosis of disease in these. The manifestation of severe epileptic encephalopathy in teenage years suggests a possible modifying effect of hormonal changes, but there was no exacerbation of clinical symptoms in pregnancy. Alcohol or toxic substances may also act as triggers of acute encephalopathy, but our information was not sufficient to explore this association.

The types of epilepsy in *POLG* deficiency and in MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) show similarities in their clinicopathological evolution, in post-mortem pathological features (Tzoulis and Bindoff, 2008; Bindoff, 2011) and in MRI. Post-mortem studies suggested that status epilepticus is the result of cortical damage resulting from energy failure (Tzoulis and Bindoff, 2008; Bindoff, 2011). Liver failure, another important prognostic factor, was also associated with epilepsy, and this was exacerbated in patients receiving sodium valproate, which led to death in six of our cases. Interestingly, liver problems were not reported in any of the patients without epilepsy, although liver failure was not always triggered by valproate therapy. If epilepsy did not develop, homozygous p.Ala467Thr patients had a milder course and remained ambulatory over decades, further confirming that the major prognostic factor in *POLG* disease is epilepsy (Tzoulis, 2006). EEG monitoring in these patients may detect early signs of epileptic activity, which would lead to the prescription of preventive antiepileptic therapy, although we are not aware of any experimental data on its efficacy. The analysis of 27 Finnish patients homozygous for p.Trp748Ser showed similar results, namely, that the disease prognosis depends predominantly on the course of the epilepsy (Hakonen *et al.*, 2007). Another study of 26 patients homozygous or compound heterozygous for either p.Ala467Thr or p.Trp748Ser (Tzoulis, 2006) suggested a more severe disease course and shorter survival in compound heterozygous patients, although the age at onset was not significantly different between the two groups. In keeping with our results, the fatal prognosis was highly dependent on the occurrence of epilepsy and liver failure, especially if patients were exposed to sodium valproate (Tzoulis *et al.*, 2006). The relative non-specificity of the histological findings in children together with the severe often fatal valproate toxicity indicates that *POLG* analysis should precede sodium valproate therapy in paediatric patients with a typical phenotype.

Peripheral neuropathy has been reported in all adult patients, but only rarely in children, suggesting either that it develops later in the disease course, unless it simply reflects that electrophysiological investigations are not frequently performed in this age group. In support of our findings, peripheral neuropathy has not been reported in other series of children with *POLG* deficiency (Hunter *et al.*, 2011; Isohanni *et al.*, 2011). Ataxia was present in most of our cases (85%) and was not a discriminating factor related to disease course or prognosis. Ataxia was largely related to the presence of severe sensory neuropathy, and all patients with sensory-axonal peripheral neuropathy had ataxia. The occasional occurrence of ataxia without neuropathy and the presence of cerebellar symptoms including nystagmus and dysarthria suggest, however, that *POLG*-related ataxia may also be due to cerebellar damage. Our data suggest that both the peripheral and central components of ataxia increase with age.

Pathogenic *POLG* mutations have been reported presenting in similar ways to primary mitochondrial DNA disease [such as MELAS, MERRF (Myoclonic Epilepsy with Ragged Red Fibres), Kearns–Sayre Syndrome], reflecting without doubt the damaging effect of the impaired mitochondrial DNA polymerase on mitochondrial DNA (Deschauer *et al.*, 2007; Tzoulis and Bindoff, 2008; Bindoff, 2011). However, some frequent mitochondrial DNA-related symptoms, such as diabetes mellitus, hearing loss or cardiomyopathy were infrequent in our cohort of patients with homozygous p.Ala467Thr. Cardiac abnormalities were only noted in two cases and were minor, which is in contrast with the frequent occurrence of MELAS-related diabetes and severe cardiac disease (Kaufmann *et al.*, 2011). It is conceivable that some tissues (heart, pancreatic beta cells, inner ear) are less sensitive to *POLG*-related damage than to single mitochondrial DNA point mutations.

Interestingly, none of the patients with homozygous p.Ala467Thr had parkinsonism. There are some reports of extrapyramidal symptoms (parkinsonism, tremor, dystonia) in *POLG*-related disease (Synofzik *et al.*, 2010; Orsucci *et al.*, 2011; Hinnell *et al.*, 2012). The role of *POLG* variants in sporadic idiopathic Parkinson disease has been intensively studied (Tiangyou *et al.*, 2006; Luoma *et al.*, 2007; Eerola *et al.*, 2010), and one study suggested that *POLG* poly-Q alleles other than the conserved 10Q allele may increase susceptibility to Parkinson's disease (Luoma *et al.*, 2007; Eerola *et al.*, 2010). However, no association was detected between common *POLG* variants and sporadic idiopathic Parkinson's disease (Tiangyou *et al.*, 2006). In our series, these symptoms were extremely rare (four cases of tremor, one of blepharospasm and no parkinsonism), indicating that extrapyramidal symptoms are rare or possibly specific for mutations of the polymerase domain (Luoma *et al.*, 2005).

Histological (ragged red fibres, cytochrome *c* oxidase fibres), biochemical (respiratory chain defect) and genetic abnormalities (multiple mitochondrial DNA deletions, mitochondrial DNA

depletion) in skeletal muscle were detected more frequently in older age (Tables 3 and 4). There was no correlation between muscle alterations and clinical severity. Muscle histology had the highest detection rate (91%), followed by multiple mitochondrial DNA deletions/depletion (60%), whereas biochemical respiratory chain analysis was the least informative feature (54%). All patients with progressive external ophthalmoparesis and/or myopathy had abnormal muscle histology, although they did not always show respiratory chain deficiency or mitochondrial DNA abnormalities, confirming that the latter changes are not present in all patients and are not prognostic, thus making their role controversial in the routine diagnosis of *POLG*-related disease (Tang *et al.*, 2011).

In this study, genetic analysis of 44 patients from different Caucasian ethnic backgrounds confirmed a founder effect for the p.Ala467Thr of this mutation, with all patients having an identical *POLG* haplotype, including the predicted promoter region polymorphism. These results also exclude that changes in the putative predicted promoter region are responsible for the variable clinical severity due to an effect on expression of the POLG protein. Increasing variability of chromosome 15 microsatellites was detected in parallel with the distance from the *POLG* gene, indicating that the p.Ala467Thr mutation is an ancient European founder and occurred in a single common ancestor.

Comparison of the chromosomal haplotype blocks with age at onset and phenotype revealed an apparent relationship between the D15S127 marker and epilepsy (Fig. 4A). Further studies are needed to define whether this result is biologically significant, or related to the limited size of our cohort, and unknown confounding factors.

We tested whether co-existing mutations in other genes acting in concert with *POLG* in mitochondrial DNA maintenance may contribute to the diverse phenotypic manifestation in our homozygous p.Ala467Thr cohort. This possibility was suggested in one patient who carried in addition to the homozygous p.Ala467Thr mutation in *POLG,* also the heterozygous p.Val289Met mutation in *ANT1* (Galassi *et al.*, 2008). The clinical presentation in this patient evolved from progressive external ophthalmoparesis to a severe neurological syndrome, comprising sensory and cerebellar ataxia and peripheral neuropathy. Unusual for the homozygous p.Ala467Thr, both parkinsonism and depression were found. We detected no evidence of additional modifying factors in our analyses of the *POLG2, PEO1* and *ANT1* genes.

A protective effect of the U (UK) mitochondrial DNA haplotype against the development of Parkinson's disease has been reported in Russian-Tatar population (Khusnutdinova *et al.*, 2008). Suissa *et al*. (2009) showed that a variant defining mitochondrial DNA background 'J' significantly increased the transcriptional efficiency and elevated mitochondrial DNA copy numbers in cybrid cells compared with other genetic background. Haplogroups U and J were suggested being more loosely coupled and therefore may be associated with less reactive oxygen species production, which could explain resistance to neurodegenerative disease (Khusnutdinova *et al.*, 2008). However, haplogroup associations may be biased by the homogeneous ethnic background or the limited number of cases. The distribution of mitochondrial DNA haplogroups in our patient cohort was similar to what would be expected from a western European population, thus indicating that mitochondrial DNA haplogroup *per se* is not likely to influence the clinical presentation of the homozygous p.A467T mutation. However, mitochondrial DNA haplogroup U was significantly less frequent in patients with epilepsy, implying that being mitochondrial DNA haplogroup U may protect against developing epilepsy and raise the possibility that haplogroup U neurons are less prone to replication errors of mitochondrial DNA. We could not detect any association between mitochondrial DNA background and other clinical features, and whole mitochondrial DNA sequencing in nine patients did not show any mitochondrial DNA variants that could modify the phenotype (B. van den Bosch, unpublished observation,). However, the number of patients was rather small and a larger study should be performed to be able to draw solid conclusions regarding the influence of other mitochondrial DNA variants besides the haplogroup variants. Also, haplogroup associations may be biased by the homogeneous ethnic background or the limited number of cases. Another possibility is that tissue-specific occurrence of mitochondrial DNA deletions, mitochondrial DNA depletion or somatic point mutations may influence the clinical course (Khusnutdinova *et al.*, 2008). Our skeletal muscle data do not support this in our cohort, however, suggesting that we still have to consider other potential modifiers such as epigenetic and environmental factors (Yu-Wai-Man *et al.*, 2011).

In summary, we report the largest series of patients homozygous for the *POLG* mutation p.Ala467Thr, and found that the clinical presentation was almost as variable as in patients carrying different pathogenic *POLG* mutations and that siblings have similar clinical presentation, suggesting that genetic or other environmental or epigenetic factors may be important modifiers of the disease course. We analysed both mitochondrial DNA haplotype and gender and found that haplotype U or U/K may protect against epilepsy and that females with p.Ala467Thr are more likely to develop epilepsy.

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Supplementary material

Supplementary material is available at *Brain* online.

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