Clinical and molecular studies of disease caused by mutations of the mitochondrial DNA polymerase gamma (POLG).

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In memory of my beloved father

Contents

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Scientific environment

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List of publications

Paper I Tzoulis C, Engelsen BA, Telstad W, Aasly J, Zeviani M, Winterthun S, Ferrari G, Aarseth JH, Bindoff LA. *The spectrum of clinical disease caused by the A467T and W748S POLG mutations: a study of 26 cases.* Brain, 2006. **129**(Pt 7): p. 1685-92.

Paper II Engelsen BA, Tzoulis C, Karlsen B, Lillebø A, Laegreid LM, Aasly J, Zeviani M, Bindoff LA. *POLG1 mutations cause a syndromic epilepsy with occipital lobe predilection.* Brain, 2008. **131**(Pt 3): p. 818-28.

Paper III Tzoulis C, Neckelmann G, Mørk SJ, Engelsen BA, Viscomi C, Moen G, Ersland L, Zeviani M, Bindoff LA. *Localized cerebral energy failure in DNA polymerase gamma-associated encephalopathy syndromes.* Brain, 2010. **133**(Pt 5): p. 1428-37.

Paper IV Tzoulis C, Papingji M, Fiskestrand T, Røste LS, Bindoff LA. *Mitochondrial DNA depletion in progressive external ophthalmoplegia caused by POLG1 mutations.* Acta Neurol Scand Suppl, 2009(189): p. 38-41.

List of important abbreviations

ADC	Apparent diffusion coefficient
ATP	Adeninosine-triphosphate
CNS	Central nervous system
COX	Cytochrome oxidase
CPM	Complex partial motor
CT	Computed tomography
DWI	Diffusion weighted imaging
EEG	Electroencephalography
EMG	Electromyography
EPC	Epilepsia partialis continua
FAD	Flavin adenine dinucleotide
FLAIR	fluid-attenuated inversion recovery
IOSCA	Infantile spinocerebellar ataxia
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	Myoclonus-epilepsy with ragged red fibres
MIRAS	Mitochondrial recessive ataxic syndrome
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy

MSCAE	Mitochondrial spinocerebellar ataxia and epilepsy
MtDNA	Mitochondrial DNA
mtSSB	Mitochondrial-DNA single strand binding proteins
NAD	Nicotinamide adenine dinucleotide
NCV	Nerve conduction velocity
OXPHOS	Oxidative phosphorylation
PCR	Polymerase-chain reaction
PEO	Progressive external ophthalmoplegia
POLG, pol γ	Polymerase-gamma
SD	Standard deviation
SDH	Succinate-dehydrogenase
SE	Status epilepticus
SKM	Skeletal muscle
SLL	Stroke-like lesions
SPM	Simple partial motor
SPS	Simple partial sensory

Abstract

Background

Mutations in the gene encoding the DNA-polymerase gamma (POLG), the enzyme that replicates and repairs the mitochondrial genome, are an important cause of human disease and disability. Over 130 pathogenic mutations have been reported and these produce a wide spectrum of disease that mainly includes syndromes with myopathy or/and encephalopathy.

Aims

To define the clinical features, natural history, pathophysiology and molecular pathogenesis of POLG-disease focusing particularly on disease caused by the mutations c.*1399G>A,* p.A467T and c.*2243G>C,* p.W748S, but including other POLG mutations causing encephalopathic disorders such as Alpers' syndrome.

Patients and methods

The studies presented herein were performed on a large group of patients (n=42) with POLG-disease comprising 36 with juvenile/adult encephalopathy, 4 with infantile encephalopathy and 2 with progressive external ophthalmoplegia (PEO). Patients were categorized according to genotype and clinical features and studied by a variety of clinical, pathological and molecular methods. Studies included thorough clinical evaluation and follow-up, clinical and electrophysiological investigation of the epilepsy, imaging methods comprising conventional magnetic resonance imaging (MRI), diffusion imaging (DWI) and magnetic resonance spectroscopy (MRS), post-mortem histological and histochemical examination and molecular studies of mitochondrial DNA in biopsy and post-mortem tissues.

Results and conclusions

The common POLG mutations, A467T and W748S, caused a clinically well-defined entity usually presenting in the mid-teens (mean 15.2 years, range 1.5-45) and characterised by progressive spinocerebellar ataxia, encephalopathy, neuropathy, migraine-like headache, myoclonus and late-onset external ophthalmoplegia. Most of our patients developed epilepsy, either at onset or during the course of their disease, and these experienced acute episodic exacerbations with rapidly progressive encephalopathy and expanding stroke-like cerebral lesions. Episode mortality was high (~50%) and in addition, several others suffered from valproate induced liver toxicity. MSCAE showed a complex epileptic semiology including a variety of clinical seizure types and frequent status epilepticus. Epilepsia partialis continua and an occipital epileptogenic focus on EEG were characteristic findings and should provide a clue for the diagnosis.

The prognosis of MSCAE depended on the genotype. Patients who were compound heterozygous (A467T/W748S) had a significantly ($p=0.003$) worse prognosis than homozygous patients with significantly lower life expectancy.

We found that MRI is a sensitive detector of disease activity in POLG-encephalopathy. Typical findings included high T2 signal lesions in the thalamus, cerebellar white matter and olivary nuclei. During exacerbation episodes both MSCAE and Alpers' patients commonly developed stroke-like cortical lesions which had a predilection for the posterior brain and showed restricted diffusion and lactate accumulation in the acute phase. Stroke-like lesions evolved dynamically mirroring clinical progression of the episode and their course had prognostic significance.

Histology of affected cortical and deep CNS areas revealed selective neuronal loss, eosinophilic necrosis and laminar cortical necrosis. Histochemistry showed reduced or absent cytochrome oxidase (COX) activity in some neurons, but was normal in blood vessels. MtDNA studies revealed tissue-specific depletion and multiple deletions. The findings of mtDNA damage and histochemical COX deficiency suggest that POLG mutations lead to secondary dysfunction of the respiratory chain, which is predicted to cause energy failure due to ATP deficiency. We present here findings suggesting that

regional chronic energy failure indeed occurs in the CNS of patients with POLG-encephalopathy. Moreover, acute episodic energy crisis may occur, which appears to be triggered and/or sustained by epileptic seizures.

1. Introduction

1.1 Historical overview

It is generally accepted today that mitochondria originated between 3.45 and 2 billion years ago as a result of an ancient endosymbiosis between an α-proteobacterial organism and an anaerobic host cell. The proteobacterial endosymbiont granted its host the ability to use atmospheric oxygen in order to produce energy from carbohydrates and fat by the process of oxidative phosphorylation (OXPHOS). The ability to perform aerobic respiration in an environment with increasing oxygen concentration (thanks to the parallel action of the primitive plant ancestors) gave the cells an evolutionary advantage and has probably been one of the key events that allowed the evolution of large multicellular animal organisms. Meanwhile, the endosymbiont adapted to its new intracellular environment, apparently translocated most of its own genes to the cell nucleus and became gradually assimilated into its host eventually becoming the organelle that today we call mitochondrion [1].

Since their discovery in the mid-late 1800s, mitochondria have been the focus of cellular, biochemical and molecular studies revealing the complexity and uniqueness of their structure and function. It was, however, not before the early 1960s that mitochondria were for the first time implicated in human disease. In 1962 the Swedish endocrinologist Rolf Luft described a young woman with a hypermetabolic syndrome and biochemical and histological findings suggesting mitochondrial dysfunction [2]. Luft syndrome was the first mitochondrial disease to be described and ironically also the rarest, since only one more case has been described [3]. In 1963 it was shown that mitochondria contain their own genome, mitochondrial DNA (mtDNA) [4, 5], which was fully sequenced in 1980 [6]. Studies have also shown that mitochondria, and therefore mtDNA, are maternally inherited and that mtDNA acquires sequence changes frequently.

The 1970's and early 1980's were marked by an increasing number of reports describing biochemical defects in the respiratory chain, but it was not until the late 1980's that the first mtDNA defects were identified. In 1988 Holt and co-workers showed that large scale mtDNA deletions can cause myopathy with progressive external ophthalmoplegia (PEO) in humans [7], while at the same time the first pathogenic mtDNA point mutation was described in Leber's hereditary optic neuropathy [8]. Thus the role of mitochondrial genetics in human disease was revealed.

In the years that followed, mtDNA mutations were found to be the cause of several distinct clinical syndromes including mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres (MERRF), maternally inherited Leigh disease, Kearns-Sayre syndrome and others. Patients with these syndromes often have complex, systemic diseases with multiple organ involvement including the central and peripheral nervous system, muscle, liver, heart, kidney, exocrine and endocrine pancreas. Due to the nature of mtDNA, these disorders are either maternally inherited, or sporadic.

In 1989, a family with autosomal dominant PEO and multiple mtDNA deletions in skeletal muscle was described [9]. Later it was revealed that the disease was caused by mutations of a nuclear gene that encoded a mitochondrial helicase called Twinkle [10]. Twinkle unwinds mtDNA to prepare it for replication and its mutations cause disease by inducing secondary damage to the mitochondrial genome. The same year, Van Goethem et al [11] reported a family with autosomal dominant PEO caused by a missense mutation in another nuclear-encoded mitochondrial protein, the polymerase gamma (POLG), which replicates and repairs the mitochondrial genome. Since the discovery of the first Twinkle and POLG pathogenic mutations the group of nuclear mitochondrial diseases has gradually increased in numbers and clinical heterogeneity as novel genes are being identified and new syndromes are constantly recognised.

The discovery of Twinkle, POLG and other nuclear genes opened a new chapter in mitochondrial medicine and defined a novel concept in clinical and molecular

genetics - that of disease caused by mutations of two genomes. The respiratory chain is controlled by both the nuclear and mitochondrial genomes and continuous cross-talk between these two genomes is necessary for proper mitochondrial function. Moreover, mutations of nuclear genes involved in mtDNA homeostasis (e.g. replication and repair) cause cellular dysfunction and disease via secondary damage of the mitochondrial genome.

Mitochondrial dysfunction has not only been associated with monogenetic diseases, it is also implicated in various neurodegenerative diseases including parkinsonism, motor-neuron disease and Alzheimer's disease, neoplasia and even the process of ageing [12, 13].

1.2 Mitochondrial structure and function

Mitochondria are the energy-producing units of the cell. They are found in most animal and plant cells, although their numbers per cell vary from a few hundreds to over 100,000 in an oocyte. Their basic form is usually that of a tubular, tortuous structure spread through the cytoplasm, much like a network. Mitochondria are surrounded by two lipid bilayer membranes: a smooth outer membrane and a highly convoluted inner membrane. The space between the two membranes is called intermembrane space and the space surrounded by the inner membrane is termed mitochondrial matrix. MtDNA is localised, transcribed and translated in the matrix. The inner membrane is highly convoluted forming multiple projections into the matrix called cristae. The energy-generating pathway, the respiratory chain, is located within the inner membrane (Figure 1). The respiratory chain generates chemical energy, adenosine-triphosphate (ATP), via the process of oxidative phosphorylation. Mitochondria are, however, involved in numerous other biochemical processes including fatty acid oxidation, heme and steroid metabolism, calcium storage and mobilisation, apoptosis and ammonia detoxification via the urea cycle[14].

1.2.1 The respiratory chain generates energy via the process of oxidative phosphorylation (OXPHOS)

The human respiratory chain consists of ~90 polypeptide subunits organised into five complexes, termed complexes - I to V, and two electron shuttle molecules called co-enzyme Q10 (CoQ, ubiquinone) and cytochrome c (Cyt-c). Thirteen of the respiratory chain subunits are encoded by the mitochondrial genome, while the remaining are encoded by the nuclear genome (Table 1, figure 1). The respiratory chain is where the process of oxidative phosphorylation (OXPHOS) takes place, which is the most important energy generating mechanism of nearly all animal cells (Figure 1).

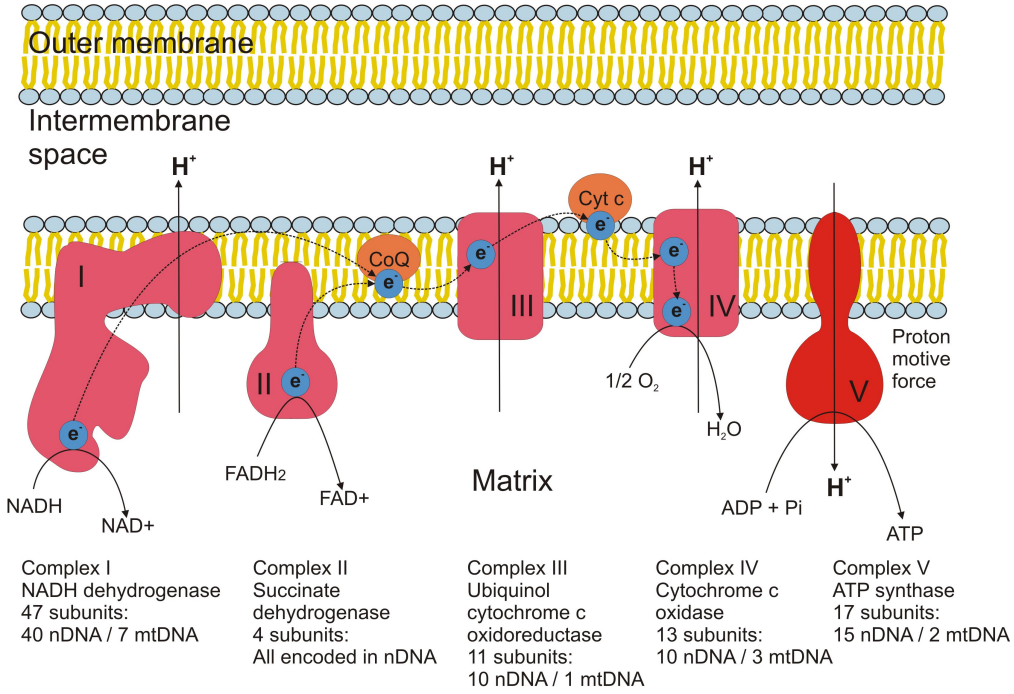


Figure 1. The respiratory chain.

Reduced cofactors such as NADH and FADH2, which are generated by glycolysis, citric acid cycle, fatty acid oxidation and other processes, are reoxidised by complexes I and II . Electrons are transferred from cofactors to complex I or II and subsequently to complexes III and IV in a series of oxidoreduction reactions before finally reacting with molecular oxygen to generate water. During this process hydrogen cations are

pumped from the mitochondrial matrix to the intermembrane space, creating an electrochemical gradient known as proton motive force. Protons are finally "allowed" to flow back into the matrix via the last complex, the ATP-synthase, which couples the flux of protons to energy formation in the form of ATP [14].

Complex	**I**	**II**	**III**	**IV**	**V**
Nuclear subunits	39	4	10	10	14
Mitochondrial subunits	7 ND1-6, ND4L	0	1 CYB	3 COX I-III	2 ATPase 6 ATPase 8
Total	46	4	11	13	16

Table 1. Composition and encoding of the respiratory chain complexes.

ND: NADH-dehydrogenase, CYB: cytochrome-b, COX : cytochrome oxidase.

1.2.2 Structure of mitochondrial DNA (mtDNA)

Mitochondria (and chloroplasts in plants) are the only organelles, other than the nucleus, that contain genetic information. Human cells contain multiple copies of mtDNA ranging from a few hundreds in a sperm cell to many thousands in an oocyte. MtDNA is inherited exclusively from the mother, although a single case of paternal transmission of mitochondrial disease has been reported [15].

MtDNA is a circular, double stranded molecule of approximately 16.5kb (Figure 2). Purine and pyrimidine content is unevenly distributed between the two strands resulting in a purine rich heavy strand and a purine poor light strand. Both strands function as templates, with the heavy strand encoding most products.

MtDNA comprises in total 37 genes encoding 13 peptides, 22 tRNAs and 2 rRNAs (Figure 2). The 13 peptides encoded in the mitochondrial genome are subunits of the respiratory chain complexes I, III, IV and V (Table 1). The remaining respiratory chain subunits are encoded by the nuclear genome along with over 1000 other proteins that are known to localise in mitochondria. Mitochondrial genes are continuous, with no intervening introns. MtDNA contains one major non-coding region (NCR) called the displacement loop. The D-loop (D-loop) contains the promoters for the transcription of the two strands and the replication origin of the heavy strand (O_H) according to some models of replication [16, 17].

1.2.3 Replication of mitochondrial DNA

Two basic models have been proposed for mtDNA replication: the asynchronous strand displacement model and the strand coupled model.

Asynchronous strand displacement

In the asynchronous strand displacement model, replication of the heavy strand starts first at O_H and proceeds gradually displacing the parental heavy strand. The displaced, single stranded parental heavy strand is covered by mitochondrial single-strand DNA-binding proteins (mtSSB), which protect it from degradation. When heavy strand replication has reached about 2/3 of the genome, the origin of replication of the light strand (O_L) is exposed and light strand replication starts, proceeding in the opposite direction. Because replication of the two strands starts at temporally distinct points, replication of the daughter molecule containing the nascent heavy strand is completed first and the two daughter molecules segregate before replication of the daughter molecule containing the nascent light strand is completed.

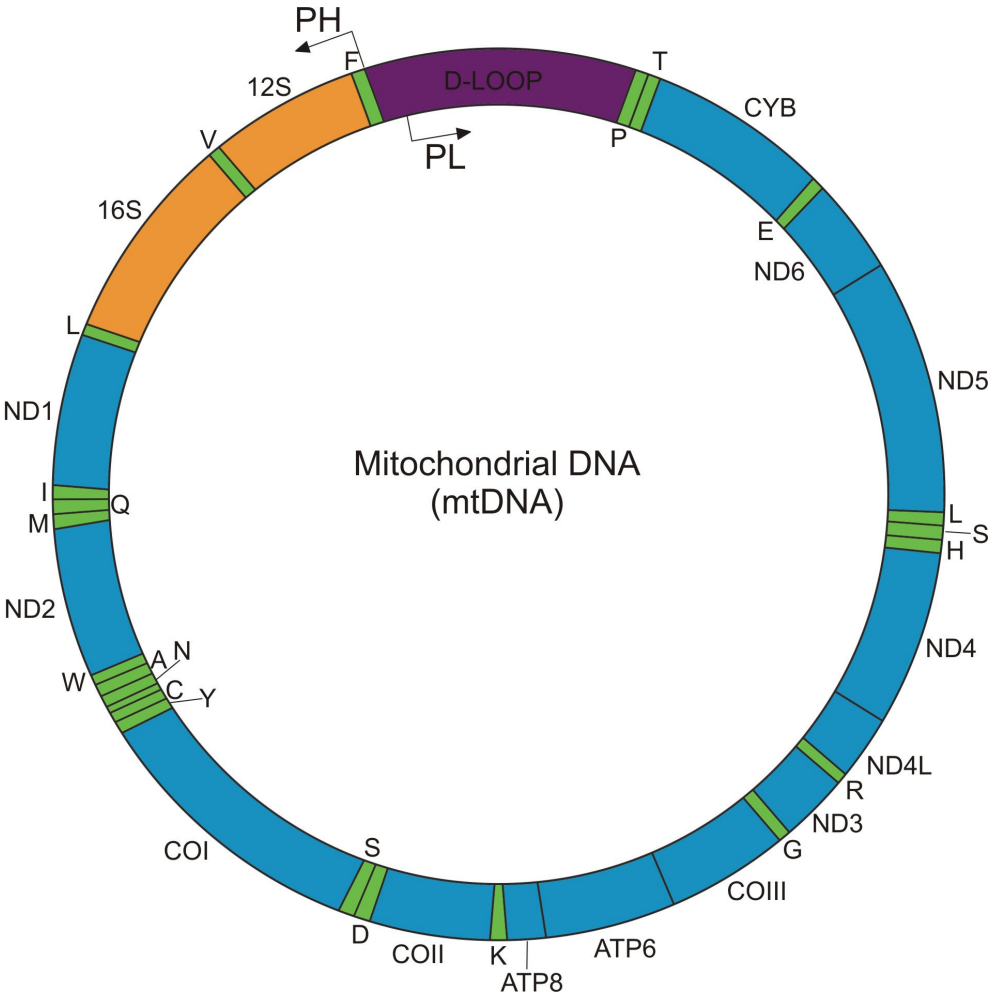


Figure 2. Mitochondrial DNA.

MtDNA encodes 13 peptides (blue): 7 subunits of complex I (ND1-6 & ND4L), 1 subunit of complex III (CYB), 3 subunits of complex IV (COI-III) and 2 subunits of complex V (ATPase6 and 8). The transfer (green) and ribosomal (orange) RNAs needed for mitochondrial translation are also encoded in mtDNA.

The segregated, still replicating, mtDNA molecule containing the new light strand will therefore contain a "gap" and is termed gapped circle [18, 19]. A modification of the asynchronous strand displacement model has been proposed which involves ribonucleotide incorporation throughout the lagging strand (RITOLS). According to this model the displaced parental heavy strand is covered by RNA instead of mtSSB. The RNA is then replaced by DNA to produce a double stranded lagging strand. It has been shown that RNA may be lost during standard extraction techniques and it has been suggested that this loss may account for the partially single stranded replication intermediate mtDNA species detected by various techniques including electron or atomic force microscopy. It is possible that both the strand-coupled and RITOLS models may occur in human cells and that cells may be able to switch between them under different conditions [20-22].

Strand coupled model

In the strand coupled model, replication of both strands starts simultaneously within a "replication zone" thought to be broader than the D-loop and daughter strand formation proceeds synchronously throughout the genome. In this model, no single stranded intermediates or gapped circles are made [18, 19].

The mitochondrial replisome

Several nuclear encoded proteins are involved in mtDNA replication and there are probably more to be discovered. The minimum number of proteins required to make a functional replisome, capable of replicating full length mtDNA *in vitro* are: the mitochondrial DNA polymerase gamma (pol γ, POLG), a helicase called Twinkle and mitochondrial single stranded binding proteins (mtSSB) [19].

1.3 The mitochondrial DNA-polymerase gamma

Polymerase gamma (POLG, Pol γ) is a DNA-dependent, DNA polymerase and the only enzyme that replicates and repairs the mitochondrial genome. The holoenzyme is a heterotrimer composed of one catalytic subunit (pol γA) of 139kDa, encoded by

POLG1 on chromosome 15q25, and a dimer of two accessory subunits (pol γB) of 53kDa encoded by *POLG2* on chromosome 17q (Figure 3).

The catalytic Pol γA shares homology with type-I (A-family) DNA polymerases such as the phage T7 polymerase and *Escherichia coli* DNA pol I. It also has unique features, such as a large spacer domain, not found in other polymerases of this family [23-25]. The structure and size of the pol γ enzyme is highly conserved in vertebrates: it has been shown that frog and murine pol γB can stimulate replication by the human pol γA homologue in primer elongation assays [26, 27]. Yeast lacks pol γB and drosophila only has one subunit, due to lack of the amino acids necessary for dimerisation, suggesting that the incorporation of pol γB into pol γ and more so its dimerisation are relatively recent evolutionary events [23]. Pol γB shares significant homology with prokaryotic class II glycyl tRNA synthetases suggesting it might have evolved from similar molecules [26, 27]

The catalytic pol γA comprises a mitochondrial leader sequence (residues 1-170), a polymerase domain (residues 441-475 and 786-1239), which replicates mtDNA, an exonuclease domain (residues 171-440), which proof-reads and repairs newly synthesised DNA in a 3'-5' direction, and a large, intervening spacer or linker region (residues 476-785) to which the accessory pol γB subunits bind (Figure 3, 4).

Pol γA has a canonical polymerase "right hand" configuration that comprises finger, palm and thumb subdomains (Figure 4). The palm (residues 816-910 and 1096-1239) contains the catalytic site and is the most highly conserved part of the molecule. Like in other DNA-polymerases, the palm is positively charged in order to stabilise the negatively charged DNA backbone and contains two magnesium-complexed aspartate residues (D^{890} and D^{1135}), which are vital for the formation of the phosphodiester bond between the 3' OH end of the growing nascent strand and the phosphate group of the incoming nucleotide.

1.3.1 POLG structure and function

The finger (residues 911-1095) and thumb (residues 441-475 and 786-815) domains are thought to have similar functions as in other DNA-polymerases. The finger domain binds to the DNA template and incoming dNTP. Once the base of the dNTP has been correctly matched with the corresponding base at the 3'-end of the template, the finger domain changes its conformation in order to push the incoming dNTP into the catalytic groove of the palm and bring it in contact with the magnesium ions that will catalyse the formation of the new phosphodiester bond. The thumb domain interacts with the nascent DNA strand and helps keep the polymerase on its template DNA, thus increasing processivity.

The exonuclease domain of pol γA repairs replication errors by 3'-5' excision and is, therefore, important for fidelity. The selectivity between forward polymerisation and excision repair is based on kinetic partitioning between the polymerase and exonuclease activities (i.e. the two processes are governed by different kinetics). After each new nucleotide is added by the polymerase at the 3'-end of the growing nascent strand, POLG will either proceed to adding the next nucleotide, or allow the exonuclease site to remove the newly incorporated nucleotide. Normally polymerisation happens at a much higher rate (~300 s^{-1}) than the slow migration of DNA to the exonuclease site (~0.2 s^{-1}). When a mismatched nucleotide is added, however, polymerisation stalls, while the rate of DNA transfer to the exonuclease active site increases strongly favouring repair [28]. While the exact mechanisms by which base mismatches are recognized by pol γ remain unknown, steric fit is thought to be important. In addition, recent work has shown that lack of hydrogen bonding may also play an important role [29].

The ~400 amino acid long spacer domain is located between the polymerase and exonuclease regions and connects to them via the two helices of the thumb subdomain (figure 4). Binding of the accessory subunits to the spacer increases holoenzyme processivity and reduces fidelity by enhancing DNA affinity and polymerization rate and simultaneously suppressing exonuclease activity [25]. The spacer comprises an intrinsic processivity subdomain (residues 475-510 and 571-785) and an accessory

interacting determinant subdomain (residues 511-570), which binds to the accessory subunit. The intrinsic processivity subdomain contains an area rich in positively charged amino acids termed the K tract (^{496}KQKKAKKVKK^{505}). When the accessory subunit binds to the accessory interacting determinant subdomain of the spacer, a conformational change exposes the positively charged K tract, which interacts with the template DNA increasing its contact length with the holoenzyme. This is believed to be one of the mechanisms by which the binding of the accessory subunit increases processivity of the holoenzyme [25].

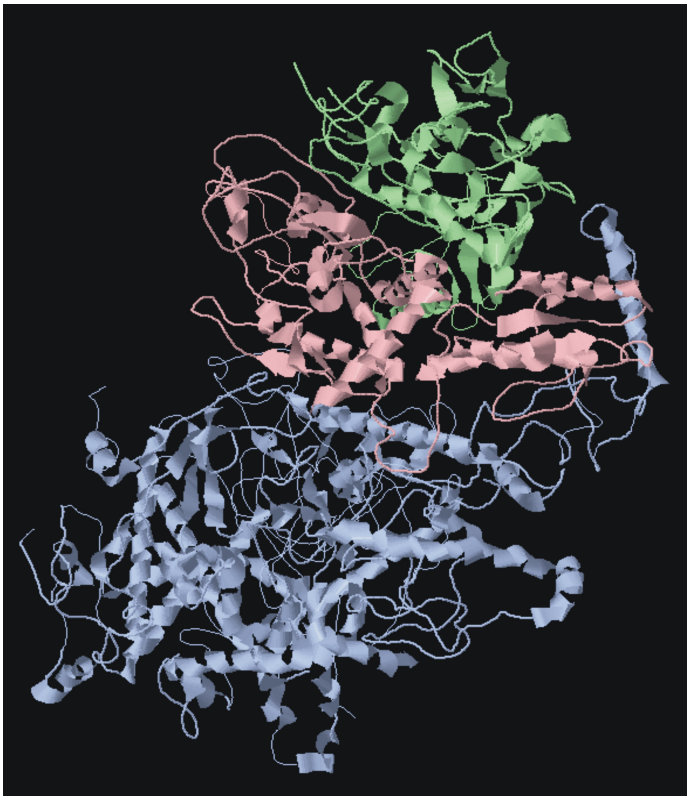


Figure 3. The structure of pol γ holoenzyme. The holoenzyme comprises one catalytic (blue) and two accessory (green and pink) subunits (protein databank ID: 3ikm) [25].

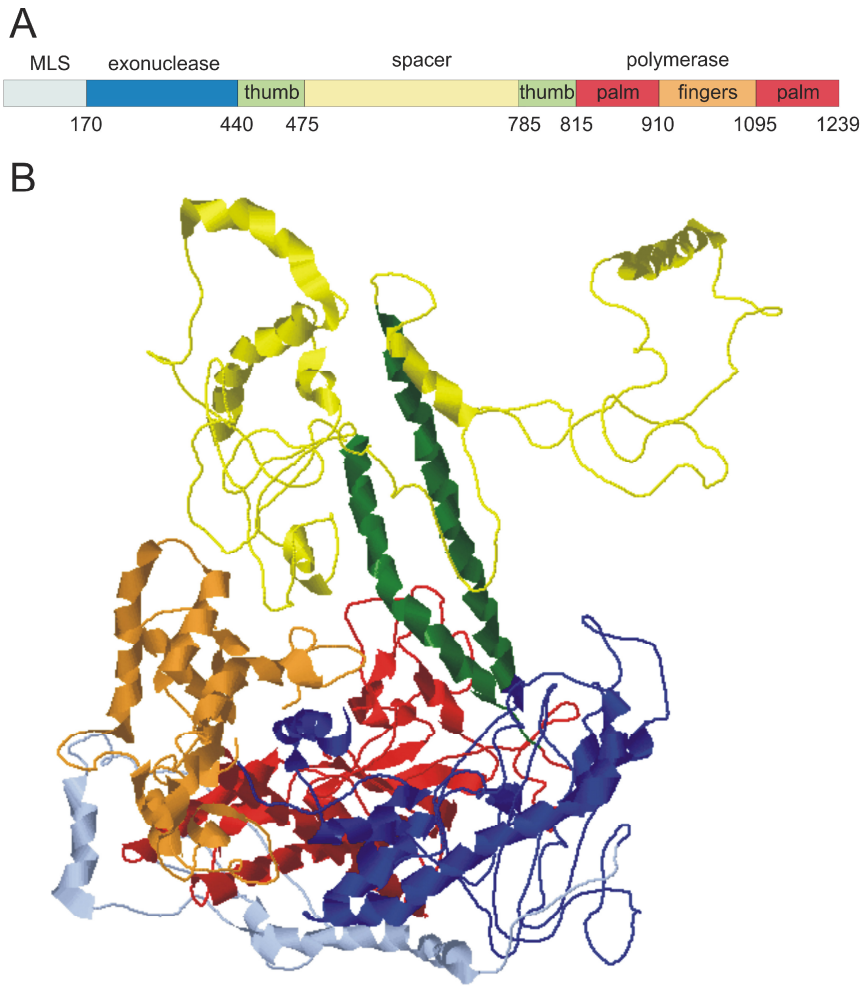


Fig 4. The structure of the catalytic pol γ subunit (pol γA). A: linearized schematic depiction. B: three dimensional figure showing secondary and tertiary protein structure when the polymerase is not bound to DNA. Alpha-helices are depicted as springs and beta-sheets as flat arrows. The catalytic subunit comprises five subdomains which, starting from the N-terminus, are: mitochondrial leading sequence (light blue), exonuclease (dark blue), palm (red), fingers (orange), thumb (green) and spacer (yellow). Protein databank ID: 3ikm [25].

1.3.2 POLG and human disease

Over 130 pathogenic mutations have been described in *POLG1*. These cause a broad spectrum of disease ranging from late onset myopathies to devastating infantile hepatoencephalopathies. Two mutations have been reported in *POLG2* causing autosomal dominant progressive external ophthalmoplegia [30, 31]. POLG-associated disease may be classified into two main groups, myopathies and encephalopathies, according to the organ system predominantly involved. The encephalopathies show significant overlap of clinical and molecular features, but may be further divided according to age of onset into an infantile type and a juvenile/adult type (Table 2).

POLG-myopathies - Progressive external ophthalmoplegia (PEO)

Over 50 *POLG1* mutations affecting all major domains of the protein cause a primarily myopathic phenotype characterized by progressive external ophthalmoplegia (PEO) with varying degrees of proximal limb weakness [32]. Inheritance is autosomal recessive or dominant according to mutation. In addition, PEO is a common clinical manifestation of many POLG mutations that cause more complex syndromes with nervous system and/or multisystem disease.

Based on reported cases, mean age of onset in this group is ~37 years, but patients may present at almost any age from early childhood to over 70 years. The autosomal recessive form has a slightly later onset (40.4 years, n=40, range 10-75, SD 16.3) than the dominant (32.8 years, n=31, range 4-66, SD 16.4). Clinically, patients have bilateral blepharoptosis and ophthalmoplegia, and most have proximal limb weakness of varying severity. Additional features may include oropharyngeal myopathy with dysphagia and dysarthria, facial myopathy, exercise intolerance, rhabdomyolysis, respiratory weakness and cardiomyopathy. Non-myopathic features may occur (PEO plus syndromes) and these include cataract, sensorineural deafness, peripheral neuropathy, ataxia, hypogonadism and parkinsonism [33-54].

POLG-encephalopathies

At least 75 *POLG1* mutations, affecting all functional domains of the catalytic POLG subunit, cause disease that involves the brain [32]. POLG encephalopathies are inherited in an autosomal recessive manner. Several distinct clinical syndromes and consistent genotype-phenotype associations are identified, but the clinical features overlap significantly and classification is not easy. Several classification systems have been proposed all with their advantages and disadvantages. One of the most clinically useful distinctions is to group POLG encephalopathies according to age of onset into infantile and juvenile/adult syndromes (Table 2).

Disease		**Inhe-ritance**	**Onset Range**	**Course**	**Myopathy**	**Encephalopathy**		**Liver disease**
						Ataxia	**Epilepsy**	
Myopathy (PEO) N=71		AR/ AD	36.5 4-75	chronic progressive	+	-	-	-
Encephalopathy	MSCAE N=73	AR	18.4 1.5-45	episodic progressive	late	+	most patients: ~65%	Valproate induced, rarely spontaneous
	Alpers' N=65	AR	0.9 0-4	episodic progressive	rare	+	+	Spontaneous

Table 2. The clinical spectrum of POLG disease. AR: autosomal recessive, AD: autosomal dominant. N: number of patients in each group. The data are from our material and review of the literature. Ages are given in years. References for POLG-myopathy: [33-54], MSCAE: [50, 55, 57-59, 62, 66, 73-78 and unpublished material], Alpers': [46, 48, 54, 55, 57-59, 61-71 and unpublished material].

Most mutations can cause infantile onset encephalopathy with frequent liver involvement and high mortality at an early age. The terms Alpers' or Alpers -

Huttenlocher syndrome are commonly used to describe these conditions [44, 46, 48, 53-71]. Some clinicians prefer to use the name Alpers' disease for pure encephalopathies and reserve the term Alpers'-Huttenlocher syndrome for cases with brain and liver disease. In this work, the term Alpers' disease is used synonymously with infantile POLG encephalopathy irrespective of liver disease.

The most common POLG mutations are the c.1399G >A, p.A467T and c.2243G > C, p.W748S and these cause a juvenile or adult onset encephalopathy syndrome, which, in its most severe form is characterised by the combination of progressive spinocerebellar ataxia, epilepsy and episodic exacerbations with stroke-like cerebral lesions. This condition has been variously called mitochondrial recessive ataxic syndrome (MIRAS) and mitochondrial spinocerebellar ataxia and epilepsy (MSCAE) [55, 72, 73].

2. Aims of the studies

The work presented in this thesis describes ongoing studies of POLG-disease in a large group of patients that now numbers 42 and includes patients with myopathy (n=2), MSCAE (n=36) and Alpers' disease (n=4). We employed a combination of clinical, histological and molecular methods in order to define clinical syndromes and study the disease mechanisms not only in the laboratory, but also dynamically in the living patient. Our findings describe the clinical spectrum and natural evolution of POLG-encephalopathy, focusing on the syndrome of MSCAE caused by the common mutations p.A467T and p.W748S, and cast light into the pathophysiology of these complex disorders.

The basic aims of this work were:

- To define the clinical spectrum and natural history of POLG-disease caused by the mutations A467T and W748S: Papers I, II, III and unpublished material.
- To characterise the clinical and electrophysiological features of the epilepsy in POLG-disease caused by the p.A467T and p.W748S mutations: Paper-II.
- To describe the central nervous system changes in POLG-encephalopathy and investigate their pathophysiology by using imaging and histology: Paper III.
- To investigate the molecular pathogenesis of POLG-disease by studying mitochondrial DNA changes in various patient tissues: Paper IV and unpublished material.

3. Patients and methods

3.1 Patients

Forty-two patients with POLG-disease comprising 40 patients with encephalopathy syndromes (36 patients with MIRAS/MSCAE and 4 with Alpers' disease) and 2 patients with myopathy (autosomal recessive PEO) were studied. Of these, 26 patients were reported in paper-I, nineteen in paper-II, thirty-two in paper-III and two in paper-IV. Five patients (CP-1C and WS-14B, 16A, 17A, 17B) have not been published (Appendix I lists the patients reported in each paper and their codes).

Patient codes for MSCAE consist of two letters, which denote the POLG mutation (AT: A467T, WS: W748S, CP: compound A467T/W748S), followed by a number describing the family and a letter describing the individual. Patients with the same number are siblings. For example patients AT-1A and AT-1B both have the A467T mutation and are siblings. Alpers' patients are called AL, followed by a number and letter (e.g. AL-1A). Again, individuals with the same number, like AL-1A and AL-1B, are siblings. The PEO patients are called A1 and B1. In papers III and IV we use the same coding system as in this thesis. We use different patient codes in each of papers I and II and provide a key in appendix I.

3.2 Clinical evaluation - papers I-IV and unpublished material

Clinical information was obtained from retrospective analysis of the clinical notes, clinical examination and follow-up of the patients during the period 2005-2010. Thirteen patients were dead at the beginning of the study. Their clinical information was obtained exclusively from the notes. Clinical assessment of the patients was performed by at least one of the neurologists involved in the studies.

3.3 Genetic investigations – papers I, III, IV and unpublished material

3.3.1 POLG analysis

Genomic DNA was isolated from blood using standard protocols and *POLG* exons 2-23 were amplified using standard procedures and AmpliTaq Gold DNA polymerase (ABI, Foster city, USA), including at least 50 bases of flanking intronic sequence. Sequencing was performed using BigDye Terminator cycle sequencing kit (v1.1, Applied Biosystems). Reference sequence for the *POLG*-gene: NM_002693.1. Nomenclature used is according to international recommendations (http://www.hgvs.org/rec.html).

3.3.2 Mitochondrial DNA studies

Total DNA was extracted from frozen skeletal muscle biopsies and postmortem tissues that had been stored at -80C by overnight incubation in proteinase-K followed either by phenol/chloroform extraction or using commercially available kits. Total mtDNA content was determined by comparing amplification in mtDNA areas least likely to be affected by deletions (12SrRNA and ND1) to a nuclear gene (18SrRNA or RNAse-P) using real time PCR with TaqMan fluorogenic probes. MtDNA deletions were detected by long-range PCR (LPCR) and quantified by real time PCR by comparing the amplification ratio in MT-ND4 and MT-ND1 genes. Detailed method description and primer/probe sequences in paper IV and upon request.

3.4 Radiological investigations – paper III

A total of 112 MRI and 11 computed tomography (CT) examinations were performed in 32 patients (28 with MSCAE and 4 with Alpers' disease). In 25 patients (21 MSCAE and 4 Alpers'), sequential examinations were available, allowing us to study lesion evolution during the chronic and acute phases of the disease. Diffusion imaging was performed in 10 patients (7 MSCAE and 3 Alpers') and apparent diffusion coefficient (ADC) values were measured in 17 stroke-like lesions. All ADC values are given in $x10^{-3}$ mm^2/sec. MR angiography (MRA) was performed on 3 patients and conventional cerebral angiography on one. Proton single voxel magnetic resonance spectroscopy (MRS) was performed in 3 patients (AT-1A, AT-1B, WS-1A). Detailed method description in paper-III.

3.5 Neurophysiology – papers I & II

A large number (295) of surface EEG recordings were performed in nineteen patients with MSCAE, the remaining patients were excluded either because they did not have epilepsy or EEG data was unavailable. Multiple sequential recordings were performed in eighteen patients and long-term video-EEG recording was done in four. Peripheral nerve studies including electromyography (EMG) and nerve conduction velocities (NCV) were performed according to standard procedure. Detailed method description in papers-I and II.

3.6 Pathology – paper III

Post-mortem examination was performed in 7 patients (AT-1A, AT-1B, AT-2A, WS-1A, WS-2A, WS-12A and AL-1B) with detailed pathological investigations of the brain, spinal cord and liver performed in 3 (AT-1A, AT-1B and WS-1A). Samples from various organs were snap-frozen in liquid nitrogen and stored at -80 °C or fixed in formaldehyde. Sections of standard areas and areas showing involvement on MRI were performed. Sections were examined by standard histology, including Hematoxylin-eosin (HE) and luxol fast blue, and immunohistochemistry with antibodies directed against glial fibrillary acidic protein (GFAP), HLA-DR/DP/DQ a microglial marker, and CD68, a monocyte marker. Double staining for cytochrome oxidase (COX) and succinate dehydrogenase (SDH) was performed in frozen brain sections of patients AT-1A and brain, spinal cord and liver of patient AL-1B as described previously [79]. Detailed method description in paper-III.

3.7 Review of the literature

The published literature on POLG (up to June 2010) obtained through PubMed (http://www.ncbi.nlm.nih.gov/pubmed) was systematically reviewed.

4. Results

4.1 POLG genetics (papers I, III, IV and unpublished material)

4.1.1 MSCAE

The MSCAE patients were either homozygous for the c.*1399G>A,* p.A467T (n=6), homozygous for the c.*2243G>C*, p.W748S (n=21), or compound heterozygous *in trans* for these two mutations, p.A467T/W748S (n=9).

4.1.2 Alpers'

The patients with Alpers' were genetically heterogeneous: AL-2A had the A467T and c.2542G>A (p.G848S) on different alleles; two brothers AL-1A and AL-1B, and another unrelated child AL-3A carried the A467T in *trans* with a previously unreported missense change: c.907G>A in exon 4. This novel mutation replaces a highly conserved glycine residue with an arginine at position 303 (G303R) in the exonuclease region of *POLG* (Figure 5). The G303R was not found in >170 other patients and controls in whom we have sequenced the entire *POLG* coding region.

4.1.3 PEO – paper IV

Both PEO patients were compound heterozygous: A1 had the c.752C>T, (p.T251I), c.1760 (p.P587L) and c.2243G>C (p.W748S). B1 had the c.2209G>C (p.737R) and c.2243G>C (p.W748S). No other mutations were found. Sequencing of the son of B1 confirmed that her mutations were *in trans.* All four mutations were known individually, but the combinations were novel.

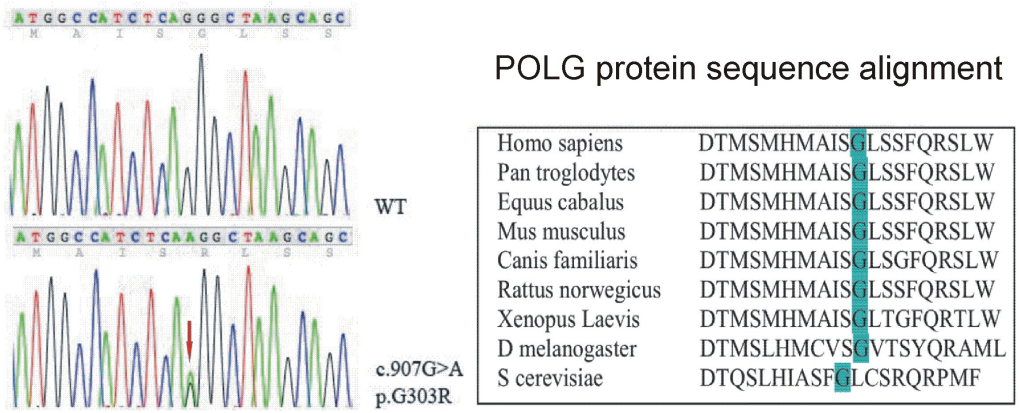


Figure 5. Novel *POLG1* mutation causing Alpers' disease. Left: chromatogram of patient AL-1A showing the novel heterozygous mutation G303R. **Right:** POLG aminoacid sequence alignment showing interspecies conservation of the 303 glycine residue. WT: wild type.

4.2 Onset, natural course and prognosis of POLG-encephalopathy (Papers I, II, III and unpublished material)

4.2.1 Onset

Individual and mean ages of onset of POLG-encephalopathy from our studies and the literature are listed in table 3 and appendix II and the distribution plots are depicted in figure 6. Mean age of onset for MSCAE in our material was 15.2 years (SD 9.8). The range was 1.5-45 years, but most patients presented during the second decade of life (22/36). Initial symptoms were in order of decreasing frequency: progressive gait unsteadiness (due to cerebellar and sensory ataxia) (14/36), epilepsy (12/36), migraine-like headache (12/36) and developmental delay (2/36).

When the patients were grouped according to epilepsy, age of onset was significantly earlier in the group with epilepsy (12.1 years, range 2-20) than in the group without epilepsy (23.1 years, range 1.5-45). The commonest initial symptom in the group with

epilepsy was seizures (12/26) and migraine-like headache (11/26), followed by ataxia (6/26). In the group without epilepsy, ataxia was the most common initial symptom (8/10). The mean age of onset for the Alpers' patients was 1.1 years and all started with epileptic seizures (Table 3, appendix II).

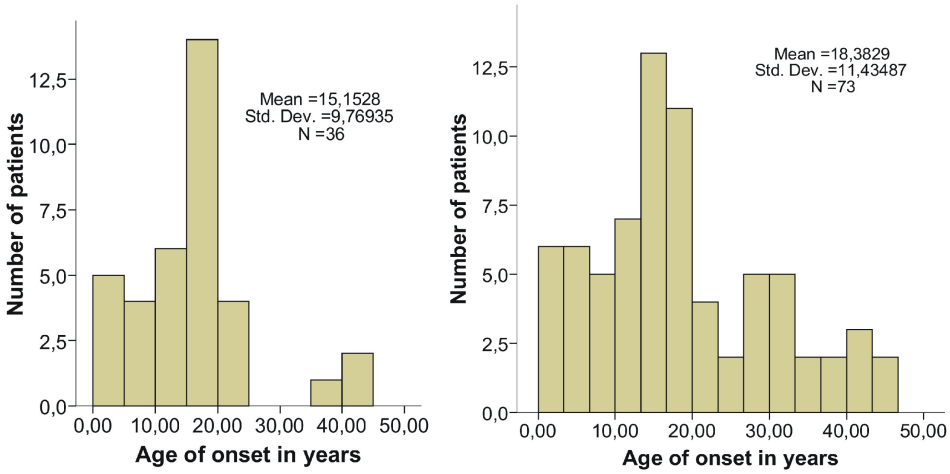


Figure 6. Distribution of recorded age of onset in MSCAE.

Left: distribution plot based on our 36 patients. Right distribution plot based on a total of 73 cases including our cases and those reported in the literature. Both plots show a wide distribution, but with most cases clustering in the second decade of life.

4.2.2 Course, prognosis and mortality

POLG encephalopathy is an invariably progressive disorder with high morbidity and mortality. In our material, mean age of death for MSCAE was 28 years (range 9-57) and median survival was 26 years. Alpers' disease was more severe with significantly shorter duration and a mean age of death of only 2.8 years. Our epidemiological findings are reproduced in an expanded material of 138 patients with POLG-

encephalopathy (73 MSCAE and 65 Alpers') including our data and cases reported in the literature (Table 3).

MSCAE

Our studies show that genotype and epilepsy are the most important prognostic factors in MSCAE and their role is confirmed in the expanded material of 73 patients (Table 3, Figure 7).

Patients with full blown MSCAE had a complex, acute-on-chronic course with acute exacerbation events superimposed on progressive worsening of the ataxia and other clinical manifestations. These exacerbation episodes were characterised by acute or subacute, rapidly progressive encephalopathy and severe epilepsy, and were often accompanied by stroke-like cerebral lesions. Episodes had high mortality (~50%) and survivors suffered permanent disability. Patients without epilepsy had a chronic, slowly progressive course with gradual worsening of the ataxia, neuropathy, ocular myopathy and other symptoms. Overall survival in the non-epilepsy group did not appear to be affected, at least until the 7th decade of life (the oldest patients in our material and the literature are in their 60s). Twenty out of our twenty-six (77%) patients with epilepsy have died, 11 of rapidly progressive encephalopathy during exacerbation episodes, 4 of encephalopathy and simultaneous liver failure, 4 of liver failure alone and 1 of chronic complications of immobilisation. None of our nine patients without epilepsy have died.

The A467T/W748S compound heterozygous genotype was associated with significantly worse prognosis (highest mortality and lowest median survival) than either the A467T or W748S homozygous genotypes (p=0.003). In our material, survival in the A467T homozygous group seemed to be better than in the W748S homozygous group, but this was not reproduced in the larger material of 73 patients including the cases from the literature (Table 3, figure 7).

Alpers' disease

Our patients with Alpers' disease had also an acute-on-chronic course with acute exacerbations superimposed on a progressive neurological deterioration. Survival and disease duration were, however, significantly shorter than in the adult syndromes (Table 3).

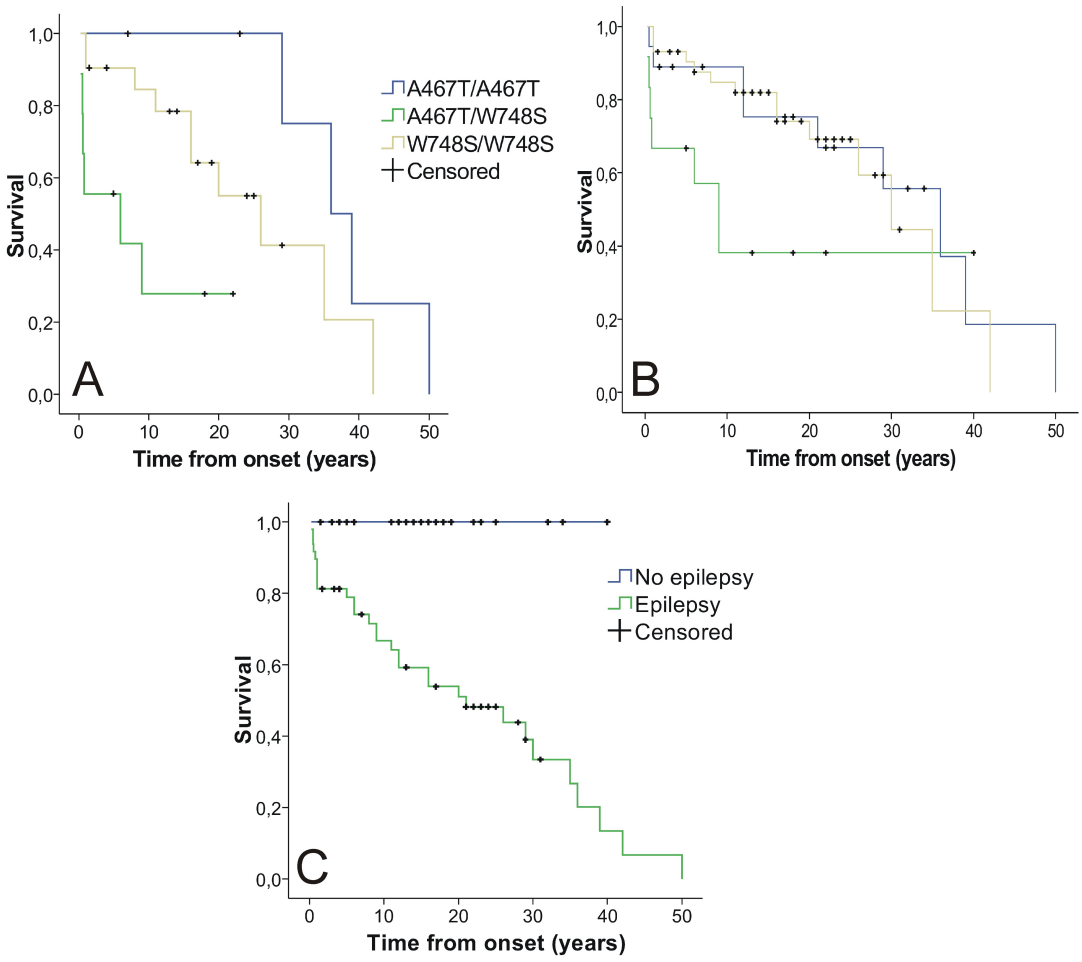


Figure 7. Survival (Kaplan-Meier) curves in MSCAE.

A: Survival according to genotype in our 36 patients with MSCAE (papers I-III and unpublished) shows that A467T homozygous patients live longest and compound heterozygous have the worse prognosis with significantly shorter survival than the

other two groups (P=0.003). **B:** Survival according to genotype in a total of 73 patients including our 36 and another 37 from the literature. In this expanded material survival is similar in the A467T and W748S homozygous, but still significantly worse in the compound heterozygous patients. **C:** Survival as a function of epilepsy in 73 patients (same as in B). Epilepsy is the most important clinical prognostic factor. Patients without epilepsy do not suffer exacerbation episodes and have a longer lifespan, at least until the 7th decade of life. Median survival for patients with epilepsy is ~30 years.

Clinical syndrome	Genotype	N	AO	AD	Median survival
MSCAE	**A467T/A467T**	6	12.5 (SD 4.3)	49.8 (n=4/6, SD 5.1)	36 (SD 5)
	W748S/W748S	21	13.3 (SD 9.8)	25.4 (n=10/21, SD 14.7)	26 (SD 6.8)
	A467T/W748S	9	20.9 (SD 10.6)	17.7 (n=6/9, SD 5)	6 (SD 6.5)
Total		36	15.1 (SD 9.7)	28 (n=20/36, SD 15.8)	26 (SD 6.7)
Full blown MSCAE	**All**	26	12.1 (SD 5.7)	28 (n=20/26, SD 15.8)	16 (SD 6.2)
No epilepsy	**All**	10	23.1 (SD 13.5)	no deaths reported 0/10	no deaths reported
Alpers'	**various**	4	1.1 (SD 0.6)	2.8 (n= 4/4, SD)	0.18 (SD 0.18)

Clinical syndrome	Genotype	N	AO	AD	Median survival
MSCAE	**A467T/A467T**	18	15.2 (SD 12)	31.8 (n=9/18, SD 19.8)	36 (SD 6.8)
	W748S/W748S	43	19.6 (SD 11.2)	25.7 (n=14/43, SD 13.2)	30 (SD 4.2)
	A467T/W748S	12	18.7 (SD 11.5)	17.1 (n=7/12, SD 4.8)	9 (SD 2.3)
Total		73	18.4 (SD 11.4)	25.5 (n=30/73, SD 14.9)	30 (SD 4.4)
Full blown MSCAE	**All**	47	13.2 (SD 7.3)	25.5 (n=30/47, SD 14.9)	20 (SD 6.9)
No epilepsy	**All**	26	27.7 (SD 11.8)	no deaths reported 0/26	no deaths reported
Alpers'	**various**	65	0.9 (SD 0.8)	2.5 (n=56/65, SD 2.5)	0.8 (SD 0.1)

Table 3. Epidemiological data of patients with POLG encephalopathy.

Our patient material (upper part) and an expanded material of 138 patients (73 MSCAE and 65 Alpers') including our patients and cases from the literature (lower

part). Age of onset (AO), age of death (AD) and median survival values (in years) are shown. Source as in table 2.

4.3 Clinical features of POLG-encephalopathy

The clinical features of our 40 patients with POLG-encephalopathy are detailed in appendix II. The full clinical spectrum of MSCAE included ataxia, sensorimotor peripheral neuropathy, myoclonus, headache with migraine-like features, late onset ptosis and external ophthalmoplegia (PEO) and cognitive decline. The majority of our patients had epilepsy and developed acute or subacute exacerbation episodes with rapidly progressive encephalopathy and worsening seizures. Liver disease developed upon exposure to anti-epileptic drugs containing valproic acid and its derivatives (sodium valproate, sodium divalproex). Spontaneous liver disease also occurred, but was rare. Our patients with Alpers'disease had infantile-onset, severe progressive-episodic encephalopathy, epilepsy and spontaneous liver disease.

4.3.1 Ataxia

Ataxia was present in all our patients with MSCAE and resulted from a combined cerebellar, spinal and peripheral sensory dysfunction. The clinical picture included nystagmus, ocular dysmetria, scanning dysarthria, truncal/gait ataxia and appendicular ataxia with intention tremor, dysmetria and dysdiadochokinesia. A sensory component was evident in the form of proprioceptive and vibratory sensory loss in the distal extremities. The gait and appendicular ataxia increased in the absence of visual input and Romberg's test was commonly positive. The ataxia was gradually progressive over years - decades and resulted in severe motor disability and wheel-chair dependence. Only one of our patients with Alpers' disease had ataxia.

4.3.2 Peripheral neuropathy

The vast majority of our MSCAE patients (97%) had a predominantly sensory peripheral neuropathy. This was clinically characterised by impairment of the superficial (light touch, pain, temperature) and deep (discriminative touch, vibration,

proprioception) sensory modalities in a distal, glove and stocking distribution and diminished tendon reflexes. The proprioceptive impairment led to sensory ataxia. Sensory symptoms started in the distal lower extremities and remained more severe there throughout the disease. Neuropathy was not seen in the Alpers' patients.

Nerve conduction studies (NCS) typically showed decreased wave amplitudes and conduction velocities in both sensory and motor nerves. Sensory nerves were more severely affected, however, and often had no measurable responses at all. Electromyography (EMG) showed moderate signs of chronic denervation including decreased/absent F-waves and increased amplitude and duration of motor unit potentials (MUP).

4.3.3 Migraine-like headache

In 12/36 patients, headache was either the presenting feature or started at the same time as the epilepsy. Headaches were episodic, could be unilateral and were often preceded and accompanied by transitory positive or negative visual phenomena such as flashes and scotomata respectively. In six of our patients headaches were initially suspected to be migraine.

4.3.4 Epilepsy

Epilepsy was present in 26/36 patients with MSCAE and was an important prognostic factor as its presence was associated in all patients with the occurrence of exacerbation episodes carrying high morbidity and mortality. Epilepsy was present in all our Alpers' patients, in whom it was also an important morbidity factor along with liver disease, and in 102/128 (80%) cases reported in the literature [44, 46, 48, 53-71]. The epileptic semiology of POLG-encephalopathy is complex and was the focus of our study presented in paper II. Patients had a variety of clinical seizure types including simple and complex partial with sensory and motor symptoms (SPS and SPM respectively) and secondary generalised tonic-clonic seizures (GTC). Partial and

generalised status epilepticus were common and associated with high morbidity and mortality.

Epilepsy usually starts early in POLG-encephalopathy, but may also start late.

In most patients with MSCAE epilepsy started early in the course of the disease. Seizure onset was accurately known in 23 patients. In 16 of these seizures were either the presenting symptom or started within one year from disease onset. In five patients, however, seizures developed 4-10 years after disease onset and another three had their first seizure >30 years after the onset of ataxia. In two of these cases (WS-1A and WS-12A), the onset of seizures was associated with a severe encephalopathy episode that proved fatal. Epilepsy was the presenting symptom in all our Alpers' patients.

Simple partial motor status epilepticus is a typical feature of MSCAE

SPM seizures occurred in all 19 MSCAE patients with epilepsy reported in paper II. The commonest clinical manifestation was continuous clonic jerking of an upper limb, shoulder, neck and head with intact consciousness. The lower limbs were also affected, but less commonly. SPM seizures were commonly prolonged and often evolved into epilepsia partialis continua (EPC), which could last for up to several months in spite of combination treatment with multiple antiepileptic drugs. Focal motor seizures of similar type, but with various degrees of impaired consciousness (CPM) also occurred, but were less common (Paper II).

Simple partial visual seizures correlate with occipital epileptic foci

Nine of the19 patients with MSCAE reported in paper II had therapy refractory SPS seizures with positive visual symptoms, usually the perception of flashing colored or white light in one visual hemifield, which occurred daily for weeks, months or even years. Visual seizures correlated with focal occipital epileptic activity on EEG examination and in most, but not all cases occipital lesions on MRI.

Status epilepticus

All MSCAE patients with epilepsy (26/36) presented in this thesis and the four patients with Alpers' disease had one or more episodes of status epilepticus (SE). Simple partial SE was often prolonged and even chronic, but not always associated with exacerbation episodes. Complex partial and especially secondarily generalized SE often marked the onset of or/and occurred during episodes of encephalopathic exacerbation.

Electroencephalography

Ictal EEG revealed focal occipital or regional occipito-temporal epileptic activity in almost all (18/19) patients studied in paper II (Figure 8). Interictal focal/regional or generalized slow wave activity was common. In the majority of patients, focal occipital activity could be correlated to symptoms of occipital origin such as positive or negative scotomas and many had occipital lesions on MRI. Focal frontal epileptic activity was also seen and correlated mostly with focal motor clinical activity and in most, but not all cases frontal lesions on MRI. Generalized epileptic activity was commonly found under generalized SE. The side of the symptoms, EEG activity and MRI lesion did not always correlate. It should, however, be noted that imaging and EEG did not always coincide in time. When we looked at EEG and imaging that were closely performed during exacerbation episodes, as presented in paper III, correlation was better, but still not complete.

Treatment of the epilepsy and status epilepticus in POLG-encephalopathy

Epilepsy in our patients with POLG-encephalopathy was often refractory and most required combination therapy. As we report in paper II, sodium channel blockers like carbamazepine, phenytoin, oxcarbazepine and lamotrigine were partly effective at least for a period of time, and well tolerated by the patients. Sodium channel blockers were often combined with a benzodiazepine or barbiturate e.g. diazepam, clonazepam or phenobarbital. Levetiracetam was also used. Clonazepam and topiramate had some effect against myoclonus, while gabapentin increased focal myoclonic activity in two patients and lamotrigine in one. Valproic acid and its derivatives have an absolute

contraindication as they cause liver failure in these patients. One patient was treated with ketogenic diet and one with plasmapheresis with no effect. Home acute seizure management with agents like rectal diazepam or buccal midazolam may help abolish seizures and is recommended. SE should be treated as aggressively as possible in order to prevent initiation and progression of the exacerbation event. We use standard protocols of intravenous benzodiazepines and fos-phenytoin, but have a low threshold for generalized anaesthesia with agents like pentothal and propofol.

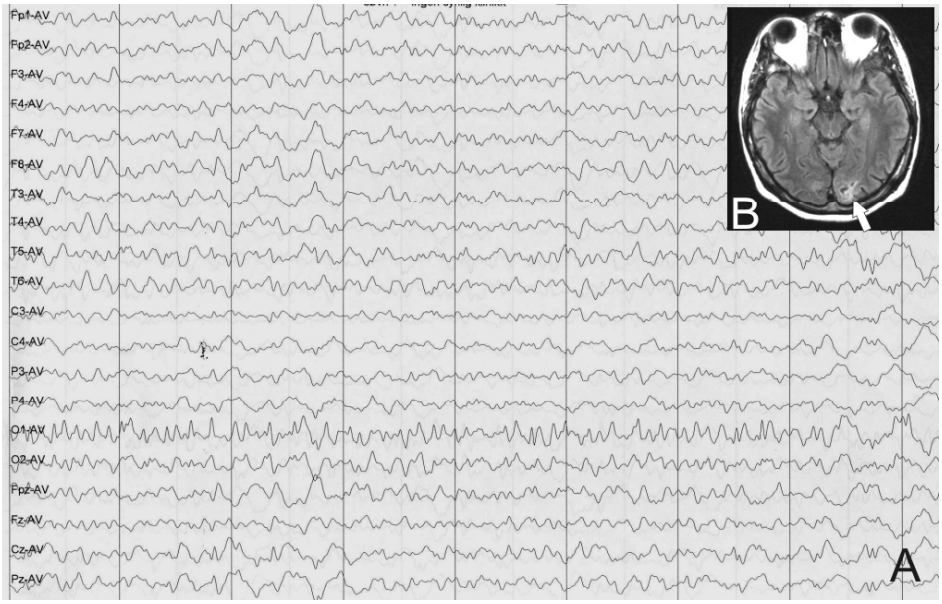


Figure 8. EEG and MRI findings in MSCAE.

A: An ictal EEG showing general slowing and epileptiform activity in the left occipital area (O1). During the recording the patient had a simple partial visual seizure with positive visual phenomena in the right visual hemifield. **B**: axial FLAIR-T2 MRI from the same period showing an old, retracted lesion in the patient's left occipital cortex.

4.3.5 Exacerbation episodes with encephalopathy and epilepsy

Irrespective of genotype, all of our patients with full-blown MSCAE (n=26) had episodes of acute or subacute exacerbation with rapid neurological deterioration that could be fatal or followed by variable degrees of recovery. The onset of exacerbation episodes was marked by either epileptic seizures or gradual mental and personality changes, including fatigue, somnolence and confusion, which could precede the onset of clinical seizures by up to several days. Episodes were associated with disturbed consciousness, ranging from confusion to deep coma and frequent seizures, including both partial and generalised status epilepticus. Cerebral imaging with CT or MRI during the course of the episode commonly revealed newly developed, infarct-like, oedematous cortical lesions. Episodes had a mean duration of 74 days (range 5-266) and were associated with significant morbidity and mortality: of 30 episodes in 26 patients, 14 (47%) proved fatal. Survivors suffered accelerated decline of motor and cognitive skills and/or cortical visual loss.

4.3.6 Progressive external ophthalmoplegia (PEO)

PEO consists of slowly progressive blepharoptosis and paresis of the extraocular muscles, which is not overcome by brain-stem mediated reflexes such as convergence, oculocephalic and Bell's. Symptoms are bilateral and usually, but not necessarily, symmetrical. More generalised myopathy, involving other muscles of the body has been reported [44, 80], but is rarely significant in the encephalopathy syndromes.

PEO was seen in 18/36 (50%) of MSCAE patients and its prevalence was age dependent. It developed late in the disease, at a mean age of 29.3 years (n=12, SD 6). PEO was more prevalent in patients with no epilepsy (67% vs. 40% in patients with epilepsy); it also appeared to be genotype dependent having the highest frequency in the A467T homozygous group (83%), followed by the W748S homozygous (45%) and compound heterozygous (25%). It did not occur in the Alpers' patients. It is,

however, likely that these phenotype and genotype-dependent differences in the prevalence of PEO are confounded by patient age and disease duration in each group.

4.3.7 Liver disease

Liver involvement was seen in 18/36 (50%) patients with MSCAE and in 12/18 cases it was associated with oral use of the anti-epileptic drug sodium-valproate. Nine patients had fulminant hepatic failure, which was preceded by use of sodium-valproate in eight and occurred spontaneously in one. The remaining nine patients had asymptomatic biochemical abnormalities including elevation of the hepatobiliary enzymes (alanine and aspartate aminotransferases, gamma-glutamyltransferase and alkaline phosphatase) and low albumin. Four of these used sodium-valproate. Only one patient (WS-12A) who used sodium-valproate for two months before her final illness did not develop liver disease. Two patients (WS-4A and CP-3A) were treated with liver transplantation. In WS-4A, transplantation was successful and she continues using sodium-valproate today, 9 years later, with no further complications. Patient (CP-3A) died shortly after the transplantation due to rejection. Liver disease was not seen in patients with no epilepsy. Two of the Alpers' patients (AL-3A, AL-4A) had biochemical liver abnormalities and one (AL-1A) developed liver failure. None of the Alpers' patients used sodium-valproate.

4.3.8 Cognitive dysfunction

Slowly progressive decline of cognitive functions was common in the MSCAE patients and often accelerated after exacerbation episodes. Cognitive dysfunction was significantly more pronounced in patients with epilepsy. We performed detailed neuropsychological evaluation in 8 of our patients and this showed cognitive dysfunction in all with lower mean performance IQ (71.8) than verbal IQ (84.3) values (P=0.001) [81]. All our patients with Alpers' disease had delayed psychomotor development.

4.3.9 Other clinical features

Four MSCAE patients developed gastrointestinal dysmotility with chronic abdominal pain, diarrhoea or pseudoobstruction symptoms. Patient WS-4A had facial dyskinesias and later developed asymptomatic, 2 Hz palatal myoclonus that correlated with the development of bilateral hypertrophic olivary degeneration on MRI. Whipple's disease was excluded in that patient by intestinal mucosal biopsy. Two patients had neurogenic deafness. One patient had cardiac dysrhythmias (supraventricular tachycardia, atrial fribrilation and bundle branch block), but no evidence of cardiomyopathy.

4.4 Neuroimaging in POLG-encephalopathy (Paper III)

We found neuroimaging abnormalities in all 32 patients with POLG-encephalopathy (28 MSCAE and 4 Alpers') who were examined during the course of their illness (Appendix III, figure 9). Only one patient (AT-2A) had a normal initial scan while 3 patients (AT-2B, CP-2A, WS-10A) had only mild trophic changes in the early stages of the disease. Imaging findings were classified into three categories based on anatomical distribution and natural evolution:

1. **Cortical stroke-like lesions (SLL) with acute/subacute onset and rapid evolution**. These developed exclusively during exacerbation episodes and evolved over days - weeks. Subsequent partial or complete regression occurred if the patient survived the episode (Figure 9E, 9F, 9H, 10).
2. **Lesions with insidious onset and stable course.** Thalamic, olivary, cerebellar white matter (WM) and some cerebellar cortical lesions developed insidiously, were often present on the patients first MRI and usually remained stable throughout the disease (Figure 9B-E).
3. **Lesions with insidious onset and slowly progressive course.** Cerebral and cerebellar atrophy were slowly progressive and mirrored the clinical progression of the ataxia and cognitive impairment. Atrophy could,

however, accelerate dramatically during severe exacerbation episodes (Figure 9A).

Acute stroke-like lesions and chronic focal lesions exhibited high T2 and low T1 signal. The most sensitive MRI sequences for detecting signal changes were T2 fluid-attenuated inversion recovery (FLAIR-T2) and DWI. All types of lesions were seen in MSCAE occurring with similar frequency in the 3 genotypes, with the exception of inferior olivary lesions that occurred exclusively in the W748S homozygous group. Stroke-like lesions did not occur in patients without epilepsy and were the only type of lesion seen in Alpers' disease. Imaging findings are summarised in Appendix III.

4.4.1 Stroke-like lesions

Stroke-like lesions were common in MSCAE and were seen in all the patients we studied with Alpers' disease. They developed exclusively during exacerbation episodes and were localised primarily in the cortex, often extending into the subcortical white matter. They appeared hypodense on CT and had high T2 and low T1 signal on MRI (Figure 9E, 9F, 9H, 10). Diffusion weighted imaging (DWI) showed restricted cortical diffusion during the acute phase (1-8 days from clinical onset of the exacerbation episode); heavy diffusion weighting (b=1000) was the most sensitive sequence for detecting new lesions and following their progression. Lesional ADC values were initially low, averaging 0.64 (range 0.53-0.79), compared with 0.84 (range 0.73-0.95) in control areas. Subsequently, ADCs gradually increased over days to weeks, exceeded the values of control areas and later decreased again towards the baseline (Figure 9F, 10). In patient AT-2A, who survived an exacerbation episode, cortical ADC had normalised after 1.5 years. In the subcortical white matter underlying the stroke-like lesion, ADCs were high in the acute phase averaging 1.22 (range 0.96-1.45) versus 0.89 (range 0.81-0.90) in unaffected subcortical white matter. Subcortical ADCs showed gradual increase followed by modest decrease, but did not normalize and remained elevated for up to 6 years, which was the longest follow-up time.

Acute stroke-like lesions did not enhance upon the administration of paramagnetic contrast. Minimal enhancement was seen in two cases only (CP-3A, WS-8A) in whom it developed late, 50 days after onset of the episode and 20 days after the discovery of the cortical lesions on MRI. In addition, patient AL-1B showed leptomeningeal, but no parenchymal enhancement. Magnetic resonance angiography (MRA) was performed in 3 patients with new cortical lesions and conventional cerebral angiography in one, and showed no abnormalities.

Stroke-like lesion distribution varied. They affected, in order of decreasing frequency, the occipital (19/23), parietal (12/23), frontal (11/23), lateral temporal (5/23) and cerebellar (4/23) cortices. The brainstem (basis pontis) was affected in one case (AT-1A). Interestingly, the medial temporal lobes and hippocampi were universally spared and, to the best of our knowledge, lesions in these regions in patients with POLG disease have not been reported in the literature.

Stroke-like lesions evolved dynamically during the course of exacerbation episodes, mirroring clinical severity and progression of the encephalopathic symptoms and seizure activity. Persistence or progressive worsening of the encephalopathy was associated with gradual lesion expansion and/or the appearance of new lesions (Figure 9H). Epileptic activity was not a prerequisite for the progression of the SLL, which often expanded inexorably, apparently also after the seizures had been successfully controlled clinically and on electroencephalography (EEG). EEG was performed during exacerbation episodes in 17 patients with acute stroke-like lesions. Twelve of these had focal epileptic activity that correlated to one or more lesion localisations. One had generalised epileptic activity, while the remaining four had focal epileptic activity that did not correlate with the location of any of their lesions. Nine patients had early EEG, performed 0-4 days from episode onset. Four of these had initially no epileptic activity, but generalised slow-wave activity that in 3/4 was maximal over the stroke-like lesion. Epileptic activity developed in these foci on later recordings.

Clinical improvement of the episodes was associated with regression of the stroke-like lesions, which was gradually replaced by focal atrophy and retraction. Lesional T2-hyperintensity often persisted chronically, suggesting gliosis. The type and degree of residual disability after episode resolution correlated with its localisation and was roughly proportional to lesion extent and severity. The commonest form of persisting disability after an episode was cortical visual dysfunction caused by occipital lesions and ranging from focal scotomas and hemianopsia to cortical blindness. Central motor sequelae were caused by frontal lesions and a general decline of cognitive functions was associated with the accelerated cerebral atrophy that often ensued after episodes. In 2 cases (CP-3A and WS-6A) an occipital SLL showed late appearing (~60 days from onset of the exacerbation) gyriform, cortical hyperintensity on unenhanced T1 sequences (Figure 9G).

4.4.2 Thalamus, brain-stem and cerebellum

Chronic MRI lesions of the thalamus, cerebellum, and inferior olivary nuclei were common in MSCAE, but were not seen in the Alpers' patients (Figure 9B-E). Thalamic lesions (Figure 9E) occurred in 18/28 MSCAE patients, were usually bilateral and showed a predilection for the posterior thalamus including the pulvinar. These lesions developed insidiously and persisted unchanged throughout the disease.

Chronic cerebellar MRI abnormalities were seen in 23/28 MSCAE patients. They comprised atrophy, small cortical/subcortical T2 hyperintensities, diffuse, symmetrical white matter T2 hyperintensities, signal changes and atrophy of the dentate nuclei (Figure 9A-C). Dentate atrophy (Figure 9B) was seen in four W748S homozygous patients all of whom also had bilateral lesions of the inferior olives (Figure 9D). Loss of the dentate T2 hypointensity was noted in a further 7 patients. No dentate or olivary abnormalities where seen in A467T homozygous patients. Cerebellar white matter lesions (Figure 9C) appeared to be more common in A467T homozygotes (33% versus 19% in the W748S) and were never seen in compound heterozygotes.

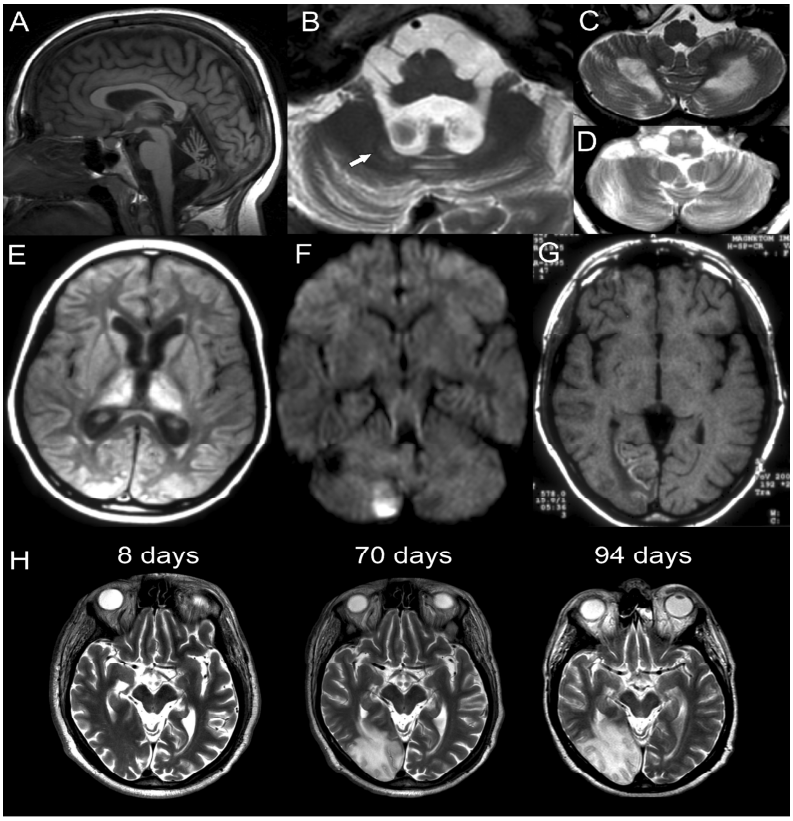
Bilateral inferior olivary lesions were seen in eight W748S homozygous patients. The inferior olivary nuclei had high T2 signal and appeared enlarged and swollen on the MRI (Figure 9D). This was best visible on T2 and proton weighted images. Four of the patients with olivary lesions had atrophy of the dentate nuclei and dentate signal change was seen in another 3 and could not be excluded in one. One patient with bilateral olivary lesions (WS-4A) exhibited palatal tremor/myoclonus, while no clinical correlate was found in the rest.

4.4.3 Atrophy

Generalised cerebral atrophy was found in 11/28 patients with MSCAE and was slowly progressive, mirroring severity and progression of cognitive impairment. Cerebellar atrophy was seen in 21/28 MSCAE patients. It affected the vermis in all and the hemispheres in most (Figure 9A). Severity of the cerebellar atrophy was proportional to the severity of ataxia. Brainstem and spinal cord atrophy was not seen. Cerebral and cerebellar atrophy often accelerated dramatically after exacerbation episodes. Atrophy of the cerebrum or cerebellum was not seen in the Alpers' patients.

4.4.4 Magnetic resonance spectroscopy

Spectra obtained from fresh cortical lesions showed significantly decreased N-acetyl-aspartate (NAA) and prominent lactate peaks (Figure 9I). Areas with normal MRI signals revealed normal spectra. Interestingly, an old cerebellar white matter lesion in patient AT-1A also revealed a normal MRS pattern.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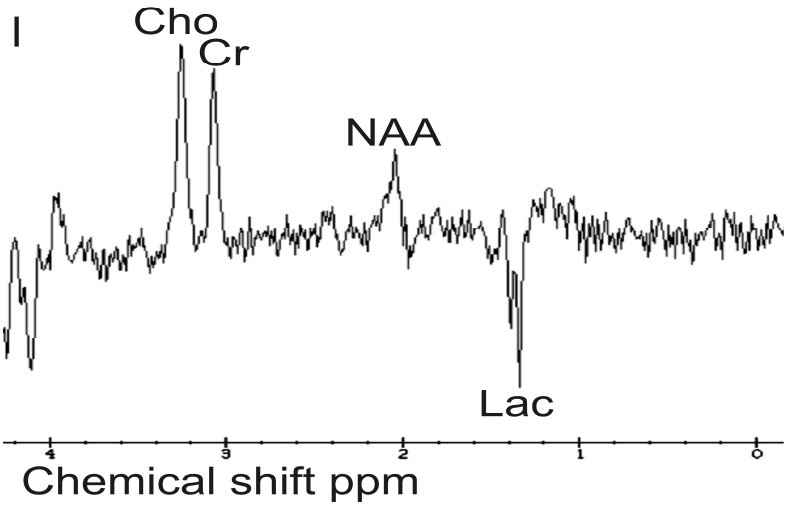
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NAA
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Chemical shift ppm

Figure 9. MRI and MRS findings in POLG-encephalopathy. A: sagital T1 image showing cerebellar atrophy in patient WS-10A. **B**: axial T2 image showing dentate atrophy (arrow) in patient WS-10A. **C**: axial T2 image showing cerebellar white matter hyperintensity in patient AT-1A. **D**: axial T2 image showing bilateral olivary lesions in patient WS-4A. The olives appear enlarged and hyperintense. **E**: axial T2 FLAIR image showing bilateral thalamic and cortical occipital lesions in patient WS-9A. **F:** axial DWI (b=1000) showing an acute SLL in the right cerebellar cortex of patient AL-1A. **G**: axial T1 image showing linear, gyriform hyperintensity in the right medial occipital cortex of patient CP-3A (laminar necrosis). **H:** Axial T2 weighted image showing the natural evolution of an occipital stroke-like lesion in patient AT-1B. Times on the images refer to intervals between episode onset and MRI. The lesion progressed inexorably, while the patient's condition gradually worsened. He eventually became comatose and died a little over 3 months after episode onset. **I:** MRS measurement in the right occipital lesion of patient AT-1B shown in H. Spectra show a decrease in N-acetyl-aspartate (NAA) and a prominent lactate peak at 1.3ppm, inverting at 144ms echo-time. Spectra from the contralateral, unaffected occipital area were normal (not shown). Cho: choline, Cr: creatine. (Modified from paper III).

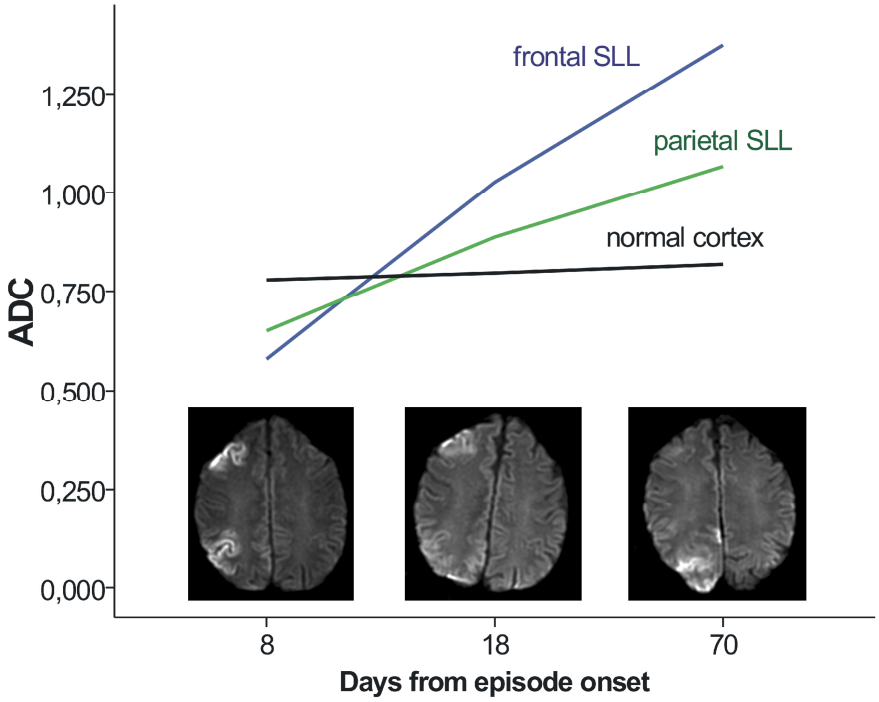

Figure10. Diffusion evolution of cortical stroke-like lesions during exacerbation episodes. Sequential ADC measurements are performed in evolving frontal and parietal stroke-like lesions (SLL) as well as in normal-looking cortex (control) of patient AT-1B during an episode. At 8 days after episode onset, both lesions have low ADCs consistent with restricted diffusion and cytotoxic oedema. Subsequently, ADC values increase, suggesting development of extracellular oedema, exceed those of control cortex and remain elevated at day 70. A new right occipital lesion appears on the scan on day 70. Representative DWI sequences (b=1000) are shown for each scan (Modified from paper III).

4.5 Pathological characterisation of POLG-encephalopathy (Paper III)

4.5.1 Histology

Pathology findings in the CNS generally correlated with imaging. The most pronounced pathological changes were found in areas with signal abnormalities and/or atrophy on ante-mortem imaging. In cortical areas affected by stroke-like lesions we found severe neuronal loss with vacuolation of the neuropil, astrocytosis and microglial activation. Of the few remaining neurons, some had normal appearance, while most exhibited intense cytoplasmic eosinophilia and pyknotic, dark nuclei (eosinophilic neuronal necrosis). Cortical neuronal loss was most severe in superficial and deep cortical layers creating a laminar pattern. The hippocampal cortex was consistently preserved. Of the deep cerebral gray structures, the thalami were affected by neuronal loss and eosinophilic necrosis, while the corpus striatum was intact. The cerebellar cortex showed selective Purkinje cell loss, eosinophilic necrosis and Bergman's gliosis. The cerebellar white matter showed sponginess and gliosis in patients AT-1A and AT-1B, who had high T2 signal MRI changes in that area. In the dentate nuclei we found neuronal loss and eosinophilic necrosis. The cerebral vasculature was unremarkable both grossly and microscopically. In the spinal cord, there was selective dorsal column degeneration, which was more pronounced in the fasciculus gracilis.

4.5.2 Immunohistochemistry

HLA-DR, DP, DQ immunostaining revealed extensive microglial activation in lesions, while monocyte/macrophage immunohistochemistry (anti MAC58) stained almost exclusively intravascular cells. In the cerebellum of patient WS-1A, we found extensive microglial proliferation forming radial, linear bands extending from the Purkinje cell layer throughout the molecular layer.

4.5.3 Histochemistry (unpublished studies)

COX/SDH histochemistry revealed no COX deficient or SDH hyperintense vessels in the cerebrum or cerebellum in either of the patients studied (Figure 11A). In patient AT-1A with MSCAE, we found a few scattered COX negative/SDH reactive neurons spread throughout the occipital cortex and, to a lesser extent, the cerebellar cortex (Figure 11A, E). Patient AL-1B with Alpers' disease had normal histochemistry in the cerebrum and cerebellum (Figure 11C), but we found COX-negative/SDH reactive neurons in the spinal cord (Figure 11B). The patient's liver showed severe COX-defect (Figure 11D).

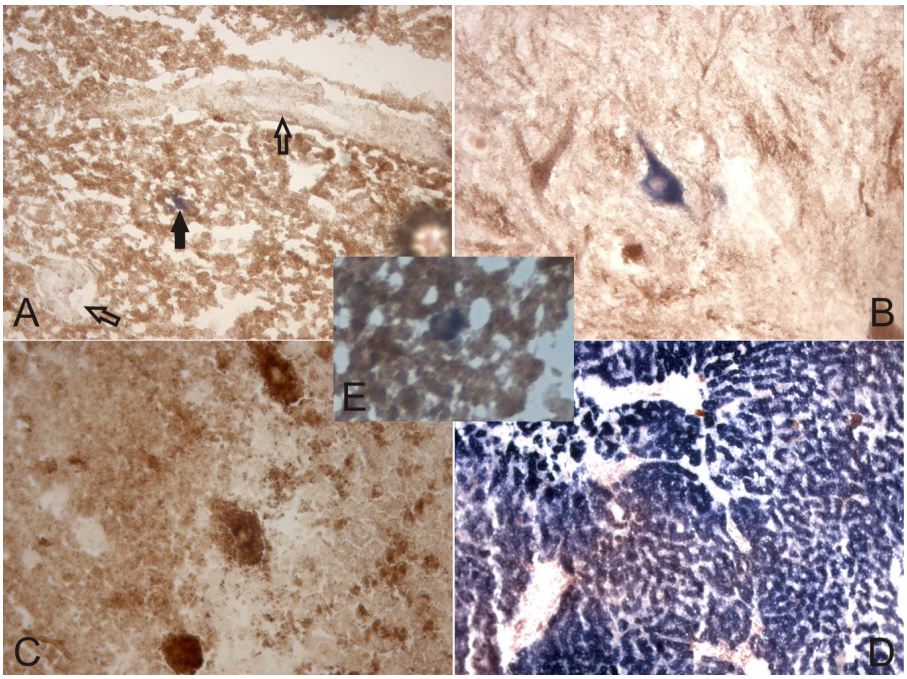

Figure 11. Histochemical staining of COX & SDH in post mortem cerebral and liver specimens.

All sections show combined cytochrome oxidase (COX) and succinate dehydrogenase (SDH) stainings of post-mortem material. **A** and **E:** occipital cortex of patient AT-2A with MSCAE showing a single COX-negative/SDH-positive neuron (black

arrowhead). **A**, shows also normal COX staining in a longitudinal and a cross section of two vessels (empty arrowheads). **B:** a single COX-negative/SDH-positive neuron in the cervical dorsal spinal horn of patient AL-1B with Alpers' disease. **C:** cerebellum of patient AL-1B, showing three Purkinje cells with normal COX staining. **D:** Nearly global COX-deficiency in the liver of patient AL-1B.

4.6 Studies of mtDNA changes in tissues of patients with POLG-myopathy and encephalopathy (Paper IV and unpublished material)

4.6.1 MtDNA changes in POLG encephalopathy

Quantification by real-time PCR showed mtDNA depletion in all skeletal muscle biopsies from 4 MSCAE and 1 Alpers' patient. Depletion was also found in the liver of three patients and the brain of two. There were normal mtDNA levels in the myocardium of MSCAE patients, but depletion was found in the heart of the one Alpers' patient studied. Long-range PCR and/or southern blotting revealed multiple mtDNA deletions in skeletal muscle from all patients examined (Table 4, unpublished data). The post-mortem tissues were not checked for deletions. Further detailed analysis of mtDNA in post mortem tissues is part of an ongoing study.

4.6.2 MtDNA changes in skeletal muscle in PEO

In a skeletal muscle biopsy of patient A1 with late onset recessive PEO, LPCR showed a typical pattern of multiple mtDNA deletions (figure 12). Real-time PCR showed a reduced ND1/ND4 ratio (average of two runs 0.77) compared with controls, giving an estimate of the percentage of mtDNA molecules with deletions involving the ND4 gene (major arc) of ~25%. The ND1/18SrRNA ratio was significantly lower than that of the controls, suggesting ~62% depletion of total mtDNA content in the patient's skeletal muscle.

Patient	Age at death	Disease duration	PEO	Liver failure	Episodic enc/thy	Tissue	Depletion	MD
AT-2A	44	29	+	-	+	Brain	-	ND
						Heart	-	ND
						Liver	-	ND
						SKM	~70%	+
WS-1A	41	35	+	+	+	Brain	-	ND
						Heart	-	ND
						Liver	~65%	ND
						SKM	~90%	+
WS-2A	24	8	-	+	+	Brain	-	ND
						Heart	-	ND
						Liver	~80%	ND
						SKM	~50%	+
WS-12A	57	42	+	-	+	Brain	~65%	ND
						SKM	~70%	ND
AL-1A			-	+	+	Brain	~65%	ND
						Heart	~65%	ND
						Liver	~75%	ND
						SKM	~90%	ND

Table 4. MtDNA studies in skeletal muscle biopsies (SKM) and post-mortem tissues of five patients with POLG encephalopathy.

MtDNA quantification was done with a Taqman based real-time PCR assay, by comparing amplification ratios in mtDNA (12SrRNA) and a nuclear gene (RNaseP).

The results are expressed as percentage of mtDNA depletion as compared to controls. ND: not done.

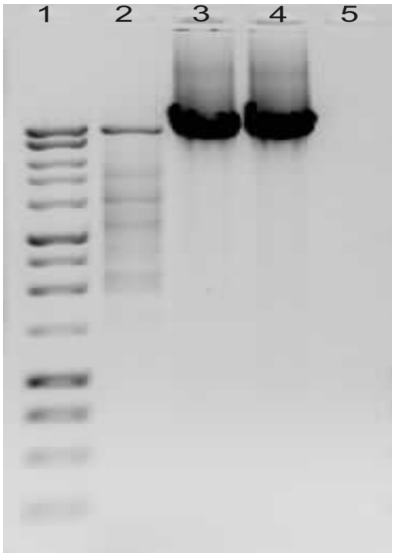

Figure 12. Multiple mtDNA deletions in PEO.

LPCR of mtDNA from a skeletal muscle biopsy of patient A1 with PEO showing multiple deletions (lane 2). The two controls show normal size bands (lanes 3 and 4).

5. Discussion

POLG mutations are an important and relatively common cause of human disease. Over 130 pathogenic mutations have been described in the gene encoding the catalytic subunit and these cause a wide spectrum of clinical disease including myopathies and encephalopathies with infantile or juvenile/adult onset.

In this work, we present detailed studies of the largest group of patients reported with POLG-disease. Our studies describe the natural history, clinical spectrum and epileptic semiology of the A467T and W748S POLG mutations, characterise the imaging and pathological features of POLG-encephalopathy and investigate the pathogenesis and pathophysiology of POLG-disease.

Our results define the syndrome of MSCAE as a recognisable clinical entity and reveal important factors affecting course and prognosis. Furthermore, we show that although POLG disease is genetically and phenotypically heterogeneous, the clinical and molecular features of the individual syndromes overlap significantly suggesting they are part of a clinical and pathophysiological continuum. Moreover, tissue specific energy failure caused by mtDNA damage plays an important role in the pathogenesis and progression of POLG-encephalopathy.

5.1 The clinical spectrum and natural history of POLG-encephalopathy, with a focus on MSCAE caused by the A467T and W748S mutations (Papers I-III)

5.1.1 The A467T and W748S POLG mutations are common causes of ataxia

The two most common POLG mutations are the c.1399G>A, p.A467T and c.2243G>C, p.W748S. These cause the syndrome of mitochondrial spinocerebellar ataxia and epilepsy (MSCAE), also known as mitochondrial recessive ataxic

syndrome (MIRAS), and are also found in compound with other mutations in recessive PEO and Alpers' disease.

Research shows that A467T and W748S were each introduced in the European populations by an ancient common founder [73, 82]. The reported carrier frequency for the A467T is 1% in Norway, 0.69% in the UK, 0.6% in Belgium, 0.5% in Sweden, and <0.2% in Finland. The carrier frequency of the W748S has been estimated to 1% in Norway and 0.8% in Finland [72, 82-84]. The high frequency of the A467T and W748S mutations in Norway and Finland places POLG mutations among the commonest causes of ataxia in these countries.

5.1.2 The A467T and W748S mutations are the main causes of MSCAE

We and others have shown that MSCAE is caused primarily by the A467T or W748S *POLG1* mutations. Most patients are homozygous for either of the mutations or compound heterozygous *in trans* (A467T/W748S). Clinical descriptions consistent with MSCAE have been reported with other *POLG1* mutations and genotypes including the p.R964C/A862T [48, 85], p.R627Q/G848S [86], p.W748S/L304R [54], p.G426S/G426S [44], p.A467T/G848S [48] and p.R627Q_Q1236H/ L965X [46]. These are, however, considerably more rare than the A467T and W748S and have been reported in single patients or families. In a review of 105 patients with MSCAE from our material and the literature, ninety-one (87%) had the A467T and/or W748S [44, 46, 48, 50, 53, 54, 56-60, 62, 66, 73, 75-78, 85-89].

5.1.3 When should the clinician suspect MSCAE?

Our data show that the syndrome of MSCAE is a clinically recognisable, well defined entity that should be considered in the differential diagnosis of juvenile or adult onset, sporadic and recessive spinocerebellar ataxia. Moreover, MSCAE should be suspected in the presence of one or more of the following additional features:

- Blepharoptosis, and/or external ophthalmoplegia
- Sensory peripheral neuropathy

- Epilepsy; especially in the presence of SPM seizures and epilepsia partialis continua, visual seizures and/or an occipital EEG focus
- Migraine-like headaches
- Acute-on-chronic course with exacerbation episodes
- One or more of the following MRI findings:
    - Stroke-like lesions developing under episodic exacerbations of the encephalopathy
    - High T2 signal lesions in the thalamus
    - Hypertrophic olivary degeneration
    - Diffuse T2 hyperintensity of the deep cerebellar white matter

<table>
<tr><th>Clinical features</th><th>Associated imaging features</th></tr>
<tr><td>Onset in teens</td><td rowspan="3">Cerebellar cortical atrophy, dentate atrophy, high T2 signal focal lesions in thalamus, cerebellar white matter and inferior olivary nuclei. No calcifications, no basal ganglia lesions.</td></tr>
<tr><td>Progressive spinocerebellar ataxia and sensory neuropathy</td></tr>
<tr><td>Ptosis and/or external opthalmoplegia</td></tr>
<tr><td>Epilepsy: especially SPM, EPC, visual seizures, frequent/severe SE</td><td rowspan="2">Acute, focal, T2 hyperintense cortical lesions, mostly occipital, but also frontal or parietal. Temporal lobes rarely affected. Lesions evolve mirroring episode severity. Associated with bad prognosis</td></tr>
<tr><td>Exacerbation episodes with epilepsy and rapidly progressive encephalopathy</td></tr>
</table>

Table 5. When should the clinician suspect MSCAE?

Common clinical features and associated MRI findings.

5.1.4 MRI is sensitive and can be specific for the diagnosis of POLG encephalopathy

According to our findings presented in paper-III, MRI detects abnormalities in the great majority of patients early in the course of the disease and sensitivity increases with disease duration. The combination of thalamic and cortical lesions, in the absence of basal ganglia involvement, is highly suggestive of MSCAE and if deep cerebellar and/or inferior olivary lesions are also present, the picture is, to the best of our knowledge, specific. In Alpers' disease, cortical SLL may often be the only finding although thalamic changes occurring early have been described [66].

5.1.5 The differential diagnosis of POLG-encephalopathy

The differential diagnosis of MSCAE includes other conditions with apparently recessive or sporadic ataxia and/or acute or episodic encephalopathy. Some of the most important clinical and radiological features that may distinguish MSCAE from other similar recessive ataxias and encephalopathies are summarised in table 5. The most important differential diagnoses will be discussed in detail.

Infantile spinocerebellar ataxia (IOSCA)

IOSCA is a recessively inherited infantile encephalopathy caused by mutations in the *C10orf2* gene that encodes the mitochondrial helicase, Twinkle. Twinkle is a functional partner of POLG in the replication of mtDNA and its mutations also cause secondary damage of mtDNA. It is therefore not surprising that Twinkle and POLG disease share significant pathophysiological and clinical similarities. Twinkle mutations cause autosomal dominant PEO, PEO plus syndromes and the syndrome of IOSCA [10, 90, 91].

IOSCA starts in infancy (commonly during the first two years of life) and is clinically characterised by muscle hypotonia, spinocerebellar ataxia, sensory neuropathy, extrapyramidal dysfunction (athetosis), PEO, hearing deficit, migraine-like headaches and female hypogonadism. Many patients develop epilepsy with seizure semiology

similar to that of POLG encephalopathy including SPM seizures, EPC and frequent SE. Moreover, like in POLG encephalopathy, epilepsy is an important negative prognostic factor as its presence is associated with crises of epileptic encephalopathy accompanied by stroke-like cortical cerebral lesions with a profile similar to that seen in MSCAE and Alpers' disease. Liver disease may occur in the most severe forms of the disease. Severity varies according to genotype. Patients with severe disease often die in infancy of severe encephalopathy, while others live into adulthood [90, 92-94].

At the molecular level, both POLG and Twinkle mutations affect mtDNA replication causing secondary damage to the mitochondrial genome. Like POLG, Twinkle mutations cause tissue specific multiple deletions and quantitative depletion of mtDNA; There are, however, differences in the tissue/organ distribution. In IOSCA depletion has been found in the liver and brain, but not in skeletal muscle, while multiple deletions have not been described. In POLG encephalopathy depletion occurs also in skeletal muscle and multiple deletions are found in both muscle and brain [72, 76, 77, 90, 95].

Distinguishing IOSCA from Alpers' disease or early onset MSCAE is not always possible on clinical grounds. Generally, POLG mutations should be tested first, as they are more common; when negative, testing Twinkle is perhaps the logical next step.

Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS)

MELAS is caused by maternally inherited mtDNA point mutations, mostly in the tRNA gene for leucine (MT-TL^{UUR}) and less frequently other tRNAs or the gene encoding subunit-5 of complex-I (MT-ND5). Severity varies according to levels of mutation heteroplasmy, tissue segregation and other factors. In its full blown form, MELAS is a multi-system disease that may include epilepsy, headache often with migraine-like feature, ataxia, peripheral neuropathy, sensorineural hearing loss, myopathy, retinopathy, diabetes mellitus, hearing impairment, lactic acidosis, heart

disease, nephropathy and other organ dysfunctions. Like POLG encephalopathy, MELAS has an acute-on-chronic course. Its clinical hallmark is the occurrence of so called stroke-like episodes (SLE), which are characterised by acute or subacute developing neurological dysfunction, accompanied by stroke-like cerebral lesions.

The stroke-like lesions of MELAS and POLG-encephalopathy have similar MRI profiles, including diffusion and spectroscopy, evolution pattern and histological appearance [79, 96]. There are, however, also important differences. While occipital involvement is common in both disorders, temporal lesions are very common in MELAS [97] but rare in POLG-encephalopathy (see section 4.4.1). The thalamus is commonly affected in POLG encephalopathy, but rarely in MELAS. Also, the basal ganglia and dentate nuclei are commonly calcified in MELAS, but calcification does not occur in POLG-encephalopathy.

COX histochemistry also differs. Mosaics of COX negative neurons and skeletal muscle fibres are seen in both conditions [72, 79]. COX negative/SDH reactive vessels, are typically present in striated muscle and brain of MELAS patients, but have not been described in POLG disease and were not seen in our patients [72, 75, 98].

Friedreich's ataxia

Friedreich's ataxia (FA) is caused by GAA trinucleotide repeat expansions in the *FXN* gene encoding frataxin, a mitochondrial protein involved in iron metabolism. Like MSCAE, Friedreich's ataxia is recessively inherited, commonly starts during the first two decades of life and is clinically characterised by spinocerebellar ataxia with distal sensory loss and decreased tendon reflexes. Unlike MSCAE however, FA consistently has pyramidal signs and is commonly associated with cardiomyopathy and diabetes mellitus. Another important diagnostic hint comes from MRI, which in FA typically shows severe atrophy of the spinal cord, often with disproportionably little atrophy of the cerebellum. In MSCAE, we did not find radiologically detectable cord atrophy, while cerebellar atrophy was common (75%).

Encephalitis

Acute viral encephalitis typically presents with acute or subacute progressive encephalopathy with epileptic seizures and progressive cerebral lesions. Especially viruses of the herpes family (including Varicella zoster) can produce symptoms and MRI changes similar to those of acute episodes of POLG-encephalopathy. Viral lesions are predominantly cortical, T2 hyperintense and may have restricted or heterogeneous diffusion profiles in the acute phase due to virus mediated cytotoxic oedema and inflammatory hypercellularity [99, 100]. Lesion localisation sometimes – but not always- helps distinguish the two conditions. Herpes simplex encephalitis commonly causes extensive oedematous lesions of the medial temporal lobes including the hippocampi. These structures were consistently spared in our patients with POLG encephalopathy.

Ischemic stroke

Acute developing exacerbation episodes with stroke-like lesions may sometimes be confused with ischemic stroke, especially when they occur early in the course of the disease before typical features have been developed or detected. Epilepsy is uncommon in the acute phase of ischemic stroke and should alert the physician to consider alternative diagnoses. Evidence of restricted lesional water diffusion on MRI cannot be used to effectively differentiate between the two as it may occur in both during the acute phase. The pattern and distribution of diffusion change in the lesion may be more informative. The diffusion profile of stroke-like lesions is usually more heterogeneous than that of ischemic infarction and may contain areas of normal or increased diffusion, especially in the underlying white matter. Moreover, the stroke-like lesion predilection for the occipital areas, distribution across vascular perfusion borders and gradual progression should raise the possibility for a metabolic cause.

5.1.6 Survival and prognosis in MSCAE: the role of genotype and epilepsy

Compound heterozygous patients have worse prognosis

Our findings presented in paper I and supplemented here with unpublished data show that patients who are compound heterozygous for the A467T and W748S mutations have a significantly worse prognosis than homozygous patients with lower age of death and significantly shorter disease duration (p=0.003). Inclusion of the reported cases in the literature supports this finding (Table 3, figure 7). Clinical picture and epilepsy frequency are similar in homozygous and compound patients and the reason(s) why the A467T/W748S genotype is associated with more severe disease remain unknown. It is possible that the two mutations have a synergistic effect mediated by interaction between the two mutant proteins in a dominant negative fashion. This suggests that an interaction between two or more catalytic POLG subunits may be taking place also under normal conditions during mtDNA replication. So far, however, studies have failed to generate evidence supporting this hypothesis [25]

The role of epilepsy

We show that the presence of epilepsy is the most important independent prognostic factor in MSCAE and divides the disease into two subgroups with differences in mortality and age of onset. Patients who have epilepsy experience during the course of their disease, severe exacerbation episodes with ~50% mortality rate and are at risk of developing liver failure if given valproate based antiepileptics. Disease progression is also generally faster in this group due to accelerated, permanent symptom worsening after each episode that the patients survive. Patients without epilepsy have significantly better survival. Interestingly patients with epilepsy appear to have an earlier age of onset (~12 years) than patients with no epilepsy (~23 years).

The reasons for this apparent phenotypical heterogeneity in MSCAE remain unknown. One possibility is that the difference is caused by genetic factors. In our material, and the cases reported in the literature, the frequency of epilepsy is similar in the three major MSCAE genotypes. The catalytic pol γA is known to be highly polymorphic and functions together with the accessory pol γB. It is possible, therefore, that one or more changes in the catalytic subunit or its partner could have a

synergistic deleterious effect. We are currently examining this question and so far have not found any changes in *POLG1* or *POLG2* showing a significant effect on the incidence of epilepsy. This is however an ongoing study and, even if it remains negative upon completion, this would not exclude influencing factors in other parts of the genome. Environmental factors could also be involved, but none has yet been identified.

The difference in age of onset is also difficult to explain. It is possible that the apparently later age of onset in patients without epilepsy simply reflects later recognition of the symptoms. Patients without epilepsy have an insidious onset and a chronic, slowly progressive course that initially may not cause sufficient concern, to the patient or their families, to warrant medical attention. Epilepsy demands early medical attention and allows other, mild findings such as a slight gait ataxia to be identified.

5.1.7 Follow-up and treatment of POLG-encephalopathy

The follow-up of patients with POLG-encephalopathy should include regular clinical evaluation in order to access disability, so that the necessary adjustments can be made in the patient's life. Compound genotype and epilepsy are associated with increased morbidity and mortality and it should be noted that epilepsy may start late during the course of the disease changing the prognosis.

Cerebral MRI should be performed at the beginning of the illness diagnostically and to be used as future reference. Regular MRI examinations have little value except during exacerbation episodes. Detecting acute stroke-like lesions under episodes and monitoring their progression has important prognostic implications. The localisation and extent of the lesions correlates with potential for recovery and resulting disability. Moreover, gradual regression or disappearance of the lesions strongly suggests potential for recovery and may influence clinical decision taking on further treatment.

Blood chemistry including liver function tests and levels of antiepileptic agents should be performed on a regular basis, at least three times a year.

No curative treatment exists for POLG disease. Symptomatic and supportive treatments should focus on controlling the epilepsy, improving quality of life and preventing chronic complications of immobilisation.

Seizure control is the cornerstone of treatment. Epilepsy is associated with significant morbidity and plays a major role in the initiation and propagation of exacerbation episodes and cortical lesions. Seizures are often refractory in POLG encephalopathy and may require high dose polytherapy. Status epilepticus should be treated aggressively with a low threshold for generalised anaesthesia. Ketamine was effective in terminating status epilepticus in one case [101]. Valproic acid derivatives are contraindicated due to high risk for liver disease. With the exception of seizure control, no therapy has shown disease modifying effect in POLG encephalopathy patients. In one study folate deficiency was found in the cerebrospinal fluid of a child with Alpers' disease and follate supplementation was associated with transient clinical improvement [61].

5.2 Imaging and pathology findings in POLG-encephalopathy reveal important elements of pathophysiology (Paper III)

5.2.1 Cortical lesions preferentially affect the posterior parts of the brain and have features consistent with local energy failure

Stroke-like lesions in POLG encephalopathy show a predilection for the posterior brain and especially the occipital lobes. This was correlated with occipital epileptic activity clinically (visual seizures) and on EEG. The reason for this regional selectivity is unclear. It is possible that it has to do with high energy requirements or/and a lower threshold for dysfunction and injury due to energy deficiency. High energy requirement, however, is unlikely to be the sole cause. The hippocampus and striatum, which are known to be highly sensitive to energy restriction due to other causes (hypoxia, ischemia, carbon monoxide, prolonged seizures), are consistently spared, as are the central motor neurons.

Stroke-like lesions consistently show restricted water diffusion in the acute phase suggesting intracellular water sequestration i.e. cytotoxic cerebral oedema. Lesional diffusion heterogeneity with the simultaneous presence of areas of normal or increased diffusion is a known phenomenon from MELAS and most probably reflects the gradual and asynchronous development and progression of various parts of the lesion. With time, as affected cells die and lyse, the trapped water is released into the extracellular compartment, were molecular motion is less restricted, and diffusion increases. Baseline overshoot is probably due to transient formation of extracellular oedema, which is then gradually absorbed into the vascular compartment. Persistent high diffusion in chronic lesions reflects retraction and cavitation with permanently increased fluid content.

Histology of stroke-like lesions shows selective neuronal loss and eosinophilic neuronal necrosis, while nearby uninvolved areas have normal neuronal density and appearance. In the absence of signs of angiopathy and ischemia, the most likely explanation for this phenomenon is neuronal ATP deficiency caused by failure of the mitochondrial respiratory chain to meet the high energy demand. This is further supported by the finding of COX-negative neurons. Epilepsy will significantly aggravate, and may even trigger SLL, either by increasing neuronal energy consumption or via direct excitotoxic effects. Our data shows that epileptic activity may be absent early in the course of an exacerbation episode and that progression can still occur even when previously documented epileptic activity disappears. Ongoing epileptic activity is not, therefore, a prerequisite for SLL expansion or clinical progression. Currently, there is insufficient data, but it appears unlikely that epileptic activity is the primary or sole mechanism behind the development and progression of exacerbation episodes and stroke-like lesions in POLG-encephalopathy.

Further support for energy depletion comes from the finding of cortical laminar necrosis (CLN). CLN is believed to represent selective damage of the cortical layers that are most sensitive to ATP deficiency; the subsequent intracortical microhemorrhage or infiltration by fat-laden macrophages accounts for the shortening

of T1 signal [102]. Neuronal energy deficiency is also supported by the histological findings of selective neuronal loss and eosinophilic necrosis in the cerebral cortex and thalamus, selective loss of Purkinje cells and dentate neurons in the cerebellum, laminar cortical necrosis and neuronal COX-deficiency. Similar histological findings, restricted water diffusion and CLN have been associated with states of secondary CNS energy deficiency such as cerebral ischemia/hypoxia [102-105], carbon monoxide (CO) poisoning [106-108], cyanide (CN) poisoning [109, 110] and hypoglycaemic encephalopathy [111-113].

5.2.2 The thalamus

Chronic MRI lesions and histopathological changes affecting the thalamus, cerebellum and brainstem in MSCAE have been reported earlier [75, 98]. Thalamic lesions are very typical of MSCAE. The suggestion that these arise secondarily to status epilepticus [114] is not supported by our studies. Seven patients with epilepsy lacked MRI evidence of thalamic involvement while 2 without epilepsy (WS-14A, WS-15A) had bilateral, prominent thalamic changes. Apparently, mitochondrial dysfunction alone is capable of producing these lesions.

5.2.3 The cerebellum

Cerebellar cortical and dentate involvement is consistent with cerebellofugal degeneration, while white matter involvement may represent general loss of the cerebellar connections. Similar MRI and histological findings in the cerebellum have been reported in patients with Kearns-Sayre syndrome (KSS) [115, 116]. Moreover, synaptic immunohistochemistry studies in KSS have shown evidence of Purkinje cell disconnection at the dentate nucleus, i.e. loss of cerebellar efferents [116]. Radial microglial proliferation in the molecular layer of the cerebellar cortex has been described in models of drug toxicity and cerebral contusion injury and is thought to represent microglial activation along the dentritic processes of injured Purkinje cells [117-119].

5.2.4 Inferior olivary lesions correlate with dentate atrophy and segregate with the W748S homozygous genotype

Olivary lesions are consistent with hypertrophic olivary degeneration. This usually occurs secondarily to de-afferentation of the inferior olives by lesions interrupting the Guillain-Mollaret triangle, i.e. the connections between the dentate nucleus of the cerebellum and contralateral inferior olive and red nucleus [120]. No signs of rubral involvement were seen in our patients. However, evidence of bilateral dentate atrophy was found in 4/8 with olivary changes and dentate involvement could not be excluded in the remaining four. Classically, this type of olivary lesions has been associated with palatal tremor/myoclonus, but this was only seen in one case [74, 121], while in the remaining seven no clinical correlate could be found. Interestingly, olivary lesions only occurred in W748S homozygotes, but the reason for this remains unclear.

5.2.5 The spinal cord shows selective degenerations of the dorsal columns

The spinal cord was radiologically intact, however, neuropathological examination showed selective degeneration of the dorsal columns consistent with the proprioceptive defect and sensory ataxia in MSCAE patients. Interestingly, changes were more severe in the fasciculus gracilis, which carries sensory input from the lower limbs. This correlates well with the clinical findings, showing more severe sensory impairment in the lower limbs. The selective involvement of long sensory axons may be associated with failure to meet high energy requirements related to axonal transport, due to the underlying mitochondrial defect.

5.3 The molecular pathogenesis of POLG disease – from POLG mutation to mtDNA damage and tissue specific energy failure (paper IV and unpublished material)

5.3.2 The common POLG mutations A467T and W748S reduce the efficiency of the polymerase

Mitochondrial DNA is necessary for the formation and function of the respiratory chain and is therefore essential for energy production in animal cells. POLG is the only molecule which replicates and repairs mtDNA and in its absence, the mitochondrial genome cannot be maintained. Homozygous POLG knock out mice die *in utero* by the eighth day of gestation and show severe mtDNA depletion and respiratory chain failure as reflected by global and total absence of histochemical COX activity [122]. In humans, several truncating POLG mutations have been described which are predicted not to allow formation of functional protein. Such mutations are asymptomatic in heterozygous carrier state and associated with severe infantile encephalopathies and encephalohepatopathies when paired *in trans* with missence mutations including the common A467T and W748S. Nonsense mutations affecting both POLG alleles have not been reported, most probably because such genotypes are lethal early in gestation.

The common A467T is one of the best studied POLG mutations in humans. The aminoacid substitution affects a highly conserved region of the molecule, which has long been considered to be in the spacer area between the polymerase and exonuclease motifs of the protein. In vitro studies have shown that the A467T mutation reduces DNA binding affinity and DNA synthesis activity of the catalytic subunit and may inhibit its interaction with the accessory subunit [45, 123]. Recent protein crystallization work suggests that the A467T mutation does not occurs in the spacer, but in the thumb subdomain of the polymerase; a structure which in nuclear DNA polymerases is important for keeping the polymerase on the DNA strand and is therefore essential for processivity [25].

The W748S mutation affects the spacer domain and reduces efficiency of DNA synthesis although binding of the accessory subunit does not seem to be affected [124]. The W748S is commonly (but not always) found *in cis* with another change, the E1143G. In vitro enzyme studies have shown that catalytic POLG subunits with the E1143G alone exhibit slightly higher polymerase activity than their wild type counterparts. Moreover, when *in cis* with the W748S, the E1143G may moderate the

deleterious effect of the latter by rescuing some of the polymerase activity [124]. It has therefore been suggested that the E1143G may have a beneficial effect in patients with W748S associated disease. The role of the E1143G alone could not be studied in our material as it always accompanied the W748S *in cis*.

5.3.3 POLG mutations lead to tissue specific mtDNA multiple deletions and/or quantitative depletion

Although the exact mechanism(s) by which POLG mutations lead to disease are still to be revealed, secondary damage of mtDNA seems to play a central role. We and others have found evidence of tissue specific multiple deletions and/or quantitative depletion of the mitochondrial genome. Quantification of mtDNA by real-time PCR in our POLG encephalopathy patients showed depletion in skeletal muscle and liver, which is consistent with other reports [60, 62, 76, 77, 95]. The most severe depletion (~80%) was seen in the liver of patients WS-2A and AL-1A, who had both spontaneous liver failure during the last part of their illness. Patients AT-2A and WS-1A, in whom we found ~ 65-70% depletion in the liver, had a mild elevation of liver function tests, but not clinical failure and they died of other causes. This suggests a high threshold for clinical liver dysfunction due to mtDNA loss and may explain the acute precipitation of liver disease by sodium-valproate, which poses an additional metabolic challenge to already compromised hepatocytes. This is supported by the findings in mouse models of mtDNA depletion, such as the MPV17 knockout mouse, where the liver shows a striking functional tolerance to severe mtDNA depletion [125, 126]. Skeletal muscle showed severe depletion in all patients. This result is more difficult to interpret because SKM was clinically mildly affected in our patients (with the exception of extraocular muscles). Moreover, depletion in muscle did not always correlate with clinical myopathy and neither did multiple deletions.

MtDNA depletion was found in brain tissue from two patients only (WS-12A and AL-1A). The results may be skewed, however, by the presence of mtDNA from non-neuronal cells. For instance, the MILON mouse, which is characterized by the

absence of mtDNA (and OXPHOS) in neurons, due to cell type-specific ablation of Tfam, shows only a moderate reduction of mtDNA content in brain homogenate, due to the presence of glial cells with normal mtDNA copy number [127]. In order to overcome this problem, single neuron studies are needed. We will be doing this as part of an ongoing project to study the molecular pathogenesis of POLG disease in tissues of patients that we collected through the years.

5.3.4 MtDNA depletion occurs in skeletal muscle of patients with PEO caused by POLG mutations

In paper IV we report mtDNA depletion in the muscle of a patient with POLG-associated PEO. Although multiple mtDNA deletions are considered a hallmark of POLG-associated PEO, depletion has, to the best of our knowledge, not been reported. Our patient's levels of depletion were significantly higher than the levels of deletion. Although this was shown in a single case only, it suggests that, in addition to multiple deletions, quantitative loss of the mitochondrial genome may play a major role in the pathogenesis of PEO caused by POLG mutations.

5.3.5 From mtDNA damage to energy failure and disease

Irrespective of whether it is deletions, depletion or both that have most impact the mitochondrial genome, mtDNA damage is predicted to affect the integrity of the respiratory chain and therefore damage OXPHOS and compromise the cell's energy generating capacity. Several of our findings, including diffusion imaging of acute lesions while they develop, histochemistry and histology of biopsy and post-mortem material, suggest that energy failure (lack of ATP) occurs in tissues of patients with POLG disease and that it is an important pathophysiological event.

Furthermore, we suggest that energy depletion in the CNS, by generating cortical neuronal dysfunction and damage, predisposes to epileptogenesis, which in turn potentiates neuronal damage by increasing the energy demands of the already metabolically challenged neurons. This results in a self-perpetuating cycle of neuronal

damage and epilepsy, which we believe is what initiates and sustains exacerbation episodes and expands cortical lesions. Other factors, like excess lactate, glutamate mediated excitotoxicity and even free radical generation may also play a role.

6. Conclusions / main points

- The common POLG mutations A467T and W748S cause a clinically well defined entity that is interchangeably called MSCAE or MIRAS. This is a complex syndrome that usually presents in the mid teens -but has a broad range form early childhood to adulthood- and is clinically characterised by progressive spinocerebellar ataxia, chronic and episodic encephalopathy and late-onset external ophthalmoplegia.

- Most Norwegian patients with MSCAE develop epilepsy at onset or during the course of their disease. All patients with epilepsy, including those who develop it late, experience acute episodic exacerbations with rapidly progressive encephalopathy and expanding stroke-like cerebral lesions. Episode mortality is high (~50% in our material) and aggressive preventive and abortive treatment of the epilepsy and status epilepticus is required. Patients with no epilepsy have milder disease with slowly progressive course, but no stroke-like episodes and although they face disability, mainly due to the ataxia, survival at least until the seventh decade and probably longer is possible.

- MSCAE has a complex epileptic semiology with a variety of clinical seizure types and frequent status epilepticus. EPC and an occipital epileptogenic focus on EEG are characteristic findings and should provide a clue for the diagnosis.

- Genotype is an important prognostic factor in MSCAE. Patients who are compound heterozygous *in trans* for the A467T and W748S mutations have a significantly worse prognosis than homozygous patients with lower median survival and age of death.

- Severe liver disease and failure may occur in MSCAE upon exposure to valproic acid derivatives, or less commonly spontaneously. Spontaneous liver failure is more common in Alpers' disease. All valproic acid derivatives are strictly contraindicated in POLG-disease.

- MRI is highly sensitive in POLG-encephalopathy and can be specific in MSCAE. Typical chronic findings in MSCAE include high T2 signal lesions in the thalamus, cerebellar white matter and olivary nuclei. Acute stroke-like cortical lesions with a predilection for the posterior brain and restricted diffusion profiles occur during episodic exacerbations in POLG-encephalopathy. These should be monitored as their evolution is important for prognosis and episode outcome.

- We and others have shown that tissue specific mtDNA damage, histochemical COX deficiency and respiratory complex defects occur in tissues of patients with POLG-disease. These findings suggest that POLG mutations lead to secondary dysfunction of the respiratory chain, which is predicted to cause energy failure in cells. Our imaging and histological findings, presented in paper III provide important evidence that energy failure indeed occurs in the CNS of patients with POLG-encephalopathy and that it plays a central role in the pathogenesis and pathophysiology of this complex disorder.

7. Future prospects

POLG is a fascinating molecule with an essential function for life and important role in human disease. Although numerous mutations and clinical syndromes have been described, still relatively little is known about the underlying mechanisms by which mutations lead to disease. Our findings suggest that energy failure due to secondary mtDNA damage and resulting respiratory chain dysfunction is an important pathophysiological event. Several questions remain however unanswered. Energy failure alone is not sufficient to explain the complete semiology, organ/region selectivity and clinical variability of POLG-disease suggesting that other mechanisms must be involved. Moreover, the type and severity of mtDNA changes seem to vary significantly in different tissue and cell types and this has not been mapped or studied sufficiently. In order to achieve this, more studies of human tissue material are required.

We are currently conducting a histological and molecular study of post-mortem and biopsy tissue-samples from various organs of patients with POLG-disease. Some of the first, yet unpublished results have been reported and discussed in this thesis and upon completion of the work we hope to achieve a better understanding of the tissue selectivity and specificity issues of POLG-disease.

Although necessary and irreplaceable, human studies have several well-known disadvantages and limitations. Human post-mortem tissues are difficult to obtain, often show terminal disease changes –giving little specific information about the processes that led to these- and are commonly degraded producing results difficult to interpret. Using cell culture and other *in vitro* systems may be useful, but cannot compensate for the lack of fresh tissue samples from affected organs. In order to overcome this problem and study POLG-disease in a living system as it arises and evolves, we have generated a transgenic mouse carrying the common POLG mutation p.A467T and are currently conducting studies in order to characterise the phenotype. Future projects will include detailed pathological, biochemical and molecular

characterisation of the mouse tissues at different stages of disease progression in order to understand the mechanisms underlying disease causation and evolution and to identify potential therapeutic targets.

Furthermore, through research collaborations, our mice will be crossed with mouse models carrying other POLG mutations. Studying the compound heterozygous offspring of such crosses will allow us study genotype-phenotype correlations and identify potential interactions between different POLG mutations.

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9. Appendices

Thesis	Paper-III	Paper-II	Paper-I
AT-1A	AT-1A	2	2
AT-1B	AT-1B	1	1
AT-2A	AT-2A	4	3
AT-2B	AT-2B	3	4
AT-3A	AT-3A		5
AT-4A	AT-4A		
CP-1A	CP-1A		10
CP-1B		6	9
CP-1C*			
CP-2A	CP-2A		7
CP-3A	CP-3A	5	6
CP-4A	CP-4A	8	12
CP-4B	CP-4B	7	11
CP-5A			8
CP-6A			
WS-1A	WS-1A		16
WS-2A	WS-2A	14	23
WS-3A	WS-3A	16	25
WS-4A	WS-4A	18	26
WS-5A	WS-5A		21
WS-6A	WS-6A	15	24
WS-7A	WS-7A	11	18
WS-7B	WS-7B	12	19
WS-8A	WS-8A		
WS-9A	WS-9A	9	15
WS-10A	WS-10A	13	22
WS-11A	WS-11A	10	17
WS-12A	WS-12A	17	20
WS-13B	WS-13B	19	13
WS-14A	WS-14A		
WS-14B*			
WS-15A	WS-15A		
WS-16A*			
WS-17A*			
WS-17B*			
WS-18A	WS-18A		
AL-1A	AL-1A		
AL-1B	AL-1B		
AL-2A	AL-2A		
AL-3A	AL-3A		

Appendix I. Key to patient codes in the different papers. Unreported cases are marked by *.

Code	POLG mutation	AO	Age now / at death*	Presenting feature	Epi	EE	MY	HA	AX	PN	PEO		Liver	
											pres	age	pres	VAL
AT-1A	A467T / A467T	15	44*	MLH	+	+	+	+	+	+	+	26	-	
AT-1B	A467T / A467T	8	47*	HA	+	+	+	+	+	+	+	30	+	-
AT-2A	A467T / A467T	16	53*	Epi	+	+	+	+	+	+	+	32	+	-
AT-2B	A467T / A467T	6	55*	Epi	+	+	+	+	+	+	+	35	-	
AT-3A	A467T / A467T	15	24	MLH	+	+	+	+	+	+	-		-	
AT-4A	A467T / A467T	15	40	PGU	-	-	+	-	+	+	+		-	
A467T homozygous, n=6			MAD		5/6	5/6	6/6	5/6	6/6	6/6	5/6		2/6	0/2
Mean		**12.5**	MAD = 50		83%	83%	100%	83%	100%	100%	83%	30.7	33 %	0
CP-1A	A467T/W748S	36	56	PGU	-	-	+	+	+	+	+	44	-	
CP-1B	A467T/W748S	18	19*	HA, Epi	+	+	+	+	+	+	-		+	+
CP-1C	A467T/W748S	40	52	PGU	-	-	-	-	+	+	+	45	-	
CP-2A	A467T/W748S	24	48	PGU	-	-	-	+	+	+	+	25	-	
CP-3A	A467T/W748S	20	20*	Epi	+	+	-	+	+	+	-		+	+
CP-4A	A467T/W748S	14	23*	HA, Epi	+	+	+	+	+	+	-		+	+
CP-4B	A467T/W748S	13	21*	Epi	+	+	+	+	+	+	-		+	+
CP-5A	A467T/W748S	10	10*	Epi	+	+	+	+	+	+	-		+	+
CP-6A	A467T/W748S	13	13*	Epi	+	+	+	+	+	?	-		+	+
Compound heterozygous,n=9					6/9	6/9	6/9	9/9	9/9	8/8	3/8		6/9	6/6
Mean		**20.9**	MAD = 17.7		67%	67%	67%	100%	100%	100%	38%	38	67 %	100%
WS-1A	W748S/W748S	6	41*	PGU	+	+	+	+	+	+	+	26	+	-
WS-2A	W748S/W748S	15	24*	MLH	+	+	+	+	+	+	-		+	-
WS-3A	W748S/W748S	17	43	MLH	+	+	+	+	+	+	+	26	-	
WS-4A	W748S/W748S	19	38	Epi	+	+	+	+	+	+	+	28	+	+

Code	POLG mutation	AO	Age now / at death*	Presenting feature	Epi	EE	MY	HA	AX	PN	PEO		Liver	
											pres	age	pres	VAL
WS-5A	W748S/W748S	12	43	MLH	-	-	-	+	+	+	+	>30	-	
WS-6A	W748S/W748S	17	32	MLH	+	+	-	+	+	+	-		-	
WS-7A	W748S/W748S	4	30*	PGU	+	+	+	+	+	?	+	30	+	+
WS-7B	W748S/W748S	2	22*	PGU	+	+	+	+	+	+	-		+	+
WS-8A	W748S/W748S	12	28*	Ataxia	+	+	+	+	+	+	+		-	
WS-9A	W748S/W748S	2	13*	DD	+	+	-	-	+	-	-		+	-
WS-10A	W748S/W748S	18	24	Epi	+	+	+	+	+	+	+	20	+	-
WS-11A	W748S/W748S	16	23	Epi	+	+	-	+	-?	+	-		-	
WS-12A	W748S/W748S	15	57*	HA, PGU	+	+	-	+	+	+	+		-	
WS-13B	W748S/W748S	8	9*	MLH	+	+	+	+	+	+	-		+	+
WS-14A	W748S/W748S	21	37	PGU	-	-	-	+	+	+	-		-	
WS-14B	W748S/W748S	16	22	PGU	-	-	-	-	+	+	-		-	
WS-15A	W748S/W748S	20	47	PGU	-	-	+	-	+	?	+		-	
WS-16A	W748S/W748S	1.5	5	DD	-	-	-	-	+	?	-		-	
WS-17A	W748S/W748S	2	18*	HA, PGU	+	+	?	+	+	+	-		+	+
WS-17B	W748S/W748S	11	12*	Epi	+	+	+	+	+	?	-		+	+
WS-18A	W748S/W748S	45	66	PGU	-	-	+	-	+	+	+		-	
W748S homozygous, n=21					15/21	15/21	12/20	16/21	20/20	16/17	10/21		10/21	6/10
Mean		**13.3**	MAD = 25.4		71%	71%	60%	76%	100%	94%	48%	26.7	48 %	60%
All MSCAE, n =36					**26/36**	**26/36**	**24/35**	**29/36**	**35/35**	**29/31**	**18/36**		**18/36**	**12/18**
Mean		**15.2**	**MAD = 28**		**72%**	**72%**	**69%**	**81%**	**100%**	**94%**	**50%**	**30.5**	**50%**	**67%**

Code	POLG mutation	AO	Age now / at death*	Presenting feature	Epi	EE	MY	HA	AX	PN	PEO		Liver	
											pres	AO	pres	VAL
AL-1A	A467T/G303R	0.9	1.08*	Epi	+	+	-	-	-	-	-		+	-
AL-1B	A467T/G303R	2	8*	Epi	+	+	-	-	+	-	-		-	-
AL-2A	A467T/G848S	0.6	0.65*	Epi	+	+	-	-	-	-	-		+	-
AL-3A	A467T/G303R	1	1.4*	Epi	+	+	-	-	-	-	-		+	-
Alpers', n=4					4/4	4/4	0/4	0/4	1/4	0/4	0/4		3/4	
Mean		1.13	MAD = 2.8		100%	100%	0	0	25%	0	0		75%	

Appendix II. Clinical features of POLG-encephalopathy. AT, WS and CP denote the genotype of the MSCAE patients (AT = A467T homozygous, WS = W748S; CP = A467T/W748S). The Alpers' patients are coded as AL. Numbers symbolise the family and letters the individual. Individuals with the same mutation and number, but different letters are siblings. AO: age at onset. Age of death is marked by "*". All ages are in years. MAD: mean age of death. Epi: epilepsy. EE: episodic exacerbations. MY: myoclonus. HA: headache. PGU: progressive gait unsteadiness. DD: developmental delay. AX: ataxia. PN: peripheral neuropathy. PEO: progressive external ophthalmoplegia. Pres: present. VAL: use of sodium valproate before liver disease.

Patient	Epilepsy & Episodes	Stroke-like lesions	Thalamus	Inferior olives	Cerebellum					Cerebral atrophy
					Cortex	WM	Dentates	Atr	Total	
AT-1A	+	BO, LP, pons	B	-	-	B	-	+	+	-
AT-1B	+	RPO, LP, BF	-	-	-	B	-	+	+	+
AT-2A	+	BF, RP	-	-	L	-	-	+	+	-
AT-2B	+	-	-	-	-	-	-	+	+	+
AT-3A	+	-	-	-	-	-	-	-	-	-
AT-4A	-	-	-	-	-	-	-	+	+	-
n=6	**83%**	**50%**	**17%**	**0**	**17%**	**33%**	**0**	**83%**	**83%**	**33%**
CP-1A	-	-	-	-	-	-	T2 loss	+	+	-
CP-2A	-	-	-	-	-	-	-	+	+	-
CP-3A	+	RO	B	-	-	-	T2 loss	+	+	-
CP-4A	+	BO, BP, RF	-	-	L	-	?	-	+	-
CP-4B	+	LTO, RO, BF, RCE	L	-	-	-	?	-	-	-
CP-6A	+	LO	L	-	-	-	-	-	-	-
n=6	**67%**	**67%**	**50%**	**0**	**17%**	**0**	**33%**	**50%**	**67%**	**0**
WS-1A	+	RO, RF	B	B	-	B	atrophy	+	+	+
WS-2A	+	RO	B	-	-	-	-	+	+	-
WS-3A	+	BO	B	B	-	-	atrophy	+	+	+
WS-4A	+	LP	L	B	B	-	atrophy	+	+	+

Patient	Epilepsy & Episodes	Stroke-like lesions	Thalamus	Inferior olives	Cerebellum					Cerebral atrophy
					Cortex	WM	Dentates	Atrophy	Total	
WS-5A	-	_	-	-	B	-	-	+	+	-
WS-6A	+	RO, RP	B	-	-	-	-	-	-	-
WS-7A	+	BO, LF, LP, BT	B	-	-	R	-	+	+	+
WS-7B	+	LO, LP, BF	B	B	-	-	?	+	+	+
WS-8A	+	BF. BP, LTO	B	-	-	-	T2 loss	-	+	-
WS-9A	+	BO, LP	B	B	-	-	T2 loss	+	+	+
WS-10A	+	RF, LO, RCE	R	B	-	-	atrophy	+	+	+
WS-11A	+	LO	B	-	-	-	-	-	-	-
WS-12A	+	_	B	-	-	B	?	+	+	-
WS-14A	-	_	B	B	-	-	T2 loss	+	+	-
WS-15A	-	_	B	-	B	-	T2 loss	+	+	+
WS-18A	-	_	-	B	B	-	T2 loss	+	+	+
n=16	**75%**	**69%**	**88%**	**50%**	**38%**	**19%**	**56%**	**81%**	**88%**	**56%**
MSCAE n=28	**75%**	**64%**	**64%**	**29%**	**29%**	**18%**	**39%**	**75%**	**82%**	**39%**
Patient	**Epilepsy & Episodes**	**Stroke-like lesions**	**Thalamus**	**Inferior olives**	**Cerebellum**					**Cerebral atrophy**
					Cortex	**WM**	**Dentates**	**Atrophy**	**Total**	
AL-1A	+	BF, RCE	-	-	-	-	-	-	-	-

AL-1B	+	BTPO	-	-	-	-	-	-	-	-
AL-2A	+	LF	-	-	-	-	-	-	-	-
AL-3A	+	BTPO, LCE	-	-	-	-	-	-	-	-
Alpers' n=4	**100%**	**100%**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Appendix III. MRI findings in 32 patients with POLG associated encephalopathy. AT, WS and CP denote the genotype of the MSCAE patients (AT = A467T homozygous, WS = W748S; CP = A467T/W748S). The Alpers' patients are coded as AL. Numbers symbolise the family and letters the individual. Individuals with the same mutation and number, but different letters are siblings. **AO:** age at onset. **AD:** age at death. **Episodes:** exacerbation episodes with epilepsy and encephalopathy. **F, P, T, O, CE:** frontal, parietal, temporal, occipital, cerebellum respectively. **R, L, B:** right, left, bilateral. **IO**: inferior olivary nuclei. **WM:** white matter,**?:** Image quality did not allow for evaluation of dentates.

8. Original Publications