Crisis in Energy Metabolism

—

Mitochondrial Defects
and
a New Disease Entity

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Göteborg 2007

Printed at Vasastadens bokbinderi, AB
Göteborg, Sweden, 2006

ISBN-10 91-628-7032-7
ISBN-13 978-91-628-7032-4

To Jonas and Joel

ABSTRACT

Impairment of energy metabolism may be associated with severe implications for affected individuals since all fundamental cell functions are energy-dependent. Disorders of energy metabolism are often genetic and associated with defects in the oxidative phosphorylation in mitochondria. This thesis addresses the pathogenesis in some mitochondrial disorders and a new disease entity associated with defects in the glycogen metabolism.

In paper I we report on a primary mutation in mitochondrial DNA. We identified a T C mutation at position 582 in the gene for $tRNA^{Phe}$ in a case of mitochondrial myopathy. The mutation alters a conserved base pairing in the aminoacyl stem of the tRNA. By analysis of single muscle fibers we showed that the level of heteroplasmy (proportion of mutant mtDNA) was higher in muscle fibers with defective cytochrome *c* oxidase (COX) activity compared to normal muscle fibers. Based on these findings we conclude that this mutation was responsible for the disease.

In paper II we investigated a 30-year-old woman, who presented with an attack of acute rhabdomyolysis. We found an isolated deficiency of COX and a novel nonsense mutation in mtDNA in the gene encoding COX subunit I. In addition to its catalytic function, our data clearly indicates an important function of subunit I for the assembly of COX. The mutation was restricted to the patient's muscle, but was not detectable in myoblasts, cultured from satellite cells isolated from affected muscle tissue. This result may have interesting implications for the natural evolution of the disease and perhaps therapy, since regenerating muscle occurs by proliferation of satellite cells.

In paper III we investigated patients with mitochondrial diseases (progressive external ophthalmoplegia, PEO) due to primary mutations in *POLG1* encoding mtDNA polymerase gamma (Pol) and secondary multiple mtDNA deletions. The results show that it is very unlikely that mtDNA point mutations contribute to the pathogenesis in PEO patients with primary *POLG1* mutations, and that the mechanism by which mutant Pol cause mtDNA deletions does not involve mtDNA point mutations as an intermediate step, as has been previously proposed.

In paper IV mtDNA alterations and pathology of muscle, brain and liver was investigated in children with Alpers-Huttenlocher syndrome (AHS), a fatal neurodegenerative disease associated with liver failure. All children had compound heterozygous missense mutations in *POLG1*. We provide evidence that AHS is a mitochondrial disease by demonstrating mtDNA alterations (reduced mtDNA copy number and multiple mtDNA deletions). Liver disease was triggered by valproate treatment in several cases possibly due to severe respiratory chain deficiency which was demonstrated in liver tissue in one case.

In paper V we report on a new disease entity "Muscle glycogen storage disease type zero" due to a homozygous stop mutation in the muscle glycogen synthase gene (*GYS1*). We performed investigations on a family where one child suffered sudden cardiac death at the age of 10 and his younger brother showed muscle fatigability and hypertrophic cardiomyopathy. In muscle there was a profound glycogen deficiency and an almost total predominance of oxidative muscle fibers.

Keywords: Energy metabolism, mitochondrial disorders, point mutation, mtDNA, multiple mtDNA deletions, Alpers-Huttenlocher syndrome, *POLG1*, Polymerase gamma, *GYS1*, glycogen synthase

ISBN 10 91-628-7032-7, ISBN 13 978-91-628-7032-4 Göteborg 2006

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. A-R. Moslemi, C. Lindberg, J. Toft, E. Holme, **G. Kollberg**, A. Oldfors. A novel mutation in the mitochondrial $tRNA^{Phe}$ gene associated with mitochondrial myopathy. *Neuromuscular disorders* 2004 Jan;14:46-50.

II. **G. Kollberg**, A-R. Moslemi, C. Lindberg, E. Holme, A. Oldfors. Mitochondrial myopathy and rhabdomyolysis associated with a novel nonsense mutation in the gene encoding cytochrome *c* oxidase subunit I. *J Neuropathol Exp Neurol*. 2005 Feb;64:123-128

III. **G. Kollberg**, M. Jansson, Å. Perez-Bercoff, A. Melberg, C. Lindberg, E. Holme, A-R. Moslemi, A. Oldfors. Low frequency of mtDNA point mutations in patients with PEO associated with *POLG1* mutations. *Eur J Hum Genet*. 2005 Apr;13:463-469

IV. **G. Kollberg**, A-R. Moslemi, N. Darin, I. Nennesmo, I. Bjarnadottir, P. Uvebrant, E. Holme, A. Melberg, M. Tulinius, A. Oldfors. *POLG1* Mutations Associated With Progressive Encephalopathy in Childhood. J Neuropathol Exp Neurol. 2006 Aug;65:758-768.

V. **G. Kollberg**, M. Tulinius, T. Gilljam, I. Östman-Smith, G. Forsander, P. Jotorp, A. Oldfors, E. Holme. Muscle glycogen storage disease 0 – A cause of sudden cardiac death. *Submitted*

ABBREVIATIONS

Acetyl-CoA	Acetyl Coenzyme A
adPEO	Autosomal dominant progressive external ophthalmoplegia
AHS	Alpers-Huttenlocher syndrome
arPEO	Autosomal recessive progressive external ophthalmoplegia
ATP	Adenosine 5'-triphosphate
bp	Base pair
cDNA	Complementary DNA
CoQ_{10}	Coenzyme Q_{10}
COX	Cytochrome c Oxidase
DGGE	Denaturing gradient gel electrophoresis
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
$FADH_2$	Flavin adenine dinucleotide
G6P	Glucose-6-phosphate
kb	Kilo base pair
kDa	Kilo Dalton
LX-PCR	Long extension polymerase chain reaction
MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes
MGSKO	Muscle-glycogen-synthase knockout
mtDNA	Mitochondrial DNA
NADH	Nicotineamid adenine dinucleotide
nDNA	Nuclear DNA
NIDDM	Non-insulin-dependent diabetes mellitus (diabetes type II)
OXPHOS	Oxidative phosphorylation
PCR	Polymerase chain reaction
PEO	Progressive external ophthalmoplegia
Pol□	Mitochondrial DNA polymerase gamma
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRF	Ragged red fibers
rRNA	Ribosomal RNA
SDH	Succinate dehydrogenase
tRNA	Transfer RNA

CONTENTS

BACKGROUND

Energy obtained from foods must be converted to chemical energy that can be utilized for cellular reactions inside a living cell. This conversion is carried out in the human body through a series of well-regulated reactions divided in three main stages. It starts with digestion of large macromolecules as proteins, carbohydrates and fats in the digestive system, which generates amino acids, glucose and fatty acids. The nutrients are then transported in the vessels to every part of the body where further degradation continues inside of the cells, beginning in the cytosol and ends up in the mitochondria where the energy metabolism is complete (Figure 1).

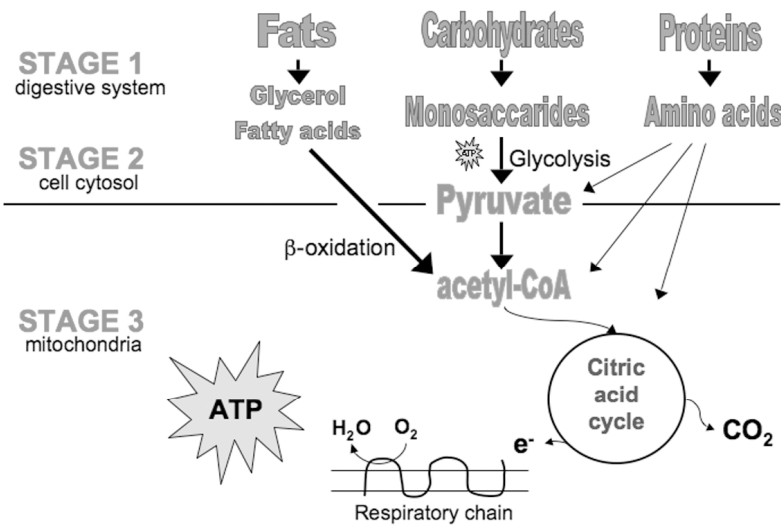


Figure 1. Schematic illustration of the three stages in the conversion of energy extracted from foods. In the first stage, large macromolecules are broken down into smaller subunits. In the second stage, which starts in the cell cytosol, these small molecules are degraded into simpler units with a central role in metabolism. Stage three includes the citric acid cycle and the respiratory chain in mitochondria. The end product in this process is ATP, a molecule that can be utilized for cellular work. Most ATP is produced in stage three but a small amount is generated in the glycolysis in stage II

Cellular energy and disease

The most important carrier and capturer of chemical energy in almost all living organisms on earth is a molecule called adenosine triphosphate, or ATP. The useful energy in ATP is bound in highly energetic phosphoanhydride bonds, which release free energy when hydrolyzed. This energy is used e.g. for synthesis of macromolecules, contraction of muscle cells, movement of individual cells from one location to another and the transport of molecules against a concentration gradient. Fundamental cell functions are dependent on energy and thus, impaired cellular energy production can affect any organ or tissue (Figure 2). The vast majority of ATP is produced inside of mitochondria, intracellular organelles present in virtually all eukaryotic cells. Indeed, diseases associated with crisis in the energy metabolism are

often multisystemic, but high energy-demanding tissues like skeletal muscle and brain are often affected in the so-called mitochondrial encephalomyopathies. [1, 2]

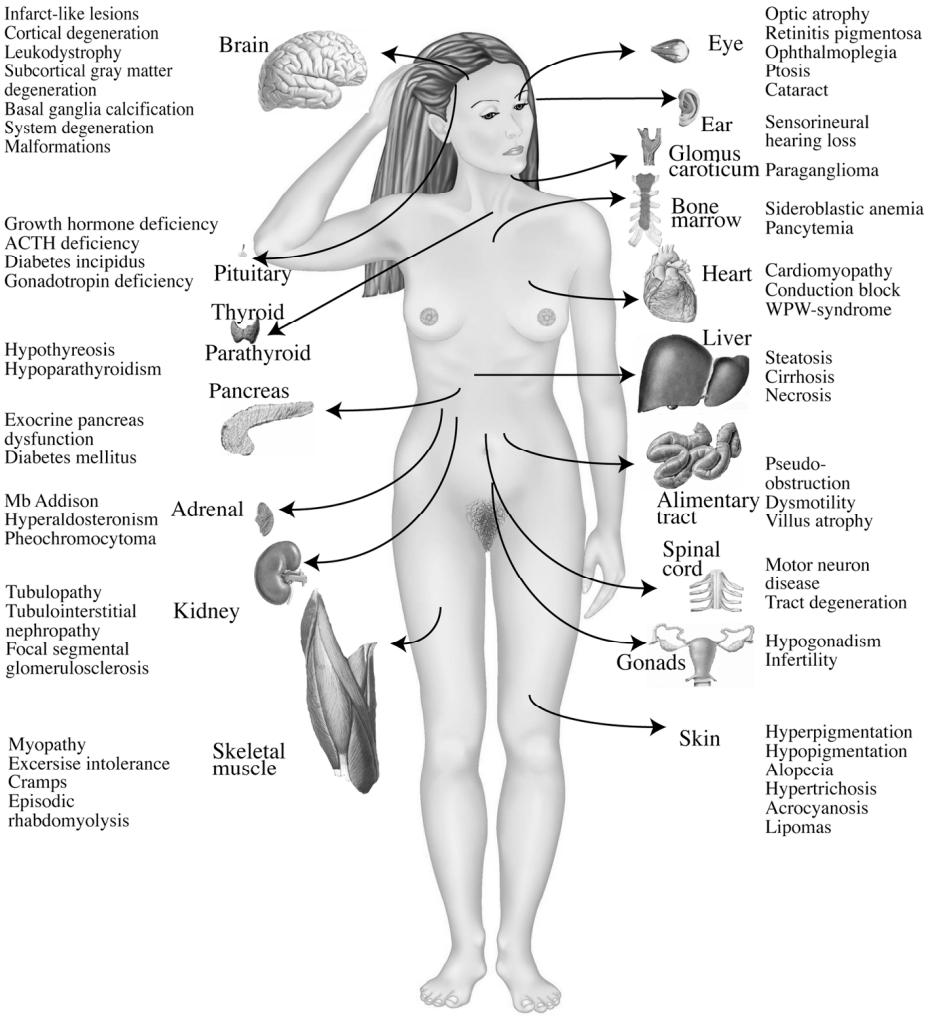


Figure 2. Summary of organ and tissue manifestations in mitochondrial diseases. Illustration by Yvonne Heijl

The turnover of ATP is rapid and there is no cellular ability of long-time storage. Thus, there is need for alternative energy storage in order to supply the energy demand between meals. Energy in mammals is efficiently stored as glycogen and fat. Defects in the capacity of energy storage as well as defects in the degradation of stored energy are associated with various metabolic diseases [3]. In addition, the energy metabolism can be affected through deficiencies of transferring substrates to its correct destiny. Perhaps the best known disease associated with a transport defect is diabetes, where the ability for glucose to enter the muscle cells from the blood is severely reduced, but defects in other transport systems can have serious implications e.g. carnitine deficiency where long-chain fatty acids cannot enter the mitochondria [4]. Moreover, certain drugs can also severely impair the energy metabolism [5].

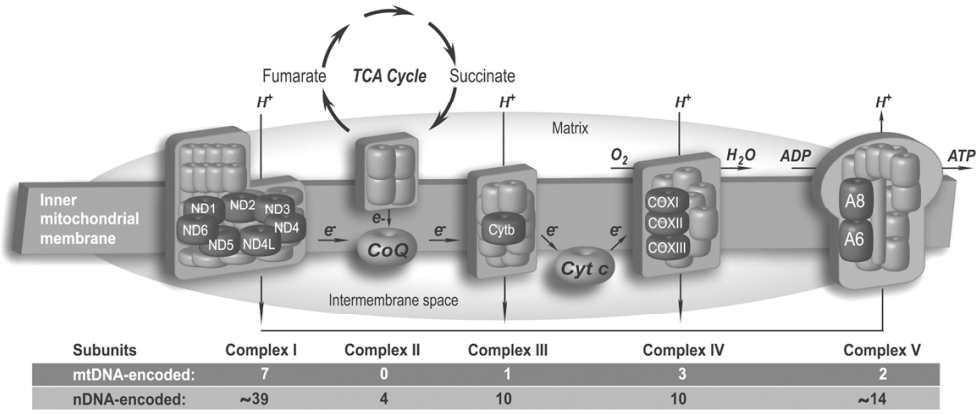
Mitochondria

The structure of mitochondria can be visualized using electron microscopy. They can vary in size and shape, but are in general oblong and 0.5 – 1 μm. Four compartments can be distinguished: the outer and inner membrane, the intermembrane space and the inner matrix. The outer membrane is permeable for molecules up to 10 000 daltons whereas the inner membrane is highly selective, and only oxygen (O_2), carbon dioxide (CO_2) and water (H_2O) can pass freely. The inner membrane has foldings, called cristae, increasing the surface, and thereby the efficiency of energy production. According to the endosymbiotic theory, mitochondria evolved from the fusion of an aerobic bacteria and an early eukaryotic cell about 1.8 billion years ago, and although mitochondria are not capable of free living today, they still have some bacterial remnants. One of the most prominent relics besides the double membrane structure is the circular, double stranded DNA molecule, discovered in 1963 [6]. The whole sequence for the human mitochondrial DNA (mtDNA) molecule was available in 1981 [7].

The ATP production in mitochondria is mediated by oxidative phosphorylation (OXPHOS), i.e. oxidation of substrates, mainly pyruvate and fatty acids, to CO_2 and H_2O. Pyruvate passes the mitochondrial membranes through a specific pyruvate carrier and forms acetyl Coenzyme A (acetyl-CoA) after decarboxylation. Acetyl CoA enters the citric acid cycle in the mitochondrial matrix. Fatty acids are activated in the outer mitochondrial membrane and carried through the inner membrane by carnitine, a compound formed from the amino-acid lysine. The specific transporter in the inner mitochondrial membrane is called carnitine-palmitoyl-transferase II. In the matrix, the fatty acids are oxidized and degraded by sequential removal of two-carbon units in the so-called β-oxidation. One molecule of acetyl-CoA is formed for each round of oxidation. Reducing agents, NADH and $FADH_2$, which are generated in the β-oxidation and in the citric acid cycle, are reoxidized in the respiratory chain. If the amount of oxygen is limiting e.g. in muscle during intense activity, pyruvate and fatty acids are not transported into the mitochondria. In order to rapidly replenish the pool of NAD+, the pyruvate forms lactate in the cytosol that is transferred to the liver where it becomes reconverted to glucose. Constantly high lactate levels in blood and/or cerebral spinal fluid, even at rest, can be a marker for mitochondrial dysfunction and impaired ATP-production.

The OXPHOS system

Five multi-subunit complexes (Complex I – V) and two loosely bound electron carriers (Coenzyme Q_{10} and Cytochrome *c*) constitute the OXPHOS system, which is embedded in the inner mitochondrial membrane (Figure 3).



Subunits	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA-encoded:	7	0	1	3	2
nDNA-encoded:	~39	4	10	10	~14

Figure 3. Schematic illustration of the respiratory chain located in the inner mitochondrial membrane. Five complexes and two electron carriers constitutes the OXPHOS system. Dark grey: mtDNA encoded subunits. Light grey: nDNA encoded subunits.

Complex I (NADH: ubiquinone oxidoreductase) consist of approximately 46 subunits, of which 7 are mtDNA encoded. Electrons from NADH are transferred to Complex I, which donates them further to CoenzymeQ_{10} (CoQ_{10}).
Complex II (succinate:ubiquinone oxidoreductase, succinate dehydrogenase), which only consists of four nuclear DNA (nDNA) encoded subunits, is a part of the citric acid cycle where it converts succinate to fumarate. In addition, Complex II passes electrons from $FADH_2$ to CoQ_{10}.
CoQ_{10} is oxidized by **Complex III** (ubiquinone: cytochrome *c* oxidoreductase), which on its turn is oxidized by cytochrome *c*. Complex III has only one subunit encoded by mtDNA, cytochrome b.
Complex IV (Cytochrome *c* Oxidase or COX) is the terminal enzyme of the electron transport chain and catalyzes the reduction of molecular oxygen to water. The three mtDNA-encoded subunits (I, II, and III) form the catalytic core of the complex. Subunit I is the largest, and is also the first intermediate in complex IV assembly [8] where it acts as a scaffold protein for the rest of the subunits [9].
As the electrons are transported along the electron transport chain, protons are pumped through the inner mitochondrial membrane into the intermembrane space by complex I, III and IV. This will build up an electrochemical gradient across the membrane and lead to the flux back of protons that drives the ATP synthesis by **Complex V** (ATP synthase). Two subunits of Complex V are mtDNA encoded.

Hence, there are two genomes contributing to the OXPHOS system: mtDNA, where all the 13 polypeptide genes encode respiratory chain subunits, and the nuclear DNA, encoding approximately 77 respiratory chain subunits. The mtDNA molecule comprises 16,569 base pairs, and in addition to the 13 proteins, mtDNA also encodes

22 tRNA genes and 2 rRNA genes necessary for the protein synthesis [7]. An approximately 1000 base pair control region (D-loop region) of non-coding DNA in the mtDNA molecule contains the heavy (H) and light (L) strand promoters and the H-strand origin of replication (OH) (Figure 4). Even though subunits encoded by mtDNA have a critical function in OXPHOS, mtDNA has a minor part in the overall function of mitochondria. Several important functions are located in the mitochondrial matrix i.e. the citric acid cycle, the β-oxidation of fatty acids, amino acid catabolism and pyruvate oxidation. All proteins necessary for these functions are encoded by nuclear DNA, synthesized in the cytoplasm and transported into the mitochondria. Moreover, transport proteins and factors necessary for the respiratory chain assembly as well as factors necessary for replication, transcription and translation of mtDNA are nuclear DNA encoded.

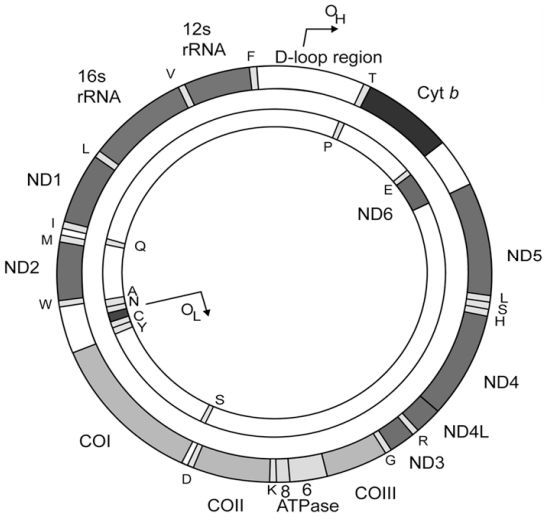


Figure 4. Schematic drawing of mtDNA with the heavy (outer circle) and light strands (inner circle). Protein coding genes: ND1-6 = NADH-dehydrogenase (complex I) subunits 1-6; Cyt b = Cytochrome b (Complex III); COI-III = cytochrome *c* oxidase (complex IV) subunit I-III; ATPase 6 and 8 = ATP synthase (complex V) subunit 6 and 8. Transfer RNA genes: short bars with corresponding amino acid letter. Ribosomal RNA genes: 12S rRNA and 16S rRNA. D-loop = Displacement loop. O_H = origin of heavy chain replication O_L = origin of light chain replication.

Mitochondrial diseases

Mitochondrial diseases are defined as disorders affecting the OXPHOS system, and the first disease coupled to defects in OXPHOS, in a woman with severe hypermetabolism, was described in 1962 [10]. The electron transport was uncoupled from the ATP synthesis and heat was generated instead of energy. When the first reports on mutations in mitochondrial DNA came 1988 [11-14], they opened a new field in the research area on metabolic disease, which generated a possibility for patients to get a correct diagnosis.

OXPHOS defects can be caused either by primary mtDNA mutations, nuclear gene mutations affecting subunits in OXPHOS or mutations in nuclear genes encoding proteins necessary for assembly of the respiratory chain, mitochondrial translation or mtDNA maintenance. The pattern of inheritance in mitochondrial diseases may vary

as a result of the dual genetic control. Diseases caused by mutations in the mtDNA molecule show maternal inheritance since mitochondrial DNA is almost exclusively inherited from mother to child [15], even though a rare exception has been reported [16]. The small amounts of paternal mtDNA that will pass into the oocyte during fertilization are normally actively degraded [17]. A Mendelian pattern of inheritance is shown in those mitochondrial diseases caused by mutations in nuclear genes.

Primary mtDNA mutations

Primary mtDNA mutations involve point mutations and rearrangements (single large-scale deletions or duplications) in the mtDNA molecule. Point mutations affect tRNA genes, rRNA genes or any of the protein-coding genes. Single large-scale deletions always encompass one or more tRNA genes. The majority of pathogenic mtDNA point mutations are in tRNA genes [18]. Each mitochondrion harbors 2 – 10 copies of mtDNA [19] and mtDNA molecules with pathogenic mtDNA mutations often co-exists with wild-type mtDNA molecules, a condition called heteroplasmy. Homoplasmy is when all mtDNA molecules are the same. Only occasionally, pathogenic mtDNA mutations show homoplasmy [13, 20, 21]. An important tool to determine the pathogenicity of a primary mtDNA mutation is to investigate the pattern of segregation for a heteroplasmic mutation in different tissues and at the single cell level. The proportion of the mutated mtDNA must reach a minimal critical level, a threshold, to cause impairment of mitochondrial protein synthesis and OXPHOS function [22, 23]. Large-scale deletions are always heteroplasmic within tissues.

Nuclear gene mutations affecting OXPHOS

Mitochondrial disorders due to nuclear gene mutations are estimated to comprise 80% of the mitochondrial diseases but reports on such diseases are so far in minority. However, this group is expanding and will certainly grow more when additional nuclear encoded proteins targeted to mitochondria, also affecting the respiratory chain, are being discovered [24].

Mutations in structural genes of the OXPHOS system encoded by nuclear DNA have only been described in genes encoding subunits of Complex I and II, whilst mutations affecting respiratory chain assembly have been described in nuclear genes encoding assembly factors of Complex III, IV and V [18]. Most frequent are reports on nuclear genes encoding assembly factors of Complex IV, *SURF1*, *SCO2*, *SCO1*, *COX10*, and *COX15*. Diseases due to defects in mitochondrial translation are rare but have been reported in severe childhood disorders causing early death [25, 26], and in cases with mitochondrial myopathy and sideroblastic anemia [27-29].

There are some mitochondrial disorders where the pathogenesis involves both the nuclear and mitochondrial genomes. Primary mutations in nuclear genes affecting stability and maintenance of mtDNA are associated with secondary qualitative or quantitative alterations of mtDNA i.e. multiple mtDNA deletions or mtDNA depletion (reduced mtDNA copy number). The first report indicating autosomal dominant inheritance of progressive external ophthalmoplegia (PEO) and mitochondrial myopathy with multiple deletions in mtDNA was published in 1989 [30]. Mutations in five nuclear genes have since then been associated with PEO and multiple mtDNA deletions; *ANT1*, encoding adenine nucleotide translocator 1 [31], *C10orf2* encoding Twinkle [32-34], *TP* encoding thymidine phosphorylase [35, 36], *POLG1* encoding the catalytic subunit of mitochondrial DNA polymerase (Pol) [37-40] and finally *POLG2*, the gene encoding the accessory subunit of polymerase , Pol [41]. Mutations in *ANT1* and *TP* are believed to cause imbalance of the nucleotide pool inside of mitochondria, whilst the gene products of *C10orf2*, *POLG1* and *POLG2* participate in mtDNA replication.

Polymerase is the only known polymerase involved in mtDNA replication [42]. It is a heterotrimer composed by one catalytic subunit and two accessory subunits [43]. The 140 kDa catalytic subunit, encoded by *POLG1*, has two functional domains; one polymerase domain in the carboxy-terminal and one exonuclease-domain with proofreading activity in the amino-terminal [44]. The 55 kDa accessory subunit, encoded by *POLG2*, increases the processivity of mtDNA replication [45] and appears to be important for mtDNA maintenance [46]. Twinkle is the helicase that unwinds mtDNA at the replication fork [47], and regulates mtDNA copy number [48].

Mitochondrial DNA depletion is a condition where the copy number of mtDNA molecules is severely reduced, down to a level that is not enough to maintain the energy production needed for cellular reactions. The onset of mtDNA depletion syndromes (MDS) is usually in infancy. A hepatocerebral form is associated with mutations in the deoxyguanosine kinase gene (*DGUOK*) [49] and a myopathic form with mutations in the tymidine kinase-2 gene (*TK2*) [50, 51]. These gene products are essential for the supply of dNTPs for mtDNA synthesis in post-mitotic tissues [52]. One syndrome, which in some reports has been associated with mtDNA depletion, is Alpers-Huttenlocher syndrome, or “progressive neuronal degeneration in childhood with liver disease”. The genetic background was until recently not known and the diagnosis was based on clinical and neuropathological findings.

INTRODUCTION AND AIMS

Paper I and II

The investigation of mitochondrial encephalomyopathies often includes muscle biopsy for morphological, biochemical and genetic investigations. A hallmark for mitochondrial diseases is the characteristic ragged red fibers (RRFs) in skeletal muscle tissue. In these fibers there is proliferation of mitochondria in the subsarcolemmal space, possibly as an attempt to compensate for the insufficient energy production [53]. The RRFs and other muscle fibers are often deficient of COX (complex IV), which can be demonstrated by enzymehistochemical techniques. The respiratory chain function can also be analyzed by spectrophotometric and oximetric methods on isolated mitochondria. The genetic investigation includes Southern blot analysis or Long extension polymerase chain reaction (LX-PCR) for identification of mtDNA deletions, and DNA sequencing for screening of point mutations.

The most common defects in human mtDNA associated with disease are point mutations in tRNA genes. All 22 tRNAs have been associated with pathogenic mutations, but most frequently affected are $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$ and $tRNA^{Ile}$ [18]. Disease-associated point mutations in mtDNA are reported in all structural genes, except *MTATP8*. Primary mtDNA mutations in tRNA genes, rRNA genes, or single large-scale deletions affect the overall mitochondrial protein synthesis whilst mutations in specific protein-coding genes generally affect the particular complex where the protein is located.

The importance of a correct evaluation of the pathogenicity of an identified mutation has major implications for the patient and for the patient's family, because of its direct consequences in the genetic counseling. Mutations in mtDNA are established within populations at a higher rate than nuclear DNA mutations and there exist a large number of fixed neutral polymorphisms with no pathogenic significance. Thus, to associate a novel mutation in mtDNA with a disease there has to be certain proofs for its pathogenicity. Five criteria have been proposed to support the deleterious role for an mtDNA mutation [54]. 1) The mutation must not be known as a neutral polymorphism and should be absent from healthy controls. 2) The mutation must affect an evolutionary conserved and functionally important site. 3) The mutation should be heteroplasmic; most pathogenic mtDNA mutations are heteroplasmic whereas nonpathogenic variants are homoplasmic (even if there are rare exceptions). 4) The proportion of mutated mtDNA should be higher in affected individuals than in their unaffected relatives. 5) The proportion of mutated mtDNA should correlate with the phenotype of single muscle fibers.

The mentioned criteria above are generally accepted and if followed properly they can often give the correct evaluation. However, McFarland et al. have improved the criteria for a more reliable determination of the pathogenicity of mtDNA tRNA mutations [55]. They introduced a scoring system for determining the likelihood that a given mtDNA sequence variant was pathogenic. Recently, the same group extended the scoring system to include evaluations of mutations in one of the seven mtDNA encoded Complex I genes [56]. The scoring system, that was based on biochemical defects, functional studies and degree of evolutionary conservation, may be extrapolated to include other mitochondrial gene mutations.

Paper I and II deal with sporadic cases of mitochondrial myopathy. In paper I the patient was a 70-year-old woman, who had experienced slowly progressive bilateral ptosis from age 50 but had had impaired balance during her entire life. The patient in paper II was a 30-year-old woman who presented with an attack of acute rhabdomyolysis (severe and widespread necrosis of skeletal muscle).

Aim

- To identify the genetic cause of mitochondrial myopathy in two patients
- To evaluate the pathogenicity of identified mtDNA mutations

Paper III

Clinical characteristics of progressive external ophthalmoplegia (PEO) and mitochondrial myopathy with multiple mtDNA deletions are weakness of the external eye muscles and exercise intolerance. Additional features are frequently reported e.g. hypogonadism, parkinsonism, cataracts, ataxia, depression and rhabdomyolysis [57]. The onset of the disease is between 18 and 40 years of age, and cases of both autosomal dominant (ad) and autosomal recessive (ar) PEO have been reported. Mutations in *POLG1* are a frequent cause of adPEO and arPEO [32, 39, 40]. Mitochondrial myopathy with COX-deficient skeletal muscle fibers is always present. Multiple mtDNA deletions are present in skeletal muscle, brain tissue and heart [58] i.e. postmitotic tissues. Single COX-deficient fibers from adPEO patients have been investigated earlier in our group by *in situ* hybridization [59]. It could be demonstrated that only one type of deletion was present in each COX-deficient muscle fiber segment. Thus, the deletions are clonally expanded in single muscle fibers. How the deletions occur is not known but they are often flanked by perfect direct repeats suggesting a strand-slippage mechanism during replication [60].

Mutations in mtDNA accumulate with age in postmitotic tissues in humans. These alterations in mtDNA in the normal aging muscle include both clonally expanded

deletions and point mutations in mtDNA and cause of COX-deficient muscle fibers [61]. The reason for this progressive damage to mtDNA is not known but an increased production of reactive oxygen species (ROS) together with an impaired antioxidant capacity in aging tissues is suggested [62]. Moreover, a knock-in mouse-model harboring a mutation in *PolgA* that abolish the proof-reading domain [63], develop mtDNA deletions as well as point mutations in mtDNA and a premature aging phenotype.

We wanted to investigate a hypothetical mechanism for the formation of deletions in patients with PEO, namely if point mutations close to the deletion breakpoints could contribute to the formation of deletions [64]. Based on the previous results from our group that mtDNA deletions are clonally expanded in COX-deficient fibers from PEO patients [59] and that clonally expanded point mutations account for many of the COX-deficient fibers in aging muscle [61], we also wanted to test the hypothesis that clonally expanded point mutations are responsible for some of the COX-deficient muscle fibers in PEO patients.

Included in this study were members of a family showing dominant inheritance of PEO and one sporadic case. The patients harbored mutations in *POLG1* that previously had been associated with adPEO and arPEO respectively.

Aim

- To study if clonally expanded point mutations in single muscle fibers contribute to the mitochondrial myopathy in individuals with PEO and multiple mtDNA deletions due to primary mutations in *POLG1*
- To test the hypothesis that mtDNA point mutations precede the formation of mtDNA deletions in these patients

Paper IV

Alpers diffuse degeneration of cerebral gray matter with hepatic cirrhosis or Alpers-Huttenlocher syndrome (AHS) [65, 66] has for long been suspected to be a mitochondrial disorder [67]. The disease is an early onset, fatal and rapidly progressive neurodegenerative disorder where the clinical features include failure to thrive, myoclonus, refractory seizures, psychomotor regression, spasticity, cortical blindness and liver dysfunction. Treatment of the seizures with valproate has sometimes accelerated the disease course to fulminant liver failure. The disease displays a recessive trait of inheritance indicating a monogenic disorder. In 1990, Harding published a review based on the clinical findings from 32 autopsied AHS-like cases [68]. The onset of disease is generally within the first year of life and it becomes

fatal 1 – 3 years after onset. However, patients with a protracted disease course have been described [69]. AHS has been associated with mtDNA depletion [70, 71] and some features are similar with the hepatocerebral mtDNA depletion syndrome caused by mutations in *DGUOK* [72]. However, children with *DGUOK* mutations don't have seizures and certain EEG abnormalities seen in AHS. The first report on *POLG1* mutations associated with AHS was published in 2004 [73].

The patients described in Paper IV are seven children with progressive encephalopathy from five unrelated families. Five of the children were previously referred to us for investigation of mitochondrial disease, and two were neurologically affected siblings of these patients. Six children had a phenotype compatible with Alpers-Huttenlocher syndrome but one of the affected siblings had a less severe phenotype without signs of liver disease.

Aim

- To systematically search for *POLG1* mutations in a group of children with Alpers-Huttenlocher syndrome (AHS)
- To study secondary effects on mtDNA (deletions/depletion) of *POLG1* mutations in muscle in AHS
- To study the morphological changes in muscle, liver and brain in *POLG1* associated AHS

Paper V

"In a starving animal there is only a little glycogen but there is always some. Without it an animal would not be able to move at all" ***Archibald Vivian Hill*** in *Muscular Activity (1926) – Awarded the Nobel Prize in Medicine 1922*

Glycogen is a branched polymer of glucose residues, and humans as well as other mammalians utilize glycogen as a readily mobilized source of glucose. In human, glycogen is most abundant in liver and skeletal muscle. Glycogen in muscle is rapidly degraded and converted to ATP when the muscle itself needs energy i.e. during exercise whilst liver glycogen serves to maintain the blood glucose homeostasis [74]. For the heart, the reliance on glycogen as an energy source is increased during an acute increase in workload [75], but the main energy source for the heart and for resting, non-activated muscle is fatty acids. The brain needs a continuous supply of glucose but is only capable of storing a small amount of glycogen for utilization during brief periods of hypoxia and hypoglycemia, and is thus dependent on the glucose that is released into the bloodstream between meals. The uptake of glucose and glycogen synthesis in skeletal muscle is stimulated either by insulin secreted by

the pancreas after a meal when the blood glucose level is high, or by glycogen depletion following exercise [76, 77]. Entering glucose has two fates: either it can be phosphorylated to glucose-6-phosphate (G6P) and enter glycolysis for ATP production. Or, at high intracellular ATP levels, glucose is converted to glucose-1-phosphate, which is further metabolized for glycogen synthesis.

The synthesis of glycogen requires several catalytic reactions and the final and rate-limiting step, incorporation of UDP-glucose into glycogen, is catalyzed by glycogen synthase encoded from *GYS1* on chromosome 19 [78] and *GYS2* on chromosome 12 [79]. *GYS1* encodes the muscle isoform, expressed in several tissues (e.g. skeletal muscle, heart, brain and kidney) and *GYS2* the liver specific isoform sometime called the M-type or the L-type glycogen synthase, respectively [80]. A specific isoform in brain, *GYS3* with 96% homology to *GYS1*, has been cloned in mice [81], but no brain isoform has been identified in human. Regulation of glycogen synthase occurs allosterically by G6P and/or by reversible covalent phosphorylation and dephosphorylation of at least nine serine residues, where the non-phosphorylated state is the most active [82, 83].

Glycogen degradation is catalyzed by glycogen phosphorylase by removal of single glucose residues from the glycogen molecule. This generates glucose-1-phosphate, which is converted to G6P that can enter the glycolysis. In the liver G6P is converted to glucose by glucose-6-phosphatase, an enzyme not expressed in skeletal muscle. G6P cannot pass trough the cell membrane and thus, there is only one-way traffic for glucose from blood to muscle. The activity of glycogen phosphorylase is hormonally regulated by covalent phosphorylation and here is the phosphorylated state the most active [84].

Virtually all proteins involved in glycogen metabolism have been associated with various glycogen storage diseases [3]. In patients suffering from any of these so-called glycogenoses, glycogen cannot be efficiently utilized as energy source, which cause exercise intolerance, cramps and myoglobinuria, due to the specific enzyme defects. A disease called “Glycogen storage disease type 0” is associated with mutations in *GYS2* and liver glycogen synthase deficiency [85]. The muscle isoform of glycogen synthase has not been directly associated with disease but disturbance of the insulin-dependent pathway of muscle glycogen synthesis has been suggested as a cause of insulin resistance and of importance for development of non-insulin-dependent diabetes-mellitus (NIDDM) or diabetes type II [74, 86-88].

Paper V deals with a family where two brothers, children of healthy consanguineous parents, had exercise intolerance and cardiomyopathy. The older brother, who had mild epilepsy, died in sudden cardiac arrest at 10 years of age when he was playing in the schoolyard. The cause of death given was hypertrophic cardiomyopathy. Some

time later, the younger brother presented with symptoms that his older brother, seen in retrospect, had before his death. He was therefore thoroughly investigated and showed muscle fatigability, hypertrophic cardiomyopathy, abnormal heart-rate response and a blood pressure drop on exercise. A muscle biopsy showed virtually complete lack of glycogen in the muscle fibers.

Aim

- To establish the genetic cause of this apparently new disease in patients with no glycogen stores in the skeletal muscle

RESULTS AND DISCUSSION

Paper I

A novel mutation in the mitochondrial $tRNA^{Phe}$ gene associated with mitochondrial myopathy

The patient was a 70-year-old woman, who had experienced slowly progressive bilateral ptosis from the age of 50. Her balance had been slightly impaired during her entire life. Her mother, sister and two of her children were free of neuromuscular symptoms, but a 39-year-old daughter suffered from fibromyalgia.

The morphological examination of muscle tissue revealed reduced COX activity in the majority of the muscle fibers and numerous RRFs. A muscle biopsy obtained from the patient's daughter with fibromyalgia was normal. The biochemical analysis of isolated mitochondria from the patient's muscle tissue showed a slight general reduction of the respiratory rate and a marked reduction of COX activity. LX-PCR analysis excluded large rearrangements of mtDNA. Sequence analysis revealed a T C substitution at position 582 in the aminoacyl acceptor stem of the $tRNA^{Phe}$. The RFLP-analysis revealed that the level of heteroplasmy was 70% in muscle homogenate. The average level in single COX deficient fibers was 86% while the average level in COX positive fibers was 15%. The mutation was only detectable in the patient's muscle and not in blood leukocytes or hair shafts. Moreover, the mutation was not detected in 100 controls and not in the muscle tissue from the daughter. The immunohistochemical staining with antibodies against two different subunits in complex IV showed reduced levels for the mtDNA encoded subunit II, but increased levels for the nDNA encoded subunit IV.

The identified novel mutation fulfills the criteria for pathogenicity of mtDNA mutations. We performed database-searches and the mutation was not previously reported as a polymorphism. Neither was the mutation identified in 100 normal controls. The mutation disrupts a conserved Watson-Crick T-A base pairing within the aminoacyl acceptor stem of the tRNA, which might affect the stability of the hairpin structure [89, 90]. The muscle tissue from the 39-year-old daughter with fibromyalgia was normal, a tissue that would have been affected if the mutation had been transmitted from her mother. Thus, there was no other family member affected than our patient. The only tissue from the patient harboring the mutation was muscle and there was a significant genotype/phenotype correlation in single muscle fibers.

We conclude that the identified T C substitution at mtDNA position 582 in the $tRNA^{Phe}$ gene is responsible for the disease. Our results on family members and the

tissue distribution indicate that this was a sporadic case and due to a somatic mutation that probably arose in myogenic stem cells during embryogenesis after germ-layer differentiation.

Paper II

Mitochondrial myopathy and rhabdomyolysis associated with a novel nonsense mutation in the gene encoding cytochrome *c* oxidase subunit I

The patient in this study was a 30-year-old woman with no family history of neurological or neuromuscular disease. From her teens she had experienced stiffness in her muscles, and her physical activity had been low since childhood. At the age of 28 she presented with generalized muscle pain and dark colored urine in an episode of rhabdomyolysis.

Two muscle biopsies were obtained from the patient about one year after the rhabdomyolysis episode. The morphological investigation revealed fibers of normal size with no ongoing regeneration or muscle fiber necrosis. The most prominent feature in the muscle tissue was a severe COX-deficiency. Only about 10% of her muscle fibers exhibited normal COX-activity and there were frequent RRFs. The biochemical investigation demonstrated an isolated COX-deficiency. LX-PCR analysis excluded large-scale rearrangements in mtDNA. Sequencing analysis of mtDNA revealed a novel nonsense mutation in *COI* encoding the largest subunit of Complex IV, COX subunit I, with 514 amino acids. The mutation at position 6708 changed a GGA codon, coding for the amino acid glycine, to an AGA termination codon, giving a predicted truncation at amino acid 269. The mutation was present at high levels in the patient's muscle but could not be identified in DNA extracted from cultured myoblasts, skin, hair shafts or peripheral blood leukocytes analyzed by PCR-RFLP analysis. The mutation could not be identified in blood leukocytes from the patient's mother. The level of heteroplasmy in single muscle fibers correlated very well with their phenotype; all COX-deficient fibers harbored more than 95% mutant mtDNA whilst the mutant load in COX-positive fibers was between 0 – 80%. In the immunohistochemical analysis we could see that only those few fibers displaying normal COX-activity also showed normal reactivity for COX subunits I, II, III and IV i.e. the reactivity for both mtDNA and nDNA encoded subunits were reduced in COX-deficient fibers. A western blot analysis revealed reduced levels of COX subunits I, II and IV in the patient compared to a normal control.

The acute breakdown of muscle tissue in rhabdomyolysis is often accompanied by myoglobinuria, characterized by dark, tea-colored urine due to increased excretion of

myoglobulin. At worst, this condition can be fatal because of renal failure. Rhabdomyolysis may occur after direct muscle trauma or infections but also as a side effect of ingested compounds or certain drugs [91]. Inherited metabolic causes include defects in the glycogen and fatty acid metabolism, where exercise is the most common inducer [92, 93]. Respiratory chain dysfunctions due to mutations in mtDNA associated with episodic rhabdomyolysis are rare. A few sporadic cases with mutations affecting subunits in Complex III and IV have been described [94-98], and three cases associated with the A3423G mutation in $tRNA^{Leu(UUR)}$ [92, 99, 100]. In patients with multiple mtDNA deletions rhabdomyolysis provoked by alcohol intake has been described [101, 102].

The fact that the mutation was restricted to the patient's muscle albeit not detectable in myoblasts, cultured from satellite cells from affected muscle tissue, may have interesting implications for the natural evolution of the disease and perhaps therapy, since regenerating muscle occurs by proliferation of satellite cells. It has been generally believed that satellite cells as well as all other cells in the myogenic line have somitic origin. However, previous work show that in the right milieu, other cells than muscle stem cells can participate to muscle regeneration [103], and cells similar to satellite cells can be isolated from the dorsal aorta of mouse embryos [104]. Different origin of satellite cells and muscle fibers can be an explanation for the lack of mutation in the satellite cells of our patient despite the high levels in mature skeletal muscle fibers. Another speculation is that the mutation actually is present in the dormant cells but is eliminated due to eradication during differentiation. Nevertheless, the rhabdomyolysis in our patient can have had some beneficial consequences since it can be expected that muscle fibers that have undergone necrosis and regeneration also have been permanently treated by endogenous gene therapy [105, 106].

The pathogenicity of this novel mutation G6708A is proven and the criteria for a pathogenic mtDNA mutation is fulfilled. Since the mutation creates a premature termination codon in an important structural gene it cannot be explained as a common polymorphism. COX subunit I has a central important catalytic function in the reduction of molecular oxygen to water in Complex IV [107]. Subunit I is also the largest subunit and acts as a scaffold protein that is in contact with the other subunits in the complex [9]. A previous study on Complex IV assembly identified COX subunit I as the first intermediate of the complex [8]. A common feature for diseases associated with primary mtDNA mutations, like the tRNA mutation described in Paper I, is reduced immuno-reactivity for mtDNA encoded subunits but increased reactivity for nDNA encoded subunits correlating to the amount of mitochondria. On the contrary, in this case, we demonstrated that the COX-deficient fibers, revealed by the enzyme-histochemical staining, had reduced levels of subunit I, reduced levels of other mtDNA encoded COX subunits but also reduced level of the nDNA encoded subunit IV. This supports the theory of an important function of subunit I for the assembly of the entire complex. There was a significant genotype/phenotype correlation in single muscle fibers, and the high level of mutant load in COX-deficient fibers is consistent

with a deleterious role for the mutation. The restriction of the mutation to the patient's muscle implies low probability that the mutation should be transmitted to her offspring.

We conclude that the identified G→A substitution at mtDNA position 6708 in the *COI* gene is responsible for the disease. We conclude that the affected subunit is important for Complex IV assembly and for the catalytic function. Moreover, our results indicate that this was a sporadic case due to a somatic mutation that probably arose in myogenic stem cells during embryogenesis after germ-layer differentiation.

Paper III

Low frequency of mtDNA point mutations in patients with PEO associated with *POLG1* mutations.

The patients in this study included four siblings from a family with dominantly inherited progressive external ophthalmoplegia (PEO) and one sporadic case of PEO. Morphological analyses of muscle tissue showed that all patients had mitochondrial myopathy with COX-deficient fibers and numerous RRFs. LX-PCR analyses revealed multiple mtDNA deletions in all patients at a similar level. The family members with adPEO harbored a mutation in *POLG1* giving the amino acid substitution Y955C, classified as a fully penetrant mutation and associated with a severe form of adPEO [39, 40]. The sporadic case had three mutations in *POLG1* previously associated with arPEO: T251I, P587L and G848S [39].

In this study we tested the hypothesis that point mutations in mtDNA contribute to the mitochondrial myopathy in addition to the large-scale deletions that are associated with ad/arPEO. We screened a large number of single, isolated COX-deficient fibers for clonally expanded point mutations, but we could not detect any mutation with the sensitive DGGE-method used. By high-fidelity PCR amplification, subcloning and sequencing ~230 000 bp of mtDNA we could also demonstrate that randomly occurring point mutations were very rare, and did not exceed the amount found in age matched controls.

We used the same method to search for clonally expanded point mutations in adPEO patients as Fayet et al. used to identify such mutations in COX-deficient fibers in aging muscle [108]. However, since none of the analyzed single COX-deficient fibers from our patients harbored clonally expanded point mutations in contrast to the analyzed fibers from aging muscle, the events leading to mitochondrial myopathy with COX-deficient fibers in adPEO and aging probably differs.

The mechanism for the formation of multiple mtDNA deletions is not known, but three models have been proposed: an illegitimate elongation model, a slipped mispairing model and a homologous recombination model [109]. All proposed models are favored by the presence of repeats in the mtDNA sequence. Indeed, the most common deletion (called the 'common deletion') is flanked by 13 bp perfectly direct repeats beginning at mtDNA position 8470 and 13447, respectively. However, the mtDNA molecule contains five direct repeat pairs ≥ 13bp and only the pair flanking the 'common deletion' is associated with disease [110]. Moreover, irrespective whether the patients have mutations in *POLG1*, *POLG2*, *C10orf2*, *ANT1* or *TP*, and display different clinical phenotypes, the mitochondrial genome integrity is lost and there are some important similarities regarding the mtDNA deletions. The pattern of deletion breakpoints seems to be the same [58, 109, 111] and the 'common deletion' is almost always present. Samuels et al. studied the sequences that flank 263 different human mtDNA deletions and found that the distribution of deletion breakpoints was similar for all investigated deleted sequences [110]. They suggested that there is a common mechanism for the formation of all deletions that is related to mtDNA replication. We investigated the theoretical hypothesis that point mutations in mtDNA, which appear secondary to a mutant polymerase would precede the formation of deletions and promote slipped mispairing [64]. Misinsertions in mtDNA were supposed to initiate large deletions between direct repeats. Although we analyzed a large amount of mtDNA fragments, we could only identify a few randomly occurring point mutations. The frequency was as low in the patients as in the controls. Our results is supported by another study of ad/arPEO patients where the authors found an age-dependent accumulation of mtDNA point mutations particularly in the D-loop but not in association with deletion breakpoints [111].

Mutant gene products of *ANT1* and *TP* cause imbalances of the mitochondrial nucleotide pool [31, 35] suggesting a toxic effect and a gain-of-function hypothesis that may explain the dominant inheritance in diseases associated with mutations in these genes. Mutations in *C10Orf2*, encoding the mtDNA helicase Twinkle, can alter its helicase activity as well as its dNTPase activity with both enhanced and decreased effect [34, 112, 113]. Thus, in addition to the direct effect on Twinkle's properties of unwinding mtDNA in the replication fork, a mutant Twinkle protein can cause an imbalance in the nucleotide pool, similar to the effect of mutations in *ANT1* and *TP*. Since polymerase □is the only polymerase for mtDNA, mutations in either *POLG1* or *POLG2* can have direct influence on the replication mechanism. Hence, mutations in any of these nuclear genes associated with multiple mtDNA deletions can induce replication stalling, impair the processivity of mitochondrial DNA polymerase □and promote the formation of deletions. Our result supports the theory of stalling during replication as the principal cause of deletions.

We conclude that it is very unlikely that point mutations in mtDNA contribute to the mitochondrial myopathy in PEO patients with primary *POLG1* mutations, and that the mechanism by which mutant polymerase cause mtDNA deletions does not involve mtDNA point mutations as an intermediate step, as has been previously proposed.

Paper IV

***POLG1* Mutations Associated With Progressive Encephalopathy in Childhood**

We identified seven children with progressive encephalopathy and *POLG1* mutation. Six of these children had a phenotype compatible with Alpers-Huttenlocher syndrome – Progressive neuronal degeneration of childhood with liver disease.

Muscle morphology showed COX-deficient fibers in four of the patients. Liver disease was present in all children except one, and the morphological changes obtained from needle biopsies showed variable pathological features from steatosis and slight fibrosis to severe degeneration with necrosis and cirrhosis. One fresh frozen liver biopsy that was available in one patient, showed marked COX-deficiency. Neuropathological investigation was performed in four patients but only parts of the brain were available in three of them. The most important neuropathological findings in three cases were neuronal cell loss, gliosis and spongiosis in the cerebral cortex in addition to cerebral white matter gliosis and spongiotic changes in the thalamus. In the cerebellum there was a severe loss of Purkinje cells with proliferation of Bergmann glia. Astrocytes of Alzheimer type II were found mainly in the cerebral cortex and basal ganglia. In one case there was marked glioses of the cerebral white matter but no obvious neural loss, spongiosis or gliosis in the investigated regions of the cerebral cortex. However, vacuolization was seen in thalamus and in cerebellum there was also loss of Purkinje cells and nerve cell loss in dentate nucleus.

LX-PCR analysis of muscle tissue revealed mtDNA deletions in four children (two sibling pairs). The quantitative PCR analysis of mtDNA copy number in muscle tissue revealed mtDNA depletion in three children.

All seven children harbored compound heterozygous mutations in *POLG1* and investigations of family members showed that the mutations segregated in the families as expected for a recessively inherited disorder. Six different mutations were identified affecting conserved amino acid residues. Three mutations were novel and none of these were present in the 200 investigated control chromosomes. Either one or both of the mutations A467T and W748S were present in all children.

The small amount of identified deletions in muscle tissue of four patients is not expected to cause any major dysfunction but since muscle is not the most affected tissue it is possible that other tissues like liver and brain with more severe pathological changes have a higher proportion of mtDNA deletions. To our knowledge multiple mtDNA deletions have not been reported in children this young and are exceptional also in adolescence. Why the effect of mutations in a housekeeping gene like *POLG1* would be tissue specific is unclear, but tissue specific mtDNA depletion is indicated in other studies on children with AHS [70, 71, 114, 115]. Depletion of mtDNA was identified in muscle tissue in three of our patients. One child had mtDNA depletion without presence of COX-deficient fibers. Barthélémy et al. demonstrated a high ratio of mitochondrial RNA to DNA in muscle from patients with mtDNA depletion compared to control muscle and Durham et al. demonstrated COX-activity in single muscle fibers despite very low density of mtDNA [116, 117]. Thus, there might be a transcriptional compensation for the mtDNA depletion. The distribution of mtDNA depletion within the muscle tissue may be important for the occurrence of COX-deficient fibers as has been demonstrated for mtDNA deletions [118].

Children with AHS suffer from refractory seizures. Thus, antiepileptic treatment is necessary and among the drugs available, valproate is a potent antiepileptic drug. However, treatment with valproate is not without danger, it can be deleterious for the patient and fatal hepatotoxicity has been reported [119]. Liver disease is a cardinal feature of AHS and these children seem to be extremely vulnerable to valproate treatment, accelerating their liver disease. There are several reports on children with AHS that deteriorated rapidly in fulminant liver failure after valproate administration [115, 120, 121]. Thus, there might be a triggering mechanism induced by this drug that may involve secondary carnitine deficiency, depression of intramitochondrial fatty acid oxidation and/or inhibition of OXPHOS [122-124]. COX-deficiency in the liver has previously been associated with Alpers-Huttenlocher syndrome [71] and by enzymehistochemical analysis we identified a severe respiratory chain insufficiency with COX-deficient hepatocytes in one of our patients where fresh frozen liver tissue was available. Since the valproate toxicity might be connected with an OXPHOS defect this indicates that the *POLG1* mutations are responsible for the respiratory chain deficiency and susceptibility for valproate in these children.

The observed neuropathological changes were characteristic for AHS in most of the investigated patients. The pathogenesis of the severe brain lesions in AHS is not clear, as is the case in the majority of mitochondrial encephalopathies. It has been hypothesized that a primary defect in the neuronal metabolism, because of mitochondrial dysfunction, causes the neuronal death [125], which may be worsened by the intractable seizures. Since epilepsy is a very energy-intensive process it is possible that areas in the brain with mitochondrial defects are vulnerable and predisposed for epileptic brain damage. An extracellular increase of excitatory amino acids can reach excitotoxic concentrations that precipitate seizures and may cause neural cell death [126]. A system for tissue-specific inactivation of mtDNA expression

in neurons of hippocampus and neocortex was generated in mice [127]. By conditional knockout of the nuclear mitochondrial transcription factor A gene (*Tfam*), which encodes a protein necessary for transcription and replication of mtDNA, the authors could show that mice with disruption of oxidative phosphorylation in the forebrain develop normally until 4 month of age and not until hereafter severe, respiratory chain-deficient neurons can be identified. Disease symptoms, coincidently with a rapidly progressive neurodegeneration, were observed at 5 month of age. Thus, respiratory chain-deficient neurons are viable for at least one month in these mice. However, neuronal cell death could be induced in 4-month-old mice if they were exposed to excitotoxic stimuli.

Another hypothesis proposed as a cause of the cerebral lesions seen in MELAS (Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [128, 129] is ischemia, especially in border zones, but some studies did not find an actual topographic correlation [130, 131]. On the other hand, in a recent report it was concluded that vascular mitochondrial dysfunction is of importance for the lesions [132]. Their study on individual neurons revealed no correlation for high mutant load in neurons harboring the 'MELAS-mutation', A3243G in mtDNA, and the neuropathological changes in different brain regions in two MELAS patients. The highest degree of heteroplasmy was observed in the vessels, which showed COX-deficiency, especially those vessels associated with regulation of the cerebral blood flow. If there is a mitochondrial angiopathy in the AHS-patients with *POLG1* mutations is not yet studied, but indeed, signs of disruption of the blood brain barrier was observed in our patients. Alzheimer type II astrocytosis was seen in some of our cases. This may be an effect of the hepatic failure [133].

The □-subunit of polymerase □ has two functional domains, one polymerase domain and one proof-reading domain with a spacer region between. The role for this spacer or linker region is not known, but it might be important for binding of the accessory subunit Pol□□, which functions as a processivity and DNA-binding factor [134]. Dominant mutations in *POLG1* affecting the polymerase domain are associated with adPEO, whilst recessive mutations are associated with arPEO, AHS or ataxic syndromes and predominantly located in the spacer or the exonuclease domain. Either one or both of the mutations A467T and W748S were present in our patients. This is a consistent finding also in all other cases of AHS with *POLG1* mutations reported to date [114, 115, 120, 121, 135, 136]. However, these mutations are not exclusively reported in AHS, but also in patients with ataxia and arPEO [40, 137-140]. Nevertheless, these mutations in compound heterozygosity with one another or with other mutations in *POLG1* cause AHS. It is suggested that the previous diagnostic criteria based on clinical and neuropathological investigations should be modified and include sequencing of *POLG1* with an initial screening for A467T or W748S [121]. In a study performed by Tzoulis et al. the clinical spectrum for 26 patients homozygous for any of these two mutations or compound heterozygous for both was investigated [141], and the compound heterozygotes were demonstrated to be more severely

affected. A recent haplotype analysis regarding the W748S change showed that people carrying this mutation originate from a common ancient founder [142].

How mutations in the spacer region of *POLG1* cause these severe phenotypes is not known. Methods for studying the structural and functional effects of Pol mutations are not yet well developed. However, one study showed that the catalytic activity of Pol is severely compromised by the A467T mutation [143] and results from other studies indicate a defect in DNA binding and processivity due to spacer region mutations [144, 145]. Mutations in *POLG2*, which cause disruption of the function for the accessory subunit, are lethal in Drosophila, leading to mtDNA depletion and diminished cell proliferation in CNS [46]. Thus, it might be speculated that spacer region mutations in *POLG1* produce a polymerase without the ability to form the heterotrimer that constitutes the functional holoenzyme.

We conclude that AHS is a recessive disorder caused by mutations in *POLG1* and either one or both of the mutations A467T and W748S are always present. Alterations of mtDNA such as multiple deletions and/or mtDNA depletion are part of the pathogenesis and cause secondary OXPHOS defects in several tissues. In the brain this is followed by intractable seizures and neuronal cell death. In the liver it will cause liver failure, which may be triggered by valproate treatment.

Paper V

Muscle glycogen storage disease 0 – A cause of sudden cardiac death.

This study deals with a family where two children, both boys, displayed symptoms of an apparently new metabolic disease with severe glycogen deficiency in muscle tissue. One child suffered sudden cardiac death at the age of 10 and his younger brother showed muscle fatigability and hypertrophic cardiomyopathy. The younger brother had an open biopsy of the vastus lateralis muscle and the older brother has been investigated post mortem. Their parents were cousins.

Morphological investigation of the muscle biopsy revealed profound deficiency of glycogen in all muscle fibers. There was a remarkable predominance of type 1 muscle fibers and an abnormal accumulation of mitochondria in most fibers. Numerous mitochondria displayed inclusions in the intermembranous space. Biochemical investigation showed an extremely high yield of isolated muscle mitochondria, whereas the respiratory rate was within normal limits for all added substrates.

Sections of paraffin-embedded heart and liver specimens prepared from paraformaldehyde-fixed post mortem samples from the older brother were stained with hematoxylin-eosin and PAS. Cardiac muscle showed myocyte hypertrophy with enlarged nuclei but no myocyte disarray or fibrosis. No glycogen was detected in cardiomyocytes by PAS staining. In contrast, the liver, which had been subjected to an identical fixing procedure contained normal amount of glycogen.

Direct sequencing of *GYS1* encoding the muscle isoform of glycogen synthase demonstrated a homozygous change compared to the reference sequence in both brothers: a C→T transition in exon 11, which changes a CGA, coding for arginine, to a TGA termination signal. The premature stop-codon is predicted to result in truncation of glycogen synthase. The mutation segregated in the family in an autosomal recessive pattern of inheritance. Analysis of mRNA showed barely detectable levels of *GYS1* transcripts in the patient's muscle. No *GYS2* transcripts were detected in muscle tissue from patient or control. However, both *GYS1* and *GYS2* transcripts were readily amplified in the control liver sample. By western blot analysis with polyclonal rabbit-anti-human-glycogen synthase-antibodies there was undetectable levels of the protein in the patient. The analysis revealed a band corresponding to the ~ 84 kDa glycogen synthase protein only in the lane loaded with muscle protein from a normal control.

GYS1 is extensively studied because of its suspected role in NIDDM [74, 86-88] and recently, even a mouse model has been created [146]. Although about 90% of the *GYS1*-null mice died soon after birth due to impaired cardiac function, the surviving muscle-glycogen-synthase knockout (MGSKO) mice share several features with our patients e.g. absence of skeletal muscle and heart glycogen, predominance of oxidative muscle fibers with accumulation on mitochondria, fast glucose clearance and cardiac enlargement [146-148]. One important difference was the ability to perform exercise. The results from strenuous exercise performance in MGSKO mice compared to their wildtype littermates were equal, whereas our patient showed severely impaired exercise capacity. However, rodents are more dependent on liver than muscle glycogen than humans for sustained muscle work [149]. The sudden death of one boy and the abnormal cardiac response to increased workload in the other boy indicate a critical role for glycogen stores in the heart for humans. This is consistent with studies showing that the heart is dependent on glycogen during bursts of activity [75].

A rare form of fasting hypoglycemia presenting in infancy or childhood, which is called Glycogen Storage Disease Type 0 is associated with different mutations in the gene encoding the liver isoform of glycogen synthase, *GYS2* [85]. The enzyme activity in these children was low or immeasurable but their glycogen content in the liver was only moderately decreased. The authors suggested some residual glycogen synthesis. Early experiments on rats showed no detectable levels of muscle glycogen synthase in the rat liver [80], but our results, as well as database searches, indicate that *GYS1* is expressed in the human liver. We suggest that the nearly normal glycogen content

observed in one of their patients with a homozygous nonsense mutation in *GYS2* actually represents *GYS1* expression and synthesis of the muscle isoform in the liver.

The epilepsy in one of the boys might be coincidental. However, one hypothetic role for the cerebral stores of glycogen is to provide energy to astrocytes for rapid glutamate neurotransmitter clearance [150]. Glycogen deficiency in the brain may result in excess of excitatory neurotransmitters and uncontrolled excitation. One previous report describes a child who died in a multiorgan failure with complete glycogen depletion in skeletal muscle tissue following status epilepticus. The authors' theory was that the increased demand for energy during status epilepticus culminated in complete depletion of glycogen reserves, leading to cellular energy exhaustion and finally to death [151]. It cannot be excluded that this may represent another case of Muscle Glycogen Storage Disease type 0 and we suggest that genetic studies should be performed.

We conclude that the homozygous nonsense mutation in *GYS1* is responsible for the disease in the two brothers. The total absence of glycogen in muscle tissue provides evidence that glycogen synthase is the sole enzyme for glycogen synthesis and that there is no alternative pathway for the synthesis of glycogen. This is in accordance with findings in the MGSKO mouse. This new inborn error of metabolism is easily revealed by routine-histochemical investigation of muscle tissue and we think that other cases might be found by a wider use of exercise tests and screening for muscle disease. In forensic investigations of sudden cardiac death or sudden death in children and adolescents with epilepsy we propose that investigation of muscle tissue should be included.

GENERAL CONCLUSIONS AND MAJOR NEW FINDINGS

- We have found two novel mutations in mtDNA in two sporadic cases of mitochondrial myopathy and proved with different methods that these mutations are pathogenic. These findings have direct importance for the genetic advice that will be given to patients harboring these mutations and their families (*Papers I and II*)

- COX subunit I is important for the catalytic function of Cytochrome *c* oxidase and is important for the assembly of entire complex IV in the respiratory chain (*Paper II*)

- Randomly occurring mtDNA point mutations are rare in normal subjects and in patients with recessive or dominant inherited PEO caused by *POLG1* mutations (*Paper III*)

- The secondary effects of *POLG1* mutations associated with the mitochondrial myopathy seen in PEO-patients are caused by deletions and not point mutations in mtDNA (*Paper III*)

- The formation of multiple mtDNA deletions in PEO-patients does not involve point mutations in mtDNA as a preceding step (*Paper III*)

- Alpers-Huttenlocher syndrome is a mitochondrial disease associated with primary mutations in *POLG1* and secondary mtDNA alterations (mtDNA deletions and mtDNA depletion) (*Paper IV*)

- Compound heterozygous recessive *POLG1* mutations can cause muscle mtDNA deletions in childhood (*Paper IV*)

- Patients with *POLG1* mutations and AHS show characteristic neuropathological features (*Paper IV*)

- Liver failure in AHS is associated with a severe OXPHOS defect (*Paper IV*)

- The new knowledge of the genetic background for AHS has made it possible to offer affected families genetic counseling and prenatal diagnosis (*Paper IV*)

– We have identified a new disease entity: "Muscle glycogen storage disease type zero" (MGSD-0). The first cases of MGSD-0 are caused by a homozygous nonsense mutation in *GYS1*, encoding the muscle isoform of glycogen synthase (*Paper V*)

– Muscle glycogen deficiency due to abolished glycogen synthesis is a new etiology for sudden childhood cardiac death (*Paper V*)

– Absence of muscle glycogen synthesis alters the metabolism in muscle tissue with changed fiber type composition and causes massive mitochondrial proliferation (*Paper V*)

– As in the mouse model of muscle glycogen synthase null mutation the lack of glycogen synthesis appears not to affect glucose tolerance in human (*Paper V*)

MATERIALS AND METHODS

Patients

The patients that were investigated in the different studies were referred to us for investigation when the clinical presentation indicated a metabolic disease.

Morphological analyses

Muscle pathology (Papers I-V)

Skeletal muscle specimens from the deltoid or vastus lateralis muscles were obtained from the patients by open muscle biopsies. The muscle specimens were immediately frozen in isopentane in liquid nitrogen prior to storage at -80°C.

a. Histochemistry (Papers I-V)

Cryostat sections of the fresh frozen skeletal muscle biopsies were investigated by light microscopy after staining with hematoxylin and eosin (HE), Gomori-Engel trichrome (GT), Sudan Black, and Periodic acid and Schiff's reagent (PAS).

b. Enzyme histochemistry (Papers I-V)

Mitochondrial respiratory chain function was studied by enzyme histochemical methods. Cytochrome *c* oxidase (COX) is a partially mtDNA encoded respiratory chain enzyme (complex IV) whilst succinate dehydrogenase (SDH), or complex II, is encoded exclusively by nuclear genes. Staining for COX activity implies incubation of cryostat sections of fresh frozen tissue in cytochrome *c* and an electron donor, 3,3'-diaminobensidine (DAB) [152]. If COX is functional, DAB is oxidized to an insoluble brown polymere. To identify SDH activity, cryostat sections of muscle are incubated in succinat and a tetrazolium salt (nitro blue tetrazolium). SDH in the tissue will reduce the tetrazolium salt to a blue insoluble product that can be identified by light microscopy [153]. For comparison of COX and SDH the stainings were performed on serial sections. Fibers were considered COX-deficient if they showed absence of COX activity concomitant with high SDH activity. In a double staining of COX and SDH the brown color corresponding to normal COX activity will cover the blue color corresponding to SDH activity. Therefore in a COX/SDH double staining COX deficient fibers appear blue (Figure 5a). The double staining facilitates the identification and isolation of single COX-deficient or COX-positive muscle fibers under dissection microscope.

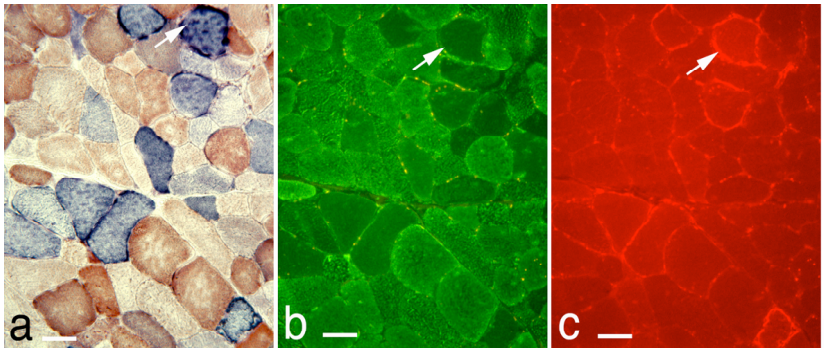


Figure 5. a) Enzyme histochemical double staining of COX/SDH. COX deficient fibers are blue and normal fibers brown. The arrow indicates a COX deficient ragged red fiber that in b) shows reduced expression for COX subunit II and c) increased expression for COX subunit IV.

c. Immuno-histochemistry (Papers I and II)

Immunohistochemical analyses were performed on cryostat sections to examine the correlation of COX-deficiency and reactivity for different subunits in COX. In paper I we used antibodies directed towards COX subunit II (mtDNA encoded) and COX subunit IV (nDNA encoded), which were generous gifts from Dr. Anne Lombes, Paris, France. In paper II, incubations were performed with antibodies against COX subunit I, II, III and IV (Molecular Probes). We used either FITC-conjugated or TexasRed™-conjugated secondary antibodies for visualization in a Nicon Eclipse E800 light microscope with filters for FITC and TexasRed™ respectively (Figure 5b and c).

d. Electron Microscopy (Paper V)

The structure of mitochondria can be identified by transmission electron microscopy (TEM). Moreover, the electron dense organelles called glycosomes containing glycogen and its associated proteins are visualized with TEM [154] (Figure 6). Samples were fixed in buffered glutaraldehyde and postfixed in Osmiumtetroxide (OsO_4). Conventional dehydration and plastic embedding was performed followed by sectioning and contrasting with uranyl acetate and lead.

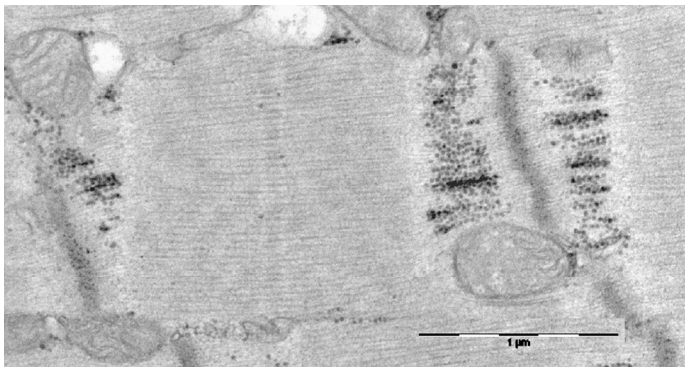


Figure 6. An electron microscopy picture showing a sarcomere in a normal muscle. The electron dense granules located adjacent to the Z-band correspond to glycogen. In the same zones are also mitochondria present. Bar = 1 µm

Neuropathology (Paper IV)

Neuropathological examination was performed in four of the children with Alpers-Huttenlocher syndrome described in Paper IV. Paraffin-embedded sections of paraformaldehyde-fixed brain specimens obtained postmortem were stained with HE and Luxol fast blue-cresyl violet. The sections were also immunostained for glial fibrillary acidic protein (GFAP).

Liver pathology (Paper IV)

Liver biopsies were obtained by needle biopsy, open biopsy or at post mortem investigation. Sections of paraffin-embedded, formalin-fixed liver specimens were investigated after staining with HE and Van Gieson stain. One liver biopsy was fresh-frozen in liquid nitrogen and subjected to enzyme histochemical investigation of COX and SDH.

Dissection of single muscle fibers (Papers I-III)

Serial sections 15 μm thick of transversely orientated muscle specimens were double stained for COX and SDH. Both blue COX-deficient muscle fibers and brown fibers displaying normal COX-activity were isolated with a sharp tungsten needle under a dissection microscope (Papers I and II). In Paper III only COX-deficient fibers were subjected to analysis. After dissection each fiber segment were lysed in 0.2 M of KOH with 5mM of dithiothreitol at 94^0 C. Neutralization buffer was added after ten minutes.

Biochemistry

Biochemical analyses were performed at the Department of Clinical Chemistry, Sahlgrenska University Hospital. Isolation of mitochondria, oximetric measurements of fresh mitochondria and spectrophotometric enzyme analyses were performed as described [155].

Molecular genetics

Total DNA was extracted from skeletal muscle (Papers I-V), heart muscle (Paper V), cell cultures (Paper II), liver tissue (Paper V) and peripheral blood (Papers I-V) using the DNeasy Tissue Kit or the DNA Blood Mini Kit (Qiagen). DNA in hair shafts (Papers I and II) and single dissected skeletal muscle fiber segments (Papers I, II and III) was analyzed after alkaline lysis. Total RNA was extracted from muscle and liver tissue (Paper V) using RNAqueous®-4PCR kit (Ambion) and complementary DNA (cDNA) was synthesized using Ready-To-Go™ You-Prime First-Strand Beads (Amersham Biosciences).

Different techniques of Polymerase Chain Reaction (PCR) (Papers I-V)

Standard PCR (Papers I-V), mismatch PCR (Paper II), Long extension (LX-) PCR (Papers I-IV), high fidelity PCR (Paper III) or quantitative PCR (Paper IV) were different PCR techniques used depending on the next application that followed i.e. DNA sequencing, transcription analysis, identification of mtDNA deletions and deletion breakpoints, restriction fragment length polymorphism (RFLP) analysis, cloning, denaturing gradient gel electrophoresis (DGGE) and quantification of mtDNA as described below under separate headings.

DNA sequencing and mutation screening (Papers I-V)

We performed mutation analyses of mtDNA (Papers I-IV) and nDNA (Papers III-V). The nuclear genes examined were: *POLG1* (Papers III and IV) and *GYS1* (Paper V). Primers for the nuclear genes were designed using MacVector® software 7.1.1. *POLG1* were amplified in 15 reactions covering the 22 coding exons with flanking intronic sequences. The 16 exons of *GYS1* were amplified in 14 reactions. Sequencing was performed using an ABI Prism 377 DNA sequencer and the Big Dye Terminator Kit (Applied Biosystems), and analyzed with MacVector® software 7.1.1.

mRNA analysis (Paper V)

Primers were designed to investigate transcription of *GYS1* and *GYS2* in liver and muscle tissue from patient and control. Primers amplifying β-actin served as cDNA quality control.

Identification of mtDNA deletions and deletion breakpoints (Papers I-IV)

LX-PCR is a sensitive albeit not quantitative method to screen for multiple mtDNA deletions. We amplified 10.8 kb fragments between mtDNA position 5447 and 16259, and if mtDNA deletions were present they appeared as smaller bands below the normal band after separation by gel electrophoresis (Figure 7). By standard PCR it is possible to amplify fragments harboring the deletion breakpoints. Primer pairs in mtDNA that normally are too far apart to form a PCR product will create fragments only in the presence of mtDNA deletions. Sequencing of these fragments will reveal the position for the breakpoint (Papers III and IV).

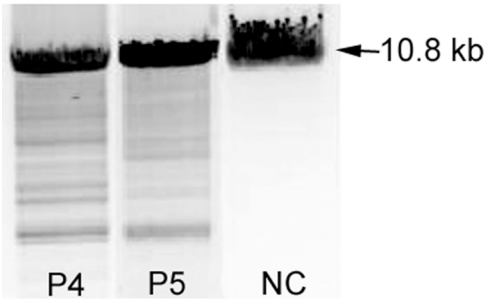


Figure 7. GelStar® staining of LX-PCR amplifications separated on a 0.6% agar gel which illustrates a normal 10.8kb amplified fragment from two of the PEO patients in Paper III. Several smaller bands, corresponding to multiple deleted mtDNA fragments are present in patients. NC = normal control

Restriction Fragment Length Polymorphism (RFLP) (Papers I, II, IV and V)

RFLP is applicable to verify if a mutation is present or absent in an individual if the mutation either creates or abolishes a restriction site. The application is suitable for screening a large control cohort for nuclear DNA mutations as we did in Papers IV and V were at least 200 control chromosomes were analyzed for the presence of identified mutations. The technique is also used to verify a mutation identified by DNA sequencing.

Regarding mtDNA mutations, RFLP can also be used in order to quantify the level of heteroplasmy of mtDNA mutations either in single muscle fiber segments or in tissue homogenate [23]. A fluorescent dye is conjugated to one of the primers, which is introduced during the PCR. After cleavage with restriction enzyme the fragments are separated by polyacrylamide gel electrophoresis and the relative amount of wild type and mutant DNA is calculated using GeneScan software (Applied Biosystems). In Paper I the mutation itself created a new restriction site but in Paper II we had to perform a mismatch PCR to introduce a restriction site in wild type DNA.

Cloning of mtDNA fragments (Paper III)

Direct sequencing of mtDNA does not detect very low levels of heteroplasmic mutations. So, to detect such 'microheteroplasmic' mutations another amplification step is needed before sequencing. To screen for randomly distributed point mutations in individual mtDNA molecules, single fragments amplified through high fidelity PCR (*Pwo* polymerase with proofreading activity), were ligated into vectors and transformed into competent *E. coli* bacteria. The high fidelity PCR was used in order to avoid PCR errors and thus get false positive results from this sensitive mutational screening method (Figure 8).

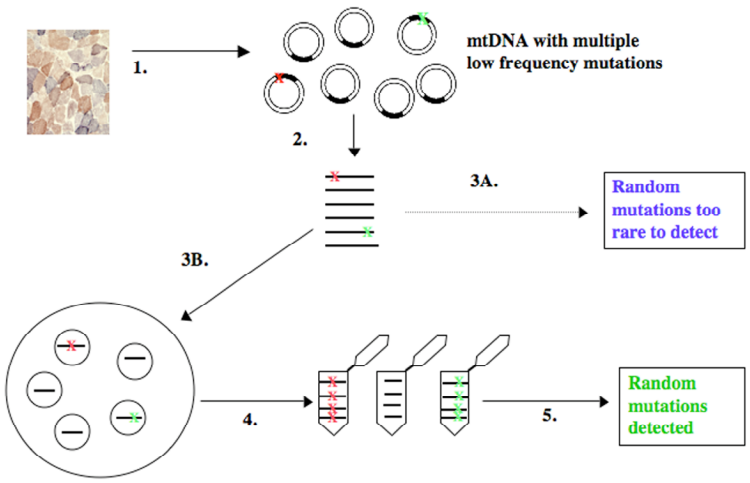


Figure 8. Illustration on the cloning procedure.

1. DNA extracted from muscle homogenate
2. Fragments of mtDNA amplified with high fidelity PCR
3. A. Direct sequencing will not reveal microheteroplasmic mutations
3. B. Fragments separated and inserts transformed into *E. coli* and colonies allowed to grow
4. Fragments amplified by PCR
5. Sequencing; random mutations will be revealed

Denaturing gradient gel electrophoresis (DGGE) (Paper III)

Mutation analysis with DGGE is based on how fragments with and without a mutation will migrate differently in a denaturing gel [61, 108]. In Paper III we used DGGE to search for clonally expanded point mutation in mtDNA in single muscle fiber segments in patients with adPEO and a primary mutation in *POLG1*.

Quantitative PCR (Paper IV)

The relative amount of mtDNA and nDNA in one single sample can be measured with multiplex quantitative real time PCR and TaqMan chemistry [156]. The target for nDNA were: the retinoblastoma susceptibility gene (RB1) with primers RB1-2672F and RB1-2750R and a FAM labelled probe RB1-2727, and for mtDNA: primers mt-8294F and mt-8436R and a VIC-labelled probe mt-8345. A quencher-dye, TAMRA, was linked to the 3'-end of both probes. The relative amount of mtDNA and nuclear DNA molecules in one sample, given by the difference in C_T value was compared with the relative amount of mtDNA/nDNA in a calibrator sample. This method is called the comparative C_T method.

Protein analyses

Protein extraction and western blot analysis (Papers II and V)

Western blot analysis was used to determine the expression of protein in the investigated tissue. Equal amounts of protein extracted from fresh frozen skeletal muscle tissue from patients and controls were loaded on 14% Tris-Glycine gels (Paper II) or 3 – 8% Tris-Acetate gels (Paper V) and electroblotted onto nitrocellulose filters. The intensity of myosin band in commasie blue staining served as control for the

amount of protein loaded on the gels. In Paper II we used monoclonal antibodies directed against the COX subunits I, II, III (mtDNA encoded) and IV (nDNA encoded) (Molecular Probes). In Paper V we used polyclonal rabbit-anti-human-glycogen synthase antibodies kindly provided by Dr. Oluf Pedersen, Copenhagen, Denmark.

Web-sources

Database searches and information about mitochondrial DNA polymorphisms and pathogenic mutations were obtained at:
MITOMAP, A human mitochondrial genome database
(http://www.mitomap.org)
mtDB – Human Mitochondrial Genome Database
(http://www.genpat.uu.se/mtDB/)
MitoAnalyzerTool, (NIST)
(http://www.cstl.nist.gov/biotech/strbase/mitoanalyzer.html)

Information about *POLG1* mutations was obtained at:
DNA Polymerase Gamma Mutation database
(http://dir-apps.niehs.nih.gov/polg/)

Information about gene expression profiles of *GYS1* and *GYS2* was obtained at
NCBI UniGene Web-site
(http://www.ncbi.nlm.nih.gov/UniGene)
UniGene Hs.386225 (*GYS1*) and UniGene Hs.82614 (*GYS2*)

GeneBank accession numbers

POLG1 NM_002693
GYS1 NM_002103
GYS2 NM_021957

POPULÄRVETENSKAPLIG SAMMANFATTNING

Energin som förbrukas i vår kropp används ifrån allt till att tillverka proteiner och nukleinsyror i enskilda celler till att driva samordnade reaktioner som gör att vi kan gå, hoppa, tänka och växa. Bränslet är en liten energirik molekyl som heter adenosintrifosfat, (ATP), som bildas i processen när maten vi äter bryts ner till små beståndsdelar och förbränns med hjälp av syret vi andas. För att driva alla energikrävande cellulära reaktioner under en dag så omsätts ungefär 40 kilo ATP hos en människa i vila. Eftersom ATP inte kan lagras krävs att vi har effektiva energireserver, glykogen och fett, som vid behov snabbt kan brytas ner och användas till ATP. Glykogen är kedjor av sammanlänkade sockermolekyler (glukos) som lagras främst i lever och muskler. Den fullständiga förbränningen av glukos och fettsyror till koldioxid och vatten, som är en syrekrävande process, sker i cellens egna energikraftverk, mitokondrier.

Mitokondrier är små organeller med dubbelt membran som finns i alla kroppens celler, men flest i dem som bygger upp energikrävande organ såsom hjärta, hjärna och skelettmuskler. Här kan det finnas flera tusen mitokondrier per cell. Mitokondrien var från början en frilevande bakterie som för ett par miljarder år sedan tog sig in i och började samarbeta med en annan cell. Olika organismer, alltifrån jäst till ryggradsdjur, har mitokondrier med samma struktur och funktion. Mitokondrier har eget DNA, och liksom hos bakterier så är det en ringformad kromosom. I varje mitokondrie kan det finnas flera mitokondrie-DNA-molekyler, och när de ska kopieras så sker det med hjälp av ett särskilt enzym som heter polymeras gamma (Polγ). Genen som kodar för Polγ heter *POLG1* och finns på en av kromosomerna i cellkärnan.

Själva ATP-tillverkningen sker över mitokondriens inre, kraftigt veckade membran där elektroner transporteras som en elektrisk ström mellan fyra proteinkomplex som samtidigt pumpar vätejoner ut från mitokondriens inre. Spänningen som byggs upp över membranet blir till slut så stark att vätejonerna strömmar tillbaks in genom ett femte komplex som roterar och bildar ATP. Om inte ATP produceras i den mängd som krävs för att hålla alla processer igång blir det energikris som kan leda till olika sjukdomar. Mitokondrier finns ju i alla celler och därför kan alla organ påverkas, men det är oftast nervsystem och muskler som drabbas hårdast när energiproduktionen inte fungerar. Sjukdomarna kan yttra sig väldigt olika. Ibland är sjukdomsdebuten sen och förloppet långsamt, men ibland går det väldigt snabbt och energikrisen leder till död i de tidiga barnaåren.

Avhandlingen handlar om att försöka förstå den bakomliggande orsaken till energikris hos patienter i olika åldrar med olika symptom och sjukdomar. Detta är viktigt inte minst för att kunna ge patienterna en korrekt diagnos, men också för att det ökar möjligheten att utveckla behandlingsmetoder till dessa oftast obotliga sjukdomar.

Vetskap om den genetiska orsaken till en sjukdom med bristande energiproduktion är helt nödvändig för att kunna ge rådgivning om ärftlighet.

De första två arbetena i avhandlingen beskriver hur vi har identifierat mutationer som sitter primärt i mitokondriers eget DNA hos patienter med misstanke om muskelsjukdom. I båda dessa fall kunde mutationerna endast identifieras i muskelvävnad vilket tyder på att de troligtvis är nya mutationer som har uppkommit spontant, tidigt under fosterlivet. Vi använde oss av flera olika metoder för att se om det var just de mutationer vi identifierat som var orsak till sjukdom. Bland annat jämförde vi enstaka muskelfibrer som visade tecken på energibrist med till synes friska fibrer, och fann en tydlig koppling mellan andelen skadat mitokondrie-DNA och funktion. Våra fynd har en direkt betydelse för den genetiska rådgivning som ges till patienterna och deras familjer.

Hos de patienter som beskrivs i det tredje arbetet fungerar inte kopieringen av mitokondriens DNA på grund av mutationer i *POLG1*. Dessa mutationer leder till att det blir så kallade deletioner i mitokondrie-DNA i bland annat hjärnceller och muskelceller (dvs den sortens celler som sällan eller aldrig delar sig). Stora delar genetisk information saknas och det blir energibrist. Hur dessa deletioner uppkommer vet man inte. Det fanns en teori om att små fel som inducerades vid själva kopieringen i sin tur ledde till att polymeraset hoppade över delar av mitokondrie-DNA så att kopian blev kortare. Vi undersökte denna teori men kunde inte finna något sådant samband.

Det fjärde arbetet handlar om en grupp barn med en svår lever- och hjärnsjukdom som kallas Alpers-Huttenlochers syndrom (AHS). Barnen med AHS är normala vid födseln men får utvecklingsstörning, svårbehandlad epilepsi med leverpåverkan och dör tidigt. Några fall av AHS har beskrivits med en senare sjukdomsdebut – upp till de tidiga tonåren. Ibland tycks sjukdomen utlösas av en infektion eller någon annan yttre påverkan. Vi kunde visa att AHS är en mitokondrie-sjukdom som beror på mutationer i *POLG1*. Denna kunskap har gjort att vi kan erbjuda drabbade familjer genetisk vägledning och prenataldiagnostik.

I det femte arbetet beskriver vi en helt ny sjukdom som också är en ny orsak till plötslig hjärtdöd hos barn. De drabbade barnen hade inget glykogen i sina muskler och vi fann att orsaken var brist på enzymet som tillverkar glykogen och det berodde på en ärftlig mutation i den sk glykogensyntasgenen *GYS1*. Tidigare studier har visat att hjärtat främst använder fettsyror för att tillverka ATP men att det behövs glykogen vid kraftig ansträngning. Därför kan bristen på glykogen kopplas till hjärtats plötsliga arbetsoförmåga under till exempel häftig lek eller idrott. Vi har givit den nya sjukdomen beteckningen ”Muscle Glycogen Storage Disease type zero”

ACKNOWLEDGEMENTS

There are so many people who have supported me and given me credit and encouragement during these years of laboratory work and thesis writing. Thank you all, it hadn't been possible without you! Especially I wish to express my sincere gratitude to:

My supervisor ***Anders Oldfors*** for giving me the opportunity to work in the field of muscle pathology research, for constructive criticism, stimulating discussions and continuous support. Thank you for being my traveling guide in science and for your enthusiasm, generosity and friendship

Ali-Reza Moslemi, my assistant supervisor and laboratory teacher. I am truly grateful for the time I could work with you and learn from you, for your generous support and for sharing your knowledge

Elisabeth Holme, Inger Nennesmo, Atle Melberg, Niklas Darin, Már Tulinius and ***Christopher Lindberg*** for collaboration and your generous support, constructive criticism and valuable discussions by telephone calls, e-mails and meetings

Monica Jansson, Åsa Pérez-Bercoff, Ingibjörg Bjarnadottir, Paul Uvebrant, Ingegerd Östman-Smith, Jan Toft, Gun Forsander, Thomas Gilljam and ***Peter Jotorp*** for valuable cooperation and co-authorship

Hermengild Barrlind and ***Monica Jacobsson*** for excellent technical assistance, support and encouragement. Your care has meant a lot to me!

Lili Seifi, Homa Tajsharghi, Gabriella Almén and ***Johanna Nilsson*** for assistance and for providing a friendly atmosphere in the lab

My friends at the 'honors table', especially ***Margareta Persson, Christina Persson, Ulla-Britt Strandlund, Lisbeth Gustafsson*** and ***Sibylle Widéhn*** (what would I do without you), but also ***Anna-Carin Ericson, Carina Karlsson, Gun Augustsson, Iréne Carlsson, Kristina Eriksén, Anette Liljeroth*** and ***Anita Fae*** for your interest and support

Kerstin Grahn, Gunilla Möllmark and ***Birgitta Börjesson*** for excellent secretarial assistance, support and nice chats

Present and former room-mates: ***Ellinor Andersson, Fredrik Persson, Mattias Andersson, Tajana Tesan, Wanzhong Wang, Karin Jennbacken, Helene Gustavsson, Tatjana Adomovic, Ahmad Hamta, Fredrik Trossö, Johanna Andersson, Melker Göransson*** and ***Annika Krona***. A special thanks to ***Marta Winnes*** and ***Gabriella Arne*** for laughs and cries at "Tre små rum" and for friendship

Britt-Marie Samuelsson and ***Ulla Höök*** for technical skills in electron microscopy

Claes Nordborg for valuable discussions

Ulric Pedersen for all your help with computers and posters

All staff at the Department of Pathology for making it joyful to work here, particularly ***Ywonne Andrén, Carola Andersson, Fredrik Enlund, Barbro Wedell, Christina Kåbjörn, Yvonne Arvidsson*** and ***Agnetha Josefsson***

Anette Ekberg and ***Ulrika Sterky*** for all your hard work collecting blood in Västerås and Göteborg

Anne Lombes and ***Oluf Pedersen*** for kindly providing important antibodies

All ***patients*** and ***their families***, and the ***Assyrian Community in Göteborg*** for your participation

All my wonderful ***friends*** for your care and encouragement! Thank you!

My house band "***Chidaruma***" for keeping me amused with live music and sometimes for not

My mother ***Inger***, I know how much you care! My Dear sister ***Maria***, thank you for always being there for me. My brother-in-law ***Kjell-Arne*** for our long friendship. My brothers, ***Anders*** and ***Thomas***, for being you and for believing in me. My uncle ***Thore***, for your endless support and for holding the family together with your fantastic research. Thank you, I love you all!

Most of all my sons ***Jonas*** and ***Joel*** for your love, support and understanding, and finally my sweet sunshine of life, my lovely granddaughter ***Andrea***. I love you all!

This work has been supported by grants from the Swedish Medical Research Council (Proj. No. 7122 and 10823), the Ländell Foundation, and the Sahlgrenska University Hospital Foundation

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