Mitochondrial biomarkers and biomarkers of mitochondrial disease

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Thesis for the degree of Philosophiae Doctor (PhD)
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Table of contents

1 Acknowledgements

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2 Scientific environment

- The Mitochondrial Medicine and Neurogenetics group, led by Professor Laurence A. Bindoff
- Department of Clinical Medicine, University of Bergen
- Department of Neurology, Haukeland University Hospital, Bergen

3 List of publications

Paper I

Varhaug KN, Vedeler CA, Myhr, Aarseth JA, Tzoulis C, Bindoff LA.

Increased levels of cell-free mitochondrial DNA in the cerebrospinal fluid of patients with multiple sclerosis. Mitochondrion. 2017

Paper II

Varhaug KN, Nido G.S., de Coo I, Isohanni, P, Suomalainen A, Tzoulis C, Knappskog P, Bindoff LA.

Using urine to diagnose large-scale mtDNA deletions in adult patients. Annals of Clinical and Translational Neurology. 2020

Paper III

Varhaug KN, Hikmat O, Nakkestad HL, Vedeler CA, Bindoff LA.

Serum biomarkers in mitochondrial disorders. Brain Communications. 2021

4 List of abbreviations

AD	Alzheimer's dementia
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
ATP	adenosine triphosphate
Bp	base pair
CK	creatinine kinase
CSF	cerebrospinal fluid
CNS	central nervous system
CoQ	coenzyme Q
COX	cytochrome c oxidase
DAMP	damage-associated molecular pattern molecule
ELISA	Enzyme-linked immunosorbent assay
FGF-21	fibroblast growth factor 21
GDF-15	growth and differentiation factor 15
H-strand	heavy strand
KSS	Kearns-Sayre syndrome
L-PCR	long-range polymerase chain reaction
LHON	Leber hereditary optic neuropathy
L/P ratio	Lactate/Pyruvate ratio

L-strand	light strand
MELAS	mitochondrial myopathy, encephalopathy lactate acidosis and stroke-like syndrome
MERRF	Myoclonus epilepsy with ragged-red fibres
MIDD	maternally inherited deafness and diabetes
MRI	magnetic resonance imaging
MS	multiple sclerosis
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
NF-L	neurofilament light chain
NMDAS	Newcastle Mitochondrial Disease Adult Scale
OXPHOS	oxidative phosphorylation
PAMP	pathogen-associated molecular pattern molecules
PD	Parkinson's disease
PEO	progressive external ophthalmoplegia
POLG	polymerase-gamma
PRR	pattern-recognition receptors
qPCR	quantitative polymerase chain reaction
RRMS	relapsing remitting multiple sclerosis
SDH	succinate dehydrogenase
TLR	toll-like receptors

5 Abstract

Background: Biomarkers are objective and reproducible tools that are essential for making the diagnosis and following disease development in clinical practice. Mitochondria are multifunctional organelles and the major site of cellular energy production. Primary mitochondrial disorders are a group of heterogeneous conditions characterised by impaired energy metabolism; however, mitochondrial dysfunction is also a feature of many other disease processes, particularly age-related neurodegenerative disorders. Thus, mitochondrial dysfunction can be both a primary disease manifestation or a secondary consequence of another disease process. Common for both is the lack of biomarkers that are robust enough to use in the clinical follow-up of patients.

Objective: To evaluate new biomarkers and new ways of using known biomarkers for the diagnosis and follow up of primary mitochondrial disorders and neurological diseases in which mitochondria or mitochondrial dysfunction may play a role.

Materials and methods: The study population in paper I were newly diagnosed patients with relapsing-remitting multiple sclerosis (MS) and an age and gender matched control group. In paper II and III the cohort constituted of patients with known, genetically confirmed primary mitochondrial disease. Paper II was an international collaborative study that included a cohort of patients with large-scale single mitochondrial DNA (mtDNA) deletions, while in paper III, the study cohort were Norwegian patients with mitochondrial disease caused by known nuclear and mtDNA mutations.

Cell-free mtDNA in cerebrospinal fluid was investigated in paper I, products from long-range PCR in urine in paper II and serum levels of fibroblast growth factor 21 (FGF-21), growth and differentiation factor 15 (GDF-15) and neurofilament light chain (NF-L) were investigated in paper III.

Results: We found that the levels of cell-free mtDNA in cerebrospinal fluid were elevated in patients with relapsing-remitting multiple sclerosis relative to controls, and that there was an inverse correlation between level of free mtDNA and disease duration.

In our studies using urine sediment cells, we found it was possible to identify large-scale single mtDNA deletions in urine in 80% of cases. In addition, this source of material also allowed us to confirm breakpoints, heteroplasmy levels and deletion size in the DNA extracted from urinary sediment cells.

We found that using a combination of serum biomarkers FGF-21, GDF-15 and NF-L, we could differentiate between subgroups of patients with mitochondrial disease. The levels of NF-L increased with the degree of central nervous system involvement and this was especially seen in patients with multisystemic and complex disease. In contrast, FGF-21 and GDF-15 were not significantly elevated in these patients, but markedly increased in those with primary myopathies.

Conclusions: These three studies provide novel information concerning the use of biomarkers in the diagnosis and management of mitochondrial disease and the use of mitochondrial biomarkers in diseases where the primary process is thought to be inflammatory.

Our findings in MS suggest that mitochondria play a role in the early disease pathogenesis. In addition, we found that changes in the level of mtDNA in cerebrospinal fluid correlated with disease activity. While this could potentially provide a novel marker for early disease activity, the need for lumbar puncture would limit its applicability.

We found that urine provides a readily available source of mtDNA for diagnostic screening of patients suspected clinically of having a single mtDNA deletion. These findings should help reduce the need for invasive muscle biopsy.

Where diagnosis is less clear, but strong suspicion of mitochondrial disease, the combined use of FGF-21, GDF-15 and NF-L provides diagnostic information, insight into differential tissue involvement and the possibility of following disease activity.

6 Introduction

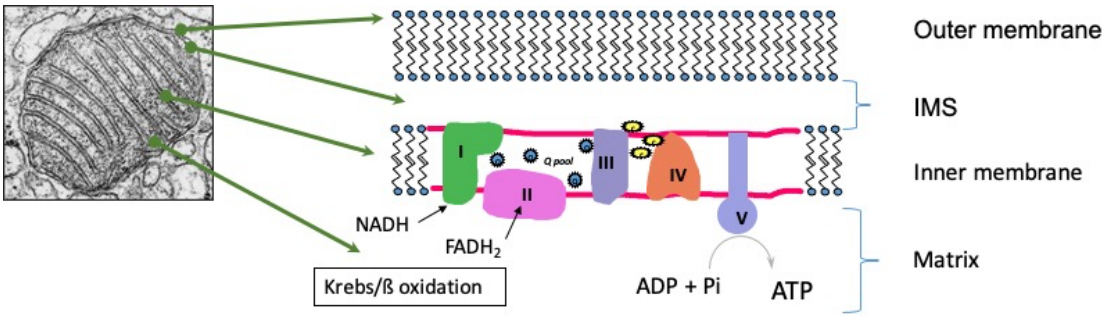
6.1 Mitochondria

Mitochondria are intracellular organelles present in all cells except mature erythrocytes (1). A mitochondrion is comprised of an outer and an inner membrane, the intermembrane space in between and the matrix within the inner mitochondrial membrane (1). The organelle is thought originally to have been a bacterium, that at some time point colonised and initiated a symbiotic relationship with primordial eukaryotic cells (2). This endosymbiosis is considered to be the explanation behind the double-membrane in mitochondria.

6.1.1 *The respiratory chain and ATP production*

Mitochondria are multifunctional organelles that are the primary drivers of energy metabolism via a system named oxidative phosphorylation (OXPHOS). The end-product of this process is adenosine triphosphate (ATP), which is the result of a condensation of adenosine diphosphate (ADP) and phosphate (3). ATP-production is performed by an enzyme pathway called the respiratory chain that is embedded in the inner mitochondrial membrane (2) (Figure 1).

Figure 1. Mitochondria and the respiratory chain. An electron microscope figure of a mitochondria with its cristae formed by the inner membrane. The respiratory chain is embedded in the inner membrane. (Courtesy of Prof. L. A. Bindoff).



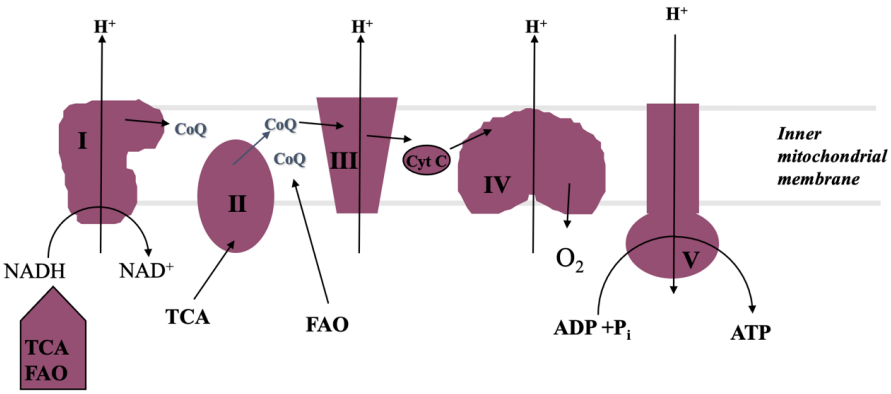
The respiratory chain consists of five complexes (I-V) and two mobile electron carriers (coenzyme Q (CoQ) and cytochrome c) (2) (Figure 2).

Mitochondrial metabolic pathways, including fatty acid ß-oxidation, generate the reduced cofactors NADH and $FADH_2$. During reoxidation of these cofactors, electrons are donated into the respiratory chain.

Complex I (NADH dehydrogenase-ubiquinone oxidoreductase) transfers electrons from NADH to CoQ. Complex II (succinate dehydrogenase-ubiquinone oxidoreductase) is not only an enzyme in the respiratory chain, but also participates in the tricarboxylic acid cycle (TCA) and oxidises succinate to fumarate with the transfer of electrons to CoQ (4). Complex III (ubiquinone-cytochrome c oxidoreductase) accepts electrons from CoQ and passes them on to cytochrome c. Finally, complex IV (cytochrome c oxidase) receives electrons from cytochrome c, transfers them to the final electron acceptor, oxygen, producing water (2, 4, 5). As electrons flow along the electron-transport chain, protons (H^+) are pumped from the mitochondrial matrix to the intermembrane space through complex I, III and IV generating an electrochemical (proton) gradient. Protons re-enter the matrix through

complex V (ATP-synthase) and the energy generated by discharging the proton gradient is used to drive the phosphorylation of ADP to ATP (2, 5).

Figure 2. The five complexes of the respiratory chain. Reduced cofactors NADH and $FADH_2$ are generated by pathways such as the tricarboxylic acid cycle (TCA) and fatty acid ß-oxidation (FAO). Reoxidation generates electrons that are transported horizontally along by coenzyme Q (CoQ) and cytochrome c (cyt C). The protons (H^+) are pumped vertically over the inner mitochondrial membrane.



6.1.2 *Mitochondrial DNA*

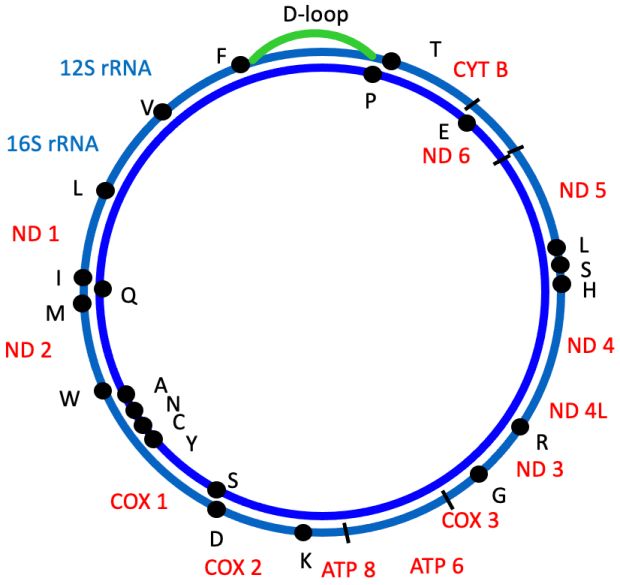
In animals, mitochondria are the only extra-nuclear organelles that have their own DNA; mitochondrial DNA (mtDNA) (Figure 3). That DNA was present in mitochondria was first recognised in 1963 and by 1981 the complete sequence of human mtDNA was known (6). Different cell types contain different numbers of mitochondria and widely different numbers of mtDNA (2, 5, 7, 8).

The mitochondrial genome is small comprising just 16,569 base-pairs. It is a circular, double-stranded molecule that encodes 2 rRNA, 22 tRNA and 13 proteins (6). The five respiratory chain complexes are multimeric protein complexes, and the 13 proteins encoded by the mtDNA, are all subunits of respiratory chain complexes: seven are complex I proteins (*MTND1-6* and *MTND4L*), one is a complex III protein (*MTCYB*), three are complex IV proteins (*MTCO1-3*) and two are subunits in complex V (*MTATP6* and *MTATP8*). The rest of the > 80 respiratory chain subunits are encoded by chromosomal genes (the nuclear DNA) and complex II is the only complex that is entirely nuclear encoded (9). The individual strands of the double-stranded mtDNA molecule are named heavy (H) strand and light (L) strand (10, 11). They are different in terms of density and mass, due to uneven nucleotide content; the H-strand is rich in guanine content, in contrast to the L-strand (11). The majority of information is encoded in the heavy strand (12 proteins, 2 rRNAs and 14 tRNAs), while the light strand encodes just one protein and 8 tRNAs (10).

The circular shape and small size distinguish the mitochondrial genome from the nuclear genome. In fact, the mitochondrial genome resembles that of bacterial DNA and it is thought that it is a remnant of the historical bacterial invasion of the primordial eukaryotic cell (the endosymbiotic theory described above) (12). The human mitochondrial genome is, however, smaller than the bacterial genome (13). It is hypothesised that evolution has deprived the human mtDNA its autonomy and reduced the size of mtDNA through gene transfer to the nucleus (12-14). In contrast to the nuclear genome, mitochondrial DNA has no introns and practically no non-coding nucleotides between the different genes (13). The largest non-coding region of mtDNA is the displacement (D)-loop and this 1kb region contains control elements for transcription and replication (11). Transcription of mtDNA is polycistronic in a bacteria-like fashion: the genes on the heavy and light strands are encoded in two large RNAs, which are subsequently cleaved into individual mRNAs and tRNAs (15). In contrast to the phase specific replication of nuclear DNA, mtDNA is continuously replicated, independent of cell cycle (11). The exact mechanism for how the replication of mtDNA is achieved remains unresolved and two main theories

exist; strand-displacement replication or strand-coupled replication. Strand-displacement theory suggests that replication starts with the H-strand and after two-thirds when the origin of replication of the L-strand is exposed, replication of the L-strand is initiated in the opposite direction. The strand-coupled replication theory suggests a bidirectional, symmetrical synthesis from multiple replication forks (11). MtDNA is also unique in the mode of inheritance and the number of genomes per cell and this is further detailed in sections below.

Figure 3. The mitochondrial genome. The heavy strand (outer light blue) and light strand (inner dark blue). Displaying the positions of genes encoding 2 rRNA (blue), 22tRNA (black) and 13 proteins (red).



6.1.3 *Nuclear-mitochondrial interaction*

While mtDNA codes for 13 proteins, the vast majority of mitochondrial proteins are encoded by nuclear genes. These nuclear encoded proteins are synthesised on cytosolic ribosomes and have to be imported in through one or both membranes. The majority of the matrix and inner membrane proteins are thus synthesised as precursor proteins and many have attached a positively charged targeting sequence, that directs the protein to the mitochondria (16). At the surface of the outer mitochondrial membrane, these precursors bind to receptor proteins and are further translocated across the outer and inner membranes into the mitochondrial matrix. Once translocated, the targeting sequence is cleaved by enzymes in the matrix (16). Inside the mitochondria, the more than 1300 nuclear-encoded proteins are involved in multiple aspects of mitochondrial function. Proteins such as the subunits in the respiratory chain are translocated and assembled with the mtDNA encoded subunits to form the multimeric complexes. Ancillary proteins such as assembly factors for the complexes are also nuclear encoded. Even the mitochondrial genome is reliant on nDNA encoded proteins and enzymes. These are essential for mtDNA replication, repair, transcription, translation and maintenance. Examples are polymerase gamma (the mtDNA polymerase), twinkle (the helicase behind mtDNA unwinding) and TFAM (for initiating transcription, replication and nucleoid packaging) (17, 18). Mitochondria are dynamic organelles. They move within the cytosol and they undergo fission and fusion. The machinery of these dynamics are also nDNA encoded (19). Thus, in summary, it is clear that mitochondrial function is absolutely dependent on a complex interplay between two genomes the nuclear and the mitochondrial.

6.1.4 *Mitochondrial role in the innate immune system*

ATP-production is not the only function of mitochondria. Complexes I and III generate small amounts of reactive oxygen species (ROS) that influence signalling pathways involved in cell proliferation, differentiation and adaption to stress (20). Mitochondria are also involved in calcium homeostasis, lipid and cholesterol

synthesis and in the synthesis of FeS clusters and heme, that are important components in redox proteins including components of the respiratory chain complexes and in DNA repair machinery, and in oxygen carriers like haemoglobin (1, 20-22). Interestingly, mitochondria also have an important role in innate immunity and inflammation.

The human immune system comprises innate and adaptive systems. In an evolutionary perspective the innate immune system is an ancient defence mechanism where similar processes are found in plants and animals (23). The cells involved are the macrophages, dendritic cells, mast cells, neutrophils, eosinophils and natural killer cells. When the immune response of the innate system is unable to handle an infection, the adaptive immune system is activated. In contrast to the innate immune system, the more sophisticated adaptive defence system is an evolutionary newcomer (23). This system comprises a defence mechanism that depends on B- and T-cells, and which following exposure to an antigen, results in a long-lasting immunologic memory. In contrast to the innate immune system, the adaptive immune system is highly individual and based on what specific infectious agent one person has been exposed to. This memory is not inherited from one generation to the next (23).

The innate immune system recognises endogenous molecules (DAMPs; damage-associated molecular pattern molecules) and exogenous molecules (PAMPs; pathogen-associated molecular pattern molecules) and initiates either a non-infectious inflammatory response or a pathogen-induced inflammatory response (24). DAMPs originate from the plasma membrane, endoplasmic reticulum, cell nucleus and cytosol. They can also arise from mitochondria and these are known as mitoDAMPs (25). Molecules or proteins that act as DAMPs or PAMPs are recognized by receptors known as pattern-recognition receptors (PRRs) (25) and complexes formed by PAMPs or DAMPs bound to PRRs initiate a variety of cascades, which result in expression of cytokines and other pro-inflammatory molecules (26). One subgroup of PRRs is the Toll-like receptors (TLR) and TLR-9 is known to bind the unmethylated CpG motifs of bacterial DNA (25, 26). Mitochondrial and bacterial DNA share many similarities, (as they probably have a similar origin) (24) and mtDNA released into

the bloodstream can act as a DAMP (24) with the potential of stimulating the Toll-like receptor 9 (24-26).

6.2 Primary mitochondrial disease

Primary mitochondrial disorders are a group of clinically heterogenous conditions characterised by deficiencies in the OXPHOS system (4, 27). The first patient described with a mitochondrial disorder had an extremely rare disease now called Luft´s disease after the clinician Rolf Luft (28) who described it. The patient´s clinical features were increased perspiration, increased thirst with normal urine volumes, low body weight despite increased appetite, and fatigue. Clinical and laboratory investigations were largely normal, except for an elevated basal metabolic rate and the patient´s metabolic rate remained elevated despite thyroidectomy. As mitochondria is the site of cellular respiration this organelle became the focus for further investigations. Biochemical studies of the patient´s skeletal muscle confirmed dysfunctional mitochondria and electron microscopy showed abnormal accumulations of mitochondria and considerable variation in mitochondrial size. By the 1970´s abnormal mitochondrial findings were also discovered in myopathies (7) and since then, mitochondrial disease has become recognised as one of the most common metabolic disorders, with a prevalence of ~ 1/5000 (29). Although there have been massive advances in the mitochondrial field over the past decades, there still exists considerable challenges. These challenges are largely summarised in the statement: "respiratory chain deficiency can give rise to any symptom, in any organ or tissue, at any age, with any mode of inheritance" (19). The variation in the mode of inheritance is due to the dual genetic control of mitochondria: mitochondrial disease can be caused by mutations in either mtDNA or genes encoded in the nuclear DNA (nDNA). To facilitate the further discussion of these disorders, sections have been divided into mtDNA linked and nDNA linked mitochondrial disease.

6.2.1 *Mitochondrial DNA linked disease*

The mitochondrial genome differs to nDNA in many ways. Firstly, it is inherited only through the maternal line: at fertilisation, sperm only contribute their nuclear DNA to the next generation, all mitochondria and thus mtDNA are derived from the oocyte giving an unique maternal inheritance pattern (2). This means that mtDNA mutations are either maternally inherited or sporadic (9).

MtDNA acquires mutations up to seven times faster than nDNA. This is thought to be a consequence of mtDNA lacking protective histone proteins and repair mechanisms, and the proximity to the reactive oxygen species (ROS) produced by the respiratory chain, hence exposing mtDNA to oxidative damage (7, 12).

Each cell contains multiple copy numbers of mtDNA molecules varying from ten´s to hundreds of thousands of copies depending on cell type. Mutations can therefore affect some or all of the mtDNA copies: the coexistence of wild type and mutant mtDNA in cells is termed *heteroplasmy*. The level of heteroplasmy can vary between tissues and even between different cells in the same tissue. Different tissues have different demands for ATP production to function adequately (5, 30). The level of mutant mtDNA (degree of heteroplasmy) that must be present to cause cellular dysfunction is called the threshold level (5, 30). This threshold will vary depending on the different tissue's energy requirements.

Changes in the level of mutant mtDNA can occur at two stages: from one generation to the next and during somatic cell division. The maternal germline forms early in foetal development and during early oogenesis there is a contraction in mtDNA copy number from $>10^5$ copies to maybe a few hundred copies. This reduction in copy number is termed the genetic bottleneck and this can change the level of heteroplasmy from one generation to the next dramatically (21, 31). The proportion of mutant mtDNA can also alter during cell division with daughter cells containing different levels of mutant mtDNA. This principal is called mitotic segregation and explains how phenotypes can change in a patient over time (32).

6.2.2 *Large-scale rearrangements of mtDNA*

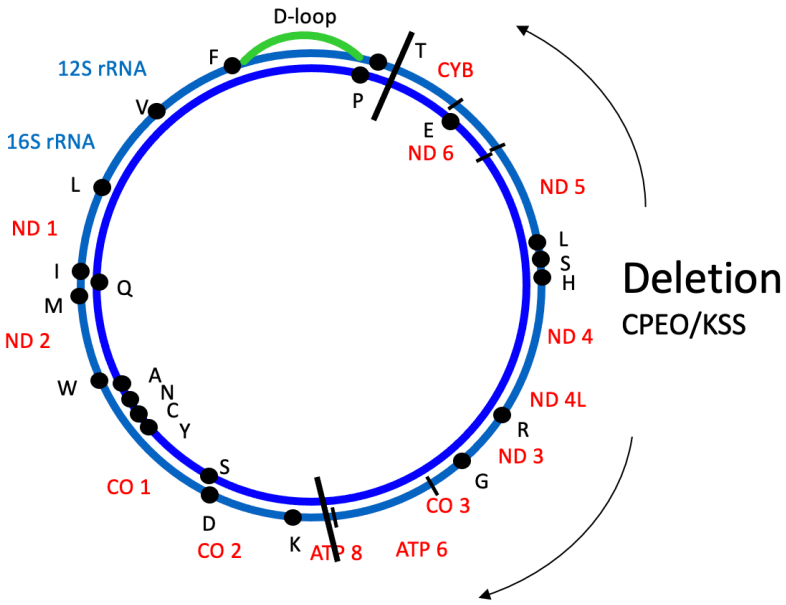
Large-scale rearrangements give rise to three classical phenotypes; Pearson disease (<5%), Kearns-Sayre syndrome (KSS) (~ 30% of the cases) and progressive external ophthalmoplegia (PEO) (~65% of the cases) (9). Pearson disease is a multisystem disorder of infancy, recognised usually by the presence of sideroblastic anaemia (33). Patients with Pearson disease that survive infancy develop KSS. KSS has been defined as onset of PEO before age 20, pigmentary retinopathy and at least one of the following features; cardiac conduction block, cerebellar ataxia and/or cerebrospinal fluid protein concentration >0.1 g/L (33). PEO is characterised by ptosis and limited eye movement, often accompanied by proximal weakness and myopathy, in addition to other non-muscular symptoms like hearing loss, ataxia and other neurological symptoms (34).

The mtDNA rearrangements are thought to arise in the oocyte, and then be transmitted to the offspring who develops the phenotype (21). Although maternal transmission has been reported, they are primarily sporadic events with a minimum prevalence of 1.5/100000 (29).

The most common of the mtDNA rearrangements are large-scale single deletions (35).

Single mtDNA deletions were discovered in 1988 and were the first pathological mutations discovered in human mtDNA (36); they contribute ~16% of all mtDNA mutations in adults (29). The majority of deletions (~85%) occur within the major arc, between the origin of H-strand replication and L-strand replication and preserve the 12S and 16S rRNA genes and origin of heavy strand replication (33, 37) (Figure 4). Although deletion size and breakpoint vary, one third of patients have what is known as the “common deletion” of 4,977 base pair (bp). This deletion is located with left breakpoint within the *ATP 8* gene at position 8,483 and right breakpoint at position 13,460 within the *ND 5* gene. At each breakpoint the common deletion is flanked by a 13 bp direct repeat (38).

Figure 4. The mtDNA illustrating the major arc, where 85% of all single deletions occur within.



In contrast to mtDNA point mutations, the clinical syndromes caused by deletions manifest a uniformity, even if the deletions themselves are heterogenous. This could be explained by the polycistronic nature of the mtDNA: the genes encoded are transcribed as two large RNAs, subsequently cleaved in individual tRNAs and mRNAs. Thus, any major deletion can affect the transcription of other genes not just those encompassed by the deletion (15).

In 1989, the first duplications of mtDNA were discovered in two patients with KSS (39). Laboratory investigations revealed a larger band in these two patients in addition to the 16.5 kb large band that represents normal mtDNA. The mtDNA

duplications were easily detectable in blood and muscle, but not in urinary epithelial cells or hair follicle cells. Further studies detected the presence of deletion dimers (combination of two deleted fragments) and monomers along with duplications (40). A re-investigation of 18 patients with either KSS or PEO and a single-deletion, showed the coexistence of mtDNA duplications, dimers and monomers in the patients with KSS, but not CPEO (41). This was confirmed in a later study, however, this study showed that also a small percentage (10%) of PEO patients had duplications present along with single deletions (42).

It is not clear how mtDNA duplications interfere with normal mitochondrial function; suggestions include both the production of abnormal products from the duplicated molecule, or an excess of normal products (43). It is also unclear what duplications do in the presence of deletions in those cases where the single deletion appears to be the dominant abnormality. It is speculated in whether the presence of duplications influences age of onset and the clinical expression of the mitochondrial disease (42) and whether the duplications in fact generate deletions (44). Another theory is that the non-pathological duplications are transmitted from mother to child, were they lead to the formation of pathological deletions and disease (45).

6.2.3 *Point mutations*

Point mutations in mtDNA can theoretically arise throughout the genome, affecting either specific respiratory chain proteins, any of the RNA species involved in mitochondrial protein synthesis or the non-coding region of the D-loop. There are however some hot spots (2, 9). More than half of the mutations are located in the mt-tRNA genes and are assumed to contribute to an overall impairment of mitochondrial translation (14). In 75% of cases the mtDNA point mutation is inherited, while in the remaining 25%, these appear to be *de novo* events (9).

The first mtDNA point mutation was described in 1988 (46). The m.11778G>A in the *MTND4* gene was found in patients with the Leber hereditary optic neuropathy (LHON) phenotype. Later the m.3460A>G (*MTND1* gene) (47) and the m.14484T>C

(*MTND6)* (48) mutations were recognised as two additional mutations commonly giving rise to LHON. LHON is the most prevalent mtDNA disorder, but the m.3243A>G mutation in the *MTTL1* gene discovered in 1990 (49) is the single most prevalent pathological point mutation (29).

One of the classical phenotypes associated with mtDNA point mutation is the syndrome of mitochondrial encephalopathy lactate acidosis and stroke-like episodes (MELAS) (50). The term stroke-like lesion is considered misleading, as these lesions have no pathological evidence of ischemia (51), but instead are thought to occur as a response to neuronal energy failure. A consensus opinion from an expert panel defined stroke-like episodes as: a subacute evolving brain syndrome driven by seizure activity in genetically determined mitochondrial disease (52).These episodes are usually characterised by a clinical prodrome, which may include nausea, vomiting, migraine-like headache and abdominal pseudo-obstruction. After hours or days, the prodrome is followed by neurological deficit including hemiplegia, hemianopia, aphasia or ataxia (53). Histopathological examination of stroke-like lesion areas shows evidence of neuronal sparing and not confluent necrosis and radiologically, lesions are not restricted to a single vascular territory as would be expected with ischaemia (50, 53). Since it is thought that these episodes are driven by seizure activity, it is crucial for these patients that their epileptic seizures are treated swiftly to limit the damage. Commonly, a combination of antiepileptic drugs is needed.

Although studies suggest that the m.3243A>G mutation causes 80% of MELAS cases (3, 14), only about 10 % of the carriers of this mutation present with the MELAS phenotype (54). A more frequent syndrome with this mutation is maternally inherited deafness and diabetes (MIDD), a phenotype that characterises about 30% of mutation carriers (54).

The m.8344A>G mutation in the *MTTK* gene, less common than the m.3243A>G mutation (29), is the predominant mutation causing the myoclonus epilepsy with ragged-red fibres (MERRF) syndrome. This syndrome was first described by Fukuhara (55), who later suggested the acronym MERRF. In adults, the epilepsy may

be milder or absent and they may present with ataxia and cervical lipomas as common additional features. As with MELAS, the clinical MERRF syndrome may occur with different mutations, but the m.8344A>G mutation is found in 80% of the cases (1).

The *MTND5* gene is another hot spot for mutations (56) and at least 15 pathological point mutations have been identified within (57). The *MTND5* gene encodes for a subunit in complex I and mutational phenotypes span from severe encephalopathies as in Leigh disease, MELAS and MERRF to LHON and isolated exercise intolerance, as seen in the m.13271T>C mutation (57). The phenotypes are seemingly independent of where within the gene the site of mutation is (57).

Apart from a few exceptions where mutations always display the same phenotype (as seen in the m.1555A>G mutation that results in deafness), mtDNA point mutations illustrate one of the major challenges in mitochondrial medicine: there is a general lack of correlation between genotype and phenotype (9). The same mutation can give rise to different phenotypes and the same phenotype can be caused by different mutations (30). This clinical diversity and poor genotype-phenotype correlation is exemplified by the range of manifestations seen with the common m.3243A>G-mutation (Table 1).

Table 1. A selection of the different phenotypes associated with the m.3243A>G mutation, and the frequency of these phenotypes. Frequency differs based on the different populations studied.

Clinical manifestations	**Frequency**	**References**
Myopathy	25% - 60%	(58-62)
Hearing impairment	48% - 77%	(54, 62-70)
Stroke-like episode	2.2% -17%	(69-71)
Cognitive decline/ Encephalopathy	50% / 21%	(69)/(71)
Epilepsy/seizures	5.4% - 25%	(54, 69-71)
Migraine	23%	(54)
Diabetes	38% - 52%	(68-70, 72)
Cardiomyopathies	20% - 88%	(73-75)
WPW/Arrhythmias	13% - 33%	(74-76)
Gastrointestinal symptoms	59% - 86%	(68, 70, 71)
Retinal abnormalities	86%	(77)
CPEO	6% - 45%	(54, 62)
Short stature	36% - 42%	(62, 69)

6.2.4 *Nuclear DNA linked mitochondrial disease*

Nuclear genes encode the vast majority of mitochondrial proteins, including most of the proteins of the respiratory chain and proteins involved in mtDNA homeostasis. Nuclear gene defects (nDNA) are inherited either as autosomal or X-linked traits. The first report of a nuclear gene mutation causing mitochondrial disease came in 1995 (78). The defect was in the gene of *SDH*, coding for a subunit of complex II and was found in two siblings with Leigh syndrome (78). Since then, the number of nuclear genes that can give rise to mitochondrial disease has grown enormously and to date, mutations involved in over 300 genes (79) have been described.

Mutations affecting nuclear genes involved in mtDNA homeostasis give rise to quantitative and/or qualitative alterations of mtDNA; quantitative loss, called mtDNA depletion, is where the number of mtDNA molecules is abnormally reduced, while qualitative alterations include point mutations and multiple deletions of mtDNA (2).

The inheritance pattern of nuclear encoded gene mutations can be autosomal recessive, dominant or X-linked and some genes can display both recessive and dominant patterns (80). It seems that recessive inheritance is associated with more severe phenotypes (9).

Table 2. Examples of nuclear DNA mutations classified according to the encoding genes' role in the mitochondria. Based on references (4, 12, 21, 81).

Nuclear DNA mutations	**Gene**
Respiratory chain subunits	*NDUFS1/2/3/7/8* *NDUFA2/6/12/8/13/11/9/10* *NDUFB10/11/3/8/9* *NDUFV1/2* *SDHA/B/C/D* *UQCRB/Q* *UQCRC2* *UQCRFS1* *CYC1* *COX4* *COX5A* *COX 6B1/7B* *ATP5F1A/D/E*
Ancillary proteins of the respiratory complexes	*NUBPL* *TIMMDC1* *ACAD9* *TMEM126B/126A*

	FOXRED1 *TMEM70* *SDHAF1/F2* *BCS1L* *LYRM7* *TTC19* *PET100* *PET117* *SCO1/2* *COA3/5/7/8* *TACO1* *SURF1* *COX10/14/15* *ATPAF2* *ICSU*
Mutations affecting mtDNA maintenance, transcription, translation or replication	*ANT1* *PEO1* *TWINKL (c10orf2)* *POLG* *TYMP*

	DGUOK *RRM2B* *SUCLA2* *SPG7* *DARS2* *PUS1* *DHX30*
Mutations affecting genes involved in the lipid milieu or matrix	*SERAC1* *TAZ* *AGK* *PITRM1*
Mutations affecting genes involved in mitochondrial dynamics	*OPA1* *MFN2* *KIF5A* *GDAP1*

Perhaps the most commonly affected nuclear gene causing mitochondrial disease is *POLG*: *POLG* encodes the catalytic subunit of polymerase gamma (POLG), the enzyme that replicates and repairs mtDNA, and mutations in this gene may account for up to 25% of patients with mitochondrial disease (82). Mutations in *POLG* can cause mtDNA depletion, multiple deletions and mtDNA point mutations (82). The

phenotype varies from mild to severe and include PEO, seizures, peripheral neuropathies, ataxia, myopathy and/or liver dysfunction (83). Early onset epileptic seizures and status epilepticus are associated with poor prognosis (84).

The *Twinkle* gene encodes a DNA helicase protein that unwinds the mtDNA replication fork and mutations leads to a similar range of phenotypes to POLG related disease with mtDNA depletion and multiple deletions being the genetic correlates. The phenotypes seen include PEO and early onset encephalopathy, but myopathy, neuropathy and cardiomyopathy may also be seen (21).

The *ISCU* gene codes for a protein that is important for iron-sulfur (FeS) clusters and thus important for the respiratory complexes I, II and III. The phenotype associated with *ISCU* mutation involves exercise intolerance and myopathy, lactate acidosis and episodic rhabdomyolysis (85).

RNA helicases are ATP-dependent enzymes involved in the RNA metabolism and subclassified in to superfamilies (86). The largest of these helicase families are the DEAD box proteins, of which DHX30 is a member (86). *DHX30* is highly expressed in the brain during neurogenesis (87) and an isoform of the protein has been found inside the mitochondria, in the vicinity of mtDNA nucleoids, suggesting a role in replication and/or transcription of mtDNA (88). The phenotype reported in patients with *DHX30* mutations have included delayed global development and intellectual disability (89).

PITRM1 encodes a protein in the mitochondrial matrix that digests the mitochondrial fraction of amyloid beta and patients identified with mutations in this gene have displayed progressive cognitive impairment, psychosis and spinocerebellar ataxia (90). In contrast to *POLG* and *Twinkle* mutations, *DHX30, ICSU* and *PITRM1* mutations are rare causes of mitochondrial disease, but are mentioned in this section as patients with these mutations are included in the study population that this thesis is based on.

6.3 Mitochondrial dysfunction in other neurological diseases

Since mitochondria are essential for all cells and tissues, many disease processes will necessarily affect these organelles, making it difficult to establish whether the mitochondrial dysfunction is primary, i.e., a specific part of a pathogenesis, or secondary to another process. If there is a link between the mitochondrial dysfunction and pathogenesis, however, future therapies that improve mitochondrial function could contribute to modifying the disease course (21).

6.3.1 *Neurodegenerative diseases*

Abnormalities in both mtDNA and the respiratory chain have been identified in several neurodegenerative diseases, particularly Parkinson's disease (PD) and Alzheimer's dementia (AD) (21). Neurodegenerative diseases are a heterogeneous group of disorders that are clinically and pathologically characterised by the death of subgroups of neurons in specific functional anatomical systems (91). Common for them all is an unknown aetiology, that age is a risk factor for developing the disease, they are progressive and that there are currently no cures (91). Among the commonest neurodegenerative disorders are PD, AD and amyotrophic lateral sclerosis (ALS) (91, 92).

Multiple sclerosis (MS) is considered to be an immune-mediated, central nervous system (CNS) inflammatory disease of unknown aetiology. Previously, the inflammatory changes and loss of myelin sheath were considered to be the cause of neuronal death and MS was therefore not defined as a neurodegenerative disorder (91). Studies suggest, however, that while inflammation appears to be the important component in relapsing-remitting multiple sclerosis, neurodegeneration is present at an early stage (93) suggesting a combined inflammatory and neurodegenerative pathogenesis of the disease.

6.3.2 *Mitochondrial impact on neurodegenerative diseases*

The link between mitochondrial dysfunction and PD has been the focus of studies for decades, and complex I deficiency especially has been associated with this disorder (94, 95). Respiratory chain deficient neurons have also been found in the hippocampal area in patients with AD (92), and in MS lesions (96, 97). A more recent link between mitochondria and neurodegeneration revolves around the mitochondria-associated membranes or "MAM", the regions of endoplasmic reticulum that are closely associated with mitochondria. These mitochondria-associated membranes are involved in multiple functions, including mitochondrial dynamics, apoptosis and calcium homeostasis (12) and these contact sites are damaged in PD, ALS and AD (98).

In addition to OXPHOS and MAM changes, further evidence of mitochondrial dysfunction in neurodegenerative disorders includes mtDNA mutations in PD, AD ALS (99) and MS (100) and mitochondrial swelling and accumulation in MS (101). Further, oxidative stress due to mitochondrial dysfunction has been implicated both in several different neurodegenerative disorders and in ageing (7).

There seems little doubt that mitochondrial dysfunction plays an important role in neurodegeneration, but whether this is a primary factor or, more likely, a secondary disturbance associated with other pathological mechanisms (92) remains unresolved. An argument against them being primary causes is the interesting fact that primary mitochondrial disorders seldom cause the typical phenotypes seen in ordinary neurodegenerative diseases (92).

6.4 Tissue and fluid biomarkers of mitochondrial dysfunction and mitochondrial disorders

A biological marker (biomarker) can be defined as an analytical tool to assess a biological parameter (102). The chosen tool must be objective and repeatable, and

may include a factor that helps to - identify a disease, stage a disease, classify the extent of disease, indicate the prognosis and/or monitor a clinical response to an intervention (102).

If we look at the available mitochondrial biomarkers there are those that do indicate the presence of mitochondrial dysfunction, but it is usually not possible to use these to differentiate between primary and secondary causes. Relevant biomarkers can be divided into those based on body fluid analysis and those based on muscle biopsy. Minimally invasive body fluid biomarkers include basal venous blood lactate and pyruvate, blood GDF-15 and FGF-21, resting CK, metabolomic studies (including serum amino acids (AA) and acyl-carnitine profiles and urine organic acids (OA))(103)). These investigations are useful for indicating the possibility of mitochondrial dysfunction, but give no precise diagnostic information. Muscle biopsy with specific staining of respiratory chain complex activities can demonstrate that a patient has mitochondrial disease and may even be used to suggest that the diseases is due to mtDNA involvement, but whether this was primary mtDNA or due to a defect in nuclear gene involved in mtDNA homeostasis would still require further genetic study. Perhaps the greatest challenge with biomarkers in primary mitochondrial disease, is however, the great clinical diversity and poor phenotype –genotype correlation.

6.4.1 *Biomarkers based on muscle biopsies*

A replicative selection against dividing cells that contain mutated mtDNA, makes the diagnosis of mtDNA disease challenging, since it means that the most easily available tissue, namely blood, may be unsuitable for identifying mtDNA mutations (104). In contrast, post-mitotic tissues such as muscle, usually harbour high levels of mutated mtDNA (105). Muscle biopsy has therefore long been considered as the gold standard for mitochondrial diagnosis (106, 107). The most common histopathological findings are subsarcolemmal accumulations of mitochondria and cytochrome c oxidase (COX) – deficient fibres (107). The first finding is often described by the term “ragged red fibre” which is due to the accumulation of a red dye in mitochondria when using the

Gomori trichrome stain (108). It reflects aggregates of abnormal mitochondria within type I muscle fibres (109, 110). Histochemical staining for SDH activity will show the same accumulation and is considered more specific as SDH is found exclusively in the mitochondria (3). In a normal muscle, stained histochemically for COX-activity, the difference in type I oxidative muscle fibres to type II glycolytic fibres will be easily visualised by the degree of coloured staining (3). COX-deficient fibres will not be stained, and a mosaic pattern of COX-activity is highly suggestive of an underlying heteroplasmic mtDNA disorder (3) while a global decrease in COX-activity is suggestive of a nuclear mutation (3). The finding of a COX mosaic is not, however, consistent as muscle biopsies from patients with the m.3243A>G mutation can show just muscle fibres with normal COX activity (111). As SDH is encoded entirely by the nuclear genome, and COX contains both nuclear and mtDNA encoded proteins, a combined staining of these two is particularly useful (32). With the combined COX/SDH stain, COX-deficient fibres will clearly stand out as intensely blue (SDH) stained (32).

The activity of OXPHOS enzymes can be assessed in fresh frozen muscle biopsies, including measurement of the individual complexes I-V, combined activities of complexes and functional studies of oxygen consumption and substrate oxidation (112). These studies are time-consuming and complex and for correct interpretation it is often necessary to ratio activities of the different complexes to a mitochondrial matrix enzyme such as citrate synthase activity and to ratio the complexes to each other e.g., complex I/II to expose abnormalities (112). Although muscle biopsy is considered one of the most important diagnostic investigations in mitochondrial disorders, it is worth mentioning that patients with mitochondrial disorders where muscle is not affected, or only mildly, can have normal muscle biopsies (113). Additionally, aging and other myopathies (such as inclusion body myositis) can have similar ragged-red fibres as found in mitochondrial disease and aging in itself can give rise to some COX-negative fibres (55, 114).

6.4.2 *MtDNA copy number*

MtDNA is present in multiple copies in each cell and the number appears dependent on degree of energy demanded e.g., heart, skeletal muscle and neurons have a high amount of mtDNA copies per cell while spleen and liver cells have low (~100 mtDNA copies per cell) (115). Based on this, mtDNA copy number has been investigated as a biomarker for mitochondrial dysfunction, not just in mitochondrial disease, but in a spectrum of diseases, including cancer, cardiovascular diseases and kidney disorders (115). In neurological disorders, mtDNA copy number has been investigated in brain tissue from different neurodegenerative diseases: e.g., in cerebellum from patients with AD and Creutzfeldt-Jakob disease (CJD), mtDNA copy number was found to be lower than controls (8). A study on tissue homogenate found mtDNA copy number lower in substantia nigra in PD patients, but not in the frontal cortex (116). Using mtDNA copy number as a biomarker in tissues is difficult. The choice of tissue investigated is important as is the copy number response to respiratory dysfunction. For example, single cells studies in dopaminergic nigral neurons (117) revealed that, as the level of mtDNA deletions increased with age, control individuals generated a compensatory increase of neuronal mtDNA copy number while PD patients did not, indicating a dysregulation of mtDNA homeostasis. Some studies, however, suggest that mtDNA copy number may have a predictive role in mitochondrial disease. For example, in LHON, where the presence of 100% mutant homoplasmy does not necessarily mean clinical penetrance and additional factors are considered necessary for clinical manifestation, it was shown that high mtDNA copy number in leukocytes appeared protective against the onset of visual impairment. Hence, in LHON mutations carriers, copy number may be a biomarker for predicting visual impairment (118). Further, mtDNA copy number in peripheral blood decreases with age and low levels have been associated with mortality in a series of chronic, often age-related, disorders, and in a general elderly population, associated with higher mortality, and poorer cognitive and physical performance (119).

6.4.3 *Heteroplasmy levels*

One of the characteristic findings associated with mtDNA mutations is the presence of heteroplasmy. Heteroplasmy is the percentage mutated mtDNA load relative to total mtDNA and in disorders due to mtDNA mutation, heteroplasmy levels are considered important. Variation in heteroplasmy has been particularly studied in patients with the m.3243A>G and the m.8344A>G mutation and studies have shown an association between level of mutant mtDNA and clinical presentation, thus giving heteroplasmy levels a value as predictors of disease progression (61, 120). Heteroplasmy levels in patients with mutated mtDNA are obtainable in most tissues, but the major challenge is the diversity of levels dependant on tissue of choice. To take an example: blood, saliva and urine are all tissues where heteroplasmy levels are readily available, but this level might not reflect levels in the untested tissue (or tissues) of interest such as kidney, brain, muscle and heart (68). Another factor is that heteroplasmy levels in some tissues declines with time (104). This particularly relates to cells such as white blood cells and is thought to reflect the selective advantage cells with low heteroplasmy have over those with high levels (104). Thus, in elderly patients the heteroplasmy levels in blood may not reflect the seriousness of their disease. Although the role of heteroplasmy level in phenotypic presentation appears important in some cases, there are exceptions. In LHON for example, even unaffected individuals can have homoplasmy of mutated mtDNA underlining the fact that heteroplasmy alone is a poor biomarker for disease burden (120).

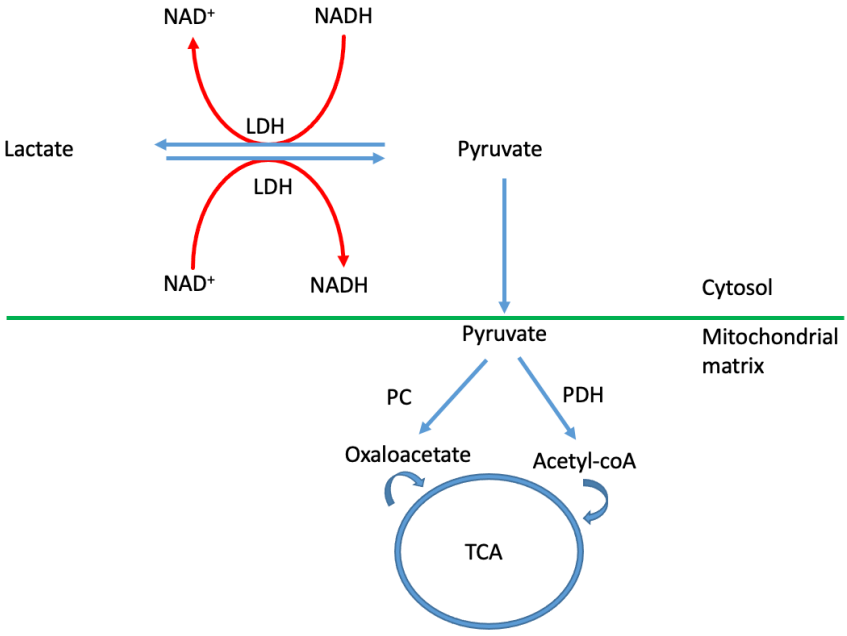
6.4.4 *Biomarkers in body fluids*

Although muscle biopsy provides one of the most valid biomarkers for mitochondrial dysfunction, obtaining tissue is invasive and, in children, it often requires general anaesthesia. More easily available biomarkers are therefore sought after, and we have focussed our work on detecting biomarkers in body fluids.

6.4.4.1 Lactate and pyruvate

Lactate is formed by reduction of pyruvate and the concurrent re-oxidation of NADH to NAD+. Lactate may be reconverted to pyruvate with the concurrent reduction of NAD+ to NADH. Both reactions are catalysed by lactate dehydrogenase. Pyruvate is produced in the cytosol by glycolysis and then imported into the mitochondrial matrix. Inside the mitochondria it is oxidised by pyruvate dehydrogenase (PDH) to form acetyl-CoA, which enters the tricarboxylic acid cycle (TCA) (2) (Figure 5).

Figure 5. A schematic figure over showing potential fates of lactate. The reactions are catalysed by lactate dehydrogenase (LDH), pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC). TCA is the tricarboxylic acid cycle.



Lactic acidosis can occur in the following conditions:

- Pyruvate accumulation.

Following transport into the mitochondrion, pyruvate can enter the TCA via conversion to acetyl-CoA by pyruvate dehydrogenase or be used in biosynthetic pathways by conversion to oxaloacetate by pyruvate carboxylase. Thus, pyruvate accumulation is due either to increased pyruvate production or decreased oxidation of pyruvate (PDH-deficiency or deficiencies in pyruvate carboxylase). In these deficiencies, an increase in blood pyruvate is a useful biomarker (106). An increased pyruvate concentration may also result in an increased lactate formation and thus lactic acidosis.

- Defects of the respiratory chain.

A respiratory chain defect usually leads to increasing failure to re-oxidise NADH and a change in the redox ratio NAD+/NADH. The conversion of pyruvate to lactate provides re-oxidised NAD+ and therefore a benefit for metabolism that is impaired by the change in redox potential. In such a setting, high levels of lactate will lead to elevated lactate/pyruvate (L/P) ratio, indicating respiratory chain disease. In contrast, in disorders that lead to pyruvate accumulation (above), lactate levels can be elevated, but the L/P ratio will either be normal or low. When lactate levels are high, this ratio is beneficial in differentiating respiratory chain deficiencies from pyruvate metabolism deficiencies, with a specificity of 100%. The sensitivity however is 31% (106).

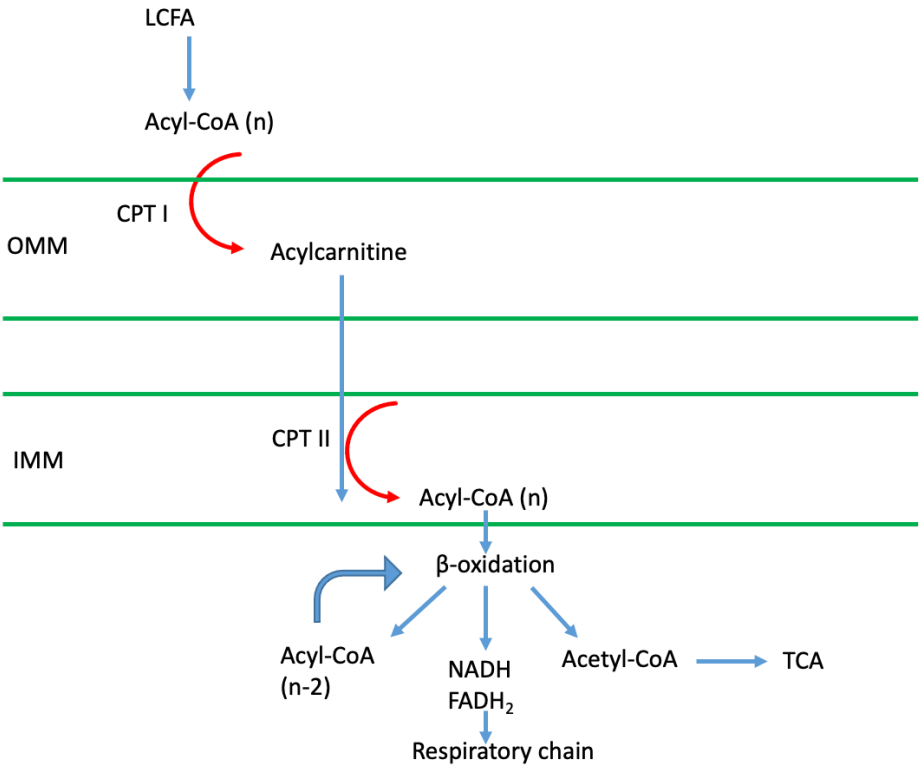
While lactate is a well-established biomarker for respiratory chain disease, the specificity and sensitivity for discriminating this from other causes are low (106). Persistent hyperlactatemia is more frequently present in children with mitochondrial disease than adults and this is also true for abnormalities of the L/P ratio. Elevated lactate is classically seen in patients with the m.3243A>G and those with mitochondrial myopathies (107). A normal resting lactate level with excessive rise following exercise (107) is also a feature of mitochondrial muscle disease. Lactate in

CSF may be raised in patients with neurological manifestation of mitochondrial disease (121), however the drawback is the need for lumbar puncture.

6.4.4.2 Fatty acids, acylcarnitine profile and plasma ketones

Fatty acid oxidation by ß-oxidation is an important source of energy. Beta oxidation takes place in the mitochondrial matrix requiring fatty acids to cross the mitochondrial inner membrane. Short- and medium-chain fatty acids enter the matrix directly where they are activated to acyl-coenzyme A (CoA) esters. Long chain fatty acids that are activated in the cytosol to acyl-CoA esters must be actively transported through the mitochondrial membrane (122) via the carnitine shuttle. The acyl-CoA is converted to an acylcarnitine by carnitine palmitoyl transferase I (CPT I) in the outer mitochondrial membrane, translocated and then reconverted to a acyl-CoA ester by CPT II in the inner mitochondrial membrane, before entering ß-oxidation (123). Each cycle ß-oxidation yields a two-carbon shortened acyl-CoA (that re-enters a new cycle of ß-oxidation), one NADH and $FADH_2$ that deliver electrons to the respiratory chain and one acetyl-CoA that enters the TCA (123) (Figure 6).

Figure 6. A schematic figure of fatty acid oxidation of long chain fatty acids (LCFA), Abbreviations: carnitine palmitoyl transferase I (CPT I), carnitine palmitoyl transferase II (CPT II), outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM).



Quantification of total and free carnitine levels and acylcarnitine esters in blood can identify a primary or secondary dysfunction of mitochondrial ß-oxidation (106). Accumulated intramitochondrial levels of acyl-CoA esters drive the CPT II in reverse

by converting the acyl-CoA´s to acylcarnitine, that is further transported out of the mitochondria and over into the bloodstream (124). Thus, patients with impaired ß-oxidation often have reduced levels of total carnitine (122), and high plasma levels of acylcarnitines.

Ketone bodies are formed in the liver from fatty acids and converted to acetyl-CoA when required as fuel (123). The ketone bodies - 3-hydroxybutyrate and acetoacetate - are maintained in equilibrium and the ratio of these reflects the NAD^+/NADH- ratio in the mitochondria (125). In the presence of a defective respiratory chain, there will be an elevation of NADH that will drive the equilibrium in the direction of acetoacetate, hence increasing the ratio (126). Changes in this ratio are, however, dependant on which of the complexes are affected, as this will determine the degree of NADH accumulation (126). In the event of a defect in the TCA cycle, post-prandial acetyl-CoA will be channelled to ketone body formation, resulting in an elevation of ketone bodies, termed paradoxical hyperketonaemia (19).

6.4.4.3 Urine organic acids

Organic acids are most easily obtained from urine. Intermediates of the TCA cycle (e.g., malate, fumarate, citrate, aconitate, isocitrate, oxoglutarate, succinate) and 3-methylglutaconic acid, which is involved in the catabolism of the amino acid leucine to give acetoacetate and acetyl-CoA, are readily detectable in urine (19, 127). Although, high levels of 3-methylglutaconic acid are associated with respiratory chain dysfunction, it is non-specific (127) and can be found in a variety of disorders, especially those involving leucine metabolism. Elevated levels of both malate and fumarate correlate with each other in mitochondrial patients, but apart from these, the other TCA intermediates correlate badly and are not sensitive biomarkers in discriminating patients with mitochondrial disease from others (128). Urinary lactate has not been found to be a good biomarker for mitochondrial patients (128).

When muscle degrades amino acids for energy needs, the resulting nitrogen is transaminated to pyruvate to form alanine. This is performed by the enzyme alanine

transaminase (ALT), which converts L-glutamate and pyruvate into α-ketoglutarate and L-alanine. Alanine may therefore be elevated in patients with pyruvate metabolism deficiencies, but is not necessarily helpful as a biomarker in patients with respiratory chain deficiencies (121).

6.4.4.4 Creatine kinase

The enzyme creatinine kinase (CK) is released from damaged muscle fibres (129). In the majority of patients with mitochondrial disorders and especially in patients with predominantly CNS involvement, serum CK is normal, or only mildly elevated (121, 130). This is also true for patients with mitochondrial myopathy such that, paradoxically, a normal CK in combination with myopathy maybe a clue to the presence of mitochondrial disease. This is in contrast to the inflammatory or dystrophic myopathies, where CK-levels often are significantly increased (1).

6.4.4.5 FGF-21 in serum

Fibroblast Growth Factor 21 (FGF-21) is a hormone-like cytokine that is secreted as a result of starvation and which leads to mobilisation of lipid stores and production of ketone bodies (131). It is involved in the intermediary metabolism of carbohydrates and lipids (132). In mitochondrial disorders, respiratory dysfunction leads to a state mimicking starvation and it is, therefore, thought that this state induces FGF-21 upregulation as a compensatory mechanism to the underlying metabolic deficiency (133). The use of FGF-21 as a biomarker in mitochondrial disease was stimulated by the discovery of FGF-21 upregulation in respiratory deficient muscle fibres, but not in other tissues, in a mouse model of mitochondrial myopathy (131). This model replicated the findings seen in patients with dominant Twinkle mutations who accumulated large-scale mtDNA deletions in skeletal muscle (129). The levels in mice decreased when mitochondrial function improved, and that, in combination with the possible association with disease severity launched FGF-21 a possible marker not only for diagnosis, but also for monitoring treatment response (129). The later finding

of increased FGF-21 RNA expression in muscle in mitochondrial patients, have underlined the assumption that the protein is secreted from muscle (134).

6.4.4.6 GDF-15 in serum

Growth and differentiation factor 15 (GDF-15) is a member of the transforming growth factor beta superfamily (135) and has a role in regulating cellular response to stress and inflammation (136). The expression of GDF-15 is influenced by cancer, non-steroid anti-inflammatory drugs, acute coronary syndrome and coronary ischemia, diabetes, antihypertensive treatment and smoking (137, 138).

GDF-15 was discovered as a potential biomarker for mitochondrial disorders in a transcriptomic study of skeletal muscle in patients with *thymidine kinase 2* mutations When compared with a control group, it was shown to be overexpressed in muscle and serum in the mitochondrial patients (137). Later studies, have identified correlations between GDF-15 and NMDAS score, heteroplasmy levels, disease severity and myocardial strain (139).

There is a positive correlation between FGF 21 and GDF-15 (140). The usefulness of these two biomarkers in identifying a mitochondrial aetiology has been replicated in several studies, and demonstrates that GDF15 and FGF21 are more robust biomarkers than the above mentioned traditional biomarkers (141).

6.4.4.7 MitoDAMPs in serum and CSF

Low levels of cell-free mtDNA are found in the CSF where it is assumed they reflect normal turnover of cells and thus mtDNA in the brain. Elevated levels of free mtDNA in CSF are also found in neurological inflammatory disorders such as anti-NMDAR encephalitis and neuromyelitis optica spectrum disorder (142, 143), and in traumatic brain injuries, indicating that mtDNA could be a mediator of a sterile, inflammatory response (144). It has been suggested that the abnormal levels of extracellular mtDNA may be involved in disease pathogenesis, potentially reflecting

the role of mtDNA as a DAMP in the innate immune system. In contrast to elevated levels suggestive of inflammation, the finding of decreased levels of cell-free mtDNA in patients with AD and PD has been suggested as a marker for this type of neurodegeneration (145-147). This appears somewhat contradictory, as it might be expected that neuronal damage should lead to elevated levels of cell-free mtDNA. One suggested explanation is that there are fewer neurons with lower mitochondrial content in these conditions, which results in lower-than-normal mtDNA levels. However, mtDNA in the CSF cannot simply reflect neuronal loss since the levels in CJD, in which massive loss occurs, are elevated (148). Evidently, the exact mechanisms that drive cell-free levels of mtDNA in CSF remain unclear, and cell-free mtDNA as a biomarker in neurological disorders has yet to be fully established. In addition, since lumbar puncture is an invasive procedure, the role of mtDNA in the CSF as a biomarker remains uncertain.

A recent study has investigated plasma levels of cell-free mtDNA in mitochondrial patients (149). This study included both mtDNA and nDNA linked mitochondrial disease and the only group with significantly higher cell-free mtDNA in plasma was those patients with MELAS (m.3243A>G). The study proposed that this could be related to inflammation in the CNS in these patients, in line with the hypothesis that mtDNA has a role in the innate immune system.

7 Aims of the thesis

The overall aim of this thesis was to evaluate new biomarkers and new ways of using known body fluid biomarkers in both primary mitochondrial disorders and neurological diseases in which mitochondria or mitochondrial dysfunction may play a role.

In **paper I,** we investigated the potential role of mtDNA in reflecting the neuroinflammatory/neurodegenerative process in relapsing-remitting multiple sclerosis by investigating the levels of cell-free mtDNA in the cerebrospinal fluid of patients and correlating this with disease state and/or disease activity.

Paper II and III investigated primary mitochondrial disorders.

In **paper II**, the aim was to investigate whether we could avoid muscle biopsy by detecting single mtDNA deletions easily and reproducibly in a more readily available body fluid, namely urine.

In **paper III,** the aim was to investigate the diagnostic utility of a combination of known mitochondrial biomarkers (FGF-21 and GDF-15) with an emerging biomarker of neurodegeneration (NF-L).

8 Materials and methods

8.1 Study populations and design

Paper I

A retrospective study of 21 patients with relapsing-remitting MS (RRMS) who had undergone diagnostic lumbar puncture at disease onset. CSF was also available from 23 age- and gender-matched controls. Controls were selected from patients who had undergone lumbar puncture for diagnostic purposes in whom inflammatory or neurodegenerative disorders were excluded. To be included the control patients had to have normal CSF white cell count, and normal MRI.

Paper II

A retrospective international study of 11 patients from three different centres (Norway, Finland, The Netherlands). All patients had a verified large-scale single deletion, with known deletion size, diagnosed from muscle biopsies.

The patient`s muscle DNA was studied in parallel with urine DNA, where available. In nine patients we had both urine and muscle samples, while in two, we only had the patient's urine sample.

Paper III

In this prospective study, 26 patients with a genetically confirmed mitochondrial disorder were successively recruited at the outpatient clinic, Department of Neurology, Haukeland University Hospital. An overview over the causative mutations is presented in Table 3.

Table 3

An overview over the different mutations in the study population in paper III. They included both nDNA and mtDNA mutations.

	MtDNA point mutations	n	Single deletions	Nuclear gene mutations	n
Genetic diagnose	*8344 A > G*	2		*POLG*	7
	3243 A > G	4		*TWINKL*	2
	13271 T > C	1		*PITRM1*	1
	5556 G > C	1		*DHX30*	1
				ICSU	1
Total		8	6		12

8.2 Source and handling of material investigated

Paper I

CSF was collected, centrifuged at 1,900 rpm at 4°C for 10 minutes, aliquoted and stored in polypropylene tubes at -80°C, until investigation.

Paper II

Urine samples were collected independent of time of day, and immediately centrifuged for 15 minutes at 2000 g, the supernatant discarded and the pellet retained. The pellet was stored at -20°C before use. A volume of 200 µl of ultra-pure water was added to the pellet, before DNA was extracted. Muscle biopsies were kept fresh-frozen at -80°C until use. For the samples sent from other centres, DNA was extracted from the tissue prior to shipping.

Paper III

Serum samples were taken following the outpatient review and stored at -80°C until analysis.

8.3 Sample preparation and DNA extraction (papers I and II)

In contrast to other studies, the use of native, untreated CSF in paper I gave no amplification irrespective of the amount used, suggesting the presence of PCR-inhibitors. We, therefore, initially tried filtering CSF using Amicon Ultra 0.5 ml, 30kDa, columns. This resulted in profound loss of mtDNA to levels below the confident detection range of our qPCR assay. To maximize DNA yield and at the same time minimize the presence of inhibitors, we therefore employed a DNA extraction protocol from 200µl CSF using the QIAamp® DNA Mini Kit (Qiagen GmbH).

The same kit was used for extracting muscle and urine in paper II.

The manufacturer`s tissue protocol was used for extracting DNA from muscle, and for the urine and CSF samples the manufacturer`s protocol for body fluids was used.

8.4 Long–range polymerase chain reaction (paper II)

Two long- range PCR reactions (L-PCR) were used to amplify mtDNA across the major arc, generating either an ~ 8 kb or a ~16 kb product in wild-type mtDNA. A control sample from a patient with a normal muscle biopsy was run together with study subjects.

The PCR products were run on a 0.7% agarose gel at 40 V for approximately 4 hours. The wells were loaded with 5µl PCR product and 1µl loading dye.

Thermal cycling 8 kb: 1 cycle: 92 °C for 2 minutes, 35 cycles: 92 °C for 10 sec, 57°C for 30 sec, 68 °C for 8 minutes and 68 °C for 7 minutes.

Thermal cycling 16 kb:1 cycle: 92 °C for 2 minutes, 10 cycles: 92 °C for 10 sec, 57°C for 15 sec, 68 °C for 16 minutes, 25 cycles: 92 °C for 10 sec, 57°C for 15 sec, 68 °C for 16 minutes (+ 20 sec elongation per cycle) and 68 °C for 7 minutes.

8.5 Quantitative polymerase chain reaction (papers I and II)

8.5.1 Determination of mtDNA copy number (paper I)

We amplified *MTND1* to quantify mtDNA and the nuclear encoded amyloid precursor protein (*APP*) to detect any nuclear DNA contamination. To ensure that we only measured cell-free mtDNA, samples showing detectable amplification of APP (CT-value < 37) were discarded (n=7). The amount of mtDNA was calculated using a standard curve derived from serial dilutions of a gel-purified human MTND1 amplicon. The serial dilutions contained 10^1-10^8 copies/µl.

8.5.2 Determination of deletion level (paper II)

We quantified the level of mtDNA deletion using quantitative PCR (qPCR). The mtDNA regions *ND1* and *ND4* were amplified together with the single-copy nuclear gene *APP*.

The reactions were run in triplicate with DNA template of ~10 ng/ul.

Thermal cycle: one cycle at 95º for 20 s, 45 cycles at 95º for 3 s and 60º for 30 s. The percentage of deletion was obtained with the ddCT method, using healthy mtDNA as calibrator.

8.6 Enzyme-linked immunosorbent assay (papers I and III)

Due to limited volume of CSF (paper I), determination of NF-L was performed on half of the samples (n=21; 10 patients and 11 controls). The concentration of NF-L in CSF was measured using the UmanDiagnostics AB, Sweden ELISA-kit, according to the manufacture`s protocol.

For paper III the concentration of NF-L was determined using the Simoa assay (UmanDiagnostics, Quanterix). FGF-21 and GDF-15 concentrations were measured in duplicate samples by ELISA according to the manufacturer´s protocol (BioVendor, Brno, Czech Republic).

8.7 DNA Sequencing (paper II)

To establish that DNA extracted from urine sediment cells could be used to define the deletion breakpoint, we chose two of the patients for Sanger sequencing. Standard

PCR reactions encompassing the deletion breakpoints were performed, and a total of 25µl with PCR-product was run on a 0.7% agarose gel. The band was extracted and PCR-product purified using the QIAquick Gel Extraction Kit (Qiagen).

PCR-products were sequenced with BigDye® Terminator v3.1 (ThermoFisher Scientific).

Next-generation sequencing was performed on the PCR product from a 16 kb L-PCR gel.

8.8 Statistical methods

Statistical analyses were performed in SPSS software (v.23.0.0.2) and Prism (v6; GraphPad). We used non-parametric tests since the data did not fit a normal distribution. A p-value of <0.05 was considered statistically significant.

The data was processed using SPSS v25 (IBM).

8.9 Ethical considerations

All three studies were approved by the Norwegian Regional Committee for Medical and Health Research Ethics: No: 2014/1371 (**paper I**), 2019/481 (**paper II)** and 2019/479 (**paper III**).

Written consent was obtained from all patients.

9 Summary of results

9.1 Paper I

Increased levels of cell-free mitochondrial DNA in the cerebrospinal fluid of patients with multiple sclerosis

Varhaug KN, Vedeler CA, Myhr KM, Aarseth JH, Tzoulis C and Bindoff LA.

The amount of cell-free mtDNA in the CSF of patients was significantly higher than controls (p=0.003, Mann-Whitney U-test): mean mtDNA concentration in patients was 188.8 copies/10µl (median 123.9, SD 205.9, range 32.7- 943.2) and 75.2 copies/10µl (median 56, SD 54.1, range 10.2- 217.7) in controls. There was no correlation between mtDNA concentration and age, sex, number of oligoclonal bands or CSF leukocyte count.

MS patient samples contained a significantly higher amount of NF-L in CSF compared to controls (p=0.005, Mann-Whitney U-test). The mean concentration in patients was 1097.9 pg/ml (SD 652.6, range 351.1-2465.1), and 525.9 pg/ml (SD 562.3, range 127.1-2116) in controls. There was no correlation between mtDNA concentration and NF-L concentration.

There was a statistically significant inverse correlation between mtDNA concentration and time interval since disease onset (p=0.007, r = - 0.57 Spearman`s correlation)

9.2 Paper II

Using urine to diagnose large-scale mitochondrial DNA deletions in adult patients.

Kristin N. Varhaug, Gonzalo S. Nido, Irenaeus de Coo, Pirjo Isohanni, Anu Suomalainen, Charalampos Tzoulis, Per Knappskog and Laurence A. Bindoff

We detected a single deletion in urine samples from 9 of 11 patients (82%), either in the 8 kb L-PCR (n= 6) and/or 16 kb L-PCR. In the remaining two patients, the band was either very weak or undetectable. In three patients the deletion was only detectable on the 16 kb gel. We therefore employed the "walking-PCR" technique that shifts primers to encompass different and larger areas until the primer pair encompasses the deletion. Using this technique, we identified large deletions, each over 7 kb, in all three remaining cases confirming our suspicion that these deletions had removed one of the 8 kb PCR primer sites.

Mean heteroplasmy level in urine was 38% ± 26 (range 8 - 84%), and 57% ± 28 (range 12 – 94%) in muscle. The level was generally lower in urinary sediment cells, but we were able to define a correlation between muscle and urine levels within our cohort (R=0.714, p=0.031(Pearson correlation)).

Sanger sequencing was performed on two patients; both had the common 4,977 bp deletion.

All deletions detected using L-PCR in both muscle and urine were also detected in both muscle and urine using NGS. However, we found that NGS generated evidence of additional deletions of varying read depth that were not found by L-PCR.

9.3 Paper III

Serum biomarkers in primary mitochondrial disorders

Kristin N. Varhaug, Omar Hikmat, Hanne Linda Nakkestad, Christian A. Vedeler, Laurence A. Bindoff

The overall mean concentration of serum NF-L was 25.70 pg/ml (SD 23.4 pg/ml). When subclassifying by genetic diagnosis, mean NF-L concentration was 43.89 pg/ml (SD 32.4 pg/ml) in patients with mtDNA point mutations, 11.70 pg/ml (SD 5.6 pg/ml) in mtDNA single deletions, and 21.39 pg/ml (SD 17.6 pg/ml) in patients with nuclear gene mutations. The difference in NF-L levels between mtDNA point mutations and single deletions was significant (p=0.024). There was a trend towards a significant difference between NF-L levels in patients with mtDNA point mutations and nuclear mutations (p=0.0585).

NF-L could differentiate between patients with mtDNA point mutations and the two other patient groups with an Area Under the Curve (AUC) 0.806. When comparing patients with point mutations to single deletions only, the AUC was 0.905.

We also looked at those patients with epilepsy, regardless of the genetic aetiology, and compared these to the remaining patients. The mean NF-L level in the epilepsy group (5 patients) was 49.74 pg/ml versus 19.7 pg/ml in the non-epilepsy group (21 patients) (p=0.015).

The group mean concentration of FGF-21 was 679.77 pg/ml (SD 630.07 pg/ml) and GDF-15 2802.43 (SD 2628.04 pg/ml). In patients with mtDNA point mutations, mean FGF-21 concentration was 735.10 pg/ml (SD 430.2 pg/ml), single deletions 1172.53 pg/ml (range SD 927.8 pg/ml) and nuclear mutations 401.1 pg/ml (SD 393.5 pg/ml). There were no significant differences between FGF-21 levels and the different underlying genetic groups (p=0.052). The subgroup means for GDF-15 concentration in mtDNA point mutations were 3027.27 pg/ml (SD 1278.0 pg/ml),

5202.59 pg/ml (SD 4281.3 pg/ml) single deletions, and 1420.54 pg/ml (SD 1087.7 pg/ml) nuclear mutations. The difference in GDF-15 levels between nuclear mutations and point mutations and single deletions were significant ($p = 0.024$ and $p = 0.006$ respectively).

There was a significant correlation between FGF-21 and GDF-15 (r=0.671, $p<0.001$, Spearman's correlation). There was no correlation between FGF-21 or GDF-15 and NF-L. However, NF-L showed a reciprocal trend to FGF-21 and GDF-15 when the patients were divided in clinical groups, from pure myopathic to multisystem disease with active cerebral damage. The difference in NF-L between these groups was significant ($p=0.012$).

10 Discussion

The overall aim of the work in this thesis was to evaluate known and new biomarkers in diseases due to primary or secondary mitochondrial dysfunction. From a neurological perspective, secondary mitochondrial dysfunction is closely related to neurodegeneration. While traditional neurodegenerative disorders (PD/ALS/AD/MS) are often easier to diagnose clinically than primary mitochondrial diseases, they share in common a lack of reliable biomarkers that can indicate prognosis or be useful in a clinical follow-up setting to predict exacerbation or measure treatment response. In primary mitochondrial disease, the challenge starts already at the time of diagnosis.

Due to the genetic and phenotypic diversity, establishing the underlying diagnosis can be difficult. Although one can make the diagnosis of mitochondrial disease without knowing the final genetic cause, this knowledge is of importance for several reasons: firstly, for genetic counselling, particularly as Mendelian and mtDNA inheritance are quite different and have different implications e.g., depending on the patient's gender; secondly, knowledge of the causative mutation gives prognostic information, e.g., the risk of developing cardiac complications, epilepsy or diabetes mellitus etc. Knowledge of the mutation, is therefore, highly desirable for any personalised medicine approach.

Another problem associated with the diagnosis of mitochondrial disease is the traditional need for invasive procedures such as muscle biopsy. MtDNA point mutations and deletions can be found in blood, although not always, but levels decline over time, due to a natural selection against mtDNA mutations in rapidly dividing cells (104). This makes blood less appropriate for use in diagnosis unless there is a strong suspicion of one specific mtDNA mutation, or a family history. In contrast, in the case of diseases caused by nuclear gene mutations, blood sampling and sequencing of candidate genes is sufficient.

In light of these problems, one of our aims was to identify biomarkers that would help point the clinician in the right direction, and hence spare the patients for unnecessary invasive procedures.

10.1 The investigation of cell-free mtDNA in cerebrospinal fluid

MS is an intriguing disease as it has both active neuroinflammatory and neurodegenerative components in its pathogenesis. The clinical course is heterogeneous and there are no available biomarkers that predict the rate of development from attack based to progressive disease, the risk of new lesions or the prospect of successful treatment response.

Originally, MS was thought of as an "outside-in disease", an immune-driven process in which systemic autoreactive immune cells invaded the CNS and led to secondary neurodegeneration. A newer theory, the "inside-out"- hypothesis, suggests that the inflammatory process is secondary to a primary pathological process in the cells of the CNS, or in the myelin-axon interaction (101). Elevated levels of cell-free mtDNA have been found in various neuroinflammatory conditions, such as encephalitis, neuromyelitis optica spectrum disease and traumatic brain injury (142-144). In contrast, decreased levels of cell-free mtDNA were found in the CSF of patients with AD and PD and this has led to the suggestion lowered mtDNA could be a marker for neurodegeneration (145-147).

In constructing our study, we hypothesised that cell-free levels of mtDNA could reflect the presence, and potentially degree, of inflammation. We recognised that, in a disease process such as MS with both inflammatory and degenerative elements, one process could cancel out the other, so we chose to investigate patients with relapsing-remitting MS early in the disease course. CSF samples were taken at time of diagnosis and before the initiation of anti-inflammatory treatment. Our population was therefore one with an expected high degree of neuroinflammation. We found that cell-free mtDNA is elevated in patients with newly diagnosed RRMS, and that this level is inversely correlated to disease duration, suggesting that this indeed reflect the ongoing inflammation that is maximal at onset.

The presence of mtDNA in the CSF could even reflect its role as a DAMP. This would be compatible with any initial process of damage and the subsequent release of cellular contents. Another explanation may be that mtDNA is actively released in

response to some stimulus, similar to the release of mtDNA by eosinophils in response to bacterial infection (150). Viewing mtDNA as a DAMP in multiple sclerosis provides us with a potential mechanism to explain the "inside-out theory" namely that inflammation is secondary to a primary intrinsic process within the neuron or other cell type such as oligodendrocytes. This process could either be due to mitochondrial dysfunction, and thus a cytodegenerative condition, or due to alterations in the myelin-axon interaction (151). The latter could then lead to either dysfunctional mitochondria that release mtDNA during breakdown, or functional mitochondria that release mtDNA actively as a response.

The finding of low levels of CSF mtDNA in AD and PD (145, 146) is interesting and may reflect what happens in specific forms of neurodegeneration. It is also possible that we are only seeing what is happening later in the disease course and levels are higher in the very early stages. The possibility that cell-free mtDNA reflects an initial phase of inflammation that later converts to a more neurodegenerative process raises the question of whether we can use mtDNA levels to follow this conversion in MS. Support for this hypothesis comes from a study, published after ours, showing that cell-free mtDNA levels were lower in post-mortem ventricular-CSF of patients with progressive MS compared with controls (152). Another possibility is that cell-free mtDNA might help us identify which therapy was appropriate for patients at different disease stage. Given the cost and possible side-effects of the newer immunosuppressive drugs, knowing whether cell-free mtDNA could predict the degree of inflammation in e.g., RRMS patients, could potentially allow immunosuppressive treatment to be personalised, saving those who have converted to a more degenerative phase from the morbidity associated with such treatments.

The need for CSF makes the investigation of cell-free mtDNA too invasive and complicated to recommend it as a routine biomarker. The possibility of studying cell-free mtDNA in blood is a much more intriguing prospect.
Recently, plasma levels of cell-free mtDNA were studied in patients with mitochondrial disease (149). Levels were significantly elevated in MELAS patients, and increased in relation to stroke-like episodes and/or status epilepticus. Not only do

these findings raise the possibility of using cell-free mtDNA as a biomarker, but they also raise the question of whether there is an inflammatory component to the process and, therefore, perhaps the possibility of a response to steroids (149).

To identify the amount of cell-free mtDNA in a fluid, one must first extract the DNA, and secondly measure the amount. Different studies use different extraction methods and different measurement methods and this lack of standardisation is a problem when comparing studies as the levels of mtDNA concentrations may vary rather significantly. In our study, the levels of mtDNA were low meaning that we had to report them in copies per 10µl volume and not copies per microlitre sample volume as others have done. Due to the low mtDNA copy number in CSF, qPCR reproducibility was also a challenge. This was particularly true when using crude CSF, but occurred also after filtration with amicon filters. In our hands, DNA extraction provided the only consistent results and while this raises the question of mtDNA released by cell lysis, we controlled for this by running a simultaneous amplification for a nuclear gene *APP*: the absence of *APP* in the same sample argues strongly against the mtDNA being released from whole cells.

Many of the current cell-free mtDNA studies are performed on blood samples, and high levels are found in healthy controls in serum and plasma, indicating a difference from CSF (153). It is possible that the yield in CSF might be improved by decreasing the volume the DNA is recovered from, using a different kit or altering the conditions for incubation with proteinase K (154). Some kits are optimalised for isolation of larger DNA fragments over 50 kb and may therefore be inadequate in isolating small mtDNA fragments (115).

The majority of studies on cell-free mtDNA are now done with digital-PCR. This was not available in our laboratory when this work was performed, but the digital droplet qPCR techniques appears both robust and accurate and any future study on cell-free mtDNA should consider this new digital tool.

10.2 The investigation of single deletions in urine

MtDNA rearrangements (single deletions/multiple deletions/duplications) can be detected by long-range polymerase chain reaction (PCR) protocols (155). Classically, skeletal muscle has been the tissue of choice for diagnosing single mtDNA deletions (3) although there are a small number of studies using urinary cells (156, 157). These studies showed that it was possible to detect rearranged mtDNA in urinary sediment cells, but suggested that deletion levels in urine were more representative of mutation levels in muscle in younger, not older patients, raising the question of whether urinary cells were an appropriate diagnostic source in adults (157). Although our numbers were not large, we identified the deletion in urine in over 80% of the adult patients we studied and, furthermore, we could use this material to identify the exact deletion size and break-points. This suggests that urinary cells are an adequate source of mtDNA in the majority of cases although confirmation of this will require a large cohort and the evaluation of more sensitive techniques, which are discussed below.

The presence of deleted mtDNA in urinary tract cells is something of an enigma. Rearrangements are not usually found in blood using standard techniques, except for patients with Pearson's syndrome and large duplications, and then only early in the disease not after conversion to KSS. Whether the increased sensitivity of next generation techniques will change this and allow us to identify rearrangements in other tissues such as blood, remains to be seen. Why cells of the urinary tract retain relatively high levels of mtDNA deletions, while others (with the exception of muscle) do not, remains, however, unresolved.

Heteroplasmy is considered a prognostic biomarker particularly in some mtDNA point mutations such as the m.3243A>G mutation. Studies have shown contradictory findings for single deletions. Some studies found no correlation between heteroplasmy and deletion size/site or disease severity or phenotype (15, 158, 159), while others did show correlation between phenotype and deletion size and site (160, 161). One study suggested that age of onset was the most important predictive factor (159). More recently, Grady and colleagues reanalysed previously published negative

data and found that there was indeed a predictive value of heteroplasmy, size and site of deletion on disease burden and progression (162).

Change in the level of heteroplasmy over time is clearly a complicating factor. In the case of point mutations, cells that retain the capacity to divide appear to lose the mutation over time. For example, studies on the m.3243A>G mutation in blood show an average annual decrease of 1-2 % in mutational load (14, 163). One hypothesis for this age-dependant decrease is “survival of the fittest” (164), i.e., cells with the lowest heteroplasmy, and therefore best energy metabolism, out-compete those with higher levels. Whether this selection pressure also affects urinary cells is unclear. Perhaps these cells are less energy demanding and thus less constrained (164). Whatever the explanation, we show that these cells retain adequate levels of mtDNA deletion to allow detection using standard methods of analysis.

Similar to previous studies (157), we found interpreting the level of heteroplasmy in urinary cells compared to skeletal muscle, problematical. Four urine samples were taken at a later time point than the muscle biopsy, which could affect the degree of heteroplasmy (21), although this is hardly the only explanation for differences between muscle and urine. As reported by Grady et al (163), urine appears to have a high *intra-individual* variability when comparing heteroplasmy in different tissues in patients carrying the m.3243A>G mutation, and this might be the case in single deletions too. The same study implied that mitochondrial copy number also affected tissue dependent disease expression in patients with m.3243A>G. We did not investigate copy number prior to publication of paper II, but did so after. It is known that mtDNA copy number declines with normal ageing in both muscle (165) and blood (119). In accordance with this, we found a significant inverse correlation between age and mtDNA copy number in muscle (Spearmans rho = -0.778, p=0.014). Intriguingly, there was no correlation between mtDNA copy number and age in urine, suggesting that the same age-related decline does not occur. Gender also appears to influence copy number and heteroplasmy in patients with the m.3243A>G mtDNA point mutation: mutation level and mtDNA copy number were highest in males. Male patients also had higher urine heteroplasmy levels meaning this must be adjusted for

sex (163). Only two males were included in our study population and they did not have the highest levels of urine heteroplasmy, but did have the highest mtDNA copy number, reflecting what is found in point mutations (163).

Long PCR is a robust technique for identifying single deletions and produces a product that can be sequenced to identify exactly the break-point. Interestingly, this technique failed to detect deletions in urine from two of our patients. The only common feature shared by these was deletion size, they had the smallest deletions in our cohort. We have no clear explanation as to why L-PCR failed in these two patients since small deletions should be easier to detect than large ones. Given that we already knew deletion size investigating muscle, we could have applied shorter amplicons or shorter elongation times, but the aim of our study was to evaluate the efficacy of L-PCR in a general clinical setting, thus we did not use prior knowledge of deletion size to alter our approach.

Next generation sequencing techniques have greater reported sensitivity than standard PCR based techniques. We therefore compared long PCR and NGS by sequencing the same 16kb PCR product produced by the L-PCR deletion analysis and found that NGS generated evidence of additional deletions of varying read depth not seen on L-PCR. This is possibly related to the initial PCR amplification and may disappear if native DNA is used, although in the case of urine, the amount of DNA available may restrict use of this tissue. Whether there are small fragments of mtDNA in urine, as are seen in human plasma (166), is also a possible explanation: low level heteroplasmy of single base changes appears common (8) and perhaps this is also true for deletions. Since investigating how robust NGS was for detecting single deletion was not the focus of our studies, clarification of these questions will have to wait.

10.3 The investigation of FGF-21, GDF-15 and NF-L in serum

Neurofilaments are cytoskeletal components of neurons that are particularly abundant in axons. Their functions include provision of structural support and maintaining size,

shape and calibre of the axons (167). Following axonal damage in the CNS, neurofilament proteins released into CSF provide an indication of the extent of axonal damage and neuronal death. The neurofilament subtype most extensively studied in this context is neurofilament light chain (NF-L). Increased NF-L levels appear to reflect ongoing neuronal damage, irrespective of the underlying pathology making it a potentially interesting biomarker for assessing neuronal damage (168).

In order to use NF-L as a biomarker, we first established what level we could use as cut-off. Data from the healthy controls used in several studies are summarised in Table 4. From the data this table is based on, it appears that a serum NF-L value of 16-20 pg/ml is normal in healthy individuals. It has been shown that NF-L increases with age and this must be taken into account (168)

Table 4: Displays normal serum NF-L levels in a heterogeneous group of healthy controls. The table is modified from Varhaug et al, 2020 (169).

Abbreviations: HC, healthy controls; ALS, amyotrophic lateral sclerosis; AD, Alzheimer's disease; MCI, mild cognitive impairment; FTD, frontotemporal dementia; PN, peripheral neuropathies; PSP, progressive supranuclear palsy.

Examined disorder	**HC (n)**	**HC mean age**	**HC serum NF-L (pg/ml)**	**Reference**
ALS	50	55	16.2	(170)
ALS	12	47	17	(171)
AD	12	86	29	(172)
FTD	28	65	19.6	(173)
MS	22	32	11	(174)
MS	42	28	10.5	(175)
PN	25	NA	6.91	(176)
PSP	12	70	17.5	(177)
Traumatic brain injury	35	31	13	(178)
Concussion	142	NA	8.47	(179)

Elevated levels of NF-L have been found in several neurodegenerative diseases, including Alzheimer`s disease (180), frontotemporal dementia (181) and motor

neuron disease (182, 183). Neurodegeneration as part of MS pathogenesis is well recognised, and there are multiple studies of NF-L in MS (168, 184, 185).

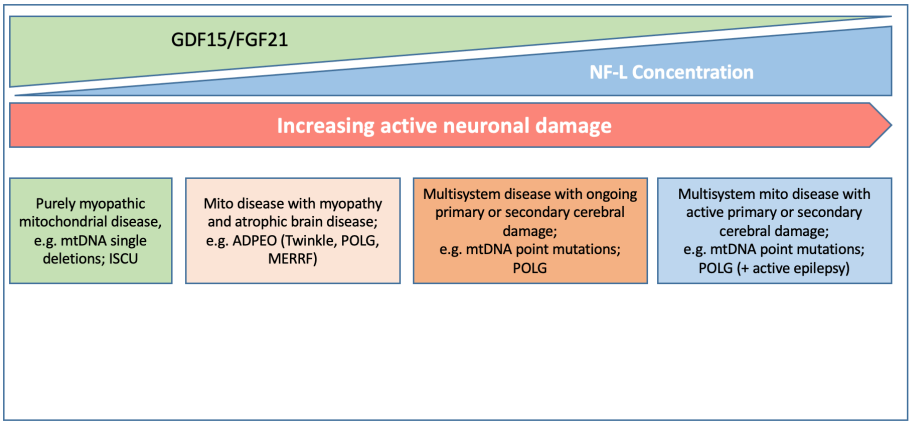
Although we found that CSF NF-L levels in RRMS were higher than controls, we found no correlation between NF-L and cell-free mtDNA levels. This could support the notion that the disease is indeed primarily a neurodegenerative process that subsequently initiates a secondary inflammatory process. The lack of correlation between these two biomarkers may reflect that the degree of secondary inflammation in patients is heterogeneous. Another explanation for this lack of association may be that NF-L determination was only performed on half of the samples. Thus, larger studies may provide additional information.

Since NF-L appears to be a general marker in neurodegenerative disorders we investigated its use in primary mitochondrial disease, alongside the more established biomarkers FGF-21 and GDF-15. The overall mean NF-L concentration in patients with mitochondrial disease was 25.70 pg/ml, which based on the cut-off we established, must be considered pathological (169) (Table 4). CNS involvement occurs in many different disease processes and an elevated NF-L will not discriminate these. Nevertheless, in a patient with suspected mitochondrial disease and little clinical evidence of cerebral involvement, an elevated level of NF-L would certainly stimulate further investigation of cerebral involvement. In those patients with known CNS involvement, NF-L could provide a way of following progression. When we classified our study cohort according to the presence and type of CNS involvement, we found that patients with multisystemic disease and clear, established involvement of the CNS, particularly those associated with point mutations such as the m.3243A>G-mutation, had the highest levels of NF-L. In contrast, in patients with single deletions, and a pure (or almost pure) myopathic phenotype, NF-L levels were normal (Figure 7).

Intriguingly, NF-L showed a reciprocal trend to FGF-21 and GDF-15. Both FGF-21 and GDF-15 were significantly elevated in patients with single mtDNA deletion, and highest in those with a significant degree of muscle involvement (Figure 7).

Correlation between these two biomarkers was also good, confirming the results of earlier studies (186). Thus, FGF-21 and GDF-15 appear useful biomarkers for mitochondrial disease in which myopathy is the major or only manifestation (186, 187), but appear less specific when muscle is not involved. In those with CNS involvement NF-L is much more sensitive and useful.

Figure 7. The figure is modified from paper III (188) showing the value of combining biomarkers. NF-L reflect the degree of cerebral damage. In cases where this is mostly slow attrition the levels of NF-L rise but less than when the damage is active. GDF-15 and FGF-21 show the reverse trend with highest levels in those patients with only or predominant muscle involvement.

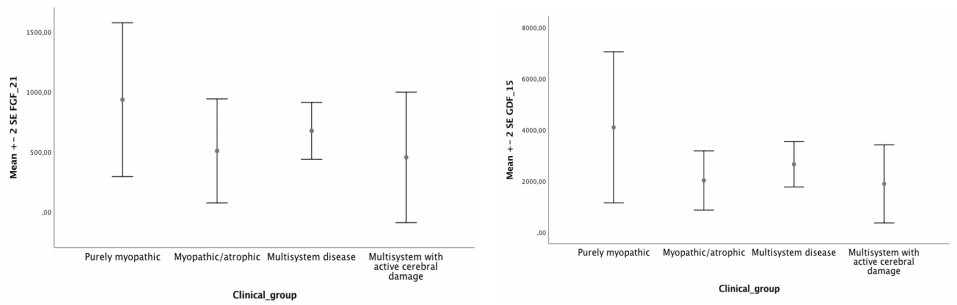


We know from earlier studies of patients with *POLG* mutations, that elevated CSF-protein is associated with worsened disease severity and with the presence of epilepsy (189). A poorer prognosis is also associated with the presence of anaemia at time of diagnosis of POLG (190). In our current studies, POLG patients with ataxia and no

epilepsy had lower NF-L levels than those who had already developed epilepsy. Since patients with POLG related disease and MELAS are at risk of developing acute lesions associated with life threatening seizures known as stroke-like episodes, being able to predict who is at risk would be an important step in biomarker development. From our studies in MS, we know that an increase in serum NF-L corresponded to presence of new radiological lesions (184). If we extend this logic to mitochondrial disease and patients at risk of stroke-like episodes, it may be possible to predict onset using NF-L levels. If this were true, these patients should have their NF-L values measured regularly: an acute increase then would trigger intervention including EEG and MRI, and the consideration of preventive treatment with anti-epileptic drugs.

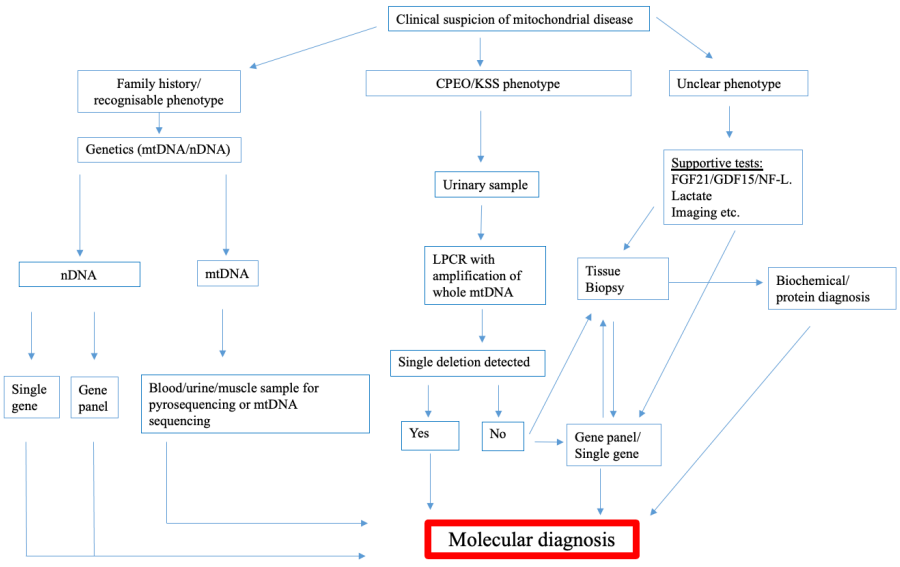
It has been suggested that GDF-15 is more sensitive than FGF-21 and not limited only to identifying those with myopathy. We found no evidence that GDF-15 was more "brain-sensitive" than FGF-21, and as shown in Figure 8, levels of GDF-15 and FGF-21 showed a similar pattern. Thus, while GDF-15 might, under certain conditions be a broader biomarker, our study suggests that it remains essentially a mitochondrial myopathy marker.

Figure 8. This figure is not included in our original manuscript, but shows the similarity in pattern between FGF-21 and GDF-15.



The results of our study suggested that NF-L, FGF-21 and GDF-15 may complement each other as diagnostic tools. Together with other biomarkers such as cell-free mtDNA (149), mtDNA copy number and heteroplasmy (163), CSF protein (189) and hemoglobin levels (190). We believe that it is increasingly possible to construct diagnostic, prognostic and treatment response algorithms to facilitate the clinical evaluation of mitochondrial patients. For example, based on our findings in paper II and paper III, we suggest that the following algorithm (Figure 9) could be useful when clinical suspicion of mitochondrial disorder is raised.

Figure 9. Algorithm for diagnosis of mitochondrial disease. The choice of diagnostic approach will differ dependant of known family history, classical phenotype (e.g., CPEO) or if the phenotype is less characteristic of a certain disease. Due to the heterogeneity and complexity of diagnosing mitochondrial disorders there is often a necessity to undergo several diagnostic steps and overlapping approaches. If genetics is negative there is often a need to return to supportive tests or tissue biopsy.



10.4 Methodological considerations

10.4.1 Study population and design (all)

Paper I

The McDonald criteria for diagnosing MS have been revised since our study of RRMS patients (191). The main differences relate to additional criteria to move patients with the clinical isolated syndrome in to the MS group. Our patients were strictly RRMS patients at time of inclusion, and therefore the revision would not have influenced the study cohort.

Paper II

We included 11 patients in this study. As it would take considerable time to gather such a sample in a prospective study, we decided on a retrospective study including only molecular verified patients with single deletions. Hence, we did not have available urinary sample form all the patients at time of diagnose. The time from which diagnosis was made in muscle to the time a urinary sample was obtained varied from 0 -18 years. This has two possible drawbacks. The time lag could potentially affect the heteroplasmy level in urine. In addition, by relying solely on detecting the deleted mtDNA, structural and pathological information provided by a muscle biopsy could be missed. In our case, we knew already the underlying diagnosis. Where the mitochondrial disease is due to a novel mutation, information obtained from a biopsy, can be essential for focussing further investigation and for establishing the diagnosis (192).

Paper III

While 26 patients is a respectable number for diseases that occur infrequently, we will still need to look at a larger population. The use of a control group in the study reported in paper III is open to discussion. The methods used had clear normal threshold values, and we thought when planning the studies, and believe still, that it is the intra-individual variation of these biomarkers that is of greatest interest.

10.4.2 Laboratory methods (all)

Determination of mtDNA copy number (paper I)

As discussed above we experienced lower levels of mtDNA copy number than reported in other studies. In addition, we were not able to use the CSF crude, but had to extract the DNA in advance to the qPCR. We believe that this obstacle will be resolved when using new digital methods.

Next-generation sequencing (paper II)

We used the sequenced PCR products when sequencing the urinary samples. As NGS is an ultra-sensitive investigation, the additional deletions detected in our material may disappear if native DNA is used.

Enzyme-linked immunosorbent assays (paper III)

Threshold values for GDF-15 depend on the kit used. We used the BioVendor kit, where the threshold has been set to 2330 pg/ml (193). For FGF-21 the threshold value is 350.0 pg/ml in mitochondrial disorders (140).

In our subgroup of patients with nuclear mutations, the mean GDF-15 level was lower than threshold. This was surprising, particularly as GDF-15 is considered a more sensitive marker for mitochondrial disorders in general, and not just those with muscle involvement (193). We believe this finding reflects the patient cohort and the fact that myopathy was not a major feature in those included. Support for this comes from the observation that FGF-21 levels were also lower than expected in this group. FGF-21 levels were similar to those reported earlier in ataxic POLG patients (134).

10.4.3 Statistical methods (all)

Small sample size restricted the choice of the statistical methods and this applies to all three papers.

In paper I, we saw high inter-individual variability in levels of cell-free mtDNA as has been shown in a study of AD that did not manage to prove any differences from controls (194). There is a risk that small studies will show significant differences due to high inter-individual variance, and that large studies are therefore necessary to overcome this effect (194). We appreciate that our sample size is small and that this risk must be considered, however, based on our median and mean values, we feel that the differences we show between patients and the control group are real.

Correlations observed in paper II must be interpreted with caution, as they are based on nine patients only.

11 Main conclusions

Our results show that mtDNA can be a marker for disease activity in early stages of MS and support previous studies suggesting that mtDNA plays a role in the innate immune system. Cell-free mtDNA remains a robust feature that could, along with NF-L, be used to follow disease activity (and potentially treatment response) in MS. Our results also support the suggestion that mitochondria play a role in the inflammatory process in MS. This is an important finding since greater understanding of the pathogenesis of MS should, in the longer term, provide us with more therapeutic options to address.

Urinary sediment cells are clearly a viable alternative to muscle biopsy for the diagnosis of single mtDNA deletion disorder, even though we may miss the structural and pathological information provided by a muscle biopsy. This conclusion may change as whole genome techniques become standard, but for now, we suggest that urine provides a significantly less invasive method for diagnosing single deletion disorder.

We have also shown that NF-L has a role as a biomarker in mitochondrial disorders, as it does in many other neurodegenerative disorders. NF-L appears useful in diagnosing mitochondrial disease with involvement of the CNS particularly in cases where this has not been clinically obvious. In addition, we believe NF-L will be a valuable tool in the follow-up of patients at risk of exacerbations with epileptic seizures or stroke-like episodes.

New biomarkers and particularly combinations of biomarkers provide new insights in to the pathogenesis of disease. Whether it is cell-free mtDNA and NF-L in the CSF in MS, deletions and heteroplasmy levels in urine or the combination of FGF-21, GDF-15 and NF-L, our studies show that these biomarkers can be used both as diagnostic tools and in clinical follow-up. We believe that they will play an important role in future natural history studies and drug trials.

12 Clinical implications and future aims

Based on our studies, we plan to use urinary sediment cells as tissue of first choice for the diagnosis of single mtDNA deletions in patients with suspected CPEO and KSS/Pearson syndrome. We will use skeletal muscle only in cases where findings in urine are insufficiently clear or the phenotype less classical. We will continue studying urine for heteroplasmy levels and also mtDNA copy number and correlate this with clinical parameters, e.g., NMDAS. We are already exploring the possibility of using next-generation sequencing in mtDNA rearrangements.

Before implementing the use of NF-L as a serum biomarker in mitochondrial patients, larger and prospective studies are needed. We hope to initiate a multicentre study that will provide greater numbers of patients in mutational and clinical subgroups. We will also correlate them to other biomarkers, such as plasma levels of cell-free mtDNA.

Since mitochondrial dysfunction plays a role in many disorders, particularly age-related neurodegenerative ones, we suggest that studies of biomarkers of mitochondrial dysfunction are essential to ascertain if they provide better targets for novel and individualised therapies. NF-L clearly has a role in many neurodegenerative disorders and FGF-21 and GDF-15 have been investigated in disease such as MS and PD (195, 196), although studies so far have failed to provide evidence that these biomarkers are specifically disturbed in these diseases. Nevertheless, it remains an intriguing possibility that the differential impact of mitochondrial dysfunction in neurodegenerative disease is one explanation for the heterogeneity we see.

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14 Papers I-III

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Short communication

Increased levels of cell-free mitochondrial DNA in the cerebrospinal fluid of patients with multiple sclerosis

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ABSTRACT

Mitochondrial DNA (mtDNA) can act as damage-associated molecular pattern molecule (DAMP) and initiate an inflammatory response. We hypothesized that the concentration of mtDNA might reflect inflammatory activity in multiple sclerosis and investigated therefore levels of cell-free mitochondrial DNA in cerebrospinal fluid of patients with relapsing-remitting multiple sclerosis. Significantly higher levels of mtDNA were found in patients compared to controls and there was an inverse correlation between disease duration and mtDNA concentration. Our study suggests that mitochondria can be involved early in multiple sclerosis, but whether this is as an initiator of the inflammatory response or part of its maintenance is unclear. Further, our study suggests that changes in mtDNA may provide a novel marker for early disease activity.

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1. Introduction

Multiple sclerosis is an immune-mediated, central nervous system (CNS) inflammatory disease of unknown etiology. Studies suggest that while inflammation appears to be the important component in relapsing-remitting multiple sclerosis, neurodegeneration is present at an early stage (Trapp et al., 1998) characterizing a combined inflammatory and neurodegenerative pathogenesis of the disease.

In view of the unpredictable and heterogeneous disease course of multiple sclerosis, ways of measuring disease activity and treatment response are highly relevant (Stuve and Racke, 2016). Neurofilaments (NF) are axonal cytoskeletal proteins that are released following axonal damage and can, therefore, be used as a marker for neuronal injury. Levels of NF-light (NF-L) are increased in the cerebrospinal fluid (CSF) and serum of patients with relapsing-remitting multiple sclerosis (Kuhle et al., 2016) and correlate with CSF lactate levels suggesting a role of mitochondrial dysfunction in the pathogenesis of multiple sclerosis (Albanese et al., 2016). Elevated levels of NF-L have also been found to decrease following natalizumab treatment (Gunnarsson et al., 2011). Despite these findings, there are no well-established blood or CSF markers to define disease activity, treatment response in multiple sclerosis or transition from a relapsing-remitting to a secondary progressive disease course.

Low levels of free mitochondrial DNA (mtDNA) are found in the CSF where it is assumed they reflect normal turnover of mtDNA in the brain. Recently, decreased levels of cell-free mtDNA were demonstrated in the CSF of patients with Alzheimer's and Parkinson's disease, suggesting mtDNA could be a marker for neurodegeneration (Podlesniy et al., 2013; Pyle et al., 2015). In contrast, elevated levels of mtDNA were found in the CSF of children with traumatic brain injury, indicating mtDNA could be a mediator for sterile inflammatory responses (Walko et al., 2014).

In the present study, we asked the question is cell-free mtDNA in the CSF of patients with relapsing-remitting multiple sclerosis elevated and if so, does this reflect disease activity.

2. Material and methods

2.1. Subjects

This retrospective study investigated 21 newly diagnosed patients with relapsing-remitting multiple sclerosis according to the revised McDonald criteria (Polman et al., 2011). Lumbar puncture (LP) was performed as part of their diagnostic assessment. None of the patients had received immunomodulatory therapy prior to LP. While all 21 patients

Abbreviations: DAMPs, damage-associated molecular pattern molecules; mtDNA, mitochondrial DNA; NF, neurofilaments; PAMPs, pathogen-associated molecular pattern molecules.

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had typical magnetic resonance imaging (MRI) findings, only 4 had gadolinium enhanced scans at the time of LP. The control group comprised 23 patients without inflammatory or neurodegenerative disease, and with normal CSF white cell count and normal MRI. Controls underwent LP for a variety of reasons including unspecific symptoms as headache, paresthesia, vertigo and fatigue. The groups were age-and gender-matched, but otherwise selected randomly. Table 1 summarizes the demographics and CSF findings.

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (No: 2014/1371), and written consent was obtained from all patients.

2.2. CSF collection and preparation

CSF was collected, centrifuged at 1900 rpm at 4 °C for 10 min, aliquoted and stored in polypropylene tubes at −80 °C in the Norwegian Multiple Sclerosis Registry and Biobank (Myhr et al., 2015). Each tube contained 500 μl CSF.

Using native, untreated CSF gave no amplification irrespective of the amount used, suggesting the presence of PCR-inhibitors. Filtering CSF using Amicon Ultra 0.5 ml, 30 kDa, columns, resulted in profound loss of mtDNA to levels below the confident detection range of our qPCR assay. To maximize DNA yield and at the same time minimize the presence of inhibitors, we therefore employed a DNA extraction protocol from 200 μl CSF using the QIAmp® DNA Mini Kit (Qiagen GmbH).

2.3. Determination of mtDNA copy number

The concentration of mtDNA was determined by quantitative polymerase chain reaction (qPCR). We amplified MTND1 to quantify mtDNA and the nuclear encoded gene amyloid precursor protein (APP) to detect any nuclear DNA contamination. To ensure that we only measured cell-free mtDNA, samples showing detectable amplification of APP (CT-value <37) were discarded (n = 7). The amount of mtDNA was calculated using a standard curve derived from serial dilutions of a gel-purified MTND1 amplicon. The serial dilutions contained 10^1–10^8 copies/μl. The following primers, probes and conditions were used. *MTND1:* forward primer: L3485–3504: 5′-CCCTAAAACCCGCCACATCT-3′, reverse primer: H3553–3532: 5′-GAGCGATGGTGAGAGCTAAGGT-3′, TaqMan® MGB probe: L3506–3529: 5′-FAM-CCATCACCCTCTACATCACCGCCC-3′. *APP*: forward primer: 5′-TGTGTGCTCTCCCAGGTCTA-3′, reverse primer: 5′-CAGTTCTGGATGGTCACTGG-3′, TaqMan® MGB probe: VIC-CCCTGAA CTGCAGATCACCAATGTGGTAG. 10 μl of CSF were used per cPCR reaction. All standard curves had amplification efficiency over 90%. PCR was performed in triplicate using an ABI 7500 Fast Real-time PCR system (v2.0.6 Life Technologies Corporation) using TaqMan ® Fast Advanced Master Mix (Thermofisher). Thermal cycling consisted of following profile: one cycle at 95 °C for 20 s, 45 cycles at 95 °C for 3 s and 60 °C for 30 s. Each run contained a negative control for both MTND1 and APP. To ensure reproducibility, 12 of the samples were replicated (27%), with results within a 5% coefficient of variation range. To avoid batch effect a random mix of controls and patient samples were always run together.

Table 1
Demographic and CSF data of subjects groups.

Variables	MS n = 21	Controls n = 23
Gender, n (%)		
Men	5 (24)	7 (30)
Women	16 (76)	16 (70)
Mean age at lumbar puncture, y (±SD)	41.9 (14.1)	42.1 (16.7)
CSF oligoclonal bands, n (%)		
Negative	0 (0)	17 (74)
1–2	1 (5)	5 (22)
3–9	3 (14)	1 (4)
>10	17 (81)	0 (0)
CSF white cell counts, n (%)		
Negative (0–3)	7 (33)	23 (100)
4–10	7 (33)	0 (0)
11–15	3 (14)	0 (0)
16–20	2 (10)	0 (0)
>20	2 (10)	0 (0)

MS = multiple sclerosis, CSF = cerebrospinal fluid, SD = standard deviation.

2.4. Determination of NF-light chain

Due to limited volume of CSF, determination of NF-L was performed on half of the samples (n = 21; 10 patients and 11 controls). The concentration of NF-L in CSF was measured using enzyme-linked immunosorbent assay (ELISA), according to the manufacture's protocol (UmanDiagnostics AB, Sweden).

2.5. Statistical analysis

Statistical analyses were performed in SPSS software (v.23.0.0.2) and Prism (v6; GraphPad). We used non-parametric tests since the data did not fit a normal distribution. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Levels of mtDNA in CSF

The amount of cell-free mtDNA in the CSF of patients was significantly higher than controls ($p = 0.003$, Mann-Whitney *U* test) (Fig. 1A): mean mtDNA concentration in patients was 188.8 copies/10 μl (median 123.9, SD 205.9, range 32.7–943.2) and 75.2 copies/10 μl (median 56, SD 54.1, range 10.2–217.7) in controls. There was no correlation between mtDNA concentration and age, sex, number of oligoclonal bands or CSF leukocyte count.

3.2. Levels of NF-L in CSF

Multiple sclerosis patient samples contained a significantly higher amount of NF-L in CSF compared to controls ($p = 0.005$, Mann-Whitney *U* test) (Fig. 1B). The mean concentration in patients was 1097.9 pg/ml (SD 652.6, range 351.1–2465.1), and 525.9 pg/ml (SD 562.3, range 127.1–2116) in controls. There was no correlation between mtDNA concentration and NF-L concentration.

3.3. Correlation with duration of symptoms

There was a statistically significant inverse correlation between mtDNA concentration and time interval since disease onset ($p = 0.007$, $r = -0.57$ Spearman's correlation) (Fig. 1C).

Too few of the patients had gadolinium-enhanced MRI examinations at the time of LP to permit correlation analysis.

4. Discussion

We found a significantly higher level of cell-free CSF mtDNA in patients with relapsing-remitting multiple sclerosis compared with controls. In addition, we found an inverse correlation between length of time symptoms had been present and levels of mtDNA. Our results suggest that mtDNA concentration may reflect early, active inflammatory activity, and that this could, potentially, provide an early marker for disease activity. Whether these findings are MS-specific, or reflect neuro-inflammation in general, will need to be investigated further.

Due to the low mtDNA copy number in CSF, qPCR reproducibility was a challenge. This was particularly true when using crude CSF, but occurred also after filtration. DNA extraction provided the only consistent results and while this raises the question of mtDNA released by

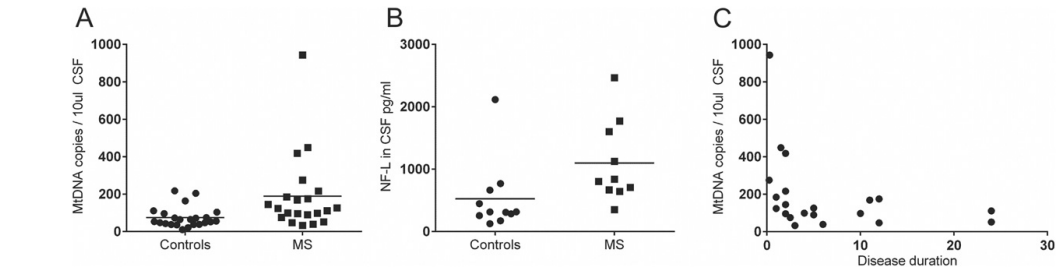

Fig. 1. A mtDNA copies/10 μl CSF in MS and controls. There is a significant difference between MS and controls. p = 0.003. When removing the outlier in the MS group the difference is still significant (p = 0.006). B NF-L levels in MS and controls shows a significantly higher amount of NF-L in CSF compared to controls. p = 0.005. C Correlation between mtDNA concentrations and disease duration. Disease duration is shown in months, and defined as the time from onset of symptoms up till the time of LP.

cell lysis, the absence of APP in the same sample argues strongly against this. The absence of nuclear gene contamination and lack of correlation with NF-L or CSF cell count suggests that the mtDNA originates from cells in the central nervous system, rather than intrathecal inflammatory cells.

The human immune system comprises innate and acquired immune systems and it is suggested that both play a role in the pathogenesis of multiple sclerosis (Witte et al., 2014). The innate immune system recognizes endogenous molecules (DAMPS) or exogenous molecules (PAMPs; pathogen-associated molecular pattern molecules) and initiates either a non-infectious inflammatory response or a pathogen-induced inflammatory response (Zhang et al., 2010). DAMPs originate from the plasma membrane, endoplasmic reticulum, cell nucleus and cytosol. They also arise from mitochondria - mitoDAMPs (Krysko et al., 2011). Molecules or proteins that act as DAMPs or PAMPs are recognized by receptors known as pattern-recognition receptors (PRRs) (Krysko et al., 2011) and complexes formed by PAMPs or DAMPs bound to PRRs initiate a variety of cascades, which result in expression of cytokines and other pro-inflammatory molecules (West et al., 2011). One subgroup of PRRs is the Toll-like receptors (TLR) and TLR-9 is known to bind the unmethylated CpG motifs of bacterial DNA (Krysko et al., 2011; West et al., 2011). Mitochondrial and bacterial DNA share many similarities, and potentially a similar origin (Zhang et al., 2010), and mtDNA released into the bloodstream can act as a DAMP (Zhang et al., 2010) with the potential of stimulating the Toll-like receptor 9 (Zhang et al., 2010; Krysko et al., 2011; West et al., 2011). Further, recent studies have identified mtDNA binding to TLR and podocyte apoptosis, supporting the view that mtDNA is a ligand responsible for activating this cell injury (Bao et al., 2016).

Since mitochondrial damage occurs in active multiple sclerosis lesions (Mahad et al., 2015), mtDNA in the CSF could reflect its role as a DAMP. This would be compatible with any initial process of damage and the subsequent release of cell contents. Another explanation is that mtDNA is actively released in response to a stimulus, similar to the release by eosinophils in response to bacterial infection (Yousefi et al., 2008). Viewing mtDNA as a DAMP in multiple sclerosis provides us with a potential mechanism to explain the “inside-out theory” which suggests that inflammation is secondary to a primary intrinsic process within the neurone or other cell such as oligodendrocytes (Witte et al., 2014). We know from other studies that mtDNA in the CSF does not simply reflect neuronal loss since the levels in Creutzfeldt-Jakob disease, in which massive loss occurs, are normal, while those in Alzheimer's disease are low (Podlesniy et al., 2016).

Thus, low levels of mtDNA in Alzheimer's disease and Parkinson's disease (Podlesniy et al., 2013; Pyle et al., 2015), may reflect specific forms of neurodegeneration. Whether this could be used to follow the conversion from primarily inflammatory to the neurodegenerative phase of secondary progressive multiple sclerosis would require a larger prospective study that also includes multiple sclerosis patients at later stages.

Author contributions

K.N.V performed the qPCR and ELISA experiment, contributed to the conception and design of the study, acquisition and analysis of data and writing and editing of the manuscript. L.A.B, contributed the original idea and design of the study, writing and editing of the manuscript. C.A.V and C.T contributed design of the study, acquisition and analysis of data and writing and editing of the manuscript. K.M.M contributed with acquisition of data and writing and editing of the manuscript. J.H.A contributed with acquisition and analysis of data

Potential conflicts of interest

Nothing to report.

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II

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RESEARCH ARTICLE

Using urine to diagnose large-scale mtDNA deletions in adult patients

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Abstract

Objective: The aim of this study was to evaluate if urinary sediment cells offered a robust alternative to muscle biopsy for the diagnosis of single mtDNA deletions. **Methods**: Eleven adult patients with progressive external ophthalmoplegia and a known single mtDNA deletion were investigated. Urinary sediment cells were used to isolate DNA, which was then subjected to long-range polymerase chain reaction. Where available, the patient's muscle DNA was studied in parallel. Breakpoint and thus deletion size were identified using both Sanger sequencing and next generation sequencing. The level of heteroplasmy was determined using quantitative polymerase chain reaction. **Results**: We identified the deletion in urine in 9 of 11 cases giving a sensitivity of 80%. Breakpoints and deletion size were readily detectable in DNA extracted from urine. Mean heteroplasmy level in urine was 38% ± 26 (range 8 - 84%), and 57% ± 28 (range 12 – 94%) in muscle. While the heteroplasmy level in urinary sediment cells differed from that in muscle, we did find a statistically significant correlation between these two levels ($R = 0.714$, $P = 0.031$(Pearson correlation)). **Interpretation**: Our findings suggest that urine can be used to screen patients suspected clinically of having a single mtDNA deletion. Based on our data, the use of urine could considerably reduce the need for muscle biopsy in this patient group.

Introduction

In humans, mitochondria are the only extra-nuclear organelles that have their own DNA; mitochondrial DNA (mtDNA). This 16.5 kb circular genome encodes 13 proteins that are subunits of respiratory chain complexes: the remaining protein subunits are encoded by genes within the nucleus. In addition to 13 proteins, the mtDNA encodes 22 tRNA and 2 rRNA that participate in mitochondrial translation. Multiple copies of mtDNA are present within each cell and a mutation in mitochondrial genome can therefore, affect some or all of the copies; coexistence of mutated and wild-type mtDNA is known as heteroplasmy.

The first pathogenic mutations in mtDNA giving rise to human disease, were identified in 1988, and were single large-scale mitochondrial deletions (single deletions).[1] Subsequently more than 150 single-nucleotide changes and rearrangements have been identified.[2]

Single deletions contribute to ~ 16% of all mtDNA mutations in adults.[3] They are primarily sporadic events with a minimum prevalence of 1.5/100 000,[3] although

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maternal transmission has been reported,[4] and the risk of transmission is higher than initially assumed.[5]

Single deletions give rise to three classical phenotypes; Pearson disease, Kearns-Sayre syndrome (KSS), and progressive external ophthalmoplegia (PEO). Pearson disease is a multisystem disorder of infancy, recognized usually by the presence of sideroblastic anemia.[6] Patients who survive infancy develop KSS. KSS has been defined as onset of PEO before age 20, pigmentary retinopathy and at least one of the following features; cardiac conduction block, cerebellar ataxia, and/or cerebrospinal fluid protein concentration> 0.1 g/L.[6] PEO is characterized by ptosis and limited eye movement, often accompanied by proximal weakness and myopathy, in addition to other non-muscular symptoms like hearing loss, ataxia, and other neurological symptoms.[7]

A replicative selection against mutations in cells that retain the ability to divide makes the diagnosis of mtDNA disease challenging, since it usually means that blood sampling is considered unsuitable for identifying mtDNA mutations. This is particularly true for mtDNA deletions, where skeletal muscle is regarded as the tissue of choice for diagnosis. Recent research has, however, shown that urine sediment cells retain sufficiently high levels of mtDNA point mutations to allow successful diagnosis of common mutations such as the m.3243A> G mutation.[8] These cells have also been used to identify single deletions and their breakpoints,[9,10] but the finding that urine mtDNA deletions levels were more representative of levels found in muscle in young patients raised questions whether these cells were appropriate for diagnosing adults with single deletions.[10] There are, however, no studies that have addressed this question systematically. Our aim was therefore to investigate: (a) how robust urine was for detecting single deletions in urinary epithelial cells of adult patients, (b) if urine could be used to map and sequence deletion breakpoints, (c) if the degree of heteroplasmy detected in urine was similar to that in skeletal muscle biopsy and (d) if we could use our data to develop a clinical algorithm for investigating patients with PEO.

Methods

Patient samples

We investigated 11 patients from three different centres (Norway, Finland, The Netherlands).

In nine patients (pts 1-5, 7-10) urine and muscle samples were available while in two (pts 6 and 11) only urine samples were available. The size of the mtDNA deletion was, however, known from diagnostic studies of muscle. Where available, the patient's muscle DNA was studied in parallel with urine DNA in all performed assays. The time from diagnosis in muscle to time of urinary sample varied from 0 up to 18 years.

Urine samples were collected independent of time of day, and immediately centrifuged for 15 min at 2000 g, the supernatant discarded and the pellet retained. The pellet was stored at −20°C before use. A volume of 200 ul of ultra-pure water was added to the pellet, before DNA was extracted using the QIAmp DNA Mini-kit (Qiagen). The manufacturer's tissue protocol was used for extracting DNA from muscle, and for the urine samples the manufacturer's protocol for body fluids was used.

Polymerase chain reactions (PCR)

Two long-range PCR reactions (L-PCR) were used to amplify mtDNA across the major arc, generating either an ~ 8 kb or a ~ 16 kb product in wild-type mtDNA. A control sample from a patient with a normal muscle biopsy was run together.

Following primers were used: 8F (8232 – 8263) 5′-TAAAAATCTTTGAAATAGGGCCCGTATTTACC-3′ and L8R (16496 – 16465) 5′ - CGGATACAGTTCACTT-TAGCTACCCCCAAGTG-3′; 15F (1650-1671) 5′-AACT-TAACTTGACCGCTCTGAG −3′ and 15R (019-001) 5′-GGGTGATAGACCTGTGATC-3′.

The PCR products were run on a 0.7% agarose gel at 40 V for approximately 4 hours. The wells were loaded with 5 μL PCR product and 1 μL loading dye.

Primer sequences for "walking-PCR" were L8R in combination with (5855-5875) TGTAAAACGACGGCCAG-TACCTCAATCACACTACTCC, (6863-6882) TGTAAAAC GACGGCCAGTATTTAGCTGACTCGCCACAC or (7713-7723) TGTAAAACGACGGCCAGTTCCTAACACTCACA ACAAAAC.

To establish that DNA extracted from urine sediment cells could be used to define the deletion breakpoint, we chose two of the patients for Sanger sequencing (pt 8 and pt 10). Standard PCR reactions encompassing the deletion breakpoints were performed, and a total of 25 μL with PCR-product was run on a 0.7% agarose gel. The band was extracted and PCR-product purified using the QIA-quick Gel Extraction Kit (Qiagen).

PCR-products were sequenced with BigDye® Terminator v3.1 (ThermoFisher Scientific).

Quantitative polymerase chain reaction

We quantified the level of mtDNA deletion using quantitative PCR (qPCR). The mtDNA regions *ND1* and *ND4* were amplified together with the single-copy nuclear gene *APP*. The following primer, probes and conditions were used. *MTND1*: forward primer: L3485-3504: 5′-

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CCCTAAAACCCGCCACATCT-3′, reverse primer: H3553-3532: 5′GAGCGATGGTGAGAGCTAAGGT-3′, TaqMan MGB probe: L3506-3529: FAM-CCATCACCCT CTACATCACCGCCC. *MTND4*: forward primer: L12087-12109: 5′-CCATTCTCCTCCTATCCCTCAAC-3′, reverse primer: H12200-12170: 5'- CACAATCTGATGTTTTGGT-TAAACTATATTT-3′, TaqMan MGB probe: L12111-12138: NED-CCGACATCATTACCGGGTTTTCCTCTTG. *APP*: forward primer: 5′TGTGTGCTCTCCCAGGTCT A-3′, reverse primer: 5′CAGTTCTGGATGGTCACTG G-3′, TaqMan MGB probe: VIC – CCCTGAACTGCAG ATCACCAATGTGGTAG. The reactions were run in triplicate with DNA template of ~ 10 ng/µl.

Thermal cycle: one cycle at 95° for 20 s, 45 cycles at 95° for 3 s, and 60° for 30 s. The percentage of deletion was obtained with the ddCT method, using healthy blood genomic control as calibrator.[11]

Next-generation sequencing

Identification of deletions in next-generation sequencing data was performed in available urine and muscle in nine of the patients, as previously described.[12] Libraries were prepared using the 16 kb PCR products and the Nextera DNA Flex Library Prep kit (Illumina), and samples were sequenced (2x150 bp) using the Illumina Nextseq 500 instrument. Raw sequencing reads were trimmed using Trimmomatic v0.39[13] with options ILLUMINACLIP:illumina.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36 and mapped against the complete human genome hg19 using bwa v0.7.15[14] with default parameters. After removal of duplicates with SAMtools v1.9[15], single-nucleotide variants (SNVs) in mtDNA were identified and filtered for each individual sample using Genome Analysis Toolkit v3.8[16] (indels were discarded and SNVs restricted to a quality score normalized by an allele depth of at least 2 and a maximum Fisher strand bias of 60). A new hg19 reference containing the alternate individual mtDNA SNVs was generated for each sample and reads were realigned with bwa. Reads with mapping quality below 30 and/or unmapped were filtered out using SAM tools. In order to identify deletions, alignments were analysed by Pindel v0.2.5[17] with options –x 5 and –A 30. Only deletions identified between MT:1,000-15,000 were kept for downstream analyses. To further reduce the false positive rate, *split reads* identified by Pindel as evidence for deletions were filtered based on a set of stringent criteria that had to be fulfilled by both the *split read* and its paired read: (1) median sequencing quality above 36, (2) no mismatches with the mtDNA sample-specific reference, (3) mapping quality above 0, (4) aligned to the mtDNA chromosome with correct paired-end orientation.

Statistical analyses

The data were processed using SPSS v25 (IBM). A *P*-value < 0.05 was considered statistically significant.

Ethics

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (No: 2019/481), and written consent was obtained from all patients.

Results

Detection of single mtDNA deletions in urine sediment cell DNA

The demographic and clinical data of the patients are summarized in Table 1. We detected a single deletion in urine samples from 9 of 11 patients (82%), either in 8 kb L-PCR (Fig. 1A) and/or 16 kb L-PCR (Fig. 1B). In the remaining two patients, the band was either very weak (pt 2), or undetectable (pt 5). Overloading the gel did not help with visualization.

In six of the patients (pts 3, 4, 7-10) the deletion was detected in both 8 kb and 16 kb L-PCR. In three patients (pts 1, 6, 11), the deletion was only detectable on the 16 kb gel, indicating that these deletions most probably encompassed one of the 8 kb PCR primer sites. To confirm this, we used a process termed "walking"-PCR, in which PCR primers are chosen to amplify regions adjacent to each of the original primers. Using this, we identified large deletions, each over 7 kb, in all three cases.

Heteroplasmy levels and deletion size

Mean heteroplasmy level in urine was 38% ± 26 (range 8 - 84%), and 57% ± 28 (range 12 – 94%) in muscle. The level was generally lower in urinary sediment cells, but we were able to define a correlation between muscle and urine levels within our cohort ($R = 0.714$, $P = 0.031$ (Pearson correlation)) (Fig. 1C). This observation must be interpreted with caution, however, since the finding is based on nine patients only.

Sequencing

In the two patients (pts 8 &10) in whom we performed Sanger sequencing; both had the common 4977 bp deletion. Pt 8 had deletion junction 8469: 13447. Pt 10 had deletion junction 8482: 13459, flanked with the 13 bp direct repeat ACCTCCCTCACCA; a known hotspot for deletion.

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Table 1. Details of phenotype, deletion size, and heteroplasmy level in adult patients investigated in this study. Deletion size in gel and NGS is based on both urine and muscle when available

Patient	Gender	Age	Phenotype	Detectable urine	Available muscle studied in parallel	Deletion size gel	Deletion size NGS	Heteroplasmy urine	Heteroplasmy muscle	Δ Heteroplasmy	Time (years) from muscle sampling to urinary sample
1	F	43	PEO	Yes	Yes	~ 7140 bp	7156 bp	84%	87%	3%	0
2	F	57	PEO	No	Yes	~ 4000 bp*	4407 bp	33%	49%	16%	0
3	F	54	PEO	Yes	Yes	~ 5-6000 bp	Not performed	8%	39%	31%	0
4	F	17	PEO	Yes	Yes	~ 5500 bp	5800 bp	45%	74%	29%	0
5	M	54	PEO	No	Yes	~ 4500 bp*	4851 bp*	26%	56%	30%	13
6	F	59	PEO	Yes	No	~ 7390 bp	7386 bp	Not performed	Not available	Not available	18
7	F	56	PEO	Yes	Yes	~ 4500 bp	4977 bp	20%	75%	55%	0
8	F	30	PEO	Yes	Yes	~ 4500 bp	4977 bp	18%	12%	−6%	10
9	F	24	PEO	Yes	Yes	~ 4500 bp	Not performed	74%	94%	20%	0
10	M	32	PEO	Yes	Yes	~ 4500 bp	4977 bp	28%	36%	8%	1
11	F	22	PEO	Yes	No	~ 8140 bp	8284 bp	Not performed	12%	Not available	0

The size marked with asterix (*) indicates that it is not detectable in urine, and is therefore based on muscle findings.
F, female; M, male; NGS, next-generation sequencing; PEO, progressive external ophthalmoplegia.

Next-generation sequencing

All deletions detected using L-PCR in both muscle and urine were also detected in both muscle and urine using NGS (Table 1). However, we found that NGS generated evidence of additional deletions of varying read depth that were not found by L-PCR.

Discussion

The first aim of this study was to evaluate if urinary sediment cells offered a robust alternative to muscle biopsy for the diagnosis of single mtDNA deletions in adults. Our multicentre study shows that single deletions are detectable in urine in over 80% of the cases who had a clinical syndrome known to be caused by deletion, and in whom deletion was present in skeletal muscle.

We failed to detect deletions in two patients. Since we wanted to evaluate the efficacy of L-PCR in the clinical setting, we did not use prior knowledge of deletion size in the skeletal muscle to generate shorter amplicons. The only common feature shared by these two individuals was deletion size; they had the smallest deletions in the cohort. Since their deletions were robustly detectable in skeletal muscle, one explanation for our failure to detect them in urinary cells is low heteroplasmy level. This, however, was not the case (Table 1). Skeletal muscle heteroplasmy levels in these two patients were not the lowest in the group (pt 2 = 49%, pt 5 = 56%; range 12–94%). Moreover, failure to detect small deletions would contradict previous studies that showed an inverse correlation between skeletal muscle heteroplasmy and deletion size [7,18,19].

Our second aim was to investigate whether heteroplasmy level in a patient´s urine was similar to that in skeletal muscle. We determined heteroplasmy level using qPCR. As clinical severity and prognosis in patients with mtDNA disorders are usually related to mutation load, the level of heteroplasmy is of clinical interest.[20] As in previous studies,[10] we found interpreting the level and impact of heteroplasmy in urinary cells problematical. Furthermore, four of the urine samples were taken at a later time point than the muscle biopsy and we know that this can affect the degree of heteroplasmy.[21] Interestingly, in contrast with muscle, urinary epithelial cells are mitotic and thus capable of eliminating cells with comprised energy metabolism levels due to mutated mtDNA. That they retain mtDNA deletions at all is an interesting phenomenon, and our results suggest that they do this to levels that reflect what is found in postmitotic skeletal muscle.

Whether heteroplasmy levels, the deletion breakpoint and size are of clinical, predictive importance in patients

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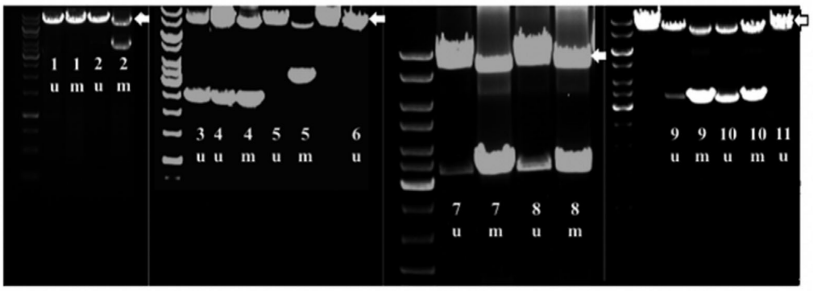
A



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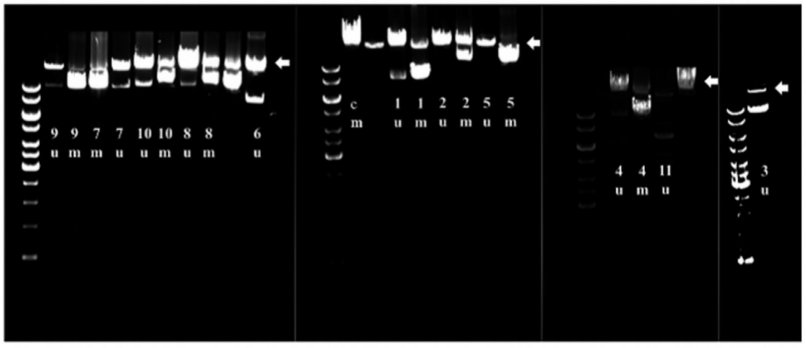

C

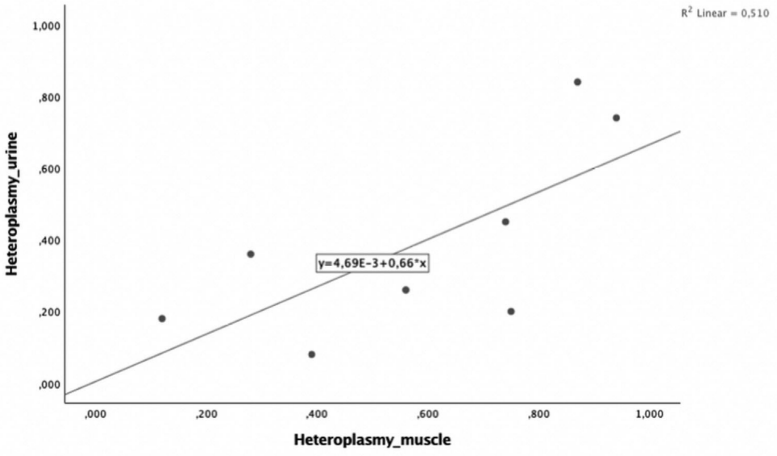

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Figure 1. Long-range PCR analysis in patients 1-11 with single deletions in urinary sediment cells (u) and skeletal muscle (m) and control patient (c). The wild type amplicon is 8kb in Figure (A), and 16kb in Figure (B), and marked with an arrow. A 10 kb DNA ladder is shown to the left of each run, and vertical lines define separate experiments. In patient 6 and 11 only urinary samples were available. Due to technical problems with patient 3 muscle, we ran the urine sample alone, and used information concerning the deletion size obtained from the diagnostic assay. (C) shows a scatterplot illustrating the correlation between percent mutant mtDNA in muscle (x-axis) and percent mutant mtDNA in urine (y-axis) within the same individual. Heteroplasmy levels were determined using qPCR as described under methods.

Table 2. An overview over the diversity in previous studies on single deletions

Reference	Correlations	Correlations not found
Zeviani et al.[23]		No relationship between size and site of deletion and biochemistry or disease severity.
Holt et al.[24]		No correlation between heteroplasmy and clinical or biochemical severity No correlation between deletions site and phenotype
Aurè et al.[22]	More severe phenotype was associated with age of onset and the presence and proportion of deletion in blood associated with more severe phenotype (same nonsignificant trend was found in urine).	No correlation between phenotype and: - Deletion size, - Site of deletion - Heteroplasmy
Yamashita et al.[25]	Deletion size correlated with: - Age at onset (inverse) - Phenotype (longer deletions in KSS) Number of deleted tRNAs correlated with: - Age at onset (inverse) - Phenotype (more in KSS) Correlation between site of deletion and age of onset	No correlation between heteroplasmy and: - Age of onset - Phenotype
Lopez-Gallardo et al.[19]	Heteroplasmy correlated with: - Age of onset (inverse) - Deletion size (inverse) (only in CPEO patients) Inverse correlation between deletion size and age of onset Correlation between site of deletion and phenotype: - Deletion involving the Mt-CYB gene correlated with KSS phenotype	
Grady et al.[18]	Heteroplasmy correlates with: - Deletion size (inverse) - Phenotype - Age of onset - COX-deficient fibre densityDisease burden and progression is predicted by: - Heteroplasmy - Deletion size - Site of deletionDeletion size correlates with: - Location of deletion - Age of onset	
Mancuso et al.[7]	Inverse correlation between heteroplasmy and: - Deletion size - Age at onset	No correlation between phenotype and: - Heteroplasmy - Deletion size Age not related to deletion size
Broomfield et al. [9]	Childhood-onset cohort. Weak correlation between heteroplasmy and age at onset	No correlation between age of onset and deletion size or site of deletion

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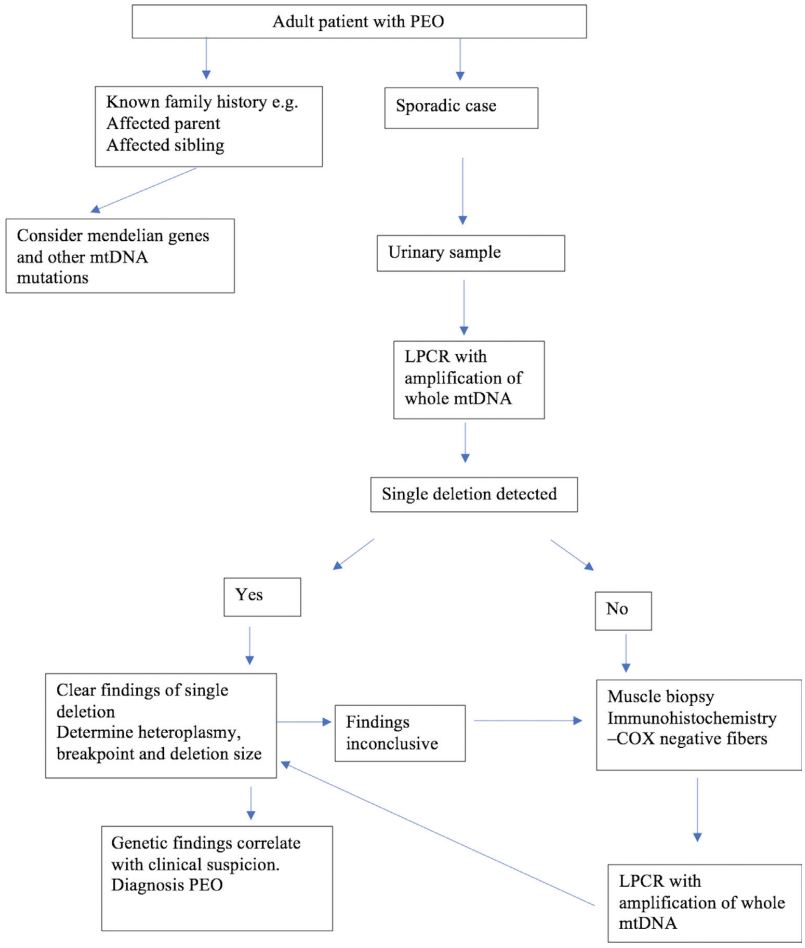

Figure 2. Proposed diagnostic algorithm in patients with progressive external ophthalmoplegia.

with single deletions remains unclear. Various studies have shown contradictory findings, and we have summarized these in Table 2. Some studies have suggested that age of onset is the most important factor.[22] More recently, Grady and colleagues reanalysed previously published data and found that there was indeed a predictive value of heteroplasmy, size, and site of deletion on disease burden and progression.[18] Regardless of the debate concerning prognostic impact of estimating deletion size, site, and heteroplasmy level, we show that this information is readily available in urine.

Both L-PCR and NGS detected mtDNA deletions in DNA extracted from urine, but we found that NGS generated evidence of additional deletions of varying read depth that were not found by L-PCR. Since we used the same 16 kb amplicon used for L-PCR, the noisier signal seen with NGS is possibly related to PCR amplification and may disappear when run on native DNA. The purpose of our study was not to perform a head-to-head comparison of these two methods, but to show that urine was a suitable tissue for diagnosis. Currently, we believe that L-PCR is robust and straightforward enough for diagnostic purposes, however, future studies may well alter this conclusion, particularly if improvements in NGS techniques make it possible to sequence without prior amplification.

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Our results suggest that urinary sediment cells are a viable alternative to muscle biopsy for the diagnosis of single mtDNA deletion disorder, even though we may miss the structural and pathological information provided by a muscle biopsy. Furthermore, although our study focused on adults with PEO, there is no reason to believe that diagnostic efficiency will be any less in children with KSS or Pearson syndrome. Our results show that urine provides evidence not only of the presence of a mtDNA deletion, but also permits the identification of breakpoints and the assessment of heteroplasmy. We would therefore recommend the following algorithm (Fig. 2): urine should be screened for single mtDNA deletions in patients with phenotypes known to be associated with this genetic defect. Muscle biopsy should be reserved for patients having high clinical suspicion but no detectable deletion in urine. We believe that this approach will reduce the need for muscle biopsy while maintaining diagnostic sensitivity.

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Author Contributions

KNV designed the research study, conducted the experiments, acquired, and analysed the data. LAB designed the research study and acquired data. GSN, CT, and PK conducted the NGS experiment and analysed the data. RdC, PI, and AS acquired data. All authors contributed to writing and editing of the manuscript.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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III

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BRAIN COMMUNICATIONS

Serum biomarkers in primary mitochondrial disorders

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The aim of this study was to explore the utility of the serum biomarkers neurofilament light chain, fibroblast growth factor 21 and growth and differentiation factor 15 in diagnosing primary mitochondrial disorders. We measured serum neurofilament light chain, fibroblast growth factor 21 and growth and differentiation factor 15 in 26 patients with a genetically proven mitochondrial disease. Fibroblast growth factor 21 and growth and differentiation factor 15 were measured by enzyme-linked immunosorbent assay and neurofilament light chain with the Simoa assay. Neurofilament light chain was highest in patients with multi-systemic involvement that included the central nervous system such as those with the m.3242A>G mutation. Mean neurofilament light chain was also highest in patients with epilepsy versus those without [49.74 pg/ml versus 19.7 pg/ml ($P = 0.015$)], whereas fibroblast growth factor 21 and growth and differentiation factor 15 levels were highest in patients with prominent myopathy, such as those with single-mitochondrial DNA deletion. Our results suggest that the combination of neurofilament light chain, fibroblast growth factor 21 and growth and differentiation factor 15 is useful in the diagnostic evaluation of mitochondrial disease. Growth and differentiation factor 15 and fibroblast growth factor 21 identify those with muscle involvement, whereas neurofilament light chain is a clear marker for central nervous system involvement independent of underlying mitochondrial pathology. Levels of neurofilament light chain appear to correlate with the degree of ongoing damage suggesting, therefore, that monitoring neurofilament light chain levels may provide prognostic information and a way of monitoring disease activity.

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Abbreviations: AUC = Area under the curve; FGF-21 = fibroblast growth factor 21; GDF-15 = growth and differentiation factor 15; MELAS = mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; mtDNA = mitochondrial DNA; NF-L = neurofilament light chain; POLG = polymerase gamma; SD = standard deviation

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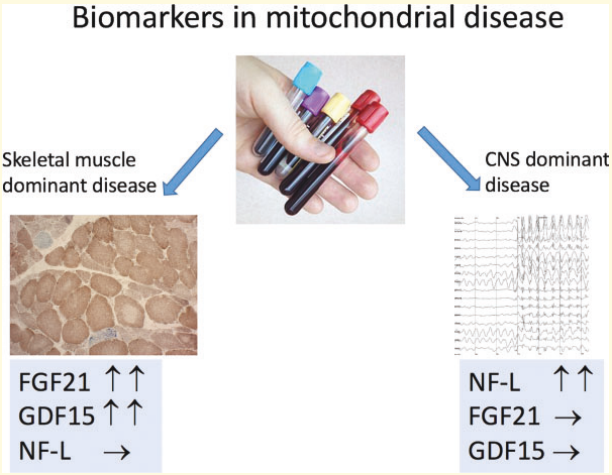
Graphical Abstract



Introduction

Mitochondrial diseases are a heterogeneous group of disorders arising from mutations in genes encoded either in the nucleus or in the mitochondrion's own multi-copy genome (mitochondrial DNA, mtDNA).

Diagnosing mitochondrial disease is challenging because of both the clinical heterogeneity and the paucity of clinically useful biomarkers. Elevated blood lactate is an inconsistent finding and diagnosis has relied heavily on muscle biopsy (Turnbull, 2011). Two new biomarkers, fibroblast growth factor 21 (FGF-21) and growth and differentiation factor 15 (GDF-15), have been described for mitochondrial disease. FGF-21 is a hormone-like cytokine secreted in response to starvation and leads to mobilization of lipid stores and production of ketone bodies (Tyynismaa *et al.*, 2010). GDF-15 is a member of the transforming growth factor beta superfamily (Weiss and Attisano, 2013), and has a role in regulating cellular response to stress and inflammation (Boenzi and Diodato, 2018). FGF-21 and GDF-15 are both primarily elevated in mitochondrial disease affecting muscle (Suomalainen *et al.*, 2011; Kalko *et al.*, 2014) although GDF-15 appears more sensitive in detecting mitochondrial dysfunction affecting other organs (Davis *et al.*, 2016).

Neurofilament light-chain (NF-L) is a neuron-specific protein. NF-L is a marker of disease activity and progression has been evaluated in a number of different neurological conditions (Varhaug *et al.*, 2019). Increased NF-L levels reflect ongoing neuronal damage, irrespective of the underlying pathology, making it a potentially interesting biomarker also in mitochondrial disorders.

Our aim was to measure these non-invasive and easily obtained serum biomarkers in a cohort of mitochondrial patients, and to evaluate diagnostic and clinical usefulness.

Materials and methods

Study design and patients

Twenty-six patients with a genetically confirmed mitochondrial disorder were recruited at the outpatient clinic, Department of Neurology, Haukeland University Hospital, Bergen, Norway.

Serum sampling and analysis

Serum samples were stored at −80°C until analysis.

The concentration of NF-L was determined using the Simoa assay (UmanDiagnostics, Quanterix). The concentrations of FGF-21 and GDF-15 were measured in duplicate samples by ELISA according to the manufacturer's protocol (BioVendor, Brno, Czech Republic).

Statistical analyses

The data were processed using SPSS v25 (IBM). Statistical differences were determined using Kruskal–Wallis and Mann–Whitney *U* non-parametric tests, with Benjamini–Hochberg adjustment. A *P*-value of <0.05 was considered statistically significant. Correlations were assessed with Spearman's correlation test. Graphs were made using Graphpad prism 8.

Ethical approval

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (No: 2019/479), and written consent was obtained from all patients.

Missing data

FGF-21 measurement was missing from one patient.

Data availability

The data that supports the findings of this study is available from the corresponding author upon reasonable request.

Results

Clinical features

Demographic characteristics are summarized in Table 1. All patients were adults, apart from one, aged 14 years. The mean age was 48.2 years [standard deviation (SD), 17.8].

NF-L

The overall mean concentration of serum NF-L was 25.70 pg/ml (SD = 23.4 pg/ml). When sub-classifying by genetic diagnosis, mean NF-L concentration was 43.89 pg/ml (SD = 32.4 pg/ml) in patients with mtDNA point mutations, 11.70 pg/ml (SD = 5.6 pg/ml) in mtDNA single deletions and 21.39 pg/ml (SD = 17.6 pg/ml) in nuclear gene mutations (Fig. 1A). The difference in NF-L levels between mtDNA point mutations and single deletions was significant ($P = 0.024$). There was a trend towards significant difference between mtDNA point mutations and nuclear mutations ($P = 0.0585$).

NF-L could differentiate between patients with mtDNA point mutations and the other two groups with an Area under the curve (AUC) of 0.806 (Fig. 2A). When compared to single deletions only, the AUC was 0.905 (Fig. 2B). We also looked at patients with epilepsy regardless of the genetic aetiology. The mean NF-L level in the epilepsy group (five patients) was 49.74 pg/ml versus 19.7 pg/ml in the non-epilepsy group (21 patients) ($P = 0.015$).

In two patients (with POLG disease and m.3243A>G-mutation), we had one blood sample taken at an earlier time point and could calculate the annual increase in the concentrations of NF-L. Normal annual increase has been previously estimated to be about 2.2%/year (Disanto *et al.*, 2017). The annual increase for the POLG-patient was 4.24%, the m.3243A>G patient 7.4%.

Table 1 Characteristics of the patients

	MtDNA point mutations	N	Single deletions	Nuclear gene mutations	N
Genetic diagnose	*8344 A>G*	2		*POLG*	7
	3243 A>G	4		*TWINKL*	2
	13271 T>C	1		*PITRM1*	1
	5556 G>C	1		*DHX30*	1
				ICSU	1
Age (mean years)	58		43	47	
Gender (female %)	63 %		100 %	83 %	
Total		8	6		12

FGF-21 and GDF-15

The group mean concentration of FGF-21 was 679.77 pg/ml (SD = 630.07 pg/ml) and GDF-15 was 2802.43 (SD = 2628.04 pg/ml). In mtDNA point mutations, mean FGF-21 concentration was 735.10 pg/ml (SD = 430.2 pg/ml), in single deletions 1172.53 pg/ml (range: SD = 927.8 pg/ml) and in nuclear mutations 401.1 pg/ml (SD = 393.5 pg/ml) (Fig. 1B). There were no significant differences between the FGF-21 levels and the different underlying genetic groups ($P = 0.052$). The sub-group mean for GDF-15 concentration in mtDNA point mutations was 3027.27 pg/ml (SD = 1278.0 pg/ml), 5202.59 pg/ml (SD = 4281.3 pg/ml) in single deletions and 1420.54 pg/ml (SD = 1087.7 pg/ml) in nuclear mutations (Fig. 1C). The difference in GDF-15 levels between nuclear mutations and point mutations and single deletions was significant ($P = 0.024$ and $P = 0.006$, respectively).

There was a significant correlation between FGF-21 and GDF-15 ($r = 0.671$, $P < 0.001$, Spearman's correlation). There was no correlation between FGF-21 or GDF-15 and NF-L. However, NF-L showed a reciprocal trend to FGF-21 and GDF-15 when the patients were divided in clinical groups, from pure myopathic to multisystem disease with active cerebral damage (Fig. 3A). The differences in NF-L were significant ($P = 0.012$) (Fig. 3B).

Discussion

Our study shows that NF-L provides important clinical information about the presence of active neuronal damage in mitochondrial disease. We also confirmed that FGF-21 and GDF-15 are useful biomarkers for mitochondrial disease in which myopathy is the major or only manifestation (Lehtonen *et al.*, 2016; Montero *et al.*, 2016). Intriguingly, NF-L showed a reciprocal trend to FGF-21 and GDF-15, suggesting that these three biomarkers may complement each other as diagnostic tools in the investigation of mitochondrial disorders.

The overall mean NF-L concentration in patients with mitochondrial disease was 25.70 pg/ml. This level is pathological (Varhaug *et al.*, 2019). When we classified our study cohort according to the presence and type of central nervous system (CNS) involvement, we observed clear differences in the concentrations of NF-L (Fig. 3). In multi-systemic disorders with CNS involvement,

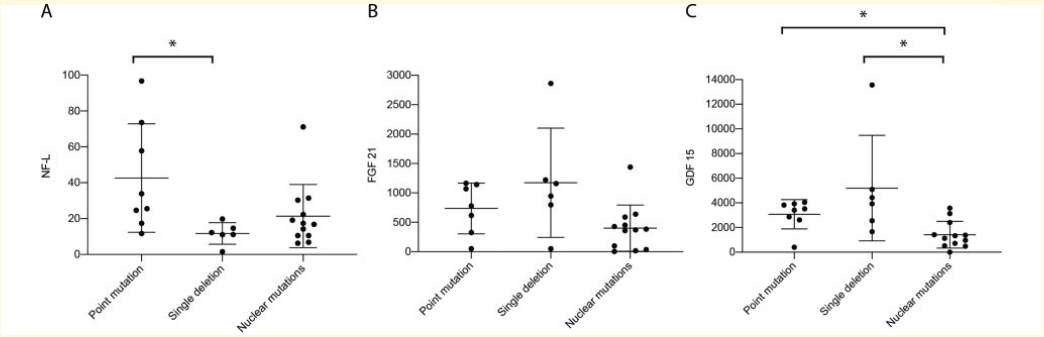


Figure 1 Biomarkers when sub-classified by genetic diagnosis. The differences in mean of the three biomarkers when the patients are genetically classified in point mutations, single deletions and nuclear mutations are shown. All values are expressed in pg/ml. The bars represent mean and standard deviation in NF-L (**A**), FGF-21 (**B**) and GDF-15 (**C**). There were significant differences between point mutations and single deletions in NF-L, and nuclear mutations and both point mutations and single deletions in GDF-15. Significant differences are marked with asterisk (*).

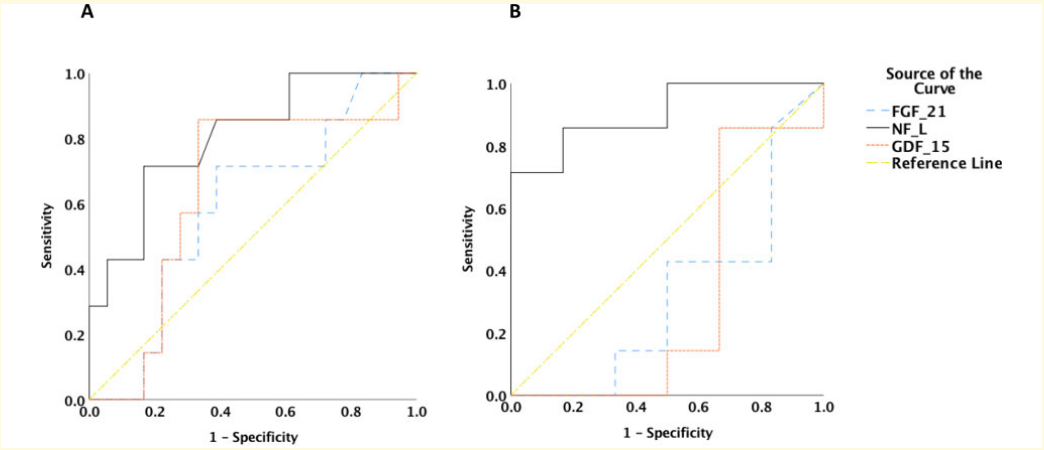


Figure 2 ROC curves for the three biomarkers. Receiver-operating characteristics curves for the three biomarkers in patients. (**A**) Point mutations versus single deletions and nuclear mutations; AUC = 0.806, ($P = 0.02$). (**B**) Point mutations versus single deletions; AUC = 0.905 ($P = 0.015$).

particularly those associated with point mutations such as the m.3243A>G-mutation, the highest levels of NF-L were observed. In contrast, in patients with single deletions, and a pure (or almost pure) myopathic phenotype, the levels of NF-L were normal.

We found significant differences in NF-L levels according to the type of CNS involvement. First, those with epilepsy had much higher NF-L levels than those without. This is of major importance since epilepsy is one of the most important prognostic factors, especially in POLG disease, and swift detection and treatment of any epileptic seizure activity is vital (Engelsen *et al.*, 2008). The stroke-like episodes which are often seen in both POLG disease and MELAS caused by m.3243A>G are medical emergencies (Ng *et al.*, 2019), and an elevated NF-L at time of diagnosis, or one that shows a rising profile, may indicate a higher risk of seizures or stroke-like episodes. We suggest, therefore, that patients with mutations known to cause severe neurodegenerative damage, such as *POLG* and m.3243A>G-mutations, should have serum NF-L levels monitored regularly (e.g. every 3 months). Rising levels should trigger further investigations including MRI, EEG

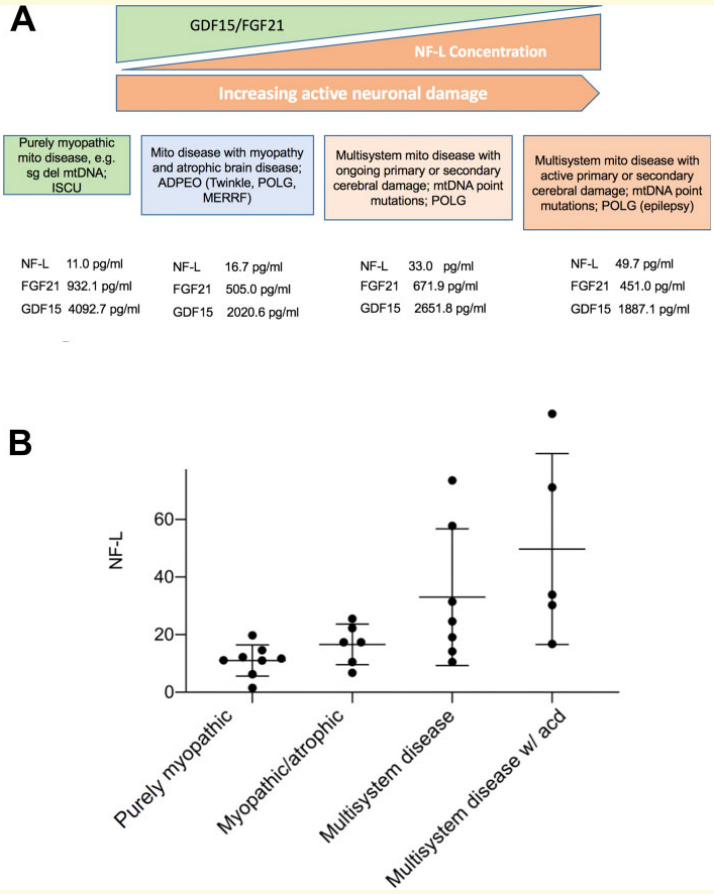


Figure 3 The biomarkers ability to differentiate clinical groups. There is a reciprocal trend in NF-L to FGF-21 and GDF-15, here illustrated by sub-classifying the patients by degree of CNS involvement. Brain involvement was sub-divided into slowly progressive atrophy, neurodegenerative disease with ongoing neuronal loss and conditions in which there would be more acute neuronal loss due to ongoing seizures or ischaemic damage (acd, active cerebral damage). The differences are most pronounced when looking at NF-L. (**B**) The differences between the clinical groups defined in (**A**) are significant ($P = 0.012$).

and consideration of whether anti-convulsant treatment should be initiated even prior to a first seizure event.

We also found differences in the levels of NF-L between patients in whom the CNS process was slowly progressive, i.e. only atrophy, compared to those in which the process was more destructive, particularly those with ongoing epilepsy. This is exemplified by looking at patients with POLG disease: those with ataxia and no epilepsy had lower levels than those who had already developed epilepsy.

A previous study in paediatric patients found that NF-L could be used as a marker for mitochondrial encephalopathies, and that it is correlated with MRI abnormalities and poor prognostic outcome (Sofou *et al.*, 2019). They also noted a marked high NF-L level in patients with MELAS. This study was, however, performed in cerebrospinal fluid and we feel that blood is a more accessible tissue and one that will better facilitate repeated measurements.

We believe that, as in other disease such as multiple sclerosis (Varhaug *et al.*, 2018, 2019), the most important use of NF-L in mitochondrial disease will be the patient's own variation. For example, in a patient with m.3243A>G-mutation there was a marked and pathological elevation of 7.4%. This reflects what we saw when comparing disease sub-groups; those with

destructive mutations such as the m.3243A>G-mutations had pathological NF-L levels that continued to rise. Interestingly, a study looking at plasma levels of cell-free mtDNA in mitochondrial patients (Maresca *et al.*, 2020) showed that these were higher in patients with MELAS than patients with mitochondrial disease due to nuclear gene mutations. They also found that levels increased over time in patients with MELAS, suggesting that cell-free mtDNA may too function as a marker of disease progression.

Previous studies have identified a threshold value of 350.0 pg/ml for FGF-21 in mitochondrial disease (Yatsuga *et al.*, 2015). The threshold for GDF-15 depends on the kit used and with the BioVendor kit, used in this study, the threshold has been set to 2330 pg/ml (Davis *et al.*, 2016). We found that patients in our sub-group of nuclear mutations had a mean GDF-15 level lower than threshold. Since GDF-15 is considered as a more sensitive marker for mitochondrial disorder, in general, and not just in mitochondrial muscle involvement (Davis *et al.*, 2016), this was surprising. The majority of our patients with nuclear gene mutations were adults with *POLG* mutations and phenotypes consisting of epilepsy, neuropathy and/or ataxia. Myopathy was not a major feature, and the low levels of GDF-15 could therefore be a reflection of this. Thus, while GDF-15 might, under certain conditions be a broader biomarker, it remains essentially a mitochondrial myopathy marker. That myopathy was not a major feature was supported by our FGF-21 results: FGF-21 levels were also lower in the group of patients with nuclear gene mutations than it could be expected, but similar to those reported earlier in ataxic POLG patients (Suomalainen *et al.*, 2011).

Both FGF-21 and GDF-15 were significantly elevated in patients with single mtDNA deletion, and high in those with a significant degree of muscle involvement. Correlation between these two biomarkers was also good, confirming the results of earlier studies (Montero *et al.*, 2016). Our impression is that these biomarkers are good when mitochondrial myopathy is present, but less specific when muscle is not involved. This is in accordance with the previous findings, showing positive correlation between FGF-21 and GDF-15, as well as with lactic acid and creatine (Maresca *et al.*, 2020). In contrast, NF-L appears much more sensitive at detecting the presence of CNS damage. We find significant differences in the levels of NF-L between the three genetic sub-groups, and the levels of AUC suggest that NF-L could be a useful tool in identifying the mitochondrial patients with more complex cerebral involvement. In combination with FGF-21 and GDF-15, NF-L, and potentially cell-free mtDNA, may contribute to narrowing the choice of diagnostic test in cases of suspected mitochondrial disease.

Mitochondrial disorders are rare and heterogenous disorders making large studies difficult. However, prospective multi-centre studies will be necessary to confirm the role of NF-L as a biomarker, and how it, together with GDF-15 and FGF-21, can be used most profitably in the investigation of mitochondrial disease. We would, however, like to suggest that NF-L be added to the list of biomarkers that should be monitored during clinical trials in mitochondrial disease (Mancuso *et al.*, 2017).

Conclusion

In conclusion, our study indicates that we now have three novel serum biomarkers for use in mitochondrial disorders. The combination of these biomarkers can be used both as diagnostic tools and in clinical follow-up. We believe that there is also an important role for these biomarkers in future natural history studies and drug trials.

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Competing interests

The authors have declared that no conflict of interest exists.

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