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CARDIOVASCULAR  
ABNORMALITIES IN  
ADULT PATIENTS WITH  
THE 3243A>G MUTATION  
IN MITOCHONDRIAL DNA

FACULTY OF MEDICINE,  
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Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium 10 of Oulu University Hospital, on May 16th, 2007, at 12 noon

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## **Majamaa-Voltti, Kirsi, Cardiovascular abnormalities in adult patients with the 3243A>G mutation in mitochondrial DNA**

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### ***Abstract***

The 3243A>G mutation in mitochondrial DNA (mtDNA), the most common cause of the syndrome of mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes, is also associated with many other phenotypes such as hearing loss, diabetes mellitus, epilepsy, cognitive decline, myopathy and cardiomyopathy. The prevalence of the mutation has been shown to be 16.3/100 000 adults in Northern Finland. The present study was performed to estimate the frequency and progression of cardiac abnormalities and to examine causes of death in patients with 3243A>G.

Left ventricular hypertrophy (LVH) was found in echocardiography in 56% of patients with 3243A>G and in 15% of age and sex-matched controls. The median thickness of the diastolic interventricular septum or posterior wall was 14 mm in the patients with LVH. The prevalence of LVH determined by echocardiography increased from 40% to 56% in 25 patients with 3243A>G during three years of follow-up, this trend being especially marked among the diabetic patients. The ultra-low-frequency (ULF) and very-low-frequency (VLF) components of the spectral analysis of heart rate variability (HRV) were lower among the patients with 3243A>G than in matched controls ( $p = 0.02$  in ULF and  $p = 0.04$  in VLF), and the short-term fractal scaling exponent in detrended fluctuation analysis of HRV was lower in the patients with 3243A>G ( $1.16 \pm 0.18$  vs.  $1.28 \pm 0.13$ ) ( $p < 0.01$ ). Survival analysis of a birth cohort from pedigrees with 3243A>G revealed excess mortality before the age of 50 years. Neurological and cardiovascular diseases accounted for 32% of all the underlying causes of death in families with 3243A>G. Death was sudden and unexpected in 31% of cases in which 3243A>G was considered to be involved in the cause of death.

The results show that cardiac abnormalities are frequent and progressive in patients with the 3243A>G mtDNA mutation and that cardiac autonomic regulation is disturbed. Patients with the 3243A>G mutation and their first degree maternal relatives died younger than was presupposed by their life expectancy at birth or at 15 years. The most common causes of death were neuropsychiatric and cardiovascular diseases.

**Keywords:** 3243A>G mutation, cardiomyopathy, cause of death, heteroplasmy, MELAS, mitochondrial DNA, phenotype



*Life is what happens to you  
while you're busy making other  
plans  
(John Lennon)*



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## Abbreviations

$\alpha_1$	short-term fractal scaling exponent
$\alpha_2$	long-term fractal scaling exponent
ADP	adenosine diphosphate
ApEn	approximate entropy
ATP	adenosine triphosphate
$\beta$	slope of the power-law relationship in RR interval data
BEHL <sub>0.5-4kHz</sub>	better ear hearing level calculated as the mean of hearing levels over the frequencies 0.5, 1, 2 and 4 kHz
CoA	coenzyme A
COX	cytochrome c oxidase
CPEO	chronic progressive external ophthalmoplegia
DCM	dilated cardiomyopathy
DM	diabetes mellitus
DNA	deoxyribonucleic acid
ECG	electrocardiography
HCM	hypertrophic cardiomyopathy
HF	high frequency
HRV	heart rate variability
LF	low frequency
LVH	left ventricular hypertrophy
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MDM	mitochondrial diabetes mellitus
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
OXPPOS	oxidative phosphorylation
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RR interval	interval between consecutive R waves
SDNN	standard deviation of RR intervals
tRNA	transfer ribonucleic acid

VLF	very low frequency
VT	ventricular tachycardia
ULF	ultra low frequency
KSS	Kearns-Sayre syndrome
LHON	Leber's hereditary optic neuropathy
MERRF	myoclonus epilepsy with ragged red fibres

## **List of original communications**

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I Majamaa-Voltti K, Peuhkurinen K, Kortelainen ML, Hassinen IE & Majamaa K (2002) Cardiac abnormalities in patients with mitochondrial DNA mutation 3243A>G. *BMC Cardiovasc Disord* 1;2: 12.
- II Majamaa-Voltti K, Majamaa K, Peuhkurinen K, Mäkikallio TH & Huikuri HV (2004) Cardiovascular autonomic regulation in patients with 3243A>G mitochondrial DNA mutation. *Ann Med* 36: 225-231.
- III Majamaa-Voltti K, Winqvist S, Remes A, Tolonen U, Pyhtinen J, Uimonen S, Kärppä M, Sorri M, Peuhkurinen K & Majamaa K (2006) A three-year clinical follow-up of adult patients with 3243A>G in mitochondrial DNA. *Neurology* 66: 1470-1475.
- IV Majamaa-Voltti K, Turkka J, Kortelainen M-L, Huikuri H & Majamaa K (2007) Causes of death in pedigrees with the 3243A>G mutation in mitochondrial DNA. Manuscript.



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# 1 Introduction

The electron transport chain located in the inner mitochondrial membrane produces energy for the cell. Mitochondrion carries its own DNA, referred to as mitochondrial DNA (mtDNA), containing genes for polypeptides in the mitochondrial respiratory chain complexes and some ribosomal ribonucleic acid (rRNA) genes and transfer RNA (tRNA) genes necessary for translation in mitochondria (Anderson *et al.* 1981). The double-stranded, circular mtDNA molecule contains 16,569 nucleotide base pairs, and one cell can carry hundreds of mitochondria with two to ten copies of mtDNA. MtDNA mutations affect the functioning of the respiratory chain, leading to impairment of the cell's energy metabolism. Organs with high energy dependence, such as the nervous system, muscle and heart, suffer first from mtDNA mutations, and the diseases related to mtDNA mutations are usually multi-organ syndromes.

The most common pathogenic point mutation in mtDNA is a transition of adenine to guanine at locus 3243 in the tRNA<sup>Leucine</sup> (tRNA<sup>L<sup>eu</sup></sup>) gene. This was first described in patients with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) (Goto *et al.* 1990). Since then the 3243A>G mutation has been related to several phenotypes such as sensorineural hearing loss, diabetes mellitus (DM), short stature, epilepsy, cognitive decline, ataxia, myopathy, cardiomyopathy and neuropathy (Taylor *et al.* 2004). Hypertrophic cardiomyopathy (HCM) (Anan *et al.* 1995) and dilated cardiomyopathy (DCM) (Vilarinho *et al.* 1997, Momiyama *et al.* 1999) have also been found in patients with 3243A>G mutation. The reports concerning cardiac manifestations and causes of death in patients with 3243A>G are based on a small number of selected patients, however, and thus the present study was performed in order to ascertain the frequency of cardiac abnormalities in a population-based cohort of patients with 3243A>G (Majamaa *et al.* 1998). The progression of cardiac manifestations was evaluated during a three-year clinical follow-up of these patients, and causes of death were evaluated in pedigrees with patients carrying this mutation.

## **2 Review of the literature**

### **2.1 Mitochondria**

Mitochondria are cytoplasmic organelles present in all cells with aerobic metabolism, their main function being to produce energy by oxidative phosphorylation (OXPHOS). The fuel for this reaction is derived from glucose metabolism in the Krebs cycle and fatty acid metabolism in  $\beta$ -oxidation. Mitochondria are also involved in calcium and iron homeostasis and are thought to be the priming site for apoptosis.

#### ***2.1.1 Structure of mitochondria***

Mitochondria are intracellular dynamic organelles of varying shape that can undergo fusion and fission, and even form branched networks (Shaw & Nunnari 2002, Karbowski & Youle 2003). Each cell contains a varying number of mitochondria depending on its need for energy metabolism. Mitochondria consist of four compartments. A limiting outer membrane enwraps the energy-transducing inner membrane, which in turn encloses a dense, protein-rich matrix. Between the inner and outer membrane there is an intermembrane space, which takes an active part in protein transport from the cytoplasm to the mitochondrial matrix and OXPHOS. The outer membrane, which is tubular or reticulated when attached to the cytoskeleton, contains lipids to 50% of its weight and is rich in porins, which form membrane channels for large molecules to pass into the intermembrane space. There are cristae bulging from the inner membrane that produce a large surface area for OXPHOS (Mannella 2006), and these are highly flexible, undergoing morphological changes in response to alterations in the metabolic state and matrix volume of the mitochondrion (Heath-Engel & Shore 2006). Separate invaginations of cristae can form dynamic junctions when this is energetically desirable (Mannella *et al.* 2001). The lipid content of the inner membrane amounts to 30% of its weight, which makes the inner membrane impermeable to charged ions such as protons,  $\text{Na}^+$  and  $\text{Cl}^-$  and leads to higher stability of the electrochemical gradient between the matrix and intermembrane space of the mitochondrion. The proteins in the inner membrane include

four components of the electron transport chain, ATP synthase and adenine nucleotide translocator (ANT) (Figure 1). The mitochondrial permeability pore (mtPTP), formed by ANT in the inner membrane and Bax and cyclophilin in the outer membrane (Wallace 1999), provide a way through both the inner and outer membrane from the matrix of the mitochondrion to the cytoplasm of the cell. The mitochondrial matrix harbours mtDNA, ribosomes, tRNAs and a number of enzymes needed for expression of the mitochondrial genome, fatty acid oxidation and the tricarboxylic acid cycle.

### ***2.1.2 Function of mitochondria***

Most of the energy in animal cells is produced by OXPHOS, in which glucose in the cytoplasm is converted to pyruvate in glycolysis and this in turn is transferred to the mitochondria and converted to Acetyl coenzyme A (CoA) by pyruvate dehydrogenase (PDH). Acetyl CoA, a substrate for the tricarboxylic acid (TCA) cycle, is also created by the  $\beta$ -oxidation of fatty acids, which are translocated across the inner mitochondrial membrane into the matrix by carnitine palmitoyltransferases (CPT I, CPT II) and carnitine-acylcarnitine translocase (CACT). The resulting fatty acyl-carnitines are then further transformed into fatty acyl-CoAs, which are substrates for  $\beta$ -oxidation in the production of acetyl-CoA. Electrons generated in the TCA cycle in the mitochondrial matrix are transferred via the electron transport chain to maintain an outflow of protons into the intermembrane space in order to create a transmembrane electrochemical gradient (Figure 1).

The OXPHOS system is composed of five complexes, which are multipolypeptide structures having subunits coded both by nuclearDNA (nDNA) and mtDNA. Complex I (NADH:ubiquinone oxidoreductase) is composed of 46 subunits, seven of which are encoded by mtDNA (Schulenberg *et al.* 2004). Complex II (succinate:ubiquinone oxidoreductase) consists of four nDNA-coded subunits (Cooper & Clark 1994). One of the eleven subunits of complex III (cytochrome bc1) is coded by mtDNA (Anderson *et al.* 1981). Complex IV (cytochrome c oxidase, COX) is composed of 13 subunits, three of which are coded by mtDNA (Anderson *et al.* 1981), and complex V (ATP synthase) has 14 subunits, including two coded by mtDNA (Cooper & Clark 1994).

Complex I transfers electrons from NADH to coenzyme Q10 in a manner coupled to the translocation of protons to the intermembrane space of the mitochondria. Complex II is a part of the TCA cycle, transferring electrons from succinate to coenzyme Q10. Complex III delivers electrons from coenzyme Q10 to cytochrome c, which in turn transfers them to complex IV which delivers electrons to oxygen in the matrix so that they produce water in combination with hydrogen in the matrix. The energy released in this process is used to translocate protons to complexes I, III and IV in the intermembrane space. The electric charge in the intermembrane space is positive, while the matrix is negative. This capacitor is depolarized by complex V, which drives protons from the intermembrane space to the matrix to produce adenosine triphosphate (ATP) from adenosine diphosphate (ADP), inorganic phosphate and water. The resulting ATP is exchanged with ADP from the cytoplasm by ANT (Figure 1).

Cellular respiration is the main source of reactive oxygen species (ROS), which are highly reactive derivatives of the oxygen molecule containing an unpaired electron. ROS include the superoxide anion ( $O^{2-}$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $OH^\cdot$ ). These accumulate in the mitochondrion if electron transport is inhibited or slowed down because of dysfunction of the respiratory chain. ROS exposure can damage mtDNA, mitochondrial and cellular proteins, lipids and nucleic acids leading to a vicious circle in which the respiratory chain is suppressed and the production of ROS increased. Eventually the excessive production of ROS leads to opening of the mtPTP, collapse of the electrochemical gradient between the matrix and intermembrane space and swelling of the inner membrane of the mitochondrion. At the same time several factors promoting cell death are released into the cytoplasm from the intermembrane space, leading to the destruction of proteins in the cytoplasm and chromatin in the nucleus. Thus the mitochondria play a key role in the initiation of apoptosis (Skulachev 1996, Fleury *et al.* 2002). ROS are also involved in carcinogenesis (Cejas *et al.* 2004), neurodegeneration (Enns 2003) and ageing (Droge 2003).

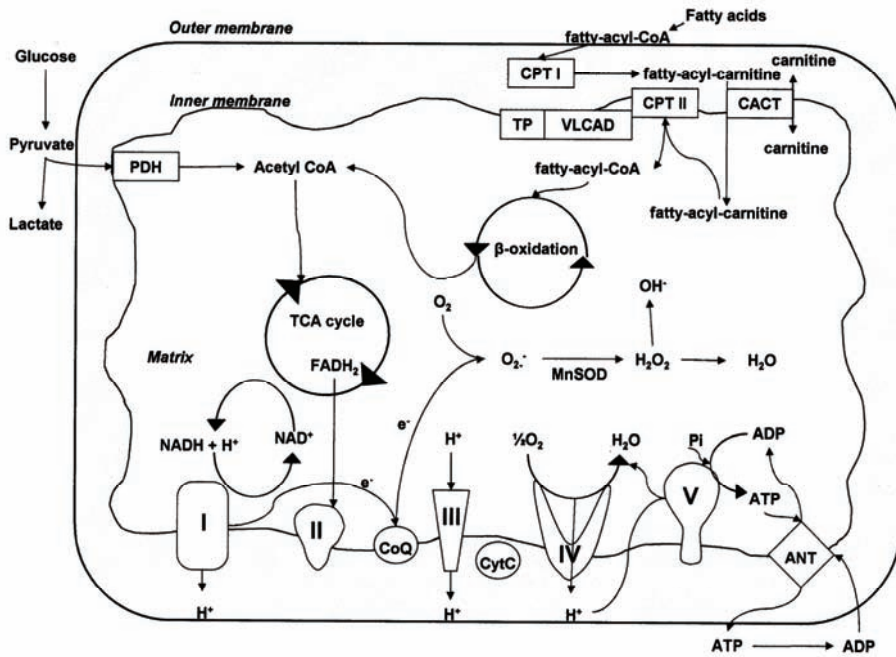


Fig. 1. Metabolic pathways in mitochondria. ADP, adenosine diphosphate; ANT, adenine nucleotide translocator; ATP, adenine triphosphate; CACT, carnitine-acylcarnitine translocase; CoA, coenzyme A; CoQ, coenzyme Q; CPT, carnitine-palmitoyltransferase; CytC, cytochrome c; FADH, reduced flavin adenine dinucleotide; MnSOD, manganese superoxide dismutase; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid; TP, trifunctional protein; VLCAD, very long-chain acyl-CoA dehydrogenase; I, complex I; II, complex II; III, complex III; IV, complex IV; V, complex V.

### 2.1.3 Mitochondrial DNA

MtDNA is a double-stranded circular structure containing 16,569 base pairs. Each mitochondrion contains two to ten such structures, leading to hundreds or thousands of copies of mtDNA in one cell. MtDNA is densely packed, containing 37 genes and no introns. Human mtDNA encodes 22 tRNAs, two rRNAs and 13 polypeptides for respiratory chain complexes. The seven mtDNA-coded polypeptides of complex I are ND1, ND2, ND3, ND4L, ND4, ND5 and ND6, while cytochrome b is the only mtDNA-coded polypeptide of complex III. Those of complex IV are COI, COII and COIII and those of complex V are ATP6 and ATP8.

The two strands of mtDNA are distinguished by their density, the heavy strand (H-strand) being rich in guanine and the light strand (L-strand) rich in cytosine (Kasamatsu & Vinograd 1974, Fernandez-Silva *et al.* 2003). The L-strand carries genes for ND6 and

eight tRNAs and the H-strand contains the rest. The regulation of transcription and replication is controlled by a 1.1 kb non-coding D-loop containing the promoters for L and H-strand transcription and the origin of replication of the H-strand. There are no introns in mtDNA, but the genes encoding proteins are separated by the genes of tRNAs. A picture of the structure of mtDNA is available on <http://www.mitomap.org/mitomapgenome.pdf>.

Together with the production of ROS in mitochondria, the lack of introns makes mtDNA vulnerable to mutations. More than 150 point mutations have so far been identified (Chinnery & Schon 2003), most of them occurring in tRNA genes and leading to impaired mitochondrial protein translation and subsequent impairment of the respiratory chain.

### **2.1.4 Genetics of mitochondrial DNA**

All the mtDNA in the cells and organs of a healthy person are homoplasmic, i.e. identical, representing the person's mtDNA haplotype, but in the cells of subjects with a mtDNA mutation may contain both normal (wild-type) mtDNA and mutant mtDNA and are called heteroplasmic. A certain amount of mutant mtDNA is needed in a cell to impair the functioning of the respiratory chain (Attardi *et al.* 1995), and this threshold differs between organs depending on their energy needs. Thus the threshold for disease is lower in tissues that are highly dependent on oxidative metabolism (DiMauro & Schon 2003).

The normal ovum contains more than 100 000 mtDNA molecules, but only about ten of them populate the primordial germ cells of an embryo (Jenuth *et al.* 1996, Jansen & de Boer 1998, Poulton & Marchington 2002). This "bottleneck" selects the mtDNA entering the primary oocyte and thus reduces the probability of mutations in mtDNA passing to offspring. It may also explain the phenotypic variation between generations and siblings, since the selection of mtDNA has occurred well before maturity of the oocyte.

Mitotic segregation also affects the redistribution of mutant mtDNA in different organs of fetus. Mitochondria containing mutant DNA are randomly redistributed to daughter cells during cell division, so that the mutation load of a previously unaffected organ can exceed the threshold level for heteroplasmy, leading to an organ disorder (DiMauro & Schon 2003).

The replication of mtDNA is under nuclear genetic control, but it takes place independently of nuclear replication (Bogenhagen & Clayton 1977). Since mtDNA lacks introns and protective histones, it undergoes spontaneous mutations 10-20 times more rapidly than nDNA (Brown *et al.* 1979, Wallace *et al.* 1987). Another special feature of mtDNA is that all the mitochondria of the zygote are those of the ovum, paternal mitochondria entering the ovum during fertilization being removed by ubiquitin-dependent proteolysis (Manfredi *et al.* 1997, Sutovsky *et al.* 2000). Thus mutations in mtDNA are inherited maternally by both male and female offspring, but only women will pass the mutation on to their children (Shoubridge 2000). The inheritance of paternal mitochondria has recently been scrutinized after the identification of a man with exercise intolerance and a 2 base pair deletion in the ND2 gene with a 90% heteroplasmy level of mutant mtDNA in muscle. The haplotype of the muscle mtDNA was identical to that of

his father, who was unaffected, while the mtDNA haplotype in his other tissues was identical to that of his mother (Schwartz & Vissing 2002). Paternal inheritance of mtDNA appears to be an extremely rare condition, however (Filosto *et al.* 2003, Taylor RW *et al.* 2003b, Schwartz & Vissing 2004).

The point mutations in mtDNA are usually maternally inherited, the most common mtDNA mutation being 3243A>G, which is often related to the MELAS syndrome, characterised by mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (Pavlakakis *et al.* 1984). 3243A>G results in defects of mitochondrial protein synthesis and subsequent reduction of complexes I and IV in the respiratory chain (Moraes *et al.* 1992). Myoclonus epilepsy and ragged red fibres (MERRF), a syndrome characterised by myoclonus, generalised epilepsy, ataxia and ragged red fibres visible in a muscle biopsy, is most often related to 8344A>G in the tRNA<sup>Lys</sup> gene (Shoffner *et al.* 1990). 3243A>G can also be found in patients with myopathy and chronic progressive extensive ophthalmoplegia (CPEO) (Moraes *et al.* 1993), which can also be related to several other point mutations in the tRNA gene (Website: [mitomap.org](http://mitomap.org) 2006). CPEO, Kearns Sayre syndrome (KSS) and Pearson bone marrow-pancreas syndrome have been associated with deletions in mtDNA (Holt *et al.* 1988). Single deletions in mtDNA present usually sporadic, suggesting that they may either arise early in development of the primary oocyte or selected in by “bottleneck” before entering the primordial germ cell. In contrast to heteroplasmy of point mutations in mtDNA the deletions are usually homoplasmic i.e. the same mutation can be found in all cells of the affected person (DiMauro & Schon 2003).

Proper functioning of the respiratory chain in mitochondria requires proteins encoded both by nDNA and mtDNA. nDNA carries the codes for most of the respiratory chain proteins and approximately 60 ancillary proteins needed for the proper assembly and functioning of respiratory chain complexes. Mutations in the nuclear genes encoding these proteins can lead to similar syndromes to those seen in patients with mtDNA mutations. nDNA-encoded factors are also essential for the integrity and replication of mtDNA. Mutations in the nuclear genes encoding these factors that relate to intergenomic communication cause diseases in the form of a reduced number of mtDNA molecules or multiple deletions in mtDNA (Hirano *et al.* 2001) by affecting the structural and assembly genes of OXPHOS, the genes involved in mtDNA maintenance and the genes affecting mitochondrial fusion and mobility (Wallace 2005). The inheritance of mutations in nuclear genes encoding proteins needed in intergenomic signalling and respiratory chain complexes obeys an autosomal dominant or recessive pattern (Shoubridge 2001b).

Populations may be divided into haplogroups based on the polymorphism of wild-type mtDNA. Two ancient macrohaplogroups, M and N, originated in Africa and the others, A, B, C, D, F, G, H, I, J, K, L, T, U, V, W and X, emerged from them. These haplogroups are differently distributed among populations on different continents (Torroni *et al.* 1996, Wallace *et al.* 1999, Finnilä *et al.* 2001, Herrnstadt *et al.* 2002, Silva, Jr. *et al.* 2002, Kong *et al.* 2003).

## 2.2 The clinical phenotype related to the mitochondrial DNA mutation 3243A>G

MELAS was introduced as a mitochondrial disorder that included mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes with migraine-like headaches, nausea, vomiting (Pavlakakis *et al.* 1984). In adults encephalopathy presents with epilepsy, cognitive decline, ataxia, myoclonus and finally psychomotor retardation. Additional symptoms described in the literature have been premature exercise-induced fatigue, cardiomyopathy, myopathy, pigmentary retinopathy, short stature as a consequence of growth hormone deficiency and sideroblastic anaemia (Hirano *et al.* 1992, Wallace 2001). Stroke-like episodes can lead to cortical blindness, hemiparesis or hemianopia and the lesions detected in magnetic resonance imaging resemble infarcts that do not correspond to the distribution of major vessels of the brain. The natural course of MELAS has been shown to include bilateral hearing impairment in late childhood or teenage years and DM, seizures, stroke-like episodes and encephalopathy in the third or fourth decade, leading to death. Laboratory findings in patients with 3243A>G include elevated lactate and pyruvate and an increased lactate/pyruvate ratio in serum and/or cerebrospinal fluid. Elevated serum alanineaminotransferase, anaemia and elevated creatine kinase may also be detected. Typical histological changes associated with mitochondrial diseases can be seen in muscle biopsies of patients with 3243A>G. Ragged red fibres can be seen when using the Gomori trichrome stain in histological analysis, where they appear as red-stained, irregular granular deposits, which contain large subsarcolemmal and intermyofibrillar collections of mitochondria, increased numbers of neutral lipid droplets and excess of glycogen. The histochemical enzyme reaction for COX may be used to evaluate mitochondrial myopathies, as COX contains subunits encoded by both the mitochondrial and the nuclear genome. The COX stain gives brown colour to normal mitochondria thus revealing their distribution in muscle cell. Extreme variability in COX activity of mitochondria of a cell indicates a probable mtDNA mutation, whereas more homogeneous distribution of COX negative mitochondria suggests defect in nDNA (Johnson *et al.* 1993). Majority of ragged red fibres is COX-deficient and contains higher proportion of mutant mtDNA than histologically or histochemically normal muscle fibres (Ozawa *et al.* 1997). Enlarged mitochondria, absence of cristae and intramitochondrial paracrystalline inclusions can be seen under electron microscopy (Taylor *et al.* 2004). Computed tomography and brain magnetic resonance imaging may reveal parieto-occipital stroke lesions, basal ganglia calcifications and cerebellar or cerebral atrophy (Lien *et al.* 2001).

The mutation 3243A>G has been related to MELAS after analysing mtDNA by molecular genetic methods (Goto *et al.* 1990). Although several other point mutations in mtDNA have also been described in patients with MELAS (Chinnery *et al.* 1999), it is clearly the one that is most commonly found in these patients (Goto *et al.* 1990, Goto *et al.* 1992). In epidemiological studies the prevalence of 3243A>G has been shown to be 0.95/100 000 adults in North East of England, when adults with suspected mitochondrial disease were examined in neurological department of a tertiary hospital during 15 years and family members of verified mutation carriers were evaluated (Chinnery *et al.* 2000). When patients with diagnoses common with 3243A>G have been screened for the

mutation in tertiary hospital in Northern Ostrobothnia in Finland the prevalence of 3243A>G has been shown to be 16.3/100 000 adults (Majamaa *et al.* 1998). The phenotypes related to 3243A>G have been shown to be variable, and MERRF (Fabrizi *et al.* 1996), MELAS with MERRF (Campos *et al.* 1996), maternally inherited CPEO (Moraes *et al.* 1993), MELAS with CPEO (Mariotti *et al.* 1995), maternally inherited mitochondrial diabetes mellitus (MDM) (van den Ouweland *et al.* 1992, Chinnery *et al.* 1997), HCM (Sato *et al.* 1994, Anan *et al.* 1995), DCM (Vilarinho *et al.* 1997), myopathy (Kärppä *et al.* 2005) and sensorineural hearing impairment with short stature (Majamaa *et al.* 1998) have all been described. Screening of pedigrees with 3243A>G has also pointed to some carriers of the mutation who have no symptoms at the time. The diversity of the phenotype in patients with 3243A>G calls for collaboration specialities within the field of medicine with respect to the diagnosis and treatment of such cases.

Diabetic patients with 3243A>G account for 0.5% - 2.8% of the diabetic population (Gerbitz *et al.* 1995, Maassen & Kadowaki 1996, Majamaa *et al.* 1998). Nearly all patients with 3243A>G develop DM or impaired glucose tolerance before the age of 70 years, and MDM manifests itself at an average age of 38 years (Maassen *et al.* 2004) and has characteristics that differ from both type 1 and type 2 DM. It is maternally inherited, and a progressive loss of insulin secretion in response to glucose is detected, whereas insulin deficiency on account of a reduction in  $\beta$ -cells is the mechanism leading to hyperglycaemia in type 1 DM and resistance to normal or elevated levels of insulin in type 2 DM. Patients with 3243A>G lack antibodies against Langerhans islet cells and usually have a low body mass index (Suzuki *et al.* 2003). Insulin resistance seems to be rare in these patients. The exact mechanism leading to impaired insulin secretion in MDM is not completely known. Normally glucose is converted into pyruvate in the pancreatic  $\beta$ -cells and the pyruvate is taken up into the mitochondria by pyruvate dehydrogenase in order to enter the TCA cycle and OXPHOS. OXPHOS increases the ATP/ADP ratio in  $\beta$ -cells, leading to closure of the  $K_{ATP}$  channel and depolarisation of the plasma membrane and subsequent opening of the  $Ca^{2+}$  channel, triggering the release of vesicles containing insulin. In carriers of 3243A>G, however, impaired pancreatic insulin secretion in response to glucose stimulation has been detected, possibly because OXPHOS function is reduced in these patients and the production of ATP is diminished, leading to a reduction in the ATP/ADP ratio that is needed for closing of the  $K_{ATP}$  channels and opening of the  $Ca^{2+}$  channels, thereby attenuating the secretion of insulin (Maassen *et al.* 2004).

There are few studies on the causes of death in patients with mitochondrial disorders. Severely affected patients with 3243A>G have been reported to have died during childhood or in early adulthood, with causes of death that have included cardiomyopathy (Terauchi *et al.* 1996, Kojima *et al.* 2003) and sudden cardiac death (Mangiafico *et al.* 2004) status epilepticus (Huang *et al.* 2002), lactic acidosis (Klopstock *et al.* 1999, Tsuchiya *et al.* 1999, Okhuijsen-Kroes *et al.* 2001), paralytic ileus (Hiel *et al.* 1998) and aortic dilatation and rupture (Tay *et al.* 2006). A case series describing 16 deceased patients with mitochondrial disorders included seven persons with 3243A>G and four affected relatives of a 3243A>G carrier. Four of these patients had died of heart failure and four of status epilepticus (Klopstock *et al.* 1999).

## 2.3 Cardiac involvement in mitochondrial diseases

Cardiomyopathy is seldom the only manifestation of mitochondrial disease, but is part of a multisystem disease involving symptoms and findings in the nervous system, sensory organs, skeletal muscles and endocrine organs. Since the function and structure of the mitochondria are under dual genetic control, mutations in both mtDNA and nDNA are related to diseases with a disturbance of energy metabolism. mtDNA contains genes for proteins essential for proper functioning of the respiratory chain reactions, while nDNA contains large numbers of genes responsible for subunits of the respiratory chain complexes and proteins needed for the integrity and assembly of mitochondria and mtDNA and the replication of mtDNA. The proteins responsible for intergenomic signalling between nDNA and mtDNA are encoded by nDNA. The structure of the lipid milieu of the mitochondrial inner membrane plays an important role in the proper function of the respiratory chain reactions, and deficiencies in  $\beta$ -oxidation have been associated with both HCM and DCM.

Ultrastructural cardiac findings in patients with mitochondrial cardiomyopathy include striking mitochondrial hyperplasia and dispersal of the sarcomeres (Vallance *et al.* 2004). An experimental model has shown that knock-out mice lacking manganese superoxide dismutase, an intramitochondrial free radical scavenging enzyme, die of DCM during the first days of life (Li *et al.* 1995), whereas knock-out mice deficient in the heart and muscle isoform of the adenine nucleotide translocator (ANT1), which transports ATP from the mitochondrion to the cytoplasm, displayed cardiac hypertrophy with mitochondrial proliferation (Graham *et al.* 1997). Mice deficient in the nucleus-encoded catalytic subunit of DNA polymerase (POLGA) needed for the translation of mtDNA developed left ventricular enlargement and hypertrophy (Trifunovic *et al.* 2004).

### 2.3.1 Point mutations in mtDNA

Cardiomyopathy has been described in patients with point mutations in the genes encoding tRNA<sup>Leu</sup> and tRNA<sup>Ile</sup>, tRNA<sup>Lys</sup> and tRNA<sup>Gly</sup>. Mutations in tRNA<sup>Leu</sup> have usually been associated with multisystem disorders such as MELAS, whereas mutations in tRNA<sup>Ile</sup> have been found in diseases that involve cardiomyopathy primarily or in combination with myopathy. The point mutations in mtDNA described in patients with cardiomyopathy are shown in Table 1. A common cardiac finding in association with these mutations is HCM, which usually manifests itself in early adulthood, although it can be present during childhood, whereupon it has a poor prognosis (Silvestri *et al.* 1994, Santorelli *et al.* 1995, Merante *et al.* 1996, Dipchand *et al.* 2001). Cardiomyopathy in relation to syndromes caused by a mtDNA mutation, such as MELAS or MERRF, seems to manifest itself during adulthood. Limited data exist on the rate of progression of cardiac symptoms and findings in patients with a mtDNA point mutation. The left ventricular posterior wall in the diastole (PWd) became thicker and left ventricular systolic function decreased in three patients during a 6.9 years follow-up of six patients with 3243A>G. (Okajima *et al.* 1998).

Echocardiographic studies have revealed symmetrical left ventricular hypertrophy (LVH) to be the most common cardiac finding in patients with the 3243A>G mutation (Sato *et al.* 1994, Anan *et al.* 1995, Okajima *et al.* 1998, Lev *et al.* 2004). Increased vacuolation, fibrosis and mitochondrial size, with an abnormal configuration of cristae, have been seen in microscopic evaluations of endomyocardial biopsy samples from such patients (Momiya *et al.* 1999, Koga *et al.* 2000), and interestingly, impaired cardiac energy metabolism as measured by phosphorus magnetic resonance imaging has been demonstrated in patients with 3243A>G with or without LVH by comparison with controls (Lodi *et al.* 2004). There are fewer reports of congestive cardiomyopathy without left ventricular dilatation or LVH (Nan *et al.* 2002) and DCM in these patients (Vilarinho *et al.* 1997). The frequency and severity of the cardiac manifestations have remained obscure, however, as the earlier reports have been based on small numbers of selected patients.

The mtDNA mutation 8344A>G in the tRNA<sup>Lys</sup> gene is the most common mutation in patients with MERRF syndrome, characterized by myoclonus epilepsy with ragged red fibers, cerebellar ataxia, dementia, and myopathy. The clinical severity of the disease can vary, with common clinical manifestations including myopathy, neuropathy, hearing loss, dementia, short stature and optic atrophy. Cardiomyopathy, pigmentary retinopathy, pyramidal signs, ophthalmoparesis, multiple lipomas and DM occur less often (Chinnery *et al.* 1997). The cardiac manifestations related to the 8344A>G mutation have been shown to be symmetric or septal LVH with diffuse motion disturbance in the left ventricular wall (Anan *et al.* 1995), but HCM has also been described in an infant with sudden death (Vallance *et al.* 2004). HCM has been shown to be the cardiac manifestation in patients with two other mutations in the tRNA<sup>Lys</sup> gene (Table 1) (Santorelli *et al.* 1996, Ozawa *et al.* 1997, Sakuta *et al.* 2002).

Leber's hereditary optic neuropathy (LHON) is one of the most common mitochondrial genetic diseases, with an estimated prevalence of 1 in 25 000 in the north-east of England (Man *et al.* 2003). This is characterized by acute or subacute impairment in both eyes, mostly in men during early adulthood or mid-life (Wallace *et al.* 1988). The mtDNA mutations 11778G>A, 3460G>A and 14484T>C are found in 95% of all LHON cases, and prolongation of the corrected QT interval (Ortiz *et al.* 1992) together with the Wolf-Parkinson-White and Lown-Ganong-Levine syndromes (Nikoskelainen *et al.* 1994, Finsterer *et al.* 2001) have been described in these patients. A patient with an accompanying mutation 12192G>A has been described as having developed HCM (Mimaki *et al.* 2003), and left ventricular abnormal trabeculation has been detected by echocardiography in two brothers with LHON and 3460G>A (Finsterer *et al.* 2001).

Reports concerning mtDNA mutations in DCM are infrequent. 3243A>G has been found abundantly (level of mutation heteroplasmy 88%) in a muscle specimen from a six-year-old boy with poor physical growth, asthenia and DCM (Vilarinho *et al.* 1997), 12192G>A has been found in two Japanese brothers with DCM and without symptoms in their nervous system or endocrine organs, and the same mutation has been found in two out of 55 patients screened for DCM (Shin *et al.* 2000). A high level of mutation 12297T>C heteroplasmy has been found in the cardiac muscle of a 36-year-old male with DCM (Grasso *et al.* 2001), and altogether 18 mutations in mtDNA have been described in patients with DCM and ultrastructural abnormalities in the cardiac mitochondria, affecting the same mitochondrial genes as mentioned above (tRNA<sup>Lcu</sup>, tRNA<sup>Llc</sup> and

tRNA<sup>Thr</sup>), but also the genes encoding individual proteins of the respiratory chain (ND1, ND2) and 12S and 16S rRNAs. Two mutations in this population had familial presentation (Arbustini *et al.* 1998). Another evaluation of the presence of the common point mutations in mtDNA (3243A>G, 3252A>G, 3260A>G, 4269A>G, 8344A>G, 8993T>G/C and 9997T>C) among unselected patients with idiopathic cardiomyopathy found none of these mutations in 52 patients with DCM and ten with HCM (Turner *et al.* 1998), whereas several point mutations altering the function of enzyme subunits of the respiratory chain were found among 45 patients with DCM after the whole mtDNA had been analysed, being present more commonly in the patients with DCM than in 62 controls without DCM (Ruppert *et al.* 2004).

*Table 1. Cardiomyopathy-related tRNA point mutations in mtDNA*

Gene	Site	Biochemical defect	Phenotype <sup>Reference</sup>
Leu	3243A>G	Complex I and IV	MELAS, HCM <sup>1),2),3)</sup>
Leu	3260A>G	Complex I and IV	CM and myopathy <sup>4),5)</sup>
Leu	3280A>G	N.R.	CM and myopathy <sup>6)</sup>
Leu	3303C>T	N.R.	CM <sup>7)</sup>
Leu	12297T>C	N.R.	DCM
Ile	4295A>G	Complex I, III, IV	HCM <sup>9), 10)</sup>
Ile	4269A>G	N.R.	DCM, encephalopathy, deafness, epilepsy <sup>11)</sup>
Ile	4300A>G	N.R.	HCM <sup>12), 13)</sup>
Ile	4320C>T	N.R.	MELAS, HCM <sup>14)</sup>
Lys	8296A>G	N.R.	HCM <sup>15)</sup>
Lys	8344A>G	Complex I, IV	MERRF, HCM <sup>3), 16)</sup>
Lys	8363G>A	Complex I, III, IV	Encephalomyopathy, sensorineural hearing loss, HCM <sup>17)</sup> , MERRF, mild CM <sup>18)</sup>
Gly	9997T>C	N.R.	HCM during childhood <sup>10)</sup>
His	12192G>A	N.R.	DCM <sup>19)</sup>

Leu: leucine; Ile: isoleucine; Lys: lysine; Gly: glycine; MELAS: Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; CM: cardiomyopathy; HCM: hypertrophic cardiomyopathy; DCM: dilated cardiomyopathy; MERRF: myoclonus epilepsy with ragged red fibres; N.R. not reported

<sup>1)</sup>(Obayashi *et al.* 1992), <sup>2)</sup>(Sato *et al.* 1994), <sup>3)</sup>(Anan *et al.* 1995), <sup>4)</sup>(Zeviani *et al.* 1991), <sup>5)</sup>(Mariotti *et al.* 1994), <sup>6)</sup>(Campos *et al.* 2003), <sup>7)</sup>(Silvestri *et al.* 1994), <sup>8)</sup>(Grasso *et al.* 2001), <sup>9)</sup>(Merante *et al.* 1996), <sup>10)</sup>(Dipchand *et al.* 2001), <sup>11)</sup>(Taniike *et al.* 1992), <sup>12)</sup>(Casali *et al.* 1995), <sup>13)</sup>(Taylor RW *et al.* 2003a), <sup>14)</sup>(Santorelli *et al.* 1995), <sup>15)</sup>(Sakuta *et al.* 2002), <sup>16)</sup>(Vallance *et al.* 2004), <sup>17)</sup>(Santorelli *et al.* 1996), <sup>18)</sup>(Ozawa *et al.* 1997), <sup>19)</sup>(Shin *et al.* 2000).

### 2.3.2 mtDNA rearrangements

Rearrangements in mtDNA include deletions and occasionally duplications. The mutation can arise during oogenesis or the early development of the embryo (DiMauro & Schon 2003). Deletion mutations in mtDNA are sporadic or inherited in either an autosomally dominant or recessive manner (Bohlega *et al.* 1996, Schroder *et al.* 2000, Kiechl *et al.* 2004). The prevalence of large-scale mtDNA deletions in the general population is 1.2-

1.6/100 000 (Chinnery *et al.* 2000, Remes *et al.* 2005). Related diseases are Kearns–Sayre syndrome (KSS), Pearson’s syndrome and CPEO.

Ophthalmoplegia (paralysis of the extra-ocular eye muscles), pigmentary retinopathy and cardiac conduction blocks are present in patients with KSS before the age of 20 years (Kearns & Sayre 1958), and both HCM and DCM have been detected in these patients (Channer *et al.* 1988, Marin-Garcia *et al.* 2002). Approximately one third of Kearns–Sayre syndrome patients harbour the same kind of mtDNA deletion (Moraes *et al.* 1989) extending over 4,977 base pairs within the region 8470-13459, often termed “the common 5-kilobase deletion”. The degree of heteroplasmy of the mutation has been shown to be higher in the cardiac conduction system than in the myocardium of a patient with both a complete atrioventricular block and congestive cardiomyopathy (Muller-Hocker *et al.* 1998).

Pearson’s bone marrow-pancreas syndrome affects mainly haematopoietic cells and the exocrine pancreas. A mtDNA deletion in bone marrow stem cells blocks the replication of these cells and leads to refractory sideroblastic anaemia, leukopenia and thrombocytopenia (Cormier *et al.* 1990, Rotig *et al.* 1990). Patients die from pancytopenia in childhood (Superti-Furga *et al.* 1993, Muraki *et al.* 1997, Toth *et al.* 1998, Muraki *et al.* 2001). Cardiomyopathy is a rare manifestation in children with Pearson’s syndrome (Krauch *et al.* 2002), although features of Pearson’s syndrome have been described in a patient with KSS (Becher *et al.* 1999).

CPEO is characterized by ptosis and weakness of the extraocular muscles, typically appearing in childhood or young adulthood. Limb muscle weakness or other cranial nerve manifestations, respiratory muscle weakness and DCM have also been described in these patients (Moslemi *et al.* 2000).

Deletions ranging from 4.4 kb to 11.1 kb were found in five children with cardiomyopathy when analysing the mtDNA of 28 children with cardiomyopathy and neurological symptoms, including KSS, seizures, ataxia developmental delay. Four of these patients had had DCM, one had had HCM and two of them had died suddenly and unexpectedly (Marin-Garcia *et al.* 2002).

### ***2.3.3 Mutations in nuclear DNA and disturbance in intergenomic signalling***

Mutations in nDNA-encoded subunits of the respiratory chain have been found in complex I and II, the related disorders being severe neurological diseases occurring during childhood, such as subacute necrotizing encephalomyopathy, or Leigh’s syndrome (Leigh 1951, Smeitink *et al.* 2004). HCM or congestive cardiomyopathy has been found in children with mutations coding for the components of respiratory chain complex I (Loeffen *et al.* 1998).

No mutations have been found in any of the nuclear-encoded subunits of OXPHOS complexes III, IV and V, but there are reports concerning mutations in nDNA coding for the ancillary proteins needed for the assembly or insertion of cofactors into complexes III (de Lonlay *et al.* 2001, Visapää *et al.* 2002) and IV (Shoubridge 2001a). nDNA carries genes coding four different factors important for the biogenesis of the cytochrome

oxidase. Mutation in the surfeit gene (SURF1) is associated with clinical phenotype of Leigh syndrome (Tiranti *et al.* 1998). Hypertrophic cardiomyopathy has been detected in children with mutations in the gene of SCO2 and COX15 (Antonicka *et al.* 2003) required for the synthesis of cytochrome oxidase (Papadopoulou *et al.* 1999). COX10 gene and SCO1 gene also affect the assembly of respiratory chain complex IV and mutations in them have been associated with tubulopathy and leukodystrophy or ketoacidotic coma and hepatopathy (Antonicka *et al.* 2003).

nDNA contains genes essential for the maintenance of the mtDNA. Impaired mtDNA stability leading to multiple deletions or even depletion of mtDNA has been detected in several clinical phenotypes involving disorders inherited in an autosomal recessive or dominant manner. A mutation in the gene encoding adenine nucleotide translocator (ANT1) has been associated with multiple mtDNA deletions resulting in CPEO (Kaukonen *et al.* 2000) and the rare Senger's syndrome with congenital cataracts, HCM, mitochondrial myopathy and lactic acidosis (Sharer 2005), and a patient with HCM has been shown to harbour a homozygous mutation in the ANT1 gene (Palmieri *et al.* 2005). Mutations in polymerase  $\gamma$  (POLG), which is essential for the replication of mtDNA (Kaguni 2004), have been found in patients with autosomal dominant and recessive CPEO, parkinsonism and premature menopause (van Goethem *et al.* 2001, Luoma *et al.* 2004), but there are no data concerning cardiac abnormalities in patients with mutations in POLG so far. Mice with the mutation have been shown to develop an enlarged, hypertrophic left ventricle with COX deficiency in some cardiomyocytes, features that have also been observed in ageing human heart (Trifunovic *et al.* 2004). The twinkle gene, the third nuclear gene affecting mtDNA maintenance, is related to autosomal dominant CPEO (Spelbrink *et al.* 2001).

Mutations in genes encoding proteins essential for the synthesis of cardiolipin, the main constituent of the mitochondrial inner membrane where the components of OXPHOS are located, have been shown to be related to Barth syndrome, characterized by mitochondrial myopathy, cardiomyopathy, growth retardation and leukopenia (Barth *et al.* 1999). Defects in nDNA-encoded genes involved in mtDNA repair are also likely to lead to mtDNA abnormalities (Hirano *et al.* 2001).

### **2.3.4 Defects in $\beta$ -oxidation**

Defects in  $\beta$ -oxidation in mitochondria lead to insufficiency in the carnitine-dependent transport of long-chain fatty acids across the mitochondrial membranes.  $\beta$ -oxidation can be impaired because of 1) deficiency of carnitine, 2) deficiency of carnitine-acylcarnitine translocase (CACT), or 3) deficiencies of carnitine palmitoyltransferases I and II (CPT I and CPT II). The cardiomyopathy involved in these defects is mostly of the dilated kind and appears during childhood or early adulthood (Antozzi & Zeviani 1997). Enzyme defects in  $\beta$ -oxidation include 1) long-chain (LCAD) and very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, 2) mitochondrial trifunctional protein deficiency and 3) defects affecting electron-transferring flavoproteins.

Mutations in genes encoding LCAD have been related to neurological symptoms, myopathy and HCM during childhood (Rocchiccioli *et al.* 1990, Bertini *et al.* 1992,

Bertrand *et al.* 1993), while mutations resulting in no residual activity of VLCAD have been related to a phenotype with early onset, high mortality and high incidence of cardiomyopathy (Andresen *et al.* 1999). In mitochondrial trifunctional protein deficiency cardiomyopathy can accompany the leading manifestations of hypoglycaemia and hepatic dysfunction (Pons *et al.* 1996). Heterozygotes for organic cation transporter gene mutations in families of patients with primary carnitine deficiency were predisposed to late onset benign cardiac hypertrophy (Koizumi *et al.* 1999). If children with defects in electron-transferring proteins survive for a few months, they may present with cardiomyopathy (Loehr *et al.* 1990).

## 2.4 Inherited cardiomyopathies

HCM is diagnosed when LVH and fibrosis of no known cause are detected. The prevalence is estimated to be 2 in 1000 people, and a figure of 3 in 100 000 people has been reported in Finnish children and adolescents (Arola *et al.* 1997). HCM is genetic in origin, although sporadic cases have also been described, and it is inherited as an autosomal dominant trait (Haugland *et al.* 1986, Watkins *et al.* 1992). More than 400 mutations causing HCM have been described in 10 genes coding for sarcomeric proteins, including  $\beta$ -myosin heavy chain, cardiac troponin T, myosin-binding protein C, alpha-tropomyosin, cardiac troponin I, myosin light chains 1-2, alpha-cardiac actin, titin, alpha-myosin heavy chain and LIM protein (Roberts & Sigwart 2005). Mutations in the gene for myosin binding protein C have been reported to be the most common cause of inherited HCM. The mutations either hinder the synthesis of the structural sarcomeric protein or lead to the production of an abnormal sarcomeric protein. Mutations in genes not coding for sarcomeric proteins have also been related to hypertrophic cardiomyopathy, e.g. that coding for the alpha2 subunit of AMP kinase, which is related to the WPW syndrome, conduction abnormalities and HCM (Arad *et al.* 2002). HCM has been related to a mutation in the phospholamban promoter, which is an endogenous inhibitor of calcium ATPase in the sarcoplasmic reticulum and a regulator of cardiac relaxation (Minamisawa *et al.* 2003)

HCM is characterized by symmetric or asymmetric hypertrophy of the left ventricular wall with increased myocyte and myofibrillar size and disarray (Spirito *et al.* 1997). Left ventricular outflow tract obstruction by a hypertrophied septum and mitral valve abnormalities can be detected by echocardiography in these patients. The phenotype of HCM is variable, ranging from mild LVH without symptoms to notable thickening of the left ventricular walls, disabling symptoms of heart failure and sudden cardiac death (Harris *et al.* 2006). Different mutations are related to different phenotypes and prognoses. Mutations in the  $\beta$ -myosin heavy chain gene are related to distinct LVH (Woo *et al.* 2003), whereas mutations in the troponin T gene are related to mild or no LVH and a high risk of sudden cardiac death (Moolman *et al.* 1997).

DCM is a familial disease in 30-50% of cases (Keeling *et al.* 1995, Grunig *et al.* 1998, Baig *et al.* 1998) and has been attributed to more than 20 mutations in genes encoding sarcomeric, cytoskeletal and nuclear proteins, and also proteins involved in the regulation of  $\text{Ca}^{2+}$  metabolism. Mutations in the gene for lamin A/C, one of the nuclear proteins

connecting the nuclear envelope, nuclear matrix and chromatin, are one of the most frequently detected mutations in patients with DCM (Fatkin *et al.* 1999, Jakobs *et al.* 2001, Hershberger *et al.* 2002, Arbustini *et al.* 2002, Verga *et al.* 2003, Kärkkäinen *et al.* 2004). The typical clinical findings in these patients are progressive conduction abnormalities, atrial fibrillation, malignant ventricular arrhythmias and the need for a permanent cardiac pacemaker or defibrillator or heart transplantation (Taylor MR *et al.* 2003, Kärkkäinen *et al.* 2004, Van Berlo *et al.* 2005). Mutations in the  $\beta$ -myosin heavy chain gene have been found in up to 10% of patients with DCM (Villard *et al.* 2005) and are associated with relatively malignant phenotypes such as DCM, which manifests itself during the first weeks after birth, sudden death related to DCM or the need for a heart transplantation (Kamisago *et al.* 2000). Mutations in the genes coding for troponins T, I and C reduce the interaction between these proteins that form a complex actively triggering actin–myosin binding under the influence of  $\text{Ca}^{2+}$ . These mutations have been shown to be related to DCM and a poor prognosis (Li *et al.* 2001, Mogensen *et al.* 2004, Murphy *et al.* 2004). Several other mutations in the genes for desmin, alpha-actinin, dystrophin, metavinculin, delta-sacoglycan, LIM-protein, Cypher/ZASP, thymopoietin, SCN5A, phospholamban, cardiac actin, alpha-tropomyosin, myosin-binding protein C, titin and telethonin have been described in a few families each. DCM has also been shown to associate with X-linked recessive mutations in the dystrophin gene in Duchenne and Becker muscular dystrophy and in the XK membrane transport protein gene in McLeod's syndrome (Cohen & Muntoni 2004).

## 2.5 Cardiomyopathy associated with diabetes mellitus

Diabetic cardiomyopathy is a clinical condition diagnosed if ventricular dysfunction is found in patients with DM in the absence of coronary atherosclerosis or hypertension (Poornima *et al.* 2006). Epidemiological studies have pointed to an increased risk of heart failure in diabetic patients even after considering their age, blood pressure, weight, cholesterol and history of coronary artery disease (Kannel *et al.* 1974, Ho *et al.* 1993). Left ventricular wall thickness has been shown to be higher in patients with adult onset DM than in age and sex-matched controls independent of blood pressure (Galderisi *et al.* 1991, Carugo *et al.* 2001, Devereux *et al.* 2001), and LVH to be related to insulin resistance in patients without DM as well (Phillips *et al.* 1998). On the other hand, patients with unexplained idiopathic DCM have a 75% greater likelihood of suffering from DM than age-matched controls (Bertoni *et al.* 2003).

There are several mechanisms leading to cardiomyopathy in patients with DM.  $\beta$ -cell failure, leading to hypoinsulinaemia and hyperglycaemia, results in altered calcium homeostasis in the myocardial cells, impaired diastolic function, altered contractile proteins and progressive systolic dysfunction in the presence of type I DM (Poornima *et al.* 2006). Insulin resistance has been shown to be the basic mechanism in type II DM. The abnormalities in lipid metabolism observed in type II diabetics include hypertriglyceridaemia and elevated levels of non-esterified fatty acids, which have been shown to exacerbate insulin resistance. The resulting hyperinsulinaemia is related to myocyte hypertrophy and eventually leads to diastolic dysfunction. Non-esterified fatty

acids are also thought to modulate myocardial contractility by opening the  $K_{ATP}$  channels (Liu *et al.* 2001).

## 2.6 Heart rate variability

Heart rate is normally determined by depolarization of the sino-atrial node, which is innervated with post-ganglionic sympathetic and parasympathetic nerve terminals. Heart rate variability (HRV) is defined as the existence of beat-to-beat fluctuations in sinus rhythm. The analysis of HRV from 24-hour electrocardiograph (ECG) recordings is a non-invasive tool for assessing cardiovascular autonomic regulation. It is easy to perform, has relatively good reproducibility and has been shown to provide prognostic information on patients with heart disease (Kleiger *et al.* 1987, Casolo *et al.* 1989, Hayano *et al.* 1990, van Hoogenhuyze *et al.* 1991, Bigger, Jr. *et al.* 1992, Bigger, Jr. *et al.* 1996, Huikuri *et al.* 1998).

Several methods have been developed for assessing features of HRV. Time domain methods can be used to analyse a sequence of successive RR intervals or RR interval differences and correlate well with vagal activity (Eckberg 1983, Hayano *et al.* 1991). Average heart rate and the standard deviation in all normal-to-normal RR intervals (SDNN) are the most widely used time domain measures. Each RR interval is recorded on a Poincaré plot as a function of the previous RR interval. Both short-term and long-term HRV can be calculated in terms of these RR intervals (Huikuri *et al.* 1990, van Hoogenhuyze *et al.* 1991, Hohnloser *et al.* 1992, Huikuri *et al.* 1996).

Frequency domain or spectral analysis quantifies the oscillatory frequency of the heart rate in addition to the amount of variability (Akselrod *et al.* 1981). Spectral analysis is visualized with a power spectrum, which reflects the amplitude of the heart rate fluctuations present at different oscillation frequencies. The total power of the power spectrum is divided into four frequency bands as follows: ultra-low frequency (ULF),  $< 0.0033$  Hz; very low frequency (VLF), 0.0033-0.04 Hz; low frequency (LF), 0.04-0.15 Hz and high frequency (HF), 0.15-0.4 Hz (Task Force 1996). The power of the HF, LF, VLF and ULF components is usually expressed in absolute units ( $ms^2$ ).

A commonly used non-linear method for analysing HRV is detrended fluctuation analysis, which quantifies the presence or absence of fractal correlation properties in the time series (Peng *et al.* 1995). Heart rate correlations have been usually defined for short-term ( $\alpha_1 < 11$  beats) and long-term ( $\alpha_2 > 11$  beats) RR interval data. Values of  $\alpha$  near 1 indicate the fractal-like behaviour seen in healthy subjects (Pikkujämsä *et al.* 1999), while there is some evidence that increased sympathetic activation is associated with impairment of the fractal dynamics of heart rate. Intravenous infusion of norepinephrine and concomitant sympathetic and vagal activation are shown to be related to reduced values of  $\alpha$  (Tulppo *et al.* 2001, Tulppo *et al.* 2005). The power law relationship reflects the density distribution of the power-spectrum over the frequency range  $10^{-4}$  to  $10^{-2}$  Hz, thus reflecting mainly fluctuations between ULF and VLF power in the power spectra. The steeper the slope of the power law relationship ( $\beta$ ) is, the greater is the ULF power relative to the VLF power (Bigger, Jr. *et al.* 1996). Approximate entropy (ApEn) can be used to quantify the regularity and complexity of time series data (Pincus 1991, Pincus &

Viscarello 1992, Pincus & Goldberger 1994), with low values indicating a more regular (less complex) signal and high values irregularity (more complex signal behaviour).

Baroreflex sensitivity is the relationship between a change in blood pressure and the RR interval in heart rate. Increased blood pressure causes vascular stretch and activates baroreceptors in the arterial walls of the carotid sinuses and aortic arch, triggering adjustments in autonomic outflow, HR and vascular resistance to buffer excessive fluctuations in blood pressure (Eckberg *et al.* 1971, Vanoli & Adamson 1994). BRS can be measured by a phenylephrine technique (Eckberg *et al.* 1971).

## 2.7 Heart rate variability and heart rate dynamics in different populations

HRV in healthy children has been shown to possess similar complexity (ApEn) and fractal correlation properties ( $\alpha_1$ ,  $\alpha_2$ ) of RR interval dynamics and a similar distribution of the lower frequencies of the power spectra ( $\beta$ ) to the HRV indices for young adults, despite the lower spectral and time-domain measures found in children. A progressive loss of complexity and alterations in long-term fractal-like heart rate behaviour have been shown from middle age to old age (Pikkujämsä *et al.* 1999), and the time and frequency domain measures of HRV have been shown to be lower in elderly people than in middle-aged or young subjects (Shannon *et al.* 1987, Hayano *et al.* 1991, Bigger, Jr. *et al.* 1995, Jensen-Urstad *et al.* 1997, Pikkujämsä *et al.* 1999). Non-linear HR dynamics seem to decline during ageing, reflecting a decrease in autonomic modulation with advancing age (Iyengar *et al.* 1996, Peng *et al.* 2002, Beckers *et al.* 2006) and a decrease in the power-law relationship has been shown to predict cardiovascular and cerebrovascular mortality in healthy aged people (Huikuri *et al.* 1998).

Cardiac autonomic regulation is reduced in patients with ischaemic heart disease, regardless of whether they have had a myocardial infarction and/or left ventricular systolic dysfunction or stable coronary artery disease (Bigger, Jr. *et al.* 1995, Bigger, Jr. *et al.* 1996, Huikuri *et al.* 1996, Huikuri *et al.* 2000, Bauer *et al.* 2006).  $\alpha_1$  has been shown to be depressed in patients with susceptibility to ventricular tachycardia after myocardial infarction (Mäkikallio *et al.* 1997) and to predict total mortality after myocardial infarction irrespective of left ventricular function (Tapanainen *et al.* 2002). Also, a decreased BRS after myocardial infarction has been shown to predict cardiac mortality (La Rovere *et al.* 1998). Impaired SDNN, VLF and LF have been observed to predict acute myocardial infarction and unstable angina pectoris in a community-based population free of clinically apparent coronary heart disease or congestive heart failure (Tsuji *et al.* 1996).

Damage to the autonomic nerve fibres that innervate the heart and blood vessels results in abnormalities in heart rate control and vascular dynamics in diabetic patients (Maser *et al.* 2003), for whom reduced HRV is the earliest indicator of cardiac autonomic neuropathy, a condition which has been shown to be related to increased total and cardiovascular mortality in such patients (Haffner *et al.* 1998, Maser *et al.* 2003). In addition, a reduction in total power in the spectral analysis has been observed to be a sensitive marker of poor glycaemic control in patients with type I DM (Mäkimattila *et al.*

2000). Comparisons of cardiac autonomic regulation between patients with type 1 or type 2 DM and healthy controls have detected a reduction in SDNN and the power spectra of HR variability in these diabetic groups (Malpas & Maling 1990, Pikkujämsä *et al.* 1998, Colhoun *et al.* 2001, Koivikko *et al.* 2005). Cardiac autonomic impairment measured with time domain measures of HRV has been shown to be present soon after diagnosis in patients with type 2 DM, and progressive worsening of autonomic cardiac function has been observed in such cases (Schroeder *et al.* 2005). Hypoglycaemia as such has been shown to be associated with a decrease in cardiac vagal activity detected through a reduction in HF power in the power spectra and short-term beat-to-beat analysis of HRV (Koivikko *et al.* 2005).

HRV has been assessed in many patient groups with LVH, in whom cardiac autonomic regulation has been shown to be depressed irrespective of the cause of LVH. Reduced SDNN has been detected in patients with LVH related to aortic valve stenosis, hypertrophic cardiomyopathy and hypertension, the decrease in SDNN being predictable from the extent of LVH (Alter *et al.* 2006). Lower values for the LF and HF components of the power spectra (Petretta *et al.* 1995) and for BRS (Ylitalo *et al.* 1997, Parati *et al.* 1998) have been detected in patients with hypertension and LVH than in healthy controls, and some data show that disturbed HRV can be normalised with treatment for hypertension, leading to a reduction in LVH (Muiesan *et al.* 1998). There is evidence, though, that the depressed HRV found in hypertensive patients with LVH seems to be related to higher blood pressure rather than to LVH (Perkiomäki *et al.* 1996).

Lower HRV and BRS indices have been found in patients with congestive heart failure than in matched healthy controls (Casolo *et al.* 1989, Grassi *et al.* 1995). Several components of HRV analysis have been shown to predict survival in patients with heart failure (Brouwer *et al.* 1996, Ho *et al.* 1997), whereas a reduced BRS has been related to an increased risk of non-sustained ventricular tachycardia (VT) (Mortara *et al.* 1997).

The ULF and VLF power spectral components have been shown in previous studies to be reduced in various disorders, e.g. among patients with a previous myocardial infarction (Bigger, Jr. *et al.* 1992, Stein *et al.* 2000), patients with Parkinson's disease (Haapaniemi *et al.* 2001) and patients with temporal epilepsy (Ansakorpi *et al.* 2002). Furthermore, reduced ULF and VLF power spectral components have been shown to be associated with an increased risk of cardiac mortality and life-threatening arrhythmic events among patients with ischaemic heart disease (Bigger, Jr. *et al.* 1992) or chronic heart failure (Guzzetti *et al.* 2005). Reduced  $\alpha_1$  has also been found among patients with heart failure (Ho *et al.* 1997, Huikuri *et al.* 2000, Mäkikallio *et al.* 2001a) and has predicted both overall mortality and sudden cardiac death in various populations (Bigger, Jr. *et al.* 1996, Ho *et al.* 1997, Huikuri *et al.* 1998, Mäkikallio *et al.* 2001b).

Cardiac transplantation abolishes cardiovascular autonomic regulation (Sands *et al.* 1989). Both the spectral and fractal components of HRV have been shown to decrease in patients with neurological illnesses such as Parkinson disease, acute ischaemic stroke or temporal epilepsy (Korpelainen *et al.* 1999, Haapaniemi *et al.* 2001, Ansakorpi *et al.* 2002). The medication provided for cardiovascular diseases may influence HRV. Beta-blocking agents have been observed to improve HRV measures both in an experimental study with healthy subjects and in patients with CAD with or without recent myocardial infarction and patients with advanced congestive heart failure (Niemelä *et al.* 1994, Lin *et al.* 2001). Angiotensin converting enzyme inhibitor has been shown to improve the

spectral components of HRV in patients with hypertension, while a calcium channel blocker had no impact on them (Tomiyama *et al.* 1998).

### **3 Purpose of the present research**

The phenotype related to the mtDNA mutation 3243A>G is variable. Some patients present with sensorineural hearing impairment and DM, while others can have MELAS syndrome. Cardiomyopathy has been frequently described in patients with 3243A>G, but the frequency and severity of the cardiac manifestations has remained obscure, as earlier reports have been based on small numbers of selected patients. The purpose of the present work was to assess the spectrum of cardiac abnormalities in patients with the heterogeneous phenotype of mtDNA mutation 3243A>G. The specific aims of the individual papers were:

1. to evaluate cardiac structural abnormalities in patients with 3243A>G by comparison with age and sex-matched controls (I),
2. to study changes in HR variability and HR dynamics in patients with 3243A>G by comparison to age and sex-matched controls (II),
3. to follow the rate of progression of clinical manifestations in patients with 3243A>G (III), and
4. to assess retrospectively the causes of death in kindreds with 3243A>G carriers (IV).

## 4 Patients

The patients with 3243A>G were selected from the target population of an epidemiological survey performed in Northern Ostrobothnia in Finland, during which 11 pedigrees with this mutation were found (Majamaa *et al.* 1998). Two additional pedigrees were ascertained in the population of the province of Central Ostrobothnia. During the epidemiological survey mtDNA of leukocytes and buccal epithelium of patients with symptoms and findings common in mitochondrial diseases were analysed for detection of the 3243A>G mutation. Adult patients with diabetes mellitus, sensorineural hearing impairment, cardiomyopathy, brain infarct, epilepsy, ataxia, and subjective visual disturbance (including field defects and double vision), an occipital lesion, basal ganglia calcifications, or cerebral white-matter changes as radiological findings were included in the screening (Majamaa *et al.* 1998). Adult patients with the 3243A>G mutation or their first-degree maternal relatives were eligible for the clinical study, the latter having a high probability of being obligatory carriers of the mutation (Chinnery *et al.* 1998). Forty nine different patients were included in at least one and fourteen of them were included in all three of the clinical studies.

Thirty-nine patients with 3243A>G underwent comprehensive cardiological examinations. Echocardiography was performed on 34 of them (I), 24-hour Holter recordings were made for 36 and cardiac catheterization was considered clinically necessary in four cases. Thirteen verified or obligatory carriers were studied by reviewing their case records and autopsy reports. Both clinical and autopsy data were available for four additional deceased cases with the mutation. Patients older than 40 years were matched with respect to age, sex and diagnosis of hypertension with a control chosen from a population-based cohort of 1200 persons 600 of whom were entitled to refund of medication for hypertension and 600 were matched controls living in Oulu (Kauma *et al.* 1998). For younger patients a matched control was chosen from the echocardiographic register at the Department of Cardiology, Oulu University Hospital. The indication for a cardiac evaluation in these cases had been palpitations, shortness of breath or suspicion of a genetic aortic disease. No signs of cardiac abnormalities had been discovered, however.

Twenty-eight subjects were examined in order to study cardiovascular autonomic regulation in patients with 3243A>G (II), together with controls chosen from the general population (Huikuri *et al.* 1996, Pikkujämsä *et al.* 1999). The mutation carriers were

matched with controls with respect to age, sex, the presence of hypertension and the use of a beta-blocker or angiotensin-converting enzyme inhibitor. All the patients with hypertension were on medication and had blood pressure values within the normal limits. Patients with DM were matched with voluntary type I diabetics living in Oulu or nearby parishes. Diabetic controls did not suffer from DM-related end-organ diseases such as retinopathy, nephropathy or atherosclerotic diseases. Left ventricular function was measured by echocardiography in all the subjects and controls.

Thirty-three adult patients with 3243A>G included in studies I and II were recruited randomly for a three-year clinical follow-up. Patients were accepted for the follow up regardless of the related clinical phenotype (III). Demographic data of the populations in the first three studies are shown in Table 2.

Lifetime analysis was conducted on pedigrees constructed after the identification of thirteen probands with 3243A>G (IV) and genealogies were constructed based on information obtained from the population registry in Finland. There were 82 complete sibships who were maternal relatives of a known carrier of 3243A>G, and complete data including date of birth and date of death or the last known date when the subject had been alive were obtained for 278 persons, including 123 deceased cases.

The cause of death was evaluated for all carriers of 3243A>G and their first-degree maternal relatives who had died after 1936, and death certificates for these people, including dates of birth and death and the immediate, underlying and contributory causes of death, were obtained from the Causes of Death Register (CDR) maintained by Statistics Finland (Statistics Finland 1968, Statistics Finland 2005). Death certificates could be retrieved for 47 subjects who had harboured 3243A>G or had been a first-degree maternal relative of a 3243A>G carrier.

All the subjects participating in the clinical studies gave their written, informed consent. The research was approved by the Ethics Committee of the University of Oulu (I-IV), permission for use of the CDR was obtained from Statistics Finland and permission to review the medical records was obtained from the Ministry of Social Affairs and Health (IV).

*Table 2. Patient characteristics in the clinical studies*

Feature	I N = 39	II N = 28	III N = 33
Men/Women N (%)	15/24 (38/62)	9/19 (32/68)	11/22 (33/67)
Age (years)	48 ± 13	41 ± 13	41 ± 13
Hypertension N (%)	10 (26)	6 (21)	12 (36)
Diabetes mellitus N (%)	20 (51)	9 (32)	16 (48)
Muscle heteroplasmy evaluated N (%)	36 (92)	26 (93)	28 (85)

## **5 Methods**

### **5.1 Cardiac examinations**

#### ***5.1.1 Electrocardiography (I, II, III)***

A 12-lead ECG was obtained for each patient included in the clinical studies and interpreted according to standard criteria. LVH was diagnosed whenever R in V<sub>5</sub> or V<sub>6</sub> + S in V<sub>1</sub> exceeded 4.5 mV (Schiller *et al.* 1989).

#### ***5.1.2 Ambulatory ECG recordings (I, II, III)***

Two-channel 24-hour ambulatory ECG recordings were performed during normal daily activities using an Oxford Medilog 4500 Holter recorder (Oxford Medical Ltd., England). A two-channel oscilloscopic display and an arrhythmia analyser were used to detect and quantify arrhythmias. Basic rhythm, average HR, number of ventricular premature beats, episodes of VT (three or more repetitive ventricular beats at a rate of  $\geq 120/\text{min}$ ) and conduction abnormalities were documented.

#### ***5.1.3 Cardiac analysis at autopsy (I)***

The weight of the heart had been documented in 12 autopsies of deceased patients with 3243A>G. In the remaining reports the heart was stated to be normal in size in four cases and hypertrophic in one. Body length was recorded in all the autopsy reports. Cardiac hypertrophy was diagnosed if the weight of the heart was one standard deviation greater than the mean weight of a normal heart stratified by sex and body length (Knight 1991).

### 5.1.4 Echocardiography (I, III)

Echocardiographic measurements were obtained and interpreted according to the standards of the American Society of Echocardiography (ASE) (Schiller *et al.* 1989). The method of Teichholz was used when measuring left ventricular volumes and ejection fractions. Left ventricular wall movement and function were also evaluated using 2-dimensional echocardiography and visual interpretation. LVH was diagnosed when the thickness of the left ventricular diastolic interventricular septum or posterior wall exceeded 12 mm in non-hypertensive patients or 14 mm in hypertensive ones (Feigenbaum 1994) or the left ventricular mass index was 134 g/m<sup>2</sup> for men or 110 g/m<sup>2</sup> for women (Hammond *et al.* 1986). Left ventricular relative wall thickness was assessed as (IVSTd + PWTd)/LVIDd (Reichek & Devereux 1982), where IVSTd is diastolic interventricular septum thickness, PWTd is diastolic posterior wall thickness and LVIDd is left ventricular end-diastolic internal diameter. Relative wall thickness was considered to be increased when the ratio was more than 0.43 (Roman *et al.* 1995). The mitral inflow velocity was measured by pulsed-wave Doppler echocardiography in the apical four-chamber view. Peak early diastolic (E) and peak late diastolic (A) velocities were measured and E/A ratio was used as rough estimate of left ventricle filling. Regurgitation of valves was estimated with pulsed-wave or colour Doppler echocardiography. Echocardiography was repeated after an interval of three years in the follow-up study (III).

### 5.1.5 Analysis of heart rate variability (II)

The lengths of consecutive RR intervals in the ECG data were noted over the whole recording time to form tachograms for the analysis of HRV. Recordings with more than 95% qualified beats for at least an 18-hour period were included in the analysis of heart rate dynamics. First automatic and then manual edition of the RR interval series was performed. Premature beats and artefacts were deleted and the resulting gaps were filled with an average value computed in the local neighbourhood to achieve more stationary data (Huikuri *et al.* 1992).

The SDNN and the average lengths of the RR interval (mean RR) were computed as standard time domain measurements for the entire recording period (Task Force 1996). To make the data more stationary, RR interval data segments of 512 samples were detrended in the frequency domain analysis of HRV (Huikuri *et al.* 1992). The power spectrum densities of HR variability were estimated by a fast Fourier transform method and quantified by measuring the areas in the following frequency bands: ULF power less than 0.0033 Hz, VLF power from 0.0033 to 0.04 Hz, LF power from 0.04 to 0.15 Hz and HF power from 0.15 to 0.40 Hz. The ULF and VLF power spectra were analysed and calculated for the entire recording period, while the LF and HF power spectra were analysed for a time window of 512 RR intervals (Task Force 1996). Natural logarithmic conversion was performed to report the results of study II. Poincaré plot was created by plotting the RR interval of a tachogram as a function of the previous RR interval for a predetermined segment length. Two diameters of the resulting ellipsoid perpendicular to

each other were measured. SD1 was assessed to show the instantaneous beat-to-beat variability in the data, while SD2 was taken as a marker of continuous, long-term RR intervals (Huikuri *et al.* 1996).

We also measured the non-linear indices of HRV by assessing the long-term power-law relationship of RR interval variability and the short-term and long-term fractal correlation properties of heart rate behaviour using detrended fluctuation analysis. Power-law scaling of RR interval variability was performed over the frequency range  $10^{-4}$  to  $10^{-2}$  Hz and a robust line-fitting algorithm of  $\log(\text{power})$  on  $\log(\text{frequency})$  was applied to the power spectrum between  $10^{-4}$  to  $10^{-2}$  Hz and the slope (exponent  $\beta$ ) of this line calculated (Bigger, Jr. *et al.* 1996, Huikuri *et al.* 1998). Detrended fluctuation analysis, in which the root-mean-square fluctuation of integrated and detrended time series was measured on a log-log scale in each observation window, was also used to quantify the fractal-like scaling properties of the RR interval data (Peng *et al.* 1995, Bigger, Jr. *et al.* 1996, Ho *et al.* 1997). Heart rate correlations  $\alpha_1$  and  $\alpha_2$  were defined (Peng *et al.* 1995, Huikuri *et al.* 2000).

ApEn, which expresses the probability of sequences of patterns being close to each other when comparisons are made between consecutive or nearby time series, was measured in order to quantify the regularity or predictability of the time series data. Time series with high regularity produce lower ApEn values, whereas random data produces higher values. Good statistical validity has been found to be associated with input variables  $m = 2$  and  $r = 20\%$  of the standard deviation of the data sets when calculating ApEn (Pincus & Viscarello 1992, Pincus & Goldberger 1994).

### ***5.1.6 Phenylephrine test (II)***

Baroreflex sensitivity (BRS) was measured with the phenylephrine test. A bolus of phenylephrine ( $2 - 2.5 \mu\text{g}/\text{kg}$ ) was injected into the cubital vein while blood pressure was measured continuously with Finapres finger cuff. (Eckberg *et al.* 1971, Airaksinen *et al.* 1993) The aim was to achieve a rise of 15 mmHg in blood pressure in three measurements. BRS was calculated as the mean of the rate-pressure responses of accepted tests. RR intervals and blood pressure were collected digitally and analysed with CAFTS software (Medikro Oy, Finland). The analysis was started from the RR interval after which the sustained increase in systolic blood pressure had begun and ended with the RR interval following the maximal blood pressure elevation. The slope of the linear relationship between the length of the RR interval (ms) and the preceding systolic blood pressure value (mmHg) in the analysis window was calculated using linear least-mean-squares fitting. Each accepted analysis included an increase of at least 15 mmHg in blood pressure and regression lines with a correlation coefficient greater than 0.7 or a significant p-value ( $p < 0.05$ ).

### **5.1.7 Neurological evaluation (I, II, III)**

A detailed interview and full neurological examination took place at the first visit (I, II) and annually during the three years of the follow up (III). The severity of the disease was scored using the Clinical Global Impression of Severity of Illness Rating (CGISIR) scale (Guy 1976), the Clinician's Interview Based Impression of Change (CIBIC) (Knopman *et al.* 1994), the Global Deterioration Scale (GDS) (Reisberg *et al.* 1982) and a modified Rankin score (Rankin 1957).

A brain MRI (III) was obtained with 1.5 T equipment (Signa Horizon, General Electric, Milwaukee, WI) at the initial assessment and after three years, using a 5 mm slice thickness and Fast Spin Echo sequence. Axial T2 and proton density, sagittal T1-weighted, coronal T1-weighted and contrast medium-enhanced T1-weighted coronal images were used.

An EEG (III) was recorded using the Braintronics amplifier (Almere, The Netherlands) and the Stellate Rhythm 10.1. Program (Westmont, Quebec, Canada) following a standardized protocol at entry and after three years. The mean and peak frequencies of the  $\alpha$  band (8-13 Hz) and the entire EEG (0.5-32 Hz) and the absolute and percentage powers in the delta, theta,  $\alpha$  and  $\beta$  bands were calculated.

A detailed cognitive examination (III) was performed at the initial assessment and after the three years of follow-up. The results were interpreted and analysed by a psychologist.

## **5.2 Audiometric measurements (I, III)**

Audiometric measurements were performed in sound-isolated cabins using Midimate 602 audiometers calibrated according to the ISO 389 standard (International Organization for Standardization 1991). Air conduction pure tone thresholds were measured at 0.125, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 kHz and bone conduction thresholds at 0.25, 0.5, 1, 2 and 4 kHz if any of the air conduction thresholds exceeded 10 dB. The better ear hearing level was calculated as the mean of the hearing levels over the frequencies 0.5, 1, 2 and 4 kHz ( $BEHL_{0.5-4kHz}$ ). A level lower than or equal to 20 dB was considered normal (Parving & Newton 1995). Hearing impairment was defined as mild when  $BEHL_{0.5-4kHz}$  was more than 20 dB but lower than 40 dB, moderate when  $BEHL_{0.5-4kHz}$  was equal to or higher than 40 dB but lower than 70 dB, severe when  $BEHL_{0.5-4kHz}$  was equal to or higher than 70 dB but lower than 95 dB and profound when  $BEHL_{0.5-4kHz}$  was equal to or higher than 95 dB (Liu & Xu 1994, Parving & Newton 1995, European Working Group on Genetics of Hearing Impairment, European Commission Directorate 1996). A difference between air and bone conduction of less than 10 dB and a  $BEHL_{0.5-4kHz}$  greater than 20 dB (Parving & Newton 1995) constituted the definition for sensorineural hearing impairment.

### 5.3 Mitochondrial DNA analysis (I, II, III)

Biopsy specimens cut from the anterior tibial muscle or vastus lateralis of the quadriceps muscle were obtained from the patients with 3243A>G as shown in Table 2. Right ventricular endomyocardial biopsies were obtained from four patients during cardiac catheterisation (I) and myocardial samples were obtained from six deceased patients at autopsy (I). Total DNA was isolated from tissue samples by the standard SDS-proteinase K method. The 3243A>G mutation was verified using Apa I restriction digestion of a 390 base pair mtDNA fragment (from nt 3150 to nt 3550) amplified by polymerase chain reaction in the presence of <sup>35</sup>S-dATP (Kobayashi *et al.* 1990). The fragments were electrophoresed through a 6% non-denaturing polyacrylamide gel, which was then dried and autoradiographed at -72°C overnight using Kodak XAR film with an intensifying screen. The intensities of the bands on the autoradiography film were analysed using a GC-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA).

### 5.4 Statistics

The data were analysed using SPSS for Windows, versions 9.0 (I, II), 10.1 (III) and 11.0 (IV) (SPSS Inc., Chicago, Illinois, USA). The Mann-Whitney U test was used to detect differences in variables with skewed distribution between two groups (I, II, III) and the Kruskal-Wallis test between several groups (I). The Wilcoxon signed rank test and the McNemar test were used when comparing paired data with skewed distribution (I, III, IV). The independent samples t-test was used to compare measured values with normal distribution between the patients with 3243A>G and their matched controls (II). The Pearson correlation coefficient was calculated in order to measure correlations between variables with a normal distribution and the Spearman correlation coefficient for variables with skewed distributions (II, III). The paired-samples t test was used to detect differences between continuous variables measured at the beginning and end of the three-year follow-up (III), and the Wilcoxon signed-rank test to estimate differences between the age at death and the respective life expectancy at birth or at the age of 15 years (IV). A p value less than 0.05 was considered significant. Kaplan-Meier survival analysis and log rank statistics were used to compare age at death with life expectancy at birth and at 15 years among the subjects included in the lifetime analysis (IV).

## **6 Results**

### **6.1 Cardiac Abnormalities in Patients with Mitochondrial DNA Mutation 3243A>G (I)**

#### ***6.1.1 Echocardiographic findings in patients with 3243A>G***

Echocardiography was performed on 34 patients with 3243A>G and 34 controls matched with them with respect to age, sex and the diagnosis of hypertension. LVH detected with echocardiography was found in 19 patients with 3243A>G (56%; 95% confidence interval (CI) 38 - 73%) and in six controls (15%; 95% CI 6 - 31%). The median thickness of the diastolic interventricular septum or posterior wall in these cases was 14 mm (range 12 - 23 mm). The left ventricular end-diastolic internal diameter in the patients with 3243A>G was smaller than in the controls (Table 3). Among the autopsies of 17 deceased patients with 3243A>G, nine had LVH, giving a frequency of 53%. The median heart weight in these patients was 472 g (range 322 – 570 g). In total, the frequency of LVH was 55% (95% CI 40 - 69%) among the 47 patients included in the clinical cohort and autopsy cohort. There was no difference in sex distribution, frequency of DM or degree of mutant heteroplasmy in muscle between the patients with and without LVH.

*Table 3. Comparison of echocardiographic findings in patients with 3243A>G and controls (I).*

Measurements	Patients with 3243A>G	Controls	p
IVSTd (mm)	12.2 ± 3.2	10.0 ± 1.7	0.003
PWTd (mm)	12.1 ± 3.7	10.0 ± 1.8	0.02
LVIDd (mm)	43.4 ± 5.8	49.6 ± 5.4	<0.001
LVH* (n)	19	6	0.002
LVMl (g/m <sup>2</sup> )	127 ± 54	102 ± 24	0.06

IVSTd: diastolic interventricular septum thickness; PWTd: diastolic posterior wall thickness; LVIDd: left ventricular end-diastolic internal diameter; LVH: left ventricular hypertrophy; LVMl: left ventricular mass index.

### **6.1.2 Arrhythmias and conduction disturbances**

The cardiovascular physical examination was normal in the majority of the patients with 3243A>G. Conduction abnormalities were the most common findings in resting ECG, involving five patients (abnormally short PQ time, n =2; 1° AV block, n =1; bundle branch blocks, n=2). Two patients had sinus tachycardia at rest with a heart rate exceeding 100 beats per minute.

Holter monitoring was performed on 36 patients, and clinically significant arrhythmias or conduction disturbances were found in 28% of cases. Five patients had more than 10 ventricular extrasystoles per hour. The arrhythmic episodes in patients with LVH included episodes of non-sustained ventricular tachycardia in one, asymptomatic sinus arrest in two, intermittent delta wave and episodes of supraventricular tachycardia in one and episodes of atrial fibrillation in three. An asymptomatic Mobitz type II atrioventricular block was found in one patient without LVH.

## **6.2 Cardiovascular autonomic regulation in patients with the 3243A>G mitochondrial DNA mutation (II)**

Demographic data on the patients examined for cardiovascular autonomic regulation are shown in Table 2. The mean ejection fraction did not differ between the patients with 3243A>G (65 ± 7.9%) and their matched controls (67 ± 8.8%). LVH was found in 14 patients with 3243A>G (50%). The men and women with 3243A>G did not differ in mean age (42 ± 14 years for men, 40 ± 13 years for women, p=0.63), but the mean BEHL<sub>0.5-4kHz</sub> was higher in the men with 3243A>G (55 ± 33 dB) than in the women (31 ± 25 dB, p = 0.04).

The power spectral density analysis of HRV revealed that the ULF and VLF components were lower among the patients with 3243A>G than in their matched controls.  $\alpha_1$  was lower in the patients than in the controls in the detrended fluctuation analysis of HRV, but  $\beta$  did not differ between the groups. The results were identical when

the patients without beta blocker or angiotensin-converting enzyme inhibitor treatment (n = 23) were analysed. The severity of the disease in terms of BEHL<sub>0.5-4kHz</sub>, Rankin score or CGI-S was not related to the disturbance of cardiac autonomic regulation. There were no significant associations between the components of HRV and the other clinical characteristics related to the 3243A>G mutation, such as DM or LVH, and neither the ejection fraction nor the degree of mutation heteroplasmy in muscle was correlated with any of the components of HRV.

### **6.3 Progression of clinical findings in patients with 3243A>G (III)**

#### ***6.3.1 Clinical features at the initial assessment***

Thirty-three adult patients with 3243A>G were recruited for the three-year clinical follow-up irrespective of their clinical phenotype at the initial evaluation (Table 2). The most common clinical findings were sensorineural hearing impairment in 22 cases (67%), cognitive decline in 16 (50%), DM in 16 (49%), short stature in 14 (42%) and LVH in 14 (42%). Epilepsy was diagnosed in six cases (18%), and three (9%) had experienced stroke-like episodes. The mean for 3243A>G heteroplasmy in muscle was  $71 \pm 12\%$ . Mild to moderate brain atrophy was detected in brain imaging in 14/31 cases (45%), all of whom had cortical atrophy. Central atrophy was present in 9 of them and cerebellar atrophy in 11. Mild white matter changes were found in 8 patients (26%) and stroke lesions were detected in three (9%). Basal ganglia calcifications were detected in four patients (13%), three of whom also had calcifications in the cerebellum. EEG at entry was normal in 22 cases (67%), mildly abnormal in seven (21%) and moderately abnormal in four (12%).

#### ***6.3.2 Clinical findings during the follow-up***

Out of the 26 patients who completed the clinical follow-up, the modified Rankin score increased in eight, the mean score being  $1.2 \pm 0.9$  at entry,  $1.3 \pm 0.8$  after two years ( $p = 0.033$ ) and  $1.5 \pm 1.0$  after three years ( $p = 0.007$ ). The CIBIC score remained constant. Two euglycaemic patients developed DM during the follow-up, and the treatment for DM had to be intensified at entry in one patient because of inadequate glycaemic control.

##### ***6.3.2.1 Cardiac findings during the follow-up***

Twenty-five patients completed the cardiac follow-up. The prevalence of LVH was 40% at entry and 56% at the end. Five of the 14 patients with LVH at the end also had dyspnoea on exertion. The increase in the thickness of diastolic interventricular septum was  $1.6 \pm 2.6$  mm and that in the diastolic posterior wall  $1.1 \pm 2.5$  mm, but the left ventricular end-diastolic internal diameter and ejection fraction did not change during the follow-up (Table 4).

The prevalence of LVH increased from three (27%) to nine (82%) among the diabetic patients who completed the follow-up ( $n = 11$ ) ( $p = 0.014$ ). The diastolic interventricular septum thickness increased by  $2.9 \pm 2.3$  mm and the diastolic posterior wall thickness by  $2.3 \pm 2.2$  mm during the three years in these cases, whereas the gain in diastolic interventricular septum thickness in the non-diabetics ( $n = 14$ ) was  $0.5 \pm 2.4$  mm and that in diastolic posterior wall thickness  $0.2 \pm 2.3$  mm. An increase in left ventricular mass index could be seen in the diabetic patients but not in the non-diabetics. The concentric type of left ventricular remodelling was found in the diabetics, since the increase in relative wall thickness was from  $0.52 \pm 0.17$  to  $0.67 \pm 0.18$  ( $p = 0.017$ ). 3243A>G heteroplasmy at the end of the follow-up was  $75 \pm 11\%$  in the diabetics and  $68 \pm 7\%$  in the non-diabetics ( $p = 0.038$ ),  $74 \pm 10\%$  in the patients with LVH and  $67 \pm 6\%$  in those without LVH ( $p = 0.051$ ). The diabetic patients ( $42.4 \pm 12.8$  years) were not significantly older at entry than the non-diabetic patients ( $38.1 \pm 13.3$  years;  $p = 0.39$ ).

Several benign arrhythmic events were detected in the Holter recordings during the follow-up. Two patients had an intermittent delta wave, and one of these had occasional episodes of supraventricular tachycardia without symptoms. Three patients with hypertension had paroxysmal atrial fibrillation. Two patients had occasional second-degree atrioventricular blocks without symptoms, but none of them needed pacemaker implantation.

### 6.3.2.2 *Other findings during follow-up*

New neurological diagnoses were infrequent in the 26 patients who completed the three-year neurological follow-up. A stroke-like episode and new epilepsy were diagnosed in one patient each. Altogether, seizures were experienced by four patients. Quantitative EEG revealed that the mean  $\alpha$  frequency in both the parietal and occipital regions was lower after three years in 13 patients (Table 4). Neuropsychological follow-up revealed that many cognitive functions had declined slightly, but no significant deterioration was observed.

$BEHL_{0.5-4kHz}$  declined by  $9.0 \pm 6.4$  dB in the sixteen patients who completed the audiological follow-up.  $BEHL_{0.5-4kHz}$  correlated with the mutation heteroplasmy in muscle at entry and at the end of the follow-up. In addition, the rate of progression of sensorineural hearing impairment during the three years correlated with mutation heteroplasmy in muscle (Pearson correlation coefficient 0.58,  $p = 0.018$ ).  $BEHL_{0.5-4kHz}$  was higher in the patients with DM, but the rate of  $BEHL_{0.5-4kHz}$  deterioration in these patients over the three years was similar to that in the non-diabetics.

An annual mortality rate of 8% was calculated, since seven patients died during the follow-up, four suddenly and unexpectedly and three after a prolonged period of deterioration. The deceased patients were more severely affected, as measured by the clinical scores, and had more 3243A>G-related diagnoses at baseline, but they were no older than those who survived. BMI was lower, sensorineural hearing impairment was more pronounced and their performance in some of the neuropsychological tests was poorer. The prevalence of hypertension, DM, coronary artery disease and LVH at entry

did not differ between those who died during the follow-up and those who remained alive.

*Table 4. Major changes in patients with 3243A>G during three years of follow-up (III)*

Measurements	N	At entry	After three years of follow-up
IVSTd (mm)	25	11.1 ± 3.0*	12.7 ± 3.0*
PWTd (mm)	25	10.8 ± 2.3†	12.0 ± 3.0†
RWT (%)	25	51 ± 16†	59 ± 24†
BEHL <sub>0.5-4kHz</sub> (dB)	16	32.2 ± 20.7‡	41.3 ± 23.2‡
Mean $\alpha$ frequency P4 (Hz)	13	10.4 ± 0.6*	10.0 ± 0.5*
Mean $\alpha$ frequency O2 (Hz)	13	10.3 ± 0.7†	10.0 ± 0.6†
Mean $\alpha$ frequency P3 (Hz)	13	10.7 ± 0.5†	10.0 ± 0.5†
Mean $\alpha$ frequency O1 (Hz)	13	10.3 ± 0.7†	10.0 ± 0.6†

\* p < 0.01, † p < 0.05, ‡ p < 0.001. IVSTd: diastolic interventricular septum thickness; PWTd: diastolic posterior wall thickness; RWT: relative wall thickness; BEHL<sub>0.5-4kHz</sub> better ear hearing level, calculated as the mean of hearing levels over the frequencies 0.5, 1, 2, and 4 kHz;  $\alpha$ : alpha band of EEG (8-13 Hz); P4: right parietal area; O2: right occipital area; P3: left parietal area; O1: left occipital area.

## 6.4 Causes of death in pedigrees with the 3243A>G mutation in mitochondrial DNA (IV)

### 6.4.1 Lifespan in families with 3243A>G

We found excess mortality before age of 50 years in the survival analysis of a birth cohort of 278 people in 11 pedigrees with 3243A>G. In that 58 out of the 147 who were 3243A>G carriers or their first degree maternal relatives had died (Figure 2A), 43 (74%) doing so at a younger age than implied by their life expectancy at birth. A similar difference was noted among those who lived longer than 15 years, among whom 31 subjects (82%) had died younger than their life expectancy at the age of 15 years would have presupposed (Fig. 2B). In contrast, the median age at death for 65 subjects who were second-degree or more distant maternal relatives of a 3243A>G carrier was similar to their life expectancy at birth, and this was also true of those who had reached the age of 15 years.

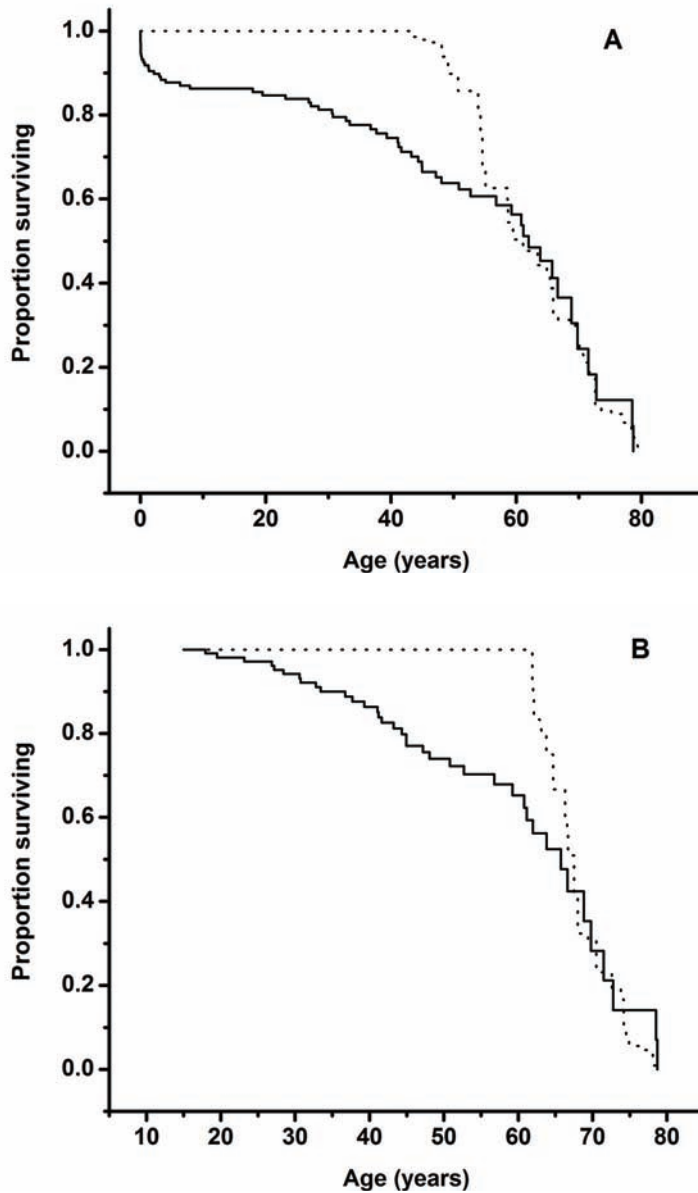


Fig. 2. A) Survival curve for 147 subjects with 3243A>G or their first degree maternal relatives. Kaplan-Meier analysis of survival of them (solid line) and matched life expectancy at birth (dotted line). Log rank analysis of the significance of the difference,  $p = 0.04$  B) Kaplan-Meier analysis of survival among 108 subjects with 3243A>G and their first degree maternal relatives who lived beyond the age of 15 years (solid line) and matched life expectancy at 15 years (dotted line). Log rank analysis of the significance of the difference,  $p = 0.01$ .

### ***6.4.2 Causes of death in families with 3243A>G***

Evaluation of the causes of death in families with 3243A>G suggested that neuropsychiatric diseases and cardiovascular diseases accounted for 30% of all immediate causes of death and for 32% of all underlying causes. These causes of death included encephalomyopathy (n = 1), epilepsy (n = 1), cerebral infarct (n = 1), cerebral haemorrhage (n = 4), DCM (n = 1), HCM (n = 1) and coronary artery disease (n = 4). Death was related to DM in two other cases. No confident statement of the cause of death could be given in eight cases, on account of either a lack of appropriate information or multiple possible causes of death identified in the review of the available information.

Involvement of the 3243A>G mutation in the death was considered likely in 34% of the 47 cases included in the analysis of cause of death. The most common underlying causes of death were neuropsychiatric diseases (encephalomyopathy n = 3, stroke n = 2, epilepsy n = 1). One patient had died of DCM, and one patient with HCM and DM had died suddenly autopsy report revealing myocardial fibrosis and coronary atherosclerosis.. Confident determination of the cause of death was not possible in five cases, but the information retrieved was consistent with the involvement of 3243A>G. Progressive cerebral dysfunction without evidence of any infection or new stroke before death had been detected in one patient who had had a stroke and subsequent epilepsy with frequent seizures several years previously. Two patients had been found dead in bed, both of whom had had cardiomyopathy, DM and epilepsy. Coronary artery disease, HCM, DM and nephropathy had been diagnosed in the fourth deceased patient, who had died suddenly and unexpectedly. Abdominal cramps, vomiting, paralytic ileus and progressive cerebral dysfunction had been the leading symptoms and findings during the last two months before death in one patient who had had stroke-like episodes eight years earlier.

Seven subjects had died suddenly and unexpectedly and five other patients had been found dead in bed. Cardiovascular diseases (n = 6) were the most common causes of death among these 12 subjects. The 3243A>G mutation was assessed to have had a role in the cause of death in five subjects who had died suddenly and unexpectedly. All of them had had DM and two of them had had epilepsy. Three of these five had had cardiomyopathy and two had had cardiomyopathy with coronary artery disease.

## 7 Discussion

### 7.1 Cardiac manifestations and clinical follow-up in patients harbouring 3243A>G

The probands included in the present work had been identified during the screening of adult patient populations with known manifestations related to 3243A>G in the province of Northern Ostrobothnia. Identification of the families of the probands resulted in 13 pedigrees with the mutation. Heteroplasmy of the mutation in muscle was measured in 85% - 93% of the patients during the clinical studies. The patients were examined systematically for cardiac symptoms and findings on an annual basis and the follow-up also included neurological, audiological and neuropsychological evaluations. Patients were accepted for the study irrespective of their clinical phenotype at entry. The survey is the largest clinical study of the occurrence of cardiac abnormalities in patients with 3243A>G, with a follow-up time amounting to 94 person-years. No comparative data yet exist for the retrospective analysis of causes of death among families with 3243A>G.

LVH was the most common cardiac finding in 55% of the verified or obligatory carriers of the 3243A>G mutation. Comparison of the patients with the mutation with matched controls showed the odds ratio for LVH to be 7.5. Clinically defined cardiomyopathy has been reported among patients with various other mtDNA point mutations, and these case reports suggest that LVH is more common than cardiac dilatation in mitochondrial diseases (Anan *et al.* 1995, Casali *et al.* 1995, Okajima *et al.* 1998, Marin-Garcia *et al.* 1998, Casali *et al.* 1999). Interestingly, knock-out mice with inactivated mitochondrial ANT1 develop HCM (Graham *et al.* 1997), suggesting that cytoplasmic ATP deficiency may be an essential contributor to the pathogenesis of this condition. A decreased concentration of ATP has been shown in cultured myoblasts harbouring 3243A>G, suggesting a role for ATP deficiency in the clinical findings of such patients (Rusanen *et al.* 2000). DCM seems to be a rare phenotype of the 3243A>G mutation (Vilarinho *et al.* 1997), an observation which was confirmed here, since only one patient out of 34 had DCM with poor left ventricular function. DCM has been shown to develop in mutant mice lacking in manganese superoxide dismutase (Li *et al.* 1995), which is one of the enzymes scavenging the ROS generated in mitochondria. This has not

been verified in the failing human heart, however. In contrast, upregulation of the catalase gene but not of manganese superoxide dismutase or glutathione peroxidase gene expression has been detected in the hearts of patients with ischaemia or DCM relative to those with a normal heart (Dieterich *et al.* 2000).

The risk of LVH was increased among patients with the 3243A>G mutation as compared with their matched controls, suggesting a role for the mutation itself, but since there was no clear difference in the proportion of the mutant genome between patients with and without LVH, estimation of the risk of LVH cannot be based solely on the degree of mutant heteroplasmy in muscle. The proportion of the mutant genome in tissues is one factor affecting the biochemical phenotype of mtDNA diseases, although nuclear modulatory factors, synergistic mtDNA mutations and the mtDNA copy number may also contribute (Rose 1998). The clinical phenotype is modulated by an imbalance between the energy demand and energy supply in a given tissue and by environmental or physiological factors such as ageing (Schon *et al.* 1997).

LVH and HCM have been described as fundamental manifestations in phenotypes related to several mtDNA mutations (Table 1). When the prevalences of the sarcomere protein gene and the genes causing lipid storage cardiomyopathy and mtDNA mutations were evaluated in a community-based study of 1862 unselected patients, 50 of them were found to have increased left ventricular wall thickness. MtDNA variants were not shown to be related to LVH in this population, whereas mutations in the sarcomere protein or lipid storage gene were found in 18% of the subjects with increased left ventricle wall thickness (Morita *et al.* 2006). On the other hand, the prevalences of mtDNA mutations in population-based studies in adults have been shown to vary from 11/100 000 to 16/100 000 (Majamaa *et al.* 1998, Man *et al.* 2003, Schaefer *et al.* 2004). Thus diagnostic mitochondrial genetic analysis in patients with familial LVH or HCM should be focused on patients with other phenotypes related to mitochondrial diseases, such as MDM, sensorineural hearing impairment and neurological and visual symptoms.

Left ventricular wall thickness increased in the present patients during the three years of follow-up, but the increase was not associated with left ventricular dilatation or any impairment of left ventricular function. More specifically, the diabetic patients with 3243A>G had an increased risk of developing LVH during the three-year period. The rate of LVH progression was not significantly correlated with 3243A>G mutation heteroplasmy in muscle, although there was a tendency for higher heteroplasmy levels to occur in patients with LVH at the end of the follow-up. Even though the pathophysiology of MDM seems to be different from that of both type 1 and type 2 DM, cardiomyopathy in all diabetic patients is characterized by a disproportionate increase in left ventricular mass (Devereux *et al.* 2000, Carugo *et al.* 2001). Progressive left ventricular dilatation and LVH has been discovered in three young patients with 3243A>G during a follow-up lasting more than five years (Okajima *et al.* 1998). An annual incidence of 3.6 cases/100 patients for DM and cardiomyopathy has been calculated for eight patients (aged 15 - 41 years) harbouring 3243A>G during seven years of follow-up (Damian *et al.* 1998). Our data are in line with this, as we found an annual incidence of 2.1/100 for DM and 4.2/100 for LVH.

We found an annual mortality rate of 8% among patients with diverse phenotypes of 3243A>G during the three years of follow-up, the deceased patients having been more severely affected than those who remained alive. Five of these patients had

cardiomyopathy, and death was sudden and unexpected in three cases, suggesting that cardiomyopathy may have contributed to it. Even a retrospective analysis of their cardiac examinations did not reveal any features predictive of sudden cardiac death (Elliott *et al.* 2000). Only one patient who had died after prolonged period of deterioration had performed symptom limited exercise test during life. Cardiomyopathy was the cause of death in three adult patients in a retrospective analysis of nine deceased patients with MELAS syndrome (Klopstock *et al.* 1999). Likewise a poor prognosis has been detected in children with mitochondrial disease and cardiomyopathy, their survival rate being only 18% at the age of 16 years, while children with neuromuscular features without cardiomyopathy have had a survival rate of 95% at the same age (Scaglia *et al.* 2004).

## **7.2 Cardiovascular risk assessment in patients with 3243A>G and causes of death in their families**

The arrhythmic events seen in our patients with 3243A>G were usually benign, and only one patient with LVH had non-sustained VT without symptoms during scheduled Holter monitoring. An intermittent delta wave suggesting pre-excitation was found in two cases and short episodes of supraventricular tachycardia without symptoms in one. WPW syndrome has been reported in patients with the 3243A>G mutation (Hirano *et al.* 1992, Anan *et al.* 1995, Okajima *et al.* 1998) and with 3460G>A, the common LHON mutation (Finsterer *et al.* 2001). A prevalence of 13.4% for WPW syndrome has been demonstrated in diabetic patients with 3243A>G (Suzuki *et al.* 2003), which is considerably higher than in our population (5%). Cardiac conduction abnormalities treated with a pacemaker have also been found in patients with mitochondrial diseases and are most often described in patients with deletion or duplication of mtDNA, leading to the Kearns-Sayre syndrome (Berenberg *et al.* 1977, Remes *et al.* 1992, Marin-Garcia *et al.* 2002). We found two 3243A>G carriers with an intermittent second-degree atrioventricular block, but neither of them needed implantation of a pacemaker.

Cardiac autonomic regulation is seriously affected in cases of cardiac disease and DM. Our patients and controls were carefully matched to avoid the effects of confounding factors related to these conditions, and differences in both spectral and fractal measures of HRV emerged between 28 patients with the 3243A>G mtDNA mutation and their matched controls. Twenty four of patients with the mutation had performed symptom limited bicycle exercise test. Twelve of them had reached less than 85 % of maximal age dependent heart rate during the test, which had had to be stopped prematurely because of fatigue, short of breath or exhaustion of legs. Coronary artery disease was not detected in any of these 24 patients since they did not have angina pectoris or ischemic findings in ECG during the exercise test. (Data not published)

The most pronounced decline was seen in the ULF and VLF components of the power spectra and  $\alpha_1$  of the patients with 3243A>G relative to the controls, whereas the decrease in HRV components was not related to DM, LVH, left ventricular systolic function, severity of the mitochondrial disease or 3243A>G mutation heteroplasmy in muscle. Our findings show that cardiovascular autonomic regulation in patients with MDM is comparable to that in matched diabetic controls, and that non-diabetic patients with

3243A>G have equally reduced indices of HRV to diabetic patients. The reduced ULF and VLF spectral components may simply reflect the subject's reduced diurnal physical activity, without any intrinsic impairment of cardiovascular autonomic function (Bernardi *et al.* 1996). Only one of the patients included in the paper II had been treated in an institution, whereas 27 of them attended an outpatient clinic and lived normal daily lives. Peripheral neuropathy has been detected in patients with mitochondrial mutations (Kärppä *et al.* 2003, Rantamäki *et al.* 2005, Santoro *et al.* 2006), but reports of patients with autonomic neuropathy are rare (Zelnik *et al.* 1996). It may be that the 3243A>G mutation has direct effects on the afferent or efferent nerve fibres or the cells of the vasomotor centre controlling autonomic regulation. Another possibility is that the mitochondrial syndrome itself may lead to secondary sympathoexcitation (Vissing *et al.* 1996), thereby resulting in changes in the indices characterizing autonomic regulation. An elevated level of circulating norepinephrine has been shown to result in a decrease in the LF component of power spectra in healthy subjects (Tulppo *et al.* 2005).

We found that the age at death was lower than the life expectancy at birth, particularly in 3243A>G carriers and their first-degree maternal relatives of both sexes. A similar finding emerged among subjects who had lived over 15 years, when comparison was made with their life expectancy at 15 years. The lifespan of second-degree or more distant relatives of a 3243A>G mutation carrier did not differ from that of the general population. The findings suggest that the 3243A>G mutation in a family increases the risk of death until middle age among the 3243A>G mutation carriers and their first-degree maternal relatives but not in more distant relatives. This may be explained by lower heteroplasmy level or absence of the 3243A>G in those who are further than first degree maternal relatives.

Awareness of symptoms and findings in diseases related to mtDNA mutations has increased only during the last couple of decades (DiMauro & Davidzon 2005), so that it was not surprising that a comparison of the clinical data with the information on death certificates revealed discrepancies in seven cases in which the clinical data had suggested involvement of 3243A>G in the cause of death. We found neuropsychiatric diseases to be the most common causes of death in these subjects, but cardiomyopathy, paralytic ileus and metabolic disturbances such as lactic acidosis and DM were also found. Similar causes of death have been described in previous case reports on deceased subjects with 3243A>G (Terauchi *et al.* 1996, Klopstock *et al.* 1999, Tsuchiya *et al.* 1999, Mangiafico *et al.* 2004). Analysis of one series of 11 deceased patients pointed to cardiopulmonary failure (36%) and status epilepticus (36%) as the most common causes of death in patients with 3243A>G (Klopstock *et al.* 1999), while respiratory failure has been found to be a common cause of death in seven subjects who had presented with various phenotypes of mitochondrial diseases during life and who had harboured a variety of mtDNA mutations (Arpa *et al.* 2003).

Sudden and unexpected death is commonly associated with cardiac diseases such as myocardial infarction, hypertrophy or DCM and inheritable arrhythmia disorders (Huikuri *et al.* 2001). The mechanism of sudden death in these diseases may be considered to be arrhythmic. Sudden and unexpected death has also been described in patients with type-1 DM (Weston & Gill 1999), type-2 DM (Kannel *et al.* 1998, Balkau *et al.* 1999, Jouven *et al.* 2005) and epilepsy (Leestma *et al.* 1997, Tellez-Zenteno *et al.* 2005), the suggested mechanisms in the case of type-1 DM being nocturnal

hypoglycaemia or arrhythmic events (Tattersall & Gill 1991). The risk of sudden death has been shown to increase in patients with type-2 DM and hyperglycaemia regardless of the presence of microvascular disease associated with DM (Jouven *et al.* 2005). The induction of hypoglycaemia has been shown to result in a reduction in cardiac vagal outflow in both type-1 diabetic patients and non-diabetic subjects, so that this condition has been suggested as making some contribution to the occurrence of dead-in-bed syndrome in patients with DM (Koivikko *et al.* 2005). Sudden and unexpected death was found in 31% of patients with 3243A>G and first degree maternal relatives to one of such in whom the cause of death was assessed as being related to 3243A>G. All these patients had had DM, four had had cardiomyopathy and two of them had had epilepsy. Cardiomyopathy, DM and epilepsy were frequent manifestations in patients with 3243A>G, so that it is not surprising that sudden death was not uncommon in these cases.

Current knowledge suggests that the screening of patients with LVH for mitochondrial mutations is not worthwhile, but that patients with other clinical manifestations of mitochondrial diseases should be screened for the 3243A>G mtDNA mutation and for cardiac abnormalities. Diabetic patients with 3243A>G have an increased risk of developing LVH, which is a risk factor for heart failure, sudden and unexpected death and all-cause mortality (Levy *et al.* 1990, Elhendy *et al.* 2003). The factors contributing to LVH in patients with 3243A>G are unknown, but treatment should include efficacious methods for controlling known risk factors for LVH such as hypertension and glycaemic control of DM. Several components of HRV measuring cardiac autonomic regulation in patients with 3243A>G are reduced compared with those in matched controls, the changes observed being parallel to those observed among patients with various cardiac and neurological disorders and among cardiac patients with an increased risk of mortality and life-threatening arrhythmic events. Indeed, we found sudden and unexpected death in almost one third of deceased subjects who were carriers of 3243A>G or first-degree maternal relatives of such.

## 8 Conclusions

1. Patients with the 3243A>G mtDNA mutation run a considerable risk of developing cardiac pathology, mainly LVH. LVH is not correlated with mutation heteroplasmy in muscle, but  $BEHL_{0.5-4kHz}$ , used as a measure of the severity of the phenotype of mitochondrial disease, was higher in patients with LVH than in those without.
2. Patients with the 3243A>G mtDNA mutation have reduced heart rate variability as measured by power spectral density analysis and detrended fluctuation analysis relative to carefully matched controls, suggesting involvement of the autonomic nervous system. The abnormalities in cardiovascular autonomic regulation are not correlated with the cardiac phenotype, but may be related to the 3243A>G mutation itself or to features of patients with 3243A>G that were not assessed here.
3. The progression of left ventricular wall thickening can be seen in diabetic patients with 3243A>G during three years of follow-up. The annual incidence of DM was 2.1/100 and that of LVH 4.6/100. Annual monitoring of fasting blood glucose should be included in the follow-up of adult patients with 3243A>G.
4. We found that persons who are 3243A>G carriers or first-degree maternal relatives of such die younger than would be presupposed by their life expectancy at birth. The most common causes of death were neuropsychiatric diseases, but cardiovascular and metabolic diseases were also found. The mode of death was sudden and unexpected in almost one third of the patients in whom 3243A>G was deemed to be related to the cause of death. These subjects had had several known risk factors for sudden death.

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