MYOPATHY AND PERIPHERAL NEUROPATHY ASSOCIATED WITH THE 3243A>G MUTATION IN MITOCHONDRIAL DNA

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Abstract

Neurological features are common in mitochondrial diseases because tissues depending upon oxidative phosphorylation bear the brunt of the pathogenesis. The 3243A>G mutation in the MTTL1 gene in mitochondrial DNA is regarded as the most frequent mitochondrial point mutation and classically presents with mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Myopathy and peripheral neuropathy have been documented in patients with mitochondrial diseases, but not properly characterised in patients with the 3243A>G mutation. We have previously determined the prevalence of patients with this mutation in a defined population in northern Finland.

The clinical spectrum and molecular aspects of myopathy and peripheral neuropathy are analysed here in a population-based cohort of patients with 3243A>G. Fifty patients were examined neurologically in order to define the frequency of myopathy and its histological, ultrastructural and clinical features. The frequency and phenotypic variability of peripheral neuropathy were determined in 32 patients and muscle computed tomography findings recorded in 24 patients. Finally, variations in mutation heteroplasmy were analysed in 10 patients using single muscle fibre PCR analysis.

The frequency of peripheral neuropathy was 22% (95% confidence interval (CI), 9–40%) and that of clinical myopathy 50% (95% CI, 36–64%). Moderate limb weakness was the most common myopathic feature, but mild weakness and external ophthalmoplegia were also present. CT scans revealed myopathic changes in 54% of the patients (95% CI, 33–76%), most frequently in the pelvic muscles. The incidence of myopathy was highest in the fifth decade of life, and higher age and male gender increased the risk of neuropathy. Muscle histology was abnormal in 72% of the cases examined (95% CI, 55–86%). The presence of intramitochondrial crystals and COX-negative fibres and variations in the size and shape of mitochondria were more common in the muscle of myopathic patients. Single muscle fibre analysis pointed to a correlation between the mutation load in ragged red fibres and in adjacent histologically normal fibres, and the proportion of 3243A>G in histologically normal muscle fibres showed a pattern compatible with random genetic drift.

The results indicate that myopathy and peripheral neuropathy are common in patients with the 3243A>G and that myopathy is highly variable in presentation. Segregation of 3243A>G in individual muscle fibres showed a complex process with random and non-random elements.

Keywords: 3243A>G mutation, heteroplasmy, MELAS, mitochondrial DNA, myopathy, peripheral neuropathy, phenotype
To my family
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Oulu, April 2004

Mikko Kärppä
Abbreviations

AD  autosomal dominant
ADP  adenosine diphosphate
AR  autosomal recessive
ATP  adenosine triphosphate
bp  base pair
CK  creatine kinase
CMAP  compound muscle action potential
CNS  central nervous system
CoA  coenzyme A
COX  cytochrome c oxidase
CT  computed tomography
CTS  carpal tunnel syndrome
DNA  deoxyribonucleic acid
EMG  electromyography
HMSN  hereditary motor and sensory neuropathy
HNPP  hereditary neuropathy with liability to pressure palsies
KSS  Kearns-Sayre syndrome
LHON  Leber’s hereditary optic neuropathy
MELAS  mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF  myoclonus epilepsy with ragged-red fibres
MNCV  motor nerve conduction velocity
mtDNA  mitochondrial DNA
MUAP  motor unit action potential
NCV  nerve conduction velocity
nDNA  nuclear DNA
PCR  polymerase chain reaction
PEO  progressive external ophthalmoplegia
RNA  ribonucleic acid
RRF  ragged red fibres
SDH  succinate dehydrogenase
SNAP  sensory nerve action potential
SNCV  sensory nerve conduction velocity
tRNA  transfer ribonucleic acid

α    alpha
β    beta
γ    gamma
List of original papers

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


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Mitochondrial DNA (mtDNA) mutations have been associated with a wide range of clinical presentations (DiMauro et al. 1985, Shoubridge 1998, Rose 1998, DiMauro & Schon 2003). These mutations may involve transfer RNA, ribosomal RNA or structural genes and can be expressed biochemically either as defects in the entire electron transport chain or as defects in individual enzymes (Wallace 1992). Mitochondrial cytopathies affect multiple organ systems, but have a predilection for organs with high oxidative metabolic activity such as the brain and muscles. Thus skeletal muscle is the tissue of choice for demonstrating a mitochondrial disease (Larsson & Oldfors 2001). Although these syndromes are often distinctive, there can be overlap between them in some patients (Pulkes et al. 1999).

The 3243A>G mutation in the MTTL1 gene is one of the most common pathogenic mtDNA point mutations and classically presents with mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (Ciafaloni et al. 1992). Its prevalence in the adult population of Northern Ostrobothnia in northern Finland has been calculated to be 16/100,000 (Majamaa et al. 1998). The high figure suggests that MELAS may constitute one of the largest group of neurogenetic diseases. This mutation may lead to many other clinical phenotypes such as diabetes mellitus, cognitive decline, epilepsy, ataxia, sensorineural hearing loss, short stature, myopathy, cardiomyopathy and peripheral neuropathy. The 3243A>G mutation is ordinarily heteroplasmic, that is, with both mutant and wild-type mtDNA to be found in the same patient. The relationship between mutation load and clinical phenotype is less obvious, however, and patients with clinical features of disease can carry different proportions of mutant mtDNA.

The clinical manifestations of muscle involvement in mitochondrial diseases include weakness, exercise intolerance, muscle cramps, myalgia and myoglobinuria. Myopathy has been suggested as the most frequent clinical feature among MELAS patients (Chinnery et al. 1997a). It is usually proximal, and atrophy is quite unusual. Peripheral neuropathy can also be one of the clinical manifestations of the MELAS syndrome (Yannikas et al. 1986, Mizusawa et al. 1991, Schröder 1993). Although there are several studies on myopathy and peripheral neuropathy in patients having mtDNA defects, no population-based survey exists of the frequency and detailed clinical, neurophysiological and histochemical features of these manifestations among patients with the 3243A>G mutation.
Correlations between the intra-tissue distribution of the 3243A>G genotype and its phenotypic expression have been presented only in case reports. The mosaic appearance of enzyme deficiency in muscle tissue is the result of replicative segregation of variable levels of mutant mtDNA in different muscle fibres (Moslemi et al. 1998). In humans, the segregation of the heteroplasmy varies between tissues from the same individual. Muscle fibre as a wide class of postmitotic cells is a favourable subject for enabling the investigation. Segregation of mutant mtDNA can be assessed both in terms of longitudinal variation (within cells) and variations in sections (among cells) (Elson et al. 2002). Single fibre analyses of muscle from patients with the 3243A>G mutation have revealed differences in mutation load between cells (Petruzella et al. 1994, Silvestri et al. 2000b). Despite the fact that ragged red fibres (RRF) usually display a higher proportion of mutant mtDNA than normal muscle fibres, the variation in the proportion of the mutant genome along individual muscle fibres is not known.

A clinical history and neurological examination are the cornerstones of the diagnosis of myopathy, but additional investigations such as muscle biopsy and electrophysiological, biochemical and genetic studies are needed for a definite diagnosis. Muscle abnormalities in computed tomography (CT) images have been recognized in neuromuscular disorders, because CT images allow the survey of a large number of muscles, including ones that cannot be assessed clinically (Bulcke et al. 1979, Nordal et al. 1988, Swash et al. 1995). Previous reports have shown that muscle CT is a safe, non-invasive and effective tool for the investigation and diagnosis of myopathies. It also provides a method for optimal muscle biopsy targeting and for following the progression of neuromuscular disorders. Although CT has been performed as a means of assessing various neuromuscular diseases, there are few radiological muscle imaging studies available concerning patients with mitochondrial myopathies and none concerning patients with the MELAS mutation (Fleckenstein et al. 1992).
2 Review of the literature

2.1 Peripheral neuropathy

The signs and symptoms of peripheral neuropathy are determined by the type of nerve that is affected (sensory, motor or autonomic). Two main types of nerve fibres are found in peripheral nerves, myelinated (1–15µm diameter) and non-myelinated (0.2–3µm diameter). Myelinated axons lie within a chain of Schwann cells, each of which forms a myelin sheath around the axon. Neuropathies are categorized as either primarily demyelinating or axonal. Neurophysiological examination plays a pivotal role in the diagnosis of neuropathies (Donofrio & Albers 1990), as their determination requires neurophysiological measurements of conduction velocities in several nerves, conduction block assessment and measurement of sensory action potentials and distal and F wave latencies. Needle electromyography in inherited neuropathies typically reveals little active denervation, with large MUAPs (motor unit action potential) consistent with distal reinnervation.

It cannot be properly determined on clinical grounds whether a peripheral neuropathy is axonal or demyelinating, although there may be some clinical clues to the type of neuropathy. Widespread loss of deep tendon reflexes even in muscles that are not particularly weak or wasted is more a feature of demyelination, while selective loss of ankle jerks in the presence of distal wasting and weakness is more typical of an axonopathy. Palpably enlarged nerves also indicate demyelinating neuropathy. The causes of axonal neuropathy are numerous, while the demyelinating form has fewer aetiologies. The classification of polyneuropathies is based on the involvement of sensory and motor nerves and on axons and myelin (Donofrio & Albers 1990). Axonal degeneration may lead to secondary breakdown of the myelin sheath, and axonal degeneration and segmental demyelination coexist in neuropathies. In fact, many neuropathies are neither purely axonal nor purely demyelinating, but rather a combination of both with a predominance of one or the other (mixed pattern).
2.1.1 Axonal neuropathy

The proteins required for axonal maintenance and regeneration must be supplied by axonal transport, because protein synthesis does not occur in axons (Droz & Leblond 1962), which are lacking in ribosomes and a rough endoplasmic reticulum. Axonal transport may be divided into orthograde transport (from the cell body to the periphery) and retrograde transport. There are two main phases of axonal transport: a fast phase composed of small, membrane-bound vesicles and tubular structures containing proteins and small molecules, and a slow phase that consists of cytoskeletal proteins such as microtubules and neurofilaments (Saper et al. 1987).

Axonal degeneration is the most common pathological reaction of peripheral nerves, and its aetiology usually includes metabolic disorders or toxins. It is presumably caused by metabolic derangement within the neurons of a kind resembling Wallerian degeneration, which is response for axonal injury, but it can also be a result of failure of the perikaryon to synthesize enzymes or structural proteins, or of regional disturbances in energy metabolism (Bosch & Mitsumoto 1996). Axonal neuropathies are characterized by reduced amplitude or absence of sensory nerve action potentials (SNAPs) and compound motor action potentials (CMAPs) with normal or slightly reduced conduction velocities. Needle electromyography of the distal muscles shows acute or chronic changes (Donofrio & Albers 1990).

2.1.2 Demyelinating neuropathy

The principal feature of demyelination is the destruction of myelin with relative preservation of the axons. The involvement of myelin is caused by immune-mediated factors or hereditary disorders of myelin metabolism in the Schwann cells. The pathological process is usually patchy along the myelinated axon. Remyelination of demyelinated segments usually follows within weeks in several kinds of Schwann cells, resulting in a decrease in the internodal length and thinner myelin sheaths of those segments. Demyelinating neuropathy can be either segmental or uniform in occurrence, the latter applying mostly to hereditary neuropathies involving mixed sensorimotor polynuropathies, while the former is typical of acquired neuropathies (Donofrio & Albers 1990). In neurophysiological examinations demyelination prolongs nerve conduction velocities (NCVs) or causes a conduction block, resulting in motor weakness and mild sensory loss. Typical neurophysiological features are dispersion of CMAPs and marked prolongation of distal and F-wave latencies (Donofrio & Albers 1990).

2.2 Inherited peripheral neuropathies

Inherited disorders of the peripheral nerve account for about 40% of patients with undiagnosed, intensively examined neuropathies (Dyck et al. 1981). They can be divided into
two main groups: primary neuropathies and neuropathies that form part of a more widespread neurological or multisystem disorder (Table 1). Inherited neuropathies may remain underdiagnosed, for several reasons, including an incomplete family history and recessive or X linked inheritance patterns. Other reasons are the occurrence of de novo mutations or the neuropathy has late onset chronic, slowly progressive course. Inherited neuropathies have some distinctive clinical features, however. They cause positive sensory symptoms (tingling, burning or pain) less frequently than acquired forms and they can produce typical skeletal deformities of the foot (e.g. pes cavus) or of the spine (e.g. kyphoscoliosis). An inherited form of neuropathy can be suspected, if a neurophysiological examination reveals neuropathy in an asymptomatic family member.

Major advances in our understanding of the molecular basis and phenotypes of inherited neuropathies have been achieved through identification of the chromosomal loci and genes by means of positional cloning (Lupski et al. 1993). Genetic tests are currently being used widely to establish the diagnosis in some hereditary neuropathies, as routine laboratory blood tests usually give normal results. Cerebrospinal fluid analysis may reveal moderate (e.g. in hereditary motor and sensory neuropathy I, HMSN type I) or marked (e.g. in Dejerine-Sottas disease, DSD) protein elevation without pleocytosis. Radiological imaging has no essential role in the diagnostic approach, but magnetic resonance imaging may show enlargement of the cauda equina and spinal ganglia. A peripheral nerve biopsy, obtained from the sural (most frequently), superficial peroneal or radial sensory nerve, is rarely needed. Such a course should be pursued only when vasculitis or amyloid neuropathy are suspected, which produce histological changes in the nerve that allow a specific diagnosis (Rosenberg et al. 2001). The management of inherited neuropathies is mainly supportive, and the prognosis in most cases of HMSN types I and II and HNPP is relatively benign. Most patients remain ambulatory and have a normal life expectancy. In contrast, occasional patients may develop a progressive course of neuropathy, and children or adolescents with DSD are often severely disabled.
Table 1. Classification of inherited neuropathies (modified after Reilly & Hanna 2002a).

<table>
<thead>
<tr>
<th>Neuropathy type</th>
<th>Main type</th>
<th>Subtype</th>
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<tr>
<td>Primary</td>
<td>HMSN</td>
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<tr>
<td></td>
<td>HNNP</td>
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<td></td>
<td>HSAN</td>
<td></td>
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<td></td>
<td>HNA</td>
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<td></td>
<td>Distal HMN</td>
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<td>Secondary</td>
<td>FAP</td>
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<td>Lipid metabolism</td>
<td>Leukodystrophies</td>
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<td>Lipoprotein deficiencies</td>
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<td>Phytanic acid storage diseases</td>
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<td>α-Galactosidase deficiency</td>
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<td>Cholestanolosis</td>
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<td></td>
<td>Sphingomyelin lipidoses</td>
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<tr>
<td>Porphyrias</td>
<td>Acute intermittent</td>
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<td></td>
<td>Hereditary coproporphyria</td>
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<td></td>
<td>Variegate</td>
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<td></td>
<td>Aminolevulinic acid dehydrase deficiency</td>
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<tr>
<td>Defective DNA-repair</td>
<td>Xeroderma pigmentosum</td>
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<td></td>
<td>Ataxia teleangiectasia</td>
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<tr>
<td></td>
<td>Cockayne syndrome</td>
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<tr>
<td>Mitochondrial disease</td>
<td>Friedreich's ataxia</td>
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<tr>
<td>Hereditary ataxias</td>
<td>Spinocerebellar ataxias</td>
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<tr>
<td>Miscellaneous</td>
<td>Giant axonal neuropathy</td>
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<td></td>
<td>Neuroacanthocytosis</td>
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<td></td>
<td>Chediak-Higashi disease</td>
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Abbreviations: FAP, familial amyloid polyneuropathies; HMN, hereditary motor neuropathies; HMSN, hereditary motor and sensory neuropathies; HNA, hereditary neuralgic amyotrophy; HNNP, hereditary neuropathy with liability to pressure palsies; HSAN, hereditary sensory and autonomic neuropathies

2.2.1 Hereditary motor and sensory neuropathy

These neuropathies are quite common, affecting both children and adults. A widely accepted classification designates Charcot-Marie-Tooth (CMT) disorders as hereditary motor and sensory neuropathies (HMSN) (Dyck et al. 1993), although both nomenclatures are in use. The genetic basis for HMSN is heterogeneous and includes autosomal dominant, autosomal recessive and X-linked forms. There are about 30 subtypes of HMSN. Most current classifications agree that the basic division should be made on electrophysiological criteria, into type I (demyelinating) and type II (axonal), and that further subclassification should be according to the inheritance pattern (Reilly & Hanna 2002a). The classification of HMSN has tendency to change because of the rapid advances in the description of the causative genes.

Symptoms of HMSN typically begin during the first or second decade of life, but the onset may occur until later in adulthood. Typical clinical features include distal muscle weakness and atrophy, impaired sensation and diminished or absent deep tendon reflexes.
There is a wide variation in clinical presentation, ranging from minimal symptoms or asymptomatic cases to severe distal atrophy and deformity causing significant neurological impairment. Symptoms most commonly involve the lower legs and feet, affecting the hands and forearms later. Electrophysiologically, the demyelinating forms have reduced motor and sensory nerve conduction velocities (NCVs), while the axonal forms have normal or near-normal NCVs (Dyck et al. 1993).

Autosomal dominant demyelinating HMSN type I can be divided into subtypes A, B, C and D. HMSN type IA is the most common form and is usually caused by a 1.5 Mb duplication in the peripheral myelin protein 22 gene (PMP-22) on chromosome 17p11.2. The PMP-22 protein is localized in the compact component of peripheral nerve myelin and is highly conserved in evolution (Patel et al. 1992). Mutations in this gene account for about 70% of all HMSN cases (Nelis et al. 1996). HMSN type IB is caused by point mutations in the human myelin protein zero (P0) gene on chromosome 1q23-23 (Hayasaka et al. 1993a). Myelin protein zero is the major structural component of peripheral nervous system myelin (about 50% by weight). The molecular defect in HMSN type IC is unknown, while HMSN type ID is caused by a point mutation in the EGR-2 gene on chromosome 10q21-q22 (Zorick et al. 1996, Warner et al. 1998). Eight autosomal recessive demyelinating HMSN subtypes have been identified to date (Reilly & Hanna 2002a).

Point mutations in the connexin 32 (Cx32) gene cause an X-linked form of HMSN (HMSNX) which has similar clinical features to HMSN type I, especially among men. The Cx32 gene is located in the proximal long arm of the X chromosome (Xq13-q21). Cx32, which is structurally similar to PMP-22, encodes a major component of gap junctions and is expressed in peripheral nerves (Bergoffen et al. 1993). HMSNX is considered to be the second most common type of HMSN after HMSN type IA (Chance & Shapiro 2002). Typical features are an early age of onset, absence of male-to-male transmission and more severe symptoms in men. Female carriers are often mildly affected.

Dejerine-Sottas disease (DSD, HMSN type III) is a severe demyelinating polyneuropathy with autosomal recessive inheritance. It appears in the first decade of life and is associated with a point mutation in the P0 or PMP-22 gene (Hayasaka et al. 1993b, Roa et al. 1993). Nerve conduction studies have shown markedly slow conduction velocities. DSD and congenital hypomyelinating neuropathy (CHN) are considered severe forms of HMSN (Warner et al. 1996). Four subtypes of DSD have been determined so far (A-D), and three in CHN (A-C).

The HMSN type II group comprises axonal neuropathies with autosomal dominant, recessive and X-linked forms. Autosomal dominant HMSN type II is genetically heterogeneous, and the causative genes for most forms have not yet been identified. AD-HMSN type II is less common than AD-HMSN type I (Harding & Thomas 1980). Four autosomal recessive forms of HMSN type II have been identified (A-D). AR-HMSN type IIA is caused by mutations in lamin A/C (LMNA), as is also the case in some myopathies such as AR-Emery-Dreifuss muscular dystrophy and limb-girdle dystrophy type 1B (Sandrelli-Giovannoli et al. 2002). One X-linked form of HMSN type II has been described, but the gene is as yet unidentified (Cowchock et al. 1985). In addition, two autosomal dominant intermediate HMSN subtypes have been described, which cannot be classified to demyelinating or axonal types.
2.2.2 Hereditary sensory and autonomic neuropathy

Hereditary sensory neuropathies (HSAN) affect the sensory neurons, causing sensory loss. This predisposes the subject to unnoticed trauma, leading to non-healing ulcerations, secondary infections and osteomyelitis and resulting in acral mutilations (acrodystrophic neuropathy) (Bosch & Mitsumoto 1996). HSAN is classified into five subtypes (HSAN types I-V), the most common of which, HSAN type I, is an autosomal dominant disease affecting the root ganglion cells and axons in peripheral sensory nerves and to some degree in the motor nerves. Sensory loss occurs in the second decade of life or later. The other HSAN forms are autosomal recessive. The loci and genes for all forms of HSAN except HSAN type II have been identified (Reilly & Hanna 2002a).

2.2.3 Hereditary neuropathy with liability to pressure palsies

Hereditary neuropathy with liability to pressure palsies (HNPP, formerly called tomaculous neuropathy) leads to recurrent, episodic demyelinating pressure nerve palsies. This autosomal dominant form of neuropathy is associated with a 1.5 Mb deletion on chromosome 17p11.2-12 which leads to reduced expression of the $PMP22$ gene (Chance et al. 1993). The deletion is the genetic reciprocal of the duplication on chromosome 17 involved in HMSN IA. Segmental demyelination and sausage-like myelin thickenings, tomaculous formations, are the pathological findings seen in peripheral nerve biopsies from HNPP patients (Madrid & Bradley 1975).

HNPP occurs during adolescence, causing attacks of acute mononeuropathy. Entrapment neuropathies such as carpal tunnel syndromes and peroneal and ulnar palsies are typical manifestations of HNPP, but it can possess atypical features such as recurrent transient sensory symptoms, scapuloperoneal syndrome or mild generalized neuropathy, especially in older individuals. Most palsies are painless and are usually followed by complete recovery. About one-fourth of the patients affected can be almost or totally free of symptoms, while one-third complain of chronic symptoms (Pareyson et al. 1996).

2.2.4 Inherited metabolic neuropathies

The inherited metabolic neuropathies are a heterogeneous group of diseases in which neuropathy is one aspect of the clinical features (Table 1). Major advances in molecular genetics have had new implications for diagnosis, screening, testing and genetic counseling in this field.

Familial amyloid neuropathy (FAP) is an autosomal dominant disease characterized by the deposition of a fibrillar protein with a beta-pleated structure in the extracellular space (Andrade 1952). It is classified into three subtypes according to the constituent fibril protein: transthyretin (TTR), apolipoprotein A-1 and gelsolin. TTR-related FAP is the most common variety (Reilly 2002b). TTR-related FAP is usually a small-fibre neuropathy
with autonomic dysfunction (Reilly & King 1993) but it can present as a large-fibre polyneuropathy (Drugge et al. 1992). Various point mutations in the TTR gene have been described. Amyloid neuropathy can also be an acquired disease secondary to B cell dyscrasia and immunoglobulin light chain deposition. The porphyrias, which result from the interruption of heme biosynthesis, are rare causes of peripheral neuropathies. Neuropathies, typically of the motor kind, are associated with four types of porphyria (Table 1).

The metachromatic leukodystrophies (MLD) are also a heterogeneous group of autosomal recessive diseases, and are characterized by an abnormal accumulation of galactosyl sulphatide in the glia and Schwann cells, causing demyelination in the central nervous system and peripheral nerves. MLD may occasionally present with peripheral neuropathy as the dominant feature (Hagberg et al. 1962, Aziz & Pearce 1968). Three types of MLD can be recognized: late-infantile, juvenile and adult-onset. More than 40 mutations in the arylsulphatase A gene have been described in patients with MLD (Gieselmann et al. 1998).

Globoid-cell leukodystrophy (Krabbes disease) is an autosomal recessive disease of infancy resulting from a deficiency in galactosylceramide β-galactosidase (Krabbe 1916, Suzuki & Suzuki 1970). The gene coding for this enzyme is situated on chromosome 14 (Suzuki et al. 1995) and several mutations have been described in this disorder (De Gasperi et al. 1996). Demyelinating sensorimotor polyneuropathy develops at six months of age and usually leads to death within two years of onset.

X-linked adrenoleukodystrophy (ALD) results from an accumulation of very-long-chain fatty acids (VLCF) because of impaired peroxisomal β-oxidation (Moser et al. 1981, Moser et al. 1992). This disorder affects the central and peripheral nervous systems and the adrenal cortex. More than 340 mutations have been identified in the gene encoding the ALD protein, which is located at Xq28 (Mosser et al. 1993, Bezman et al 2001). Mild axonal, and occasionally demyelinating, peripheral polyneuropathy with spastic paraplegia are features of adrenomyeloneuropathy (AMN), which is one of the several phenotypes of ALD.

An accumulation of the branched-chain fatty acid phytanic acid, brought about by a deficiency in the peroxisomal enzyme phytanoyl-CoA hydroxylase (Jansen et al. 1997, Mihalik et al. 1997), causes classical Refsum’s disease. This is an autosomal-recessive disorder which affects children or adolescents, and is usually accompanied by progressive sensorimotor demyelinating polyneuropathy.

Hereditary high-density lipoprotein deficiency, or Tangier disease, is a very rare autosomal recessive disease characterized by the deposition of cholesteryl esters in many tissues, including the peripheral nerves. It is caused by mutations in the gene encoding the adenosine triphosphate binding cassette transporter (ABC-1) (Brooks-Wilson et al. 1999, Remaley et al. 1999). The most common clinical manifestation is peripheral neuropathy, which is often asymptomatic but may be of the asymmetric mononeuritic type or a slowly progressive symmetric polyneuropathy.

Bassen-Kornzweig disease, or abetalipoproteinaemia, is also a rare autosomal recessive disorder with the absence of plasma lipoproteins containing apolipoprotein B (Apo B). It is caused by mutations in the gene encoding a microsomal triglyceride transfer protein (Wetterau et al. 1992, Sharp et al. 1993). Although the main neurological feature is progressive ataxia, peripheral sensorimotor polyneuropathy is occasionally seen with a stocking-glove pattern (Wichman et al. 1985).
Fabry’s disease, or α-galactosidase A deficiency, results from a deficiency in the lysosomal hydrolase α-galactosidase (Brady 1966, Kornreich et al. 1989), and is an X-linked disorder characterized by the accumulation of neutral glycosphingolipids, particularly in the vascular lysosomes. The usual manifestation is sensory neuropathy with a burning pain, which can be triggered by physical exertion or emotional stress.

Cholestanolosis (cerebroretinoid xenanthomatosis) results from a defect in the sterol 27-hydroxylase, leading to the accumulation of cholestanol and cholesterol in many tissues, especially the nervous system (Thomas 1993). The phenotype is of variable severity and predominantly sensory and mainly axonal peripheral neuropathy is one of its features (Argov et al. 1986). Sphingomyelin lipidoses (Niemann-Pick disease) are classified into four types, I-IV, types I and II being true sphingomyelin lipidoses with the accumulation of sphingomyelin in many tissues (Thomas 1993). Segmental demyelination causing peripheral neuropathy can occur in type I.

Defects in DNA repair or replication after damage by ultraviolet irradiation or various chemical carcinogens can lead to xeroderma pigmentosum (XP). This is a rare autosomal recessive disorder that can be divided into seven subgroups. Eight genes have been linked to XP (Cleaver et al. 1999). The neurological form of XP includes central nervous system symptoms, and axonal sensory peripheral neuropathy may be present.

Patients with Cockayne’s syndrome usually suffer from sensitivity to sunlight and central nervous system deficiencies. Demyelination is seen both in the central nervous system and in the peripheral nerves. Ataxia telangiectasia is an autosomal recessive disorder characterized by oculocutaneous telangiectasia, immunological deficiency, chromosomal instability and neurological complications, in which peripheral neuropathy is caused by loss of large myelinated fibres.

### 2.2.5 Epidemiology of hereditary neuropathies

Peripheral neuropathy is one of the most common neurological diagnoses in clinical practice, affecting about 2–8% of the population (Martyn & Hughes 1997) and about 13% of those aged over 55 years (Monticelli & Beghi 1993). Inherited neuropathies are common disorders that may remain undiagnosed. The prevalence of hereditary motor and sensory neuropathies has been estimated to range from 4.7 to 36/100,000 (Hughes 1995), and has been found to be 20.1/100,000 in Sweden, with the demyelinating form accounting for 16.2/100,000 (Holmberg 1993). The prevalence of the most common form, HMSN type I, is estimated roughly at 1/5000 to 1/6000, making it one of the most common inherited diseases (Dyck et al. 1993).

The prevalence of hereditary liability to pressure palsies is difficult to estimate because of unrecognized cases and unaffected carriers (Hughes 1995). If the unequal crossing-over hypothesis is correct, the prevalence of HNPP should be close to that of HMSN type I (Pareyson et al. 1996). The prevalence of HNNP is 16/100,000 in Finland (Meretoja et al. 1997). The prevalence of symptomatic amyloid neuropathy in northern Sweden has been estimated to be 31/100,000 but the mutation prevalence to 1,500/100,000 (Holmberg et al. 1994). The frequency of HSAN is unknown. The incidence of metachromatic leukodystrophy is estimated to be 1/40,000–50,000 (Farrel 1981, Gustavson & Hagberg
1971), and similar rates have been described for adrenoleukodystrophy in men (Sereni et al. 1993, Bezman et al. 2001). The incidence of Fabry’s disease has been estimated at 1/117,000 births (Miekle et al. 1999).

2.3 Inherited metabolic myopathies

Metabolic disorders causing myopathy or neuropathy bear a clinical resemblance to symptoms caused by mitochondrial DNA defects. Myopathy is especially prominent in primary disorders of energy metabolism and may occur at any age. Typical symptoms are exercise intolerance, muscle pain, fatigue, cramps, contractures and myoglobinuria. Metabolic myopathies involve impairment of the maintenance of ATP levels, resulting in exercise intolerance. Symptoms can improve during sustained exercise because of the shift to the use of free fatty acids in patients with glycogenoses (the “second wind” phenomenon). The course of the disease may be episodic or chronic progressive, or both.

Muscle uses predominantly fatty acids at rest, but during maximal work their energy is derived from anaerobic glycolysis. The pattern of muscle fuel utilization during exercise is determined by the type, intensity and duration of the activity. Local stores of ATP and its derivation from the hydrolysis of phosphocreatine are sufficient only for a few seconds, and this is followed by ATP production by anaerobic oxidation for the next 30 seconds. After that it is aerobic oxidative phosphorylation, the main mode of energy production in muscles, that is the primary source during sustained exercise. Oxidative energy production continues after the depletion of glycogen, and free fatty acids are the major energy supply allowing for prolonged exercise. Free fatty acids and glucose are produced in the liver (Brooke & Cwik 1996, DiMauro & Musumeci 2002) and delivered by the blood.

Metabolic myopathies have been categorized into three main groups: disorders of carbohydrate metabolism, or glycogenoses, disorders of lipid metabolism and a miscellaneous group. Metabolic myopathies can also be divided into two groups according their clinical presentation (Figure 1): diseases characterized by acute, recurrent and reversible muscle dysfunction causing exercise intolerance, myalgia with or without painful muscle cramps and myoglobinuria, and diseases with fixed muscle weakness, sometimes simulating muscular dystrophies (DiMauro & Musumeci 2002). Analysis of specific metabolic products in the blood and urine can be used to diagnose metabolic myopathies, e.g. patients with carnitine deficiency have markedly decreased plasma carnitine levels. Serum creatine kinase is generally elevated in glycogenoses and also rises markedly in other metabolic myopathies in cases of rhabdomyolysis. Non-invasive near-infrared spectroscopy or phosphate magnetic resonance spectroscopy can be used to detect or monitor a metabolic defect, and provocative tests such as the forearm ischaemic test, dynamic exercise test or monitored prolonged starvation test may be helpful in the investigation of metabolic disorders. Molecular tests are available for the common mutations associated with medium-chain acyl-Coa dehydrogenase deficiency and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency, and for analyses of most other metabolic myopathies in research laboratories (Renaud & Clarke 2003).
Fig. 1. Metabolic myopathies causing either fixed weakness or exercise intolerance and myoglobinuria. PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; LDH, lactate dehydrogenase; MCAD, medium-chain acyl-coenzyme A; SCAD, short-chain acyl-coenzyme A dehydrogenase; GA, glutaric aciduria type II (multiple acyl-CoA dehydrogenase deficiency); TG, triglyceride; CPT, carnitine palmitoyltransferase; VLCAD, very long-chain acyl-coenzyme A dehydrogenase; TP, trifunctional protein; SCHAD, short-chain 3-hydroxyacyl-coenzyme A dehydrogenase; CoQ10, coenzyme Q10 (modified after DiMauro & Musumeci 2002).

### 2.3.1 Disorders of carbohydrate metabolism

The disorders of carbohydrate metabolism which cause myopathy are currently classified according to the enzyme defect in the metabolic pathway (Table 2).
Table 2. Classification of disorders of carbohydrate metabolism associated with myopathy (modified after Renaud & Clarke 2003).

<table>
<thead>
<tr>
<th>Location of biochemical defect</th>
<th>Enzyme defect</th>
<th>Inheritance</th>
<th>Gene locus</th>
<th>Major clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogenolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McArdle disease</td>
<td>Myophosphorylase</td>
<td>AR</td>
<td>11q13</td>
<td>E, R</td>
</tr>
<tr>
<td>Cori-Forbes disease</td>
<td>Debrancher enzyme</td>
<td>AR</td>
<td>1p21</td>
<td>C, G, H</td>
</tr>
<tr>
<td>PBK deficiency</td>
<td>Phosphorylase b kinase</td>
<td>AR</td>
<td>16q12-q13</td>
<td>E, H, R</td>
</tr>
<tr>
<td>Glycolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tarui disease</td>
<td>Phosphofructokinase</td>
<td>AR</td>
<td>12q13.3</td>
<td>A, E, R</td>
</tr>
<tr>
<td>PGK deficiency</td>
<td>Phosphoglycerate kinase</td>
<td>XR</td>
<td>Xq13</td>
<td>A, P, R</td>
</tr>
<tr>
<td>PGM deficiency</td>
<td>Phosphoglycerate mutase</td>
<td>AR</td>
<td>7p13-p12.3</td>
<td>E, R</td>
</tr>
<tr>
<td>LDH deficiency</td>
<td>Lactate dehydrogenase</td>
<td>AR</td>
<td>11p15.4</td>
<td>E, R</td>
</tr>
<tr>
<td>Glycogen synthesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andersen disease</td>
<td>Glycogen branching enzyme</td>
<td>AR</td>
<td>3p13</td>
<td>C, G, H</td>
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<tr>
<td>Lysosomal glycogen storage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pompe disease</td>
<td>Lysosomal α-glucosidase</td>
<td>AR</td>
<td>17q25.2-q25.3</td>
<td>C</td>
</tr>
<tr>
<td>Danon disease</td>
<td>LAMP-2</td>
<td>AD, AR, XD</td>
<td>Xq24</td>
<td>C, P</td>
</tr>
</tbody>
</table>

Abbreviations: A, haemolytic anaemia; AD, autosomal dominant; AR, autosomal recessive; C, cardiomyopathy; E, exercise intolerance; G, hypoglycaemia; H, hepatomegaly; LAMP-2, lysosome-associated membrane protein-2; LDH, lactate dehydrogenase; P, psychomotor retardation; PBK, phosphorylase b kinase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; R, rhabdomyolysis; XD, X-linked dominant; XR, X-linked recessive.

2.3.1.1 Defects in glycogenolysis

Phosphorylase kinase (PhK) regulates glycogen metabolism by activating glycogen phosphorylase. Because of the complexity of the enzyme, which is composed of four subunits, the clinical and genetic presentations are heterogeneous. Four clinical variants can be distinguished on the basis of tissue involvement, two of them involving skeletal muscle. PhK defects can be inherited in autosomal or X-linked fashion. A myopathic variant of PhK deficiency, with exercise intolerance and muscle cramps, resembles a milder form of myophosphorylase deficiency, but the ischaemic forearm exercise test is normal (DiMauro et al. 1997).

McArdle’s disease is caused by a deficiency in muscle myophosphorylase, encoded by a gene on chromosome 11 (Lebo et al. 1984). Considerable molecular heterogeneity is observed, in that at least 16 mutations have been reported (Andreu et al. 1998a). Exercise intolerance is a major clinical feature, accompanied by muscle cramps, myalgia or stiffness. Most patients have mild fixed proximal muscle weakness, and the “second wind” phenomenon is common. Myoglobinuria is seen in approximately half of the patients. A diagnosis is generally made in the second or third decade of life, but a history of exercise intolerance from childhood is often present.
The severity of symptoms and amount of exercise needed to precipitate them may vary considerably from patient to patient. McArdle’s disease is the second most common metabolic cause of recurrent myoglobinuria in adults after carnitine palmitoyltransferase II (CPT II) deficiency. Because intracellular glycogen cannot be used, there is no rise in venous lactate or it is abnormal, which means that the ischaemic forearm test is useful but not specific for screening purposes. A histological examination may reveal the diagnosis by means of a specific stain and an increase in glycogen.

Cori-Forbes disease, caused by a deficiency in the debrancher enzyme, can be divided into two forms, in both of which patients present with hepatomegaly and fasting hypoglycaemia in infancy (DiMauro et al. 1997). Slowly progressive distal muscle weakness often develops in the third or fourth decade, usually accompanied by exercise intolerance, myoglobinuria and peripheral neuropathy.

2.3.1.2 Defects in glycolysis

Phosphofructokinase deficiency, or Tarui’s disease, caused by a deficiency in muscle phosphofructokinase (PFK), is clinically indistinguishable from McArdle’s disease. PFK is the rate-limiting enzyme of glycolysis and converts fructose 6-phosphate to fructose 1.6-diphosphate, so that a deficiency, which is inherited as an autosomal recessive trait, will block glycolysis. Exercise intolerance accompanied by muscle cramps appears to worsen with high carbohydrate intake (Dunaway et al. 1988) and the second wind phenomenon has been described in some patients. Attacks are often accompanied by nausea, vomiting and muscle pain. Mild jaundice and arthritis due to myogenic hyperuricaemia may occur in some patients. Increased bilirubin levels and reticulocyte counts, reflecting mild haemolytic anaemia, are differential diagnostic tests with respect to McArdle’s disease. A severe, early-onset myopathy with respiratory failure has also been described (Swoboda et al. 1997).

Phosphoglycerate kinase (PGK) deficiency is a rare, X-linked recessive disorder. Several point mutations have been described, and clinical presentation may be highly heterogeneous (Tsujino et al. 1995a). Myopathy and haemolytic anaemia may be accompanied by central nervous system symptoms such as mental retardation and seizures. Phosphoglycerate mutase deficiency is marked by exercise intolerance, cramps and recurrent myoglobinuria (DiMauro et al. 1981a).

Lactate dehydrogenase (LDH) deficiency may be asymptomatic or cause exercise intolerance, cramps, fatigue and myoglobinuria. LDH, a tetrameric enzyme composed of muscle-specific (LDH-A) and cardiac (LDH-B) subunits, converts pyruvate to lactate in the final step of the glycolytic pathway (Kanno et al. 1983). Pyruvate is markedly elevated in the ischaemic forearm exercise test, while no lactate is produced.

Aldolase A is the isoenzyme present in erythrocytes and skeletal muscle. A case report exists describing aldolase A deficiency as leading to haemolytic anaemia, exercise intolerance, myopathy and mild developmental delay (Kreuder et al. 1996).
2.3.1.3 Defects in glycogen synthesis

Branching enzyme deficiency (Andersen disease) in glycogen synthesis can appear in four clinically heterogeneous forms. The enzyme defect can be silent or affect predominantly the liver, heart, skeletal muscle or brain (DiMauro et al. 1997). One subtype leads to progressive liver dysfunction, one to cardiomyopathy, one to progressive upper and lower motor neuron involvement (adult polyglucosan body disease, APBD) and a neuro-muscular form causes slowly progressive muscle weakness and atrophy.

2.3.1.4 Lysosomal glycogenoses

Acid maltase deficiencies (AMD) involve lysosomal acid maltase or acid $\alpha$-glucosidase (Engel et al. 1973). Acid maltase is a lysosomal enzyme involved in the degradation of glycogen, and a deficiency results in the deposition of undegraded glycogen both outside and within lysosomes. More than 50 mutations have been reported in the gene encoding acid maltase. Pompe’s disease, the infantile form, presents with hypotonia, muscle weakness and macrognlossia, along with rapidly progressive hypertrophic cardiomyopathy. Death results within the first two years of life due to cardiac or respiratory failure. In childhood AMD the onset of muscle weakness occurs in late infancy or early childhood, and death, following respiratory failure, ensues in the second decade.

Slowly progressive proximal myopathy resembling polymyositis or limb-girdle myopathy is characteristic of the adult form of AMD, which can be distinguished clinically on the basis of the early and selective involvement of the respiratory muscles. The onset of symptoms occurs in the third decade, and respiratory failure is the most common cause of death. An accumulation of glycogen is seen, and this may cause the formation of intracranial aneurysms (Makos et al. 1985). Histological examination reveals vacuoles in the muscles, the extent of vacuolation is largely proportional to the clinical severity of the disease.

The deficient enzyme in Danon disease is lysosome-associated membrane protein-2 (LAMP-2), which is a lysosomal structural protein rather than a glycolytic enzyme (Nishino et al. 2000a), and this is no longer considered a lysosomal glycogen storage disease. Because of the X-linked dominant mode of inheritance, the patients are men, and women are affected by a milder disease. Danon disease consists of paroxysmal muscle weakness, hypertrophic cardiomyopathy and mental retardation (Danon et al. 1981). The myopathy is usually mild mostly, involving the neck and shoulder girdle. Two forms of the disease have been described, early-onset and late-onset (Amato 2000).

2.3.1.5 Treatment of glycogenoses

There are no effective treatments for glycogenoses. Prevention is based on identification of the underlying metabolic defect or the precipitating exogenous substances. Patients
must adapt their lifestyle to limited exercise tolerance and avoid situations that might lead to myoglobinuria and thus to acute renal insufficiency. A high-protein diet, pyridoxine and creatine have been suggested, but no therapy has proved to be beneficial. Maintenance of normoglycaemia has proved to be useful in cases of a debranching enzyme defect, and an intravenous injection of purified acid α-glucosidase has been found to improve the clinical and metabolic features of acid maltase deficiency (Kikuchi et al. 1998). Efforts have been made to develop gene therapy for McArdle’s disease by transferring an adenoviral recombinant with human myophosphorylase complementary DNA into phosphorylase-deficient sheep and human myoblasts (Pari et al. 1999).

2.3.2 Disorders of lipid metabolism

At least 21 enzymes and transporters are involved in the oxidative metabolism of fatty acids in mitochondria, many of them associated with distinct clinical disorders, including acute and chronic myopathies (Table 3). The importance of recognizing these clinical entities lies in the fact that most of the related potentially fatal disorders are treatable. Short and medium-chain fatty acids are dependent on esterification with carnitine through the carnitine cycle for transport into mitochondria, while free long-chain fatty acids are initially esterified with coenzyme A (CoA) in the outer mitochondrial membrane by long-chain acyl-CoA synthetase. Carnitine palmitoyl transferase I (CPT I), located in the inner aspect of the outer mitochondrial membrane, catalyzes the trans-esterification of long-chain acyl-CoAs to long-chain acylcarnitines, with the release of free coenzyme A. This permits the transport of long-chain fatty acyl groups across the mitochondrial membrane. CPT II is a key enzyme in the carnitine cycle, which consist of four elements. The disorders of lipid metabolism may be subdivided into those involving the carnitine cycle and those affecting the intramitochondrial β-oxidation of fatty acids. All the disorders of fatty acid metabolism are inherited as autosomal recessive traits.
Table 3. Classification of disorders of fatty acid metabolism (modified after Renaud & Clarke 2003).

<table>
<thead>
<tr>
<th>Location of defect</th>
<th>Enzyme defect</th>
<th>Inheritance</th>
<th>Gene locus</th>
<th>Main clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnitine cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMCT</td>
<td>OCTN2</td>
<td>AR</td>
<td>5q33.1</td>
<td>C, D, G, H, M, R</td>
</tr>
<tr>
<td>CPT I</td>
<td>CPT I</td>
<td>AR</td>
<td>11q13, 22q13.3</td>
<td>G, H</td>
</tr>
<tr>
<td>CPT II</td>
<td>CPT II</td>
<td>AR</td>
<td>1p32</td>
<td>C, E, M, R</td>
</tr>
<tr>
<td>CACT</td>
<td>CACT</td>
<td>AR</td>
<td>3p21.31</td>
<td>G</td>
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<tr>
<td>Fatty acid oxidation</td>
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<tr>
<td>VLCAD</td>
<td>VLCAD</td>
<td>AR</td>
<td>17p11.2-p11.1</td>
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<td>LCAD</td>
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<td>AR</td>
<td>2q34-q35</td>
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</tr>
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<td>MCAD</td>
<td>MCAD</td>
<td>AR</td>
<td>1p31</td>
<td>D, G</td>
</tr>
<tr>
<td>SCAD</td>
<td>SCAD</td>
<td>AR</td>
<td>12q22-qter</td>
<td>M</td>
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<tr>
<td>Multiple acyl-CoA dehydrogenase</td>
<td>ETF/ETF-Q10O</td>
<td>AR</td>
<td>15q23-q25, 19q13.3, 4q32-qter</td>
<td>G, H, M</td>
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<td>TP/LCHAD</td>
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<td>AR</td>
<td>2p23</td>
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<tr>
<td>SCHAD</td>
<td>SCHAD</td>
<td>AR</td>
<td>4q22-26</td>
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</tbody>
</table>

Abbreviations: AR, autosomal recessive; C, cardiomyopathy; CACT, carnitine-acylcarnitine translocase; CoA, coenzyme A; CPT, carnitine palmitoyltransferase; D, sudden death; E, exercise intolerance; ETF, electron transfer flavoprotein; ETF-Q10O, ETF-coenzyme Q oxidoreductase; G, hypoglycaemia; H, hepatomegaly; LCAD, long-chain acyl-CoA dehydrogenase; LCHAD, long-chain hydroxyacyl-CoA dehydrogenase; M, myopathy; MCAD, medium-chain acyl-CoA dehydrogenase; OCTN2, sodium ion-dependent carnitine transporter; PMCT, plasma membrane carnitine transporter; R, rhabdomyolysis; SCAD, short-chain acyl-CoA dehydrogenase; SCHAD, short-chain hydroxyacyl-CoA dehydrogenase; TP, trifunctional protein; VLCAD, very long-chain acyl-CoA dehydrogenase.

2.3.2.1 Carnitine cycle disorders

Autosomal recessive plasma membrane, i.e. primary systemic, carnitine transporter deficiency causes proximal myopathy and progressive hypertrophic or dilated cardiomyopathy in childhood, with reduced plasma, liver and muscle carnitine concentrations (Tein et al. 1990). Cardiac function responds dramatically to carnitine supplementation. Hypoketotic hypoglycaemia, acute encephalopathy and hepatomegaly have also been reported. Myopathy may be manifested as a slowly progressive proximal weakness or hypotonia.

Primary myopathic carnitine deficiency is characterized by decreased muscle carnitine but normal serum carnitine, causing progressive proximal muscle weakness, sometimes with a fluctuating course. Fatigue, exercise-related pains or cardiomyopathy are occasionally reported. Symptoms occur during childhood. Secondary carnitine deficiency may accompany metabolic disorders, hepatic cirrhosis, malnutrition, pregnancy or iatrogenic factors (hemodialysis, valproate therapy), for instance, and in this secondary form serum carnitine is decreased, and often that in muscle, too (Pons & Vivo 1995, Brivet et al. 1999).

Carnitine palmitoyltransferase I (CPT I) deficiency leads to classical infantile hepatic disease with hypoketotic hypoglycaemia, encephalopathy and hepatomegaly, but little
involvement of the skeletal or cardiac muscles. A deficiency in CPT II, which is situated in carnitine/acylcarnitine translocase in the inner mitochondrial membrane and releases free carnitine, is one of the most common metabolic abnormalities in muscle and was first described in two brothers with exercise-induced myoglobinuria (DiMauro & DiMauro-Melis 1973). It is more common in males than in females. CPT II deficiency appears in three subtypes; a rare lethal neonatal form, an infantile hepatomuscular form and, the most common, an adult myopathic form involving recurrent episodes of rhabdomyolysis and myoglobinuria without painful cramps precipitated by prolonged fasting or often strenuous exercise. Clinical examination findings and CK values are normal between attacks. The precipitating factors may also include cold exposure, high fever or lack of sleep. The patients are predominantly men, suggesting that gene expression may be hormonally regulated (Montermini et al. 1994).

CTP II deficiency may be indistinguishable from some fatty acid oxidation disorders such as very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD). The ischaemic forearm exercise test, routine laboratory blood test and needle EMG are normal. Muscle histology may reveal some lipid droplets, but it is usually normal. The diagnosis can be made by analysing muscle CPT II activity in or identifying the most common mutation, Ser113Leu (Kaufmann et al. 1997), which accounts for 60% of mutant alleles (Taroni et al. 1993). Carnitine-acylcarnitine translocase (CACT) exchanges acylcarnitine and carnitine across the inner mitochondrial membrane. CACT deficiency occurs in the neonatal period, with hyperammonemia, hypoglycaemia, hypotonia and cardiac arrhythmias (Brivet et al. 1999).

2.3.2.2 Fatty acid oxidation disorders

These disorders may be difficult to distinguish from carnitine cycle disorders. The best screening laboratory test is determination of the profile of acylcarnitines in plasma by tandem mass spectrometry (DiMauro & Musumeci 2002). Very long-chain acyl-CoA dehydrogenase (VLCAD) and trifunctional protein (TP) prepare long-chain fatty acids for β-oxidation in the mitochondrial matrix, and deficiencies in these cause hypoketotic hypoglycaemia with liver dysfunction, cardiomyopathy and sudden infant death. Hypotonia and progressive myopathy may also occur (DiDonato 1997).

Short-chain acyl-CoA dehydrogenase (SCAD) deficiency presents with metabolic acidosis, poor feeding, vomiting and hypotonia. Developmental delay, myopathy and seizures have also been described (Turnbull et al. 1984). The condition includes hyperketotic rather than hypoketotic hypoglycaemia, and congenital multicore myopathy and progressive external ophthalmoplegia have also been reported (Tein 1999). Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency causes episodic hypoketotic hypoglycaemia induced by fasting or infection in childhood. Lethargy, vomiting, encephalopathy, seizures and sudden death are involved in this disorder, but myopathy is unusual (Lafolla et al. 1994).

Multiple acyl-CoA dehydrogenase deficiency (glutaric aciduria type II), which results from electron transfer flavoprotein (ETF) or ETF-coenzyme Q10 oxidoreductase deficiency, possesses three clinical subtypes: a severe neonatal form characterized by hypoto-
nia, hepatomegaly, facial dysmorphism and multiple congenital anomalies and early
death, a milder form with a longer survival period and without anomalies, and an adult
form with myopathy and exertional myalgias (Mongini et al. 1992). Multisystem triglyceride
storage disorder (Chanarin disease) is characterized by massive triglyceride storage
in all tissues, with ataxia, steatorrhoea, ichthyosis, nystagmus and progressive limb weakness (Chanarin et al. 1975). The biochemical foundation for this disorder lies in an inability to degrade endogenously synthesized triglycerides.

2.3.2.3 Treatment of disorders of fatty acid metabolism

The most important aspect is avoidance of prolonged fasting, and a high-carbohydrate,
low-fat diet with fat-soluble vitamins is beneficial. Medium-chain triglyceride oil is used
for patients with long-chain fatty acid disorders, but is contraindicated in MCAD defi-
ciency. Carnitine supplementation is important for patients with plasma membrane car-
nitine transporter deficiency, and riboflavin in multiple acyl-CoA dehydrogenase and
SCAD deficiency. One should avoid aspirin in β-oxidation disorders, and valproic acid
because of the risk of carnitine depletion. Genetic counselling is essential for the screen-
ing of family members and the prevention of fatal attacks by the early initiation of treat-
ment (Renaud & Clarke 2003).

2.3.3 Miscellaneous disorders

Adenylate deaminase deficiency (AMPD) causes various symptoms, the most common
being cramps, stiffness or muscle pain. Some patients have muscle weakness, paraesthesia
or periodic paralysis, but others are asymptomatic. Case reports have shown that
patients who were homozygous for mutations in the myophosphorylase or phosphofruc-
tokinase gene were also homozygous for the AMPD-1 mutation (Tsujino et al. 1995b). A
specific histochemical stain can confirm a diagnosis of AMPD even though a muscle
biopsy will often be normal.

2.4 Mitochondria

2.4.1 Structure of mitochondria

Mitochondria are approximately the size of bacteria (1–2 μm by 0.5–1.0 μm) and are
located in the cytoplasm of cells. They are more numerous in type I and IIA muscle fibres
than in the type IIB fibres and are normally located between myofibrils adjacent to the Z
disc, beneath the sarcolemma, at the poles of nuclei and at motor end plates (Carpenter &
Three-dimensional reconstructions indicate that skeletal muscle mitochondria are of a complex branched shape, varying in size and complexity (Kayar et al. 1988) and can undergo rapid, characteristic changes as a consequence of muscle use, as in exercise training or under environmental conditions such as microgravity (Howald et al. 1985, Hoppeler & Flück 2003).

Structurally, mitochondria have four compartments: an outer membrane, an inner membrane, the intermembrane space and the matrix. The outer membrane, which separates the organelle from the cytoplasm, differs from the inner membrane in function and in its lipid and protein content (Ernster & Kuylenstierna 1970). The lipid content of the outer membrane is about 50% and that of the inner membrane about 30% by weight. Most of the lipids are phospholipids. Of the neutral lipids, cholesterol is more abundant in the outer membrane than in the inner membrane. Ubiquinone is present only in the inner membrane (Lee & Martens 1994). The inner membrane is folded into cristae, which increase its surface. It is relatively rich in protein, and about 70% of the membrane proteins are integral proteins. Where the outer membrane is rich in porin, and thus relatively permeable to most ions, the inner membrane has a very low permeability, so that charged ions (Na\(^+\), Cl\(^-\)) cannot diffuse through it.

The matrix contains enzymes that metabolize pyruvate and fatty acids, the citric acid cycle enzymes, some chaperone proteins, enzymes for the expression of the mitochondrial genome, mitochondrial DNA, ribosomes and transfer RNAs. The most common marker enzymes of the mitochondrial compartments are monoamine oxidase (outer membrane), respiratory chain enzymes e.g. cytochrome oxidase (inner membrane), adenylate kinase or creatine kinase (intermembrane space) and malate dehydrogenase (matrix).

### 2.4.2 Oxidative phosphorylation and other biochemical properties

Mitochondria are intimately involved in cellular homeostasis in that they perform numerous tasks that are essential to the cell (Figure 2). The most important is the generation of energy by producing adenosine triphosphate (ATP), but pyruvate oxidation, the tricarboxylic acid cycle and metabolism of fatty acids, amino acids and steroids are also crucial. The biochemical process of aerobic metabolism takes place in the following steps: transport, substrate use, the tricarboxylic acid cycle, the electron transport chain and oxidation-phosphorylation coupling. Mitochondria generate energy in the form of ATP by oxidative phosphorylation. The respiratory chain (incorporating the electron transport chain and the oxidative phosphorylation system) is situated in the inner mitochondrial membrane and consists of five multimeric protein complexes and contains about 100 protein subunits. Reducing equivalents (electrons) are carried along the respiratory chain to create an efflux of protons from the matrix across the inner membrane and produce a transmembrane proton gradient. Energy created by this electrochemical gradient is used by complex V to synthesize ATP from ADP. Furthermore, mitochondria participate in the intracellular calcium ion dynamics and Ca\(^{2+}\) activates certain dehydrogenases in the mitochondrial matrix.

The biochemical defect entailed in a mitochondrial disease can manifest itself in several steps along the biochemical pathway. First, the defect may be localized in substrate
transport to the mitochondrion, as in carnitine or CPT deficiencies. Second, it can affect mitochondrial substrate utilization in fatty acid oxidation, ketone synthesis or pyruvate oxidation. Third, defects can be present in the citric acid cycle, e.g. fumarase or aconitase deficiencies. Fourth, a defect may affect any complex of the respiratory chain, and finally, fifth, it may affect oxidation-phosphorylation coupling. The small mitochondrial genome may contain several mutations, causing a wide variety of clinical syndromes.

**Fig. 2. Metabolic pathways in mitochondria.** Respiratory chain complexes and components are situated in the grey box. ADP, adenosine diphosphate; ANT, adenine nucleotide translocator; ATP, adenosine triphosphate; CACT, carnitine-acylcarnitine translocase; CoA, coenzyme A; CoQ, coenzyme Q; CPT, carnitine palmitoyltransferase; Cyt c, cytochrome c; DIC, dicarboxylate carrier; ETF, electron-transfer flavoprotein; ETF-DH, electron-transfer dehydrogenase; FADH₂, reduced flavin adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PDHC, pyruvate dehydrogenase complex; 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 and V, complex V (ATP synthase).

### 2.4.3 Mitochondrial DNA

Mitochondria are the only organelles of the cell besides the nucleus that contain their own DNA (mitochondrial DNA, mtDNA) and their own properties for synthesizing RNA and
proteins. MtDNA codes for less than 10% of total mitochondrial protein, a small but vital set of proteins. The mitochondrial genome has been completely sequenced and all 37 genes have been identified. Mitochondrial DNA is a circular, double-stranded structure of 16,569 base pairs (Anderson et al. 1981, Andrews et al. 1999), most of the information in which is encoded in the heavy strand, which harbours genes for 12S RNA, 16S RNAs, 14 tRNAs and 12 proteins, while the light strand codes for eight tRNAs and one protein. Mitochondrial DNA is highly polymorphic, and any two individuals may differ by up to 60 base pairs (Chinnery & Schon 2003a) and any two Finns by an average of 30 base pairs (Finnilä et al. 2001).

Of the 37 genes, 24 are needed for mitochondrial DNA translation (22 transfer RNAs and two ribosomal RNAs) and the remaining 13 encode polypeptides, the RNA genes being interspaced between the protein-encoding genes. The D-loop, a 1.1 kb non-coding region, is involved in the regulation of transcription and replication of the genome but not directly in the synthesis of respiratory chain polypeptides. All the polypeptides are subunits of the respiratory chain: seven subunits of complex I (ND1, 2, 3, 4, 4L, 5 and 6), one subunit of complex III (cytochrome b), three subunits of complex IV, or cytochrome c oxidase (CO1, CO2, CO3), and two subunits of complex V, or ATP synthase (A6 and A8). Complex II (succinate dehydrogenase) is completely nucleus-encoded, as are the majority of the mitochondrial respiratory chain polypeptides (Shoubridge 2001).

Each mitochondrion contains two to ten copies of mtDNA, and as each cell contains multiple mitochondria, this means that there are thousands of copies of mtDNA in each cell. Over 150 point mutations have so far been identified in mtDNA (Chinnery & Schon 2003a, Figure 3). Although they may affect ribosomal RNA or protein-encoding genes, most of them occur in tRNA genes. A reduced quantity of functional tRNAs will impair mitochondrial protein translation. The point mutations are usually maternally inherited. On the other hand, a genetic defect may affect mitochondrial function though large-scale rearrangements in the mtDNA, including both large deletions and, less commonly, duplications of single segments. Approximately one-third of the mtDNA deletions involve an identical 4977 base-pair segment, often referred to as the common deletion. Single deletions are usually sporadic, suggesting that they may be selected out during rapid division of the germ cell line (Ballinger et al. 1992). Duplications of mtDNA were initially reported in patients with Kearns-Sayre syndrome (KSS) with deletions (Poulton et al. 1989).

Wild-type mtDNA can be subdivided into various genetic groups, i.e. haplogroups, based on a characteristic pattern of polymorphism that occurs within the normal population (Torroni et al. 1996), and the haplogroup background may modify the phenotypes of pathogenic mutations (Torroni et al. 1997). There is evidence that environmental factors may affect the manifestation of specific symptoms in mitochondrial disease, e.g. Leber hereditary optic neuropathy (LHON) may proceed to visual failure due to heavy smoking, alcohol intoxication or starvation (Riordan-Eva et al. 1995, Sadun 1998).
2.4.4 Mitochondrial genetics

Mitochondrial genetics differs from that of nuclear DNA in several aspects, and these properties are responsible for the unusual genetic features of mitochondrial diseases. First, mtDNA does not contain introns, and therefore the genome is tightly packed with information. Second, mtDNA is maternally inherited, i.e. only the mother contributes to the mitochondrial genome of the offspring. Third, mtDNA undergoes spontaneous mutations between 10 and 20 times more rapidly than nuclear DNA, possibly due to the lack of protective histones and introns and the absence of effective DNA repair systems. Fourth, the mitochondrial genetic code differs from the universal nuclear genetic code. Fifth, each cell contains hundreds or thousands of copies of mtDNA, while nuclear DNA contains two copies of each autosome. Sixth, normal recombination does not occur between individual mtDNA molecules. Seventh, cells may harbour both normal (wild-type) and mutant (or polymorphic) mtDNA, a situation known as heteroplasmy.
2.4.4.1 Maternal inheritance and the genetic bottleneck effect

All mitochondria in the zygote derive from the ovum, and during the formation of the zygote, mtDNA is derived exclusively from the oocyte. Therefore, a mother carrying an mtDNA mutation will pass it on to all her children, but only her daughters will transmit mtDNA to their progeny. The transmitted level of heteroplasmy is determined at an early stage in oogenesis in the case of a heteroplasmic female developing in utero. The term genetic bottleneck refers to a restriction in the number of mitochondrial genomes during early oogenesis (Jenuth et al. 1996, Poulton et al. 1998). The random redistribution of mtDNA in early oogenesis can change the proportion of mutant mtDNAs received by daughter cells (random genetic drift). Mutation load can vary remarkable between generations and between siblings.

Although maternal inheritance is one of the distinctive rules of mitochondrial genetics, paternal inheritance of a mtDNA mutation restricted to the skeletal muscle has been reported in a patient with exercise intolerance and a microdeletion in the ND2 gene (Schwartz & Vissing 2002). It has been pointed out in two additional studies, however, that paternal transmission of mtDNA is a rare phenomenon unrelated to the pathogenesis of muscle-restricted mtDNA mutations (Taylor et al. 2003, Filosto et al. 2003).

2.4.4.2 Heteroplasmy and the threshold effect

In normal subjects all mtDNAs are identical, i.e. homoplasmic, while heteroplasmy implies a mixture of mutant and normal (wild-type) mtDNA within the same cell. While most disease-causing mutations are heteroplasmic, homoplasmic mutations also occur in non-pathogenic polymorphisms and in cases of Leber’s hereditary optic neuropathy (LHON). Large studies have shown a relationship between the percentage level of mutant mtDNA in the skeletal muscle (Chinnery et al. 1997a) and other tissues (White et al. 1999) and the severity of the related mitochondrial disease. This relationship is less clear for the individual patient, however. It has been suggested that the amount of wild-type mtDNA may be more important than the proportion of mutant and wild-type mtDNA (Moraes et al. 1995).

Heteroplasmy in itself does not impair respiratory chain function unless it exceeds a certain threshold (typically 70–90%). This is known as the threshold effect. On the other hand, very low levels of heteroplasmy may cause mitochondrial dysfunction in vivo (Chinnery et al. 2000a). Post-mitotic cells with high metabolic activities in the skeletal muscle, central nervous system, heart, liver and kidney are particularly sensitive to respiratory chain deficiency. The variable heteroplasmy of mtDNA mutations produces an extensive range of disease severity, whereas tissue-specific gene expression, tissue distribution and the threshold effect explain the variable involvement of multiple organ systems.
2.4.4.3 Mitotic segregation and relaxed replication

There are two other mechanisms in addition to the genetic bottleneck that can change the proportion of mutant mtDNA in an individual tissue during life, relaxed replication and mitotic segregation. The random redistribution of heteroplasmic mtDNA during cell division can alter the proportion of mutant mtDNAs received by daughter cells. The random drift mechanism may also permit a decrease in mutation load within a segment in muscle fibre (Elson et al. 2001). Partly as a result of segregation with variable levels of mutant mtDNA, enzyme deficiency is manifested in a characteristic mosaic pattern in muscle histochemistry.

Unlike nuclear DNA, which replicates only once during each cell cycle, mtDNA is not replicated in absolute synchrony. Continuous recycling occurs even in non-dividing tissues such as skeletal muscle and brain (Bogenhagen & Clayton 1977). MtDNA replication is therefore relaxed, i.e. independent of the cell cycle. Possible differences in rates of replication between mutant and wild-type mtDNA may explain the late onset and progression of some mitochondrial disorders (Chinnery & Samuels 1999).

2.4.5 Nuclear mutations and defects in intergenomic communication

Although mitochondria contain their own DNA, most of the genes necessary for mitochondrial functions are located in the nuclear genome. The fact that the respiratory chain is under dual genetic control makes mitochondrial disorders interesting, involving both Mendelian and mitochondrial genetics. Mutations in structural components or ancillary proteins of respiratory chain may be due to defects in nuclear DNA as well as defects in intergenomic signalling affecting respiratory function or the membrane lipid milieu. Numerous diseases have been described which involve nuclear genes encoding the respiratory chain subunits (Wallace 1999, Leonard & Schapira 2000) and genes encoding other mitochondrial enzymes, such as CPT II and a subunit of the PDH complex (Chun et al. 1993). Mutations have also been described in nuclear genes which encode the proteins necessary for respiratory chain complex assembly, such as SURF-1 (Tiranti et al. 1998) and SCO2 (Papadopoulou et al. 1999). Nuclear gene defects may also be responsible for classic mitochondrial phenotypes with an absence of mtDNA mutations, such as CPEO, MERRF or KSS (Pulkes et al. 2003). Nuclear genes are similarly important for maintaining the mitochondrial genome, including those encoding the mitochondrial DNA polymerase γ (POLG1) (van Goethem et al. 2001) or the essential factors needed for mitochondrial transcription and translation (Larsson et al. 1998).

Because mitochondria play an essential role in maintaining life through energy production and mediating cell death by apoptosis, it is clear that mitochondrial dysfunction is associated with numerous diseases and ageing. There is also a group of "new" mitochondrial diseases, in which defective oxidative phosphorylation is due to mutations in nuclear genes encoding non-respiratory chain proteins (Graeber & Müller 1998). These proteins are involved in mitochondrial biogenesis and cause impaired energy metabolism, and thereby neurological disease (Schapira 2002). These diseases include Friedreich’s ataxia (Campuzano et al. 1997), Leigh syndrome associated with SURF-1 gene mutations.
(Tiranti et al. 1998), hereditary spastic paraplegia (Casari et al. 1998), dystonia (Truong et al. 1990), Huntington disease (Gu et al. 1996, Tabrizi et al. 1999), Wilson’s disease (Gu et al. 2000) and dominant optic atrophy (Alexander et al. 2000).

Furthermore, evidence of mitochondrial dysfunction has been found in some common neurodegenerative diseases. Mitochondrial abnormality has been detected in Parkinson disease (Bindoff et al. 1991a, Schapira et al. 1998), parkinsonism (Casali et al. 2001), Alzheimer disease (Parker et al. 1994) and motor neuron disease (Wiedemann et al. 1998). Whether the mitochondrial dysfunction implied in these diseases is primary or secondary is still undefined. Defects in intergenicomic communication cause diseases, in the form of either a reduction in the number of mtDNA molecules (mtDNA depletion syndromes) or multiple deletions, Mendelian traits passed on by either autosomal dominant or recessive inheritance (Bohlega et al. 1996, Zeviani et al. 1989).

Ophthalmoplegia associated with other clinical features is the hallmark of these multiple deletions (Hirano & DiMauro 2001). Some families with AD-PEO have mutations in Twinkle, which is a mitochondrial protein, whereas some families have mutations in the mitochondrial adenine nucleotide translocator 1 (ANT1) (Kaukonen et al. 2000, Spelbrink et al. 2001). A mutation in the nuclear gene encoding thymidine phosphorylase has been found in patients with multiple mtDNA deletions, and thymidine phosphorylase dysfunction, resulting in elevated concentrations of thymidine in the blood, causes mitochondrial neurogastrointestinal encephalomyopathy (Nishino et al. 1999).

### 2.5 Diagnosis and evaluation of mitochondrial disease

#### 2.5.1 History and physical examination

Mitochondrial disorders can manifest themselves at any age in any organ or tissue with wide clinical diversity, neuromuscular symptoms being the most common. They can have single organ or multisystem involvement, and any combination of neurological disease and extraneurological involvement should raise the suspicion of a mitochondrial disorder. A medical and family history, together with a careful physical examination of the patient, may reveal subtle clues pointing to a defined mitochondrial disease. Certain feasible features should be borne in mind concerning MELAS, for example, such as premature exercise-induced fatigue, migrainous headaches, diabetes mellitus, short stature and sensorineural hearing loss. Consensus diagnostic criteria were proposed in 1996 (Walker et al. 1996) and in 2002 (Bernier et al. 2002, Wolf & Smeitink 2002).

#### 2.5.2 Laboratory tests

Routine blood tests, including a complete blood count, serum electrolytes, glucose, liver function tests, urea, nitrogen and creatine kinase (CK), should be performed routinely.
CK is often normal or slightly elevated in mitochondrial myopathies. A glucose tolerance test may be useful for evaluating the diagnosis of mitochondrial disease, and a lumbar puncture analysis may show elevated cerebrospinal fluid (CSF) protein, especially in cases of KSS. CSF may also reveal an elevated lactate or pyruvate level even if the patient has normal serum levels of these. Patients with mitochondrial disease usually have elevated intermediary metabolites such as lactate, pyruvate and alanine in their blood or urine, and these provide indirect evidence of mitochondrial impairment. In practice, only lactate levels are helpful when investigating patients with mitochondrial disease.

2.5.3 Biochemical tests

The measurement of individual respiratory chain complexes is a useful approach for investigating diseases caused by mitochondrial dysfunction (Birch-Machin et al. 1994). The activities of respiratory chain enzyme complexes can be measured by polarographic methods in freshly isolated muscle mitochondria or disrupted mitochondria in tissue homogenates (Rustin et al. 1991). Isolated monoenzymopathies can be measured in some mitochondrial disorders such as infantile COX deficient myopathies. Coenzyme Q deficiency can be measured reliably to confirm or disprove this diagnosis (Hirano et al. 2002). Biochemical tests are also useful for diagnostic purposes, but not for monitoring disease severity because they require repeated muscle biopsies. They are also susceptible to differences in laboratory techniques, and their interpretation may be influenced by questions of age and fitness.

2.5.4 Molecular genetics

In patients with a defined clinical mitochondrial syndrome, it may be possible to carry out a specific molecular genetic test on DNA extracted from blood (leukocytes). This is a good approach in cases of homoplasmic mutations (such as LHON), but where heteroplasmic mutations or large-scale deletions are concerned, pathogenic mtDNA mutations may not be detectable in the blood by conventional techniques. The percentage of mutant DNA is almost always higher in muscles than in leukocytes (Suzuki et al. 1997), and therefore, if a mitochondrial disease is suspected, a muscle biopsy should be performed, especially if the blood DNA test analysis has been negative. A skin biopsy can also be considered. Buccal mucous membrane or hair follicle samples may be feasible non-invasive alternatives to muscle or skin biopsy.

The mutation must be considered pathogenic if it fulfils specific criteria: it must affect a site that has been conserved during evolution, it must be situated in a region that is functionally important, it should not be a known polymorphism, it should be heteroplasmic and the heteroplasm should correlate with the severity of the disease, and it should segregates with the disease biochemically. Molecular genetic methods should be performed to detect specific mtDNA or nuclear gene mutations. Known point mutations in mtDNA can be diagnosed by methods based on the polymerase chain reaction (PCR), while
Southern blot analysis or long-template PCR are the most appropriate techniques to identify mtDNA rearrangements. Mutation heteroplasmy can also be determined in an individual muscle fibre by single muscle fibre mtDNA analysis (Petruzella et al. 1994, Moraes & Schon 1996). Direct sequencing can be used to detect new mtDNA mutations.

2.5.5 Muscle biopsy

2.5.5.1 Histochemistry

As mitochondrial diseases commonly affect muscles, muscle biopsy is one of the cornerstones of mitochondrial myopathy diagnosis, even when there may be no signs or symptoms of myopathy (Morgan-Hughes 1994). Three main human muscle fibre types can be delineated according the myosin heavy chain isoforms involved: I, IIA and IIX/IIB. Type I implies oxidative slow-twitch fibres and types IIA and IIX/IIB fast-twitch fibres. The percentage of the fibre volume occupied by mitochondria varies from 6% in type I to 4.5% in type IIA and 2.3% in type IIX/IIB (Howald et al. 1985). Fibres also differ in the availability of high-energy phosphates. Resting phosphocreatine is higher in fast than in slow fibres (Söderlund & Hultman 1991, Sahlin et al. 1997), while the resting ATP content is fairly similar in both (Greenhaff et al. 1993) and remains relatively constant in both during exercise (Sahlin et al. 1997), although ATP consumption is markedly higher in fast fibres than in slow ones (Sahlin et al. 1998). The morphological hallmark of a mitochondrial defect is the ragged red fibre (RRF), which contains large subsarcolemmal and intermyofibrillar collections of mitochondria. These aggregates are seen in fresh frozen sections, where they appear as red-stained, irregular granular deposits in the modified Gomori trichrome method. RRFs also contain increased numbers of neutral lipid droplets and an excess of glycogen. RRFs display a higher proportion of mutant mtDNA than histologically normal muscle fibres (Petruzella et al. 1994, Ozawa et al. 1998, Mita et al. 1998, Houshmand et al. 1999).

RRFs are also occasionally seen in non-mitochondrial diseases such as dermatomyositis, inclusion body myositis and polymyalgia rheumatica (Grau et al. 1994) and also in muscular dystrophies, probably reflecting secondary changes. On the other hand, no difference has been shown in the frequency of RRFs between patients with polymyositis and age-matched controls (Rifai et al. 1995). RRFs are not seen in many primary mitochondrial diseases affecting metabolic pathways other than the respiratory chain, such as carnitine palmitoyl transferase deficiency. They are not present in cases of Leber’s optic atrophy (Brown et al. 1992), Mendelian Leigh’s disease or NARP (DiMauro et al. 1987), and they are not seen in children under five years, even in the presence of a mitochondrial disease (Carpenter & Karpati 2001). Longitudinal sections have revealed that RRFs have segmental abnormalities, reflecting a segmental deficiency in oxidative phosphorylation.

Two histochemical assays are used as sensitive indicators of mitochondrial disease, one for succinate dehydrogenase and the other for cytochrome c oxidase. Succinate dehydrogenase (SDH), the enzyme that catalyzes the conversion of succinate to fumarate in
the tricarboxylic acid cycle, consists of two large subunits, which form complex II. Use of a SDH stain reveals a checkerboard pattern in a normal muscle section, while highly oxidative type I fibres show a darker mitochondrial network and type II fibres a light blue network stain. A modification of the SDH reaction with phenazine methylsulphate has the advantage that only fibres with excess mitochondria are stained, but not normal fibres. This renders the modified SDH the best single screening reaction for mitochondrial disease, because it shows not only developed ragged red fibres but also ragged red equivalents (Carpenter & Karpati 2001). Most RRFs show an intense blue SDH reaction. SDH cytochemistry is also useful for the diagnosis of complex II deficiency, and excessive SDH staining may be seen within the blood vessel walls of MELAS patients (Hasegawa et al. 1991).

Cytochrome c oxidase (COX), complex IV of the respiratory chain, is composed of thirteen subunits, the three largest being encoded by mtDNA. The COX stain gives a brown checkerboard pattern corresponding to the distribution of mitochondria in muscle. Extreme variability of in cytochrome c oxidase activity is an indicator of a probable mitochondrial DNA abnormality, and cytochrome c oxidase defects in muscle with a homogeneous distribution are more likely to be associated with defects of the nuclear genome (Johnson et al. 1993). As in SDH, type I fibres stain darker, due to their more abundant mitochondrial content. In fibres with a critical dose of certain mutations in mitochondrial transfer RNAs, the mitochondria become totally COX-negative, and these are characteristic of mitochondrial disease. The COX stain is useful for demonstrating COX deficiency due to mutations in either an mtDNA gene or a nuclear gene (Zhu et al. 1998). COX-negative fibres have a tendency to accumulate with age, and up to 5% are observed in the muscle of healthy elderly individuals, although their presence would suggest a mitochondrial disease in a young person (Müller-Höcker 1990). Double staining for SDH and COX is sensitive method for visualizing COX-deficient RRFs (Sciacco et al. 1994). Clinical progression of mitochondrial myopathy has been shown to be associated with a random accumulation of COX-negative muscle fibres (Chinnery et al. 2003b).

Abnormal lipids can be seen in disorders of mitochondrial fatty acid oxidation, and occasionally in respiratory chain disorders. Several immunological probes can be used to perform immunocytochemical studies of mitochondria. Antibodies are available against mtDNA-encoded subunits of the respiratory chain complexes (such as subunit I of complex I and subunit II of complex IV) and nDNA-encoded subunits (such as subunit IV of complex IV). Histological examination of muscle samples from patients with a mitochondrial disorder does not usually reveal signs of inflammation, muscle fibre degeneration, necrosis or atrophy (Morgan-Hughes 1994), although deleted mitochondrial genomes may contribute to age-related fibre atrophy (Lee et al. 1998). Dystrophic changes have been found in some patients (Vissing et al. 1998).

2.5.5.2 Ultrastructural changes

Characteristic ultrastructural findings include subsarcolemmal accumulations of abnormal mitochondria of various shapes and sizes. Proliferation of mitochondria is also seen between myofibrils. Giant mitochondria and cristae alterations are common findings. The
changes are most prominent in RRFs, although similar, less widespread changes are found in non-RRFs. Intramitochondrial crystals are the most striking ultrastructural abnormality in mitochondrial disease. Two types of crystals (also called paracrystalline inclusions) are present: type I, which are found in the intercristal space or outer membrane and are more common because they occur in type I fibres, where mitochondria are normally more numerous (Carpenter & Karpati 2001), and type II, which are larger and often situated in type II fibres. Both types contain creatine kinase (Stadhouders et al. 1994). Other common ultrastructural abnormalities in mitochondria are an excess of matrix, osmiophilic densities in the matrix and lipid globules. Osmiophilic densities contain phospholipid, lipoprotein, glycoprotein and probably calcium and COX (Carpenter & Karpati 2001).

2.5.6 Exercise testing and near-infrared spectroscopy

Exercise testing is a qualified non-invasive technique for diagnosis and monitoring myopathy in mitochondrial disease. Measurement of venous oxygen levels during aerobic forearm exercise is an easy screening test for mitochondrial disease (Taivassalo et al. 2002). Near-infrared spectroscopy (NIRS) is a non-invasive method for monitoring oxygen availability and utilization by tissues as reflected in the near-infrared spectra of haemoglobin. It is capable of demonstrating the dynamic process of oxygen delivery and consumption in the exercising limb, and has proved to be useful for measuring the severity of myopathy and exercise intolerance in patients with mitochondrial myopathy (Abe et al. 1997, van Beekvelt et al. 1999). NIRS may also be useful for evaluating the effect of coenzyme Q10 supplementation on patients with encephalomyopathy, including MELAS (Abe et al. 1999).

2.5.7 Magnetic resonance spectroscopy

Phosphorus magnetic resonance spectroscopy (MRS) has a high sensitivity and specificity for mitochondrial myopathies (Matthews et al. 1991, Chinnery et al. 2001). It provides a non-invasive means of examining oxidative phosphorylation in the muscle tissue of affected patients by demonstrating an impaired rate of resynthesis of high-energy phosphate compounds following exercise. It has to be remembered, however, that the reduced phosphocreatine/inorganic phosphorus ratio found at rest in most cases of mitochondrial myopathy may also be seen in other neuromuscular diseases (Argov & Bank 1991).
2.5.8 Clinical investigations

Routine examinations such as an electrocardiogram should be obtained for all patients, and Holter monitoring should be considered. Specific clinical investigations can be used to monitor specific phenotypes of mitochondrial disease. Pure tone audiometry can be used to measure hearing loss (Uimonen et al. 2001), and echocardiography to document cardiac abnormalities in patients with 3243A>G mutation (Majamaa-Voltti et al. 2002). A clinical examination may be useful in prospective studies for documenting the natural history of mitochondrial diseases. Electromyography usually shows a proximal myopathic pattern in cases of mitochondrial myopathy, but it may be also normal. Nerve conduction findings may show axonal neuropathy. An EMG and single fibre electromyography may reveal alterations in patients without clinical signs of myopathy or neuropathy (Girlanda et al. 1999).

2.5.9 Radiological evaluation

Brain imaging by computed tomography (CT) or magnetic resonance imaging (MRI) may reveal basal ganglia calcifications or atrophy and stroke-like lesions, often in posterior region (Jackson et al. 1995). Multiple symmetric lesions in the basal ganglia, mesencephalon and brainstem are suggestive of Leigh syndrome (Zeviani et al. 1996). Imaging of muscles by CT, MR and ultrasound has been shown to be a sensitive method for evaluating muscle involvement in various neuromuscular diseases (Bulcke et al. 1979, Bulcke & Herpels 1983, Hawley et al. 1984, Calo et al. 1986). In primary myopathies, muscle CT scans reveal small, focal hypodense areas, which may progress to form widespread areas of low attenuation. The most severe abnormalities of this kind have been observed in muscular dystrophies (Swash et al. 1995). In neurogenic diseases, muscle CT can reveal atrophic changes, although muscles may also contain small punctate areas of decreased attenuation (Swash et al. 1995) or even severe hypodensity in hereditary neuropathies (Marconi et al. 2001). Muscle CT has been used to examine patterns of muscle involvement in various neuromuscular diseases, but not in mitochondrial myopathies.

2.6 Neuromuscular manifestations of mitochondrial disease

2.6.1 Peripheral neuropathy

2.6.1.1 Polyneuropathy

Peripheral neuropathy is not uncommon among patients with mitochondrial disease (Gemignani et al. 1982, Mizusawa et al. 1988, Eymard et al. 1991, Chu et al. 1997, Zans-
sen et al. 1998), and it varies in severity, from asymptomatic to moderate. Involvement is usually mild, with a chronic course. Electrophysiological studies have shown axonal neuropathy (Yannikas et al. 1986, Pezeshkpour et al. 1987, Mizusawa et al. 1991), and demyelinating (Goebel et al. 1986, Uncini et al. 1994, Rusanen et al. 1995) or mixed types of neuropathy (Molnar et al. 1996, Sciaccio et al. 2001) have been observed in some patients. These results have been confirmed by histopathological studies on sural nerve biopsies (Yiannakas et al. 1986). Furthermore, abnormalities detected by thermal threshold tests have suggested that small-diameter nerve fibres are preferentially affected (Sue et al. 1997).

The aetiology of peripheral neuropathy in mitochondrial diseases is not fully known. The mutant load in the peripheral nerves has been observed to be similar to that in other tissues such as skeletal muscle (Chu et al. 1997, Fadic et al. 1997). Abnormal mitochondria in Schwann cells suggest that dysfunction in these cells may lead to demyelination or secondary axonal damage (Schröder & Sommer 1991), while an increase in mitochondria in the vasa nervorum shows that mitochondria play a significant role in the pathogenesis of peripheral mitochondrial neuropathy (Molnar et al. 1995).

Mitochondrial polyneuropathy is usually of a sensory rather than a motor type. Mild distal weakness may be present, but proximal or diffuse weakness is a sign of coexistent myopathy (Peyronnard et al. 1980). Autonomic neuropathy has been reported as a rare feature in mitochondrial disease (Enzi et al. 1985; Zelnik et al. 1996). Polyneuropathy is a major feature in the syndrome of multiple symmetric lipomatosis (Enzi et al. 1985), and radiculopathy has been reported in a case with possible KSS (Groothuis et al. 1980) and a case with acanthocytosis (Mukoyama et al. 1986).

2.6.1.2 Sensory ataxic neuropathy

Sensory ataxic neuropathy is usually paraneoplastic in origin but it has also been found to be a predominant clinical feature associated with multiple mtDNA deletions. Patients have a significant loss of vibratory and joint position sense in the lower distal limbs, areflexia and an ataxic gait. Some patients also have PEO and dysarthria, representing the syndrome known as SANDO (sensory ataxic neuropathy, dysarthria and ophthalmoplegia) (Fadic et al. 1997), while some have myoclonic epilepsy (van Domburg et al. 1996) or multiple lipomas (Calabresi et al. 1994). SANDO patients have multiple mtDNA deletions in muscles and nerves and these have been shown to be caused by polymerase gamma mutations (van Goethem et al. 2003). Other recessive POLG1-mutant patients with sensory neuropathy and ataxia may present without PEO or other muscle symptoms and lack overt findings of multiple mtDNA deletions (B Udd, personal communication).

2.6.1.3 Neuropathy, ataxia and retinitis pigmentosa syndrome

Peripheral neuropathy commonly occurs in the syndrome of neuropathy, ataxia and retinitis pigmentosa (NARP), which is associated with a T>G missense point mutation at posi-
tion 8993 in the ATPase6 gene (Bardosi et al. 1987, Holt et al. 1990). This leads to a reduction in ATP synthesis by altering the membrane proton channel of the mitochondrial ATPase (Tatuch et al. 1992). NARP is also characterized by seizures, cognitive decline and neurogenic muscle weakness. The onset of symptoms may be subtle in young adults and the course is gradually progressive. If the degree of heteroplasmacy is very high (higher than 90-95%), the phenotype is a more devastating and earlier-onset disease, Leigh’s encephalopathy (Tatuch et al. 1992). At the same locus 8993 another point mutation T>C causes a syndrome without retinitis pigmentosa and may even present late in life with sensory motor neuropathy with or without ataxia (B Udd, personal communication).

2.6.1.4 Mitochondrial neurogastrointestinal encephalomyopathy

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal-recessive multisystem mitochondrial disorder including PEO, severe gastrointestinal dysmotility, cachexia, diffuse leukoencephalopathy and peripheral neuropathy (Hirano et al. 1994). Gastrointestinal dysmotility includes diarrhea, recurrent nausea and vomiting and pseudo-obstruction, while the peripheral neuropathy is of the sensorimotor type and mild to moderate in severity, and electrophysiological studies reveal both axonal and demyelinating features. Approximately two-thirds of the patients have lactic acidosis at rest (Nishino et al. 2000b). The syndrome begins in childhood and may lead to early death in some cases. MNGIE is associated with multiple deletions and depletion of mtDNA due to mutations in the gene encoding thymidine phosphorylase (TP) (Hirano et al. 1998). Point mutations have also been described (Verma et al. 1997). Since TP catabolises thymidine, loss of TP function increases thymidine levels (Nishino et al. 1999).

2.6.2 Myopathy

2.6.2.1 Exercise intolerance and muscle weakness

Myopathy is a common manifestation of many mitochondrial diseases. The clinical manifestations of muscle involvement in mitochondrial diseases include weakness, exercise intolerance, muscle cramps and myalgia. As with most myopathies, proximal muscles are most commonly affected. Patients with mitochondrial myopathies are generally of slender build, with decreased muscle bulk (Hirano et al. 2002). Exertional fatigue or intolerance is a cardinal feature of impaired oxidative phosphorylation in mitochondrial myopathies, and may be the sole manifestation of an mtDNA mutation (Andreu et al. 1999a) or in a part of multisystem mitochondrial disorder (Wibrand et al. 2001). Exercise intolerance or limb weakness can be manifested in several generations (Heiman-Patterson et al. 1997) and has been reported to be present in 23% of patients with defined mitochondrial disease (Petty et al. 1986).

The myopathy associated with mitochondrial disease is usually mild proximal and symmetric. It may occur alone, but in most cases it is part of a syndrome. Exertional weakness or myalgias with exertional headache or nausea have been linked to suspected mitochondrial disease (Morgan-Hughes et al. 1990). Atypical presentations of mitochondrial myopathy can also involve subacute myalgia and elevated serum CK (Cohen et al. 1998), or acute respiratory failure due to respiratory muscle weakness (Barohn et al. 1990, Cros et al. 1992). An iatrogenic form of mtDNA depletion is zidovudine (antiviral drug) myopathy with RRFs (Dalakas et al. 1990, Arnaudo et al. 1991).

### 2.6.2.2 Cardiomyopathy

Cardiac involvement is common in mitochondrial diseases and may be manifested in various ways. Cardiomyopathy is most commonly associated with point mutations in tRNA genes (Tanaka et al. 1990, Zeviani et al. 1991, Bruno et al. 1999), but may be present in autosomal dominant or recessive diseases with multiple deletions (Suomalainen et al. 1992) or single large-scale deletions (Moslemi et al. 2000). Families with multiple mtDNA deletions present with severe hypertrophic cardiomyopathy, PEO and proximal muscle weakness (Bohlega et al. 1996). The common 4977 base-pair deletion accumulates preferentially in the cardiac conduction system, causing conduction blocks (Müller-Höcker et al. 1998). The frequency of cardiomyopathy has been observed to be very low in a meta-analysis on 184 reported cases (Chinnery et al. 1997a), while structural and functional abnormalities of the heart were common in patients with the 3243A>G mutation. Left ventricular hypertrophy (LVH) was diagnosed in 56% of patients (Majamaa-Voltti et al. 2002).

### 2.6.2.3 Progressive external ophthalmoplegia

The most pathognomonic feature of mitochondrial myopathy is probably progressive external ophthalmoplegia (PEO) (Petty et al. 1986), typically in childhood or young adulthood. It is not known whether the cause of PEO is neurogenic, myogenic or a combination of these (Rowland et al. 1997). PEO is characterized by ptosis and weakness of the extraocular muscles, however, eye movements are limited, transient or persistent diplopia occurs only in a minority of patients. Most PEO patients also suffer from limb
muscle weakness or other cranial nerve manifestations, and some have respiratory muscle weakness. Kearns-Sayre syndrome is characterized by ophthalmoplegia, pigmentary retinopathy and cardiac conduction blocks (Kearns & Sayre 1958), and PEO is a hallmark of KSS caused by mtDNA deletions, although it has also been reported in cases of partial mtDNA duplication (Dunbar et al. 1993), maternally inherited point mutations (Hamman et al. 1993, Chinnery et al. 1997b), sporadic point mutations (Seibel et al. 1994) and multiple mtDNA deletions (Zeviani et al. 1989, Servidei et al. 1991, Bohle et al. 1996), in autosomal-dominant PEO (AD-PEO) (Zeviani et al. 1989) and in autosomal-recessive PEO (AR-PEO) (van Goethem et al. 2003).

2.6.2.4 Encephalomyopathy

Patients with encephalomyopathy have various central nervous system features involving muscle weakness. The onset of symptoms is highly variable, from infancy to the seventh decade, but usually in childhood. MELAS and MERRF syndromes are the classic forms of mitochondrial encephalomyopathy, and an overlap between them has been described (Campos et al. 1996, Nakamura et al. 1995). Leigh syndrome, or subacute necrotizing encephalomyopathy, is a progressive disease of infancy that leads to death. Hypotonic muscle weakness is one feature of the spectrum of predominantly central nervous system symptoms. An outstanding defect in complex IV is the most common biochemical alteration in Leigh syndrome, although deficiencies in other respiratory chain complexes have reported. An adult-onset form with milder symptoms has also been described (Chalmers et al. 1997). Two forms of encephalomyopathy are associated with deficiencies in CoQ, one causing recurrent myoglobinuria and the other manifested as a cerebellar syndrome (Ogasahara et al. 1989, Sobreira et al. 1997).

2.6.2.5 Infantile encephalomyopathy

Infantile myopathy is characterized by hypotonic weakness, severe lactic acidosis, psychomotor delay and respiratory and feeding difficulties. There are two subtypes, fatal and benign, the fatal one being the more common, leading to death due to cardiorespiratory failure. In benign, or reversible infantile myopathy, infants develop severe myopathy and lactic acidosis soon after birth, but improve dramatically and are virtually normal by the age of 2-3 years (DiMauro et al. 1981b). While the benign form is associated with defects only in complex IV, perhaps due to a nuclear mutation (Zeviani et al. 1987), the fatal form has been reported to involve defects in all respiratory chain complexes and the pyruvate dehydrogenase complex (Bindoff et al. 1991b). Mutations of the nuclear COX assembly gene, SCO2, have also been reported (Papadopoulos et al. 1999), as has severe mtDNA depletion (Moraes et al. 1991) as the cause of the fatal infantile form.
2.6.2.6 Myoglobinuria

Recurrent myoglobinuria is found in several mitochondrial disorders, typically ones presenting with attacks of myalgia, muscle cramps and stiffness of variable frequency. As in metabolic myopathies as a whole, attacks are precipitated by prolonged exertion, fasting or fever and are frequently accompanied by exercise intolerance. Symptoms typically occur in childhood or young adulthood. Myoglobinuria associated with metabolic myopathies is discussed above, and recurrent myoglobinuria has also been described with multiple deletions in mtDNA (Ohno et al. 1991), a microdeletion in the COX III gene (Keightley et al. 1996) or a deficiency in coenzyme Q (Hirano et al. 1996).

2.6.2.7 Rhabdomyolysis

Hereditary causes of rhabdomyolysis include inherited deficiencies in glycogenolytic enzymes and mitochondrial lipid metabolism. Rhabdomyolysis associated with disorders in lipid metabolism develops after a prolonged period of strenuous exercise, in contrast to rhabdomyolysis with glycogenolytic disorders. The most common hereditary disorder causing rhabdomyolysis has proved to be CPT I and II deficiency (Tonin et al. 1990), and it has been described in connection with deficiencies in carnitine (Prockop et al. 1983), long-chain 3-hydroxyacyl-coenzyme A dehydrogenase (Stanley 1987) and short-chain L-3-hydroxyacyl-coenzyme A dehydrogenase (Tein et al. 1991). Episodic weakness with rhabdomyolysis and myoglobinuria can be a rare manifestation of mitochondrial myopathy (Chinnery et al. 1997c, Melberg et al. 1998).

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

2.7.1 Genetics

Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) is associated most commonly with a 3243A>G mutation in the gene encoding tRNA^Leu(UUR) (MTTL1) in mtDNA (Goto et al. 1990, Goto et al. 1992). The observed frequencies of the various symptoms in a meta-analysis were as follows: recurrent strokes 48%, CPEO 28%, diabetes mellitus 15%, pigmentary retinopathy 15%, deafness 44%, dementia 27%, epilepsy 50%, myopathy 53%, short stature 15%, lipomata 1%, optic atrophy 1%, neuropathy 5%, ataxia 24% and myoclonus 8% (Chinnery et al. 1997a). The phenotypic variability can be partly explained by varying degrees of heteroplasmy in tissues (Kauffman et al. 1996), but the pathogenetic mechanism behind this clinical heterogeneity has not been fully elucidated.
In vitro studies with hybrid cell lines have shown that the 3243A>G mutation causes a mitochondrial respiratory chain defect and impairs mitochondrial protein synthesis if a threshold of about 85% is exceeded (Chomyn et al. 1991, Kobayashi et al. 1991, Boulet et al. 1992, King et al. 1992). A clear relationship has been found between the level of mutant mtDNA in muscle, but not in blood, and the frequency of clinical features (Chinnery et al. 1997a). Although 3243A>G is the most common mutation associated with MELAS phenotype (occurring in about 80% of cases), at least 14 other pathogenic point mutations have been described. Thus mitochondrial genetics can involve cases in which one phenotype can cause multiple genotypes and vice versa.

It has been suggested that an accumulation of somatic mutations initiated by 3243A>G may contribute to the progressive mitochondrial dysfunction found in MELAS (Kovalenko et al. 1996). Fibroblasts from MELAS patients have elevated levels of ionized calcium and cannot normally sequester calcium influxes (Moudy et al. 1995). Calcium overload and oxidative stress are thought to be crucial events in the pathogenesis of human mitochondrial encephalomyopathies (Graeber & Müller 1998, Wong & Cortopassi 1997). Several studies have shown that 3243A>G may also lead to MELAS overlap syndromes, in which patients also have other phenotypes such as chronic external ophthalmoplegia (CPEO), myoclonic epilepsy and ragged red fibers (MERRF), diabetes, sensorineural hearing loss or myopathy alone (Chen et al. 1993, Hammans et al. 1995, Campos et al. 1996). Muscle histochemistry in MELAS patients usual shows more COX-positive RRFs (i.e. with preservation of COX staining) than COX-negative RRFs, in contrast to MERRF or KSS patients. The amounts of both wild-type and mutant mtDNAs in RRF are increased in MELAS, and there are no differences in the amount of total or mutant mtDNA between the muscle fibre types (Mita et al. 1995).

2.7.2 Epidemiology

The prevalence of pathogenic point mutations or deletions in mitochondrial DNA in adults has been estimated to be 6.6/100,000. Overall, 12.5/100,000 adults or children either had mtDNA disease or were at risk of developing it (Chinnery et al. 2000b). The frequency of 3243A>G in the adult population of northern Finland was calculated to be at least 16.3/100,000 (Majamaa et al. 1998). The high prevalence of the common MELAS mutation in the adult population suggests that mitochondrial disorders constitute one of the largest diagnostic categories of neurogenetic diseases. These studies reflect the minimum prevalence of mitochondrial disease within the population and it is likely that the prevalence figures will increase as the genotypic and phenotypic spectrum of mitochondrial disorders broadens.

2.7.3 Clinical features

The classic phenotype of 3243A>G is characterized by stroke-like episodes with migraine-like headaches, nausea, vomiting and lactic acidosis (Pavlakis et al. 1984, Cia-
The clinical features that accompany the mutation are very variable in terms of age of onset, severity and involvement of organs, however, and additional features such as sensorineural hearing loss, external ophthalmoplegia, ptosis, cardiomyopathy, myopathy, peripheral neuropathy and cognitive decline may also occur. Peripheral neuropathy may occur in both its axonal and demyelinating forms (Barak et al. 1995, Rusanen et al. 1995, Fang 1996, Sue et al. 1997). Various endocrine and metabolic abnormalities are found in association with this syndrome, including diabetes mellitus, hypothalamic-pituitary dysfunction and growth hormone deficiency. The full MELAS syndrome occurs infrequently in more than one member within a pedigree, and maternal relatives may frequently be oligosymptomatic or asymptomatic.

Myopathy, usually manifested in the form of progressive proximal limb weakness (DiMauro & Moraes 1993), is thought to be one of the most frequent clinical features in patients who harbour the 3243A>G mutation, and estimates of its frequency have varied from 25% (Deschauer et al. 2001) to 61% (Hammans et al. 1995). A meta-analysis has suggested a frequency of 53% (Chinnery et al. 1997a). Progressive extraocular muscle weakness, ptosis and proximal limb weakness are typical features of myopathy associated with the 3243A>G mutation (Moraes et al. 1993, Fang et al. 1993), and rhabdomyolysis can occur (Hara et al. 1994). Hypertrophic cardiomyopathy has been reported in association with 3243A>G (Silvestri et al. 1997), and cardiac involvement has been described as being common in patients with 3243A>G (Majamaa-Voltti et al. 2002).
3 Aims of the research

The purpose of this work was to study skeletal muscle and peripheral nerve changes in patients carrying the 3243A>G mutation in their mitochondrial DNA within a defined population in northern Finland.

The specific aims were:

1. to characterize the frequency of peripheral neuropathy and its phenotypic variability among patients,
2. to define the frequency and histological, ultrastructural and clinical features of myopathy associated with 3243A>G,
3. to analyse the features of 3243A>G mitochondrial disease detectable in muscle computed tomography,
4. to investigate variations in the mutant genome in different longitudinal segments along muscle fibres of various types and to study the segregation of the mutation in a family by means of single muscle fibre analysis.
4 Subjects and methods

4.1 Patients (I–IV)

The patients with the 3243A>G mutation, aged 16–73 years, were from seventeen families ascertained in the population of the provinces of Northern and Central Ostrobothnia and Kainuu in northern Finland (Table 4). A total of 50 patients, 18 men and 32 women, were included in the series (as reported in Paper 2), five of whom participated in all four studies, 16 in three, 19 in two and 10 in one. Early development had been normal in every case. All the patients were examined neurologically and their clinical features and symptoms were evaluated. The research was approved by the Ethics Committee of the Medical Faculty of the University of Oulu and all the examinations were carried out after obtaining informed consent from the patients.

Table 4. Demographic data on the patients discussed in Papers I–IV.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Patients (n)</th>
<th>Men/women (n)</th>
<th>Age (years)</th>
<th>Heteroplasmy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>32</td>
<td>10/22</td>
<td>46 ± 13 (22–69)</td>
<td>65 (36–91)</td>
</tr>
<tr>
<td>II</td>
<td>50</td>
<td>18/32</td>
<td>45 ± 14 (16–70)</td>
<td>69 (30–94)</td>
</tr>
<tr>
<td>III</td>
<td>24</td>
<td>8/16</td>
<td>47 ± 16 (19–73)</td>
<td>74 (53–94)</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>3/7</td>
<td>38 ± 10 (24–62)</td>
<td>75 (48–94)</td>
</tr>
</tbody>
</table>

*Values are means ± standard deviation (range). Heteroplasmy was assessed in a muscle homogenate.*

4.2 Clinical tests (I–IV)

The cases were assigned to three groups on the basis of the global clinical severity of the disease, a mild disease being recorded when the patient was asymptomatic or had symptoms and signs that were not disabling, while those who were moderately affected had symptoms that interfered with daily activities and those who were severely affected suffered from disabling encephalopathy (IV).
Symptoms of neuropathy were evaluated by means of the Neuropathy Symptom Score (NSS) (Dyck et al. 1980), which is based on the number of symptoms abstracted from the neurological history (scale 0–17), so that all scores ≥1 were considered abnormal. The Neuropathy Disability Score (NDS) is based on a detailed evaluation of neurological deficits (on a scale 0–280, where NDS ≥2 is considered abnormal). The severity of neuropathy was staged according to the criteria previously published for diabetic neuropathy (Dyck et al. 1980, Dyck 1988), except that the quantitative sensory examination (QSE) and quantitative autonomic examination (QAE) were not performed (I).

For a diagnosis of polyneuropathy it was required that two or more abnormalities should be found in nerve conduction (NC), a neurological examination assessed with the NDS or neuropathic symptoms assessed with the NSS. The patient was classified as having no neuropathy when the minimal criteria for neuropathy were not fulfilled (Dyck 1988), asymptomatic neuropathy when there were at least two abnormalities in NC and NDS but NSS was 0, and symptomatic neuropathy if symptoms were also present (NSS ≥1) (I).

Patients’ physical activity and susceptibility to exercise intolerance were determined by means of a structured interview. Muscle strength was assessed bilaterally according to the modified MRC grading system (scale 0–5) (MRC 1986), in which grades 5, 4 and 3 were subdivided into quarters. The strength of the facial and mandibular muscles, shoulder abduction, flexion and extension of the neck, the elbows, the wrists, the fingers, the hips and the knees, pronation and supination of the forearms and ankle dorsiflexion and plantar flexion was evaluated (III).

A composite Medical Research Council score was also used (Katirji 2002), in which ten muscle functions were tested on each side (arm abduction, elbow flexion, elbow extension, wrist extension, fingers abduction, hand grip, hip flexion, knee extension, knee flexion and ankle dorsiflexion). A normal muscle examination in terms of the composite MRC was one that gave a total score of 100 (II). The time required to stand up from the supine position was measured, and gait and stepping onto a 30-cm high footstool were observed (Cwik & Brooke 1996). A stepping test was defined as abnormal if hesitation, hip dip, jump or hand support was observed, and gait was defined as abnormal if waddling or any ambulatory disturbance due to muscle weakness was observed (II).

Functional capacity was estimated using a modified Rankin scale on which functional outcome was assigned a grade from 0 (no symptoms or disability) to 5 (severe disability). A seven-item test was used, including assessment of gait, climbing stairs, rising from a chair, sitting from supine, standing from sitting, and function of the proximal and distal upper limbs (Mahjneh et al. 2001) (I–III).

4.3 Laboratory tests (I–III)

Venous blood lactate at rest and serum creatine kinase were measured (II, III). Routine laboratory tests were performed to exclude other causes of peripheral neuropathy (I) or myopathy (II, III). Diabetes mellitus was diagnosed according to the WHO criteria (Harris et al. 1985).
4.4 Muscle biopsy (I–IV)

Muscle specimens were obtained from the anterior tibial muscle or vastus lateralis of the quadriceps muscle under local anaesthesia. The specimens were frozen in isopentane chilled with liquid nitrogen and stored at –70°C. The frozen muscle specimens were then cut into 10-µm sections and mounted on polylysine-coated slides.

4.4.1 Histochemistry

4.4.1.1 Double stain and other stains (II–IV)

Frozen sections of muscle were stained with haematoxylin and eosin (HE) and modified Gomori trichrome, and for NADH-tetrazolium reductase and ATPase after incubation at pH 9.4, 4.6 and 4.3 (II–IV). Specimens were also stained for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) (Sciacco & Bonilla 1996). Blue fibres were considered COX-negative and fibres with a brown colour COX-positive. Ragged red fibres (RRF) were identified in sections stained with modified Gomori trichrome or for SDH. The percentages of COX-negative fibres and RRFs in each muscle specimen were obtained by counting between 200–500 muscle fibres in each biopsy (II, IV).

4.4.1.2 Isolation of single muscle fibres (IV)

Single muscle fibres were dissected out from double stained sections with a sharp tungsten needle (Oldfors et al. 1995), the target fibre in each case being hooked out apart from the adjacent muscle fibres under a microscope. The isolated fibres were then placed directly into 5 µl of a solution containing 200 mM KOH and 50mM dithiothreitol for incubation.

4.4.2 Ultrastructural analysis (II)

Fresh tissue was fixed in 4% formaldehyde/1% glutaraldehyde buffered to pH 4 with phosphate buffer, post-fixed in OsO₄ and embedded in Epon LX-112 (Electron Microscopy Sciences, Fort-Washington, PA). Ultra-thin sections were contrasted with uranyl-acetate and lead citrate and examined in a Philips LS electron microscope (Philips Export B.V., Eindhoven, The Netherlands). Findings were documented photographically.
4.5 Molecular methods (I–IV)

4.5.1 Analysis of muscle heteroplasmia (I–IV)

The 3243A>G mutation heteroplasmia in total muscle DNA was determined by restriction fragment analysis using ApaI restriction digestion of a 390bp mtDNA fragment (from nt 3150 to nt 3550), amplified by the polymerase chain reaction in the presence of 35S-ATP (Kobayashi et al. 1990). The fragments were then electrophoresed through a 6% non-denaturing polyacrylamide gel, which was dried and autoradiographed at –72°C overnight with Kodak XAR film, which was then analyzed using a Bioimage scanner and image processing apparatus (Millipore, Ann Arbor, Michigan).

4.5.2 Analysis of heteroplasmia in single muscle fibres (IV)

To analyse the proportions of mutant and wild-type mtDNA, each single muscle fibre sample was incubated for 10 min at 94°C in 5 µl of a solution of 200 mM KOH and 50 mM dithiothreitol. Five µl of neutralization buffer (900 mM Tris-HCl, pH 8.3, 300 mM KCl and 200 mM HCl) was added to this solution. A portion of the lysate obtained from a single muscle fibre was then taken for DNA amplification. A fragment spanning the nucleotides (nts) 3187 and 3410 of mtDNA was amplified by PCR using a forward primer corresponding to nts 3187–3206 of the light strand and a reverse primer corresponding to nts 3410–3391 of the heavy strand. DNA was amplified in 35 cycles of denaturation at 94°C for 60 s, primer annealing at 49°C for 60 s and primer extension at 72°C for 60 s in the presence of 1.0 U of DNA polymerase (Dynazyme; Finnzymes, Finland). For quantification of the percentage of mutation, 10 pmol of 6-FAM-labelled forward primer and 1.0 U DNA polymerase were added to each PCR reaction before the last cycle. The amplified fragments were inspected visually for quality after gel electrophoresis in 1.5% agarose and then digested with 10 U of the restriction enzyme ApaI overnight at 37°C. ApaI cleaves mutant mtDNA into two fragments of 186 and 37 bp, while the wild-type mtDNA remains intact (223 bp).

The FAM-labelled restriction fragments were electrophoresed through a 4% polyacrylamide gel using a model 377 DNA sequencer (Applied Biosystems) and analysed with the Genotyper version 2.0 software (Applied Biosystems). A wild-type sample was included in each electrophoresis. The proportions of mutant and wild-type mtDNAs were calculated from the peak areas of cleaved and uncleaved mtDNAs. The results are given as means of two measurements made on each fibre, and the results were accepted if the difference between the two measurements was 2 percentage units. The proportions of mutant and wild-type mtDNAs in the muscle homogenate were analysed after extracting DNA from muscle fibre sections containing thousands of fibres.
4.6 Electrophysiological examinations (I, III)

Nerve conduction (NC) examinations were performed by conventional techniques. Motor and sensory nerve conduction velocities (MNCV and SNCV) were measured using surface recordings. SNCV measurements were performed on the median, radial, ulnar, superficial peroneal and sural nerves on both sides by antidromic methods. The amplitudes of the compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs) were analysed using peak-to-peak measurements. Skin temperature was measured after each nerve stimulation. The F-wave latencies from the median, ulnar and deep peroneal nerves were measured. The examination remained incomplete for four patients, but in each case polyneuropathy could be ruled out (I).

Values differing from the mean of the controls, 62 healthy subjects, by 2.5 standard deviations were considered abnormal. The control values were corrected for the effect of age and height of the subject and the skin temperature of the measurement area. The electrophysiological diagnosis and classification of polyneuropathy were based on previously published criteria (Donofrio & Albers 1990) (I).

A concentric needle EMG examination was performed on the right side, including the anterior tibial, medial gastrocnemius, lateral vastus femoris, biceps femoris, semitendinosus, semimembranosus, gluteus maximus, iliopsoas, trapezius, biceps brachialis, triceps brachialis and first dorsal interosseus muscles. Due to patient incompliance, the hamstring muscles could not be examined in six patients, the first dorsal interosseus muscle in three patients and the trapezius in two patients. In addition, EMG results were available for four patients who had been examined previously, except that the gluteal and hamstring muscles had not been examined in these cases. For a diagnosis of myopathy it was required that myopathic findings should have been detected in the EMG of at least one muscle (III).

4.7 Muscle computed tomography (III)

The patients underwent multi-slice computed tomography (Toshiba Aquilion, Nasu, Japan 2000, 4 x 5 mm slices, 120 kV, 150 mAs, 0.5 s rotation time, standard algorithm, 256 x 256 matrix) in the supine position. A total of 38 muscles were visualised bilaterally at six transverse levels (Bulcke et al. 1979), including 1 = neck muscles (focusing point: the fourth cervical vertebra), 2 = the shoulder girdle (middle of the fossa glenoidalis), 3 = lumbar muscles (fourth lumbar vertebra), 4 = the pelvic girdle (5 cm above the symphysis), 5 = thighs (midpoint between the greater trochanter and the centre of the knee joint) and 6 = lower legs (largest diameter of the lower leg). In addition, the gluteal region was scanned at three levels in four patients, three of them with clinical myopathy. Six healthy and physically active controls (women aged 24, 40 and 53 years and men aged 25, 41 and 51 years) were scanned using the same protocol.

The CT images were evaluated with regard to intramuscular signal density and degree of muscle atrophy. Myopathic CT findings were graded in terms of four patterns (Bulcke et al. 1981, Schwartz et al. 1988): 0 = normal: a muscle with normal attenuation, 1 = spot-like: a muscle with several small hypodense areas, 2 = moth-eaten: a muscle with
multiple patchy areas of low attenuation with little or no reduction in muscle area, and 3 = washed-out: a muscle with widespread low attenuation or complete replacement with low attenuation tissue. Muscle atrophy was determined on the basis of a reduction in the cross-sectional area of the muscle and was also graded in four patterns: 0 = normal, 1 = mild: simple atrophy of the muscle corresponding to a slight reduction in size, 2 = moderate: simple atrophy corresponding to a 50 % reduction in muscle size, and 3 = severe: marked atrophy of the muscle or its disappearance, accompanied by an increase in subcutaneous tissue. The scans were analysed by two neurologists and one neuroradiologist, and a consensus was required.

4.8 Statistical analysis (I–IV)

The Mann Whitney U and Fischer exact tests were used to detect differences between groups (I–III). Bonferroni correction for multiple comparisons was applied by multiplying the observed $p$ value by the number of tests, and $p$ values <0.05 after correction were considered significant. The $p$ values shown are the uncorrected ones (II). The paired samples t-test was used to detect differences in the degree of heteroplasmy between histologically different subgroups of fibres and between mothers and daughters, and ANOVA (analysis of variance) to detect variation among sisters (IV). The variability in mutation load between morphologically normal fibres at the ten levels was examined using the Kruskal-Wallis test (IV). The correlation between patient age and mutation load was assessed by Pearson correlation analysis (IV).
5 Results

5.1 Peripheral neuropathy associated with 3243A\(\rightarrow\)G (I)

5.1.1 Electrophysiological findings

Seven out of 32 patients (22%; 95% confidence interval (CI), 9–40%) fulfilled the criteria for polyneuropathy in the electrophysiological examinations (I; Table 1). Mixed axon loss and demyelinating sensorimotor neuropathy was the most common type of polyneuropathy, while one patient presented with uniform demyelinating sensorimotor polyneuropathy. Polyneuropathy was more of the sensory than the motor type (I; Table 2). The examinations revealed a carpal tunnel syndrome (CTS) in three patients (9.4%), two of whom had both classic symptoms and positive provocative tests, whereas the third was asymptomatic. One of them had diabetes mellitus as a predisposing factor, but other aetiologies of CTS were ruled out by the laboratory tests. Electrophysiological examination did not reveal neuropathy in 22 patients.

5.1.2 Clinical peripheral neuropathy

The seven patients with electrophysiological abnormalities presented with symptoms of neuropathy, the median value for their neuropathy symptom scores (NSS) being 5 (I; Table 3). The polyneuropathic patients showed significantly more neurological deficits in the detailed clinical examination (neuropathy disability score, NDS) than the other patients, their median score being 79, while those without neuropathy had a median score of 3. The patients with polyneuropathy also presented with other clinical features of a mitochondrial syndrome (I; Table 2). Their functional disability was considered more serious than that among the other patients, as reflected by a significant difference in the Rankin scores between these groups (I; Table 3). The degree of 3243A\(\rightarrow\)G mutation heteroplasmY in muscle was not correlated with the presence of neuropathy, but higher age
and male gender did increased the neuropathy risk. The frequency of diabetes mellitus
did not differ between the groups. Proximal myopathy was confidently diagnosed in six
out of the seven patients with polyneuropathy, and ragged red fibres were found in five of
them. No fibre type grouping or other neurogenic changes in muscle histochemistry were
found among the patients with polyneuropathy.

5.2 Myopathy associated with 3243A>G (II, III)

5.2.1 Phenotypic features of mitochondrial myopathy

Twenty-five out of the 50 patients (50%; 95% CI, 36–64%) presented with myopathic
findings (II; Table 1), either mild or moderate in severity. Moderate limb weakness,
which was symmetric and proximal, was observed in thirteen patients (26%; 95% CI, 13–
39%), three of whom also had extraocular muscle weakness. Four patients (8%) had
extraocular muscle weakness and eight patients (16%) were found to have mild proximal
limb weakness. The age at onset of myopathy could be determined from the medical his-
tory in 16 cases, while the onset was defined as the age at clinical examination in nine
cases. The highest incidence of myopathy was found in the fifth decade of life, and the
cumulative incidence by the age 70 years was 0.77 (II; Figure 1).

Nineteen patients (47%) reported taking active physical exercise, while 20% exercised
occasionally and the remaining patients (33%) not at all. No difference in physical activ-
ity was found between the 13 myopathic patients with moderate limb weakness and the
remainder. The patients with myopathy were significantly slower in standing up from the
supine position than the non-myopathic patients. Their gait and stepping onto the foot-
stool were frequently abnormal (II; Table 2). The myopathic patients with moderate limb
weakness were slightly older than those without limb weakness, and there was a correla-
tion between myopathy and neuropathy (p < 0.001) among the 50 patients, but not
between myopathy and other clinical features such as short stature, ataxia, lactic acidosis,
diabetes mellitus, cardiomyopathy, epilepsy or stroke-like episodes.

5.2.2 Laboratory findings and muscle heteroplasmy

Serum CK activity was mildly elevated in 14 patients (five men, nine women; range of
CK activity 171–654 U/l). Interestingly, the patients with higher CK often had lactic aci-
dosis (p = 0.007), diabetes (p = 0.003) or CNS manifestations (p = 0.004). Venous blood
lactate at rest was elevated in 47% of the patients and was higher among the men (mean
2.0 mmol/l, range 0.6–4.5) than among the women (mean 1.6 mmol/l, range 0.5–5.1).
Increased blood lactate was associated with high mutation heteroplasmy (p = 0.002) and
diabetes (p < 0.001). No association was found between the presence of clinical myopa-
thy and elevated lactate or raised CK activity.
Mutation heteroplasmy in muscle varied from 30% to 94% (mean heteroplasmy 69% ± 14%), but no difference was found between the myopathic and non-myopathic patients or between the patients with mild and moderate myopathy. Heteroplasmy was higher among the patients with CNS manifestations (79%; 95% CI, 73–85%) than among those without CNS manifestations (65%; 95% CI, 59–71%), and higher among those with diabetes (77%; 95% CI, 73–82%) than among those without (61%; 95% CI, 54–68%).

5.2.3 Histological and ultrastructural results

Muscle histology was abnormal in 26 out of 36 patients (72%; 95% CI, 57–88%). The findings in the pathological samples consisted of myopathic changes in nine cases (25%), atrophic changes in eight (22%), fat infiltration in three (8%) and inflammatory changes in one (3%). The mean proportion of the COX-negative fibres in the muscle of the 16 patients who were analysed was 1.8% (range 0–5.0%), and a significant difference in this proportion was found between patients patients with and without myopathy regardless of its severity (II; Table 2). RRFs were absent in 34% of the muscle samples, few in number in 43%, moderate in 9% and numerous in 14%. Most of the RRFs were COX-positive. The presence of numerous RRFs did not correlate with the clinical severity of myopathy, but it did correlated with features such as diabetes mellitus (p = 0.001), epilepsy (p = 0.006) and lactic acidosis (p = 0.001). Ultrastructural examination of the muscle samples revealed that variations in mitochondrial size and shape were common among patients with limb weakness (II; Figure 2). Furthermore, both type I and type II crystals were present more frequently in these patients.

5.3 Muscle computed tomography pattern in 3243A>G patients (III)

5.3.1 Clinical features

Twenty-four patients with the 3243A>G mutation were selected for muscle computed tomography patterns. The clinical phenotype was quite variable among these patients (III; Table 1). A definite myopathic weakness was found in clinical examination in six cases (two men, four women), three of whom had a slightly waddling gait and difficulties in climbing stairs and rising from a supine position as a consequence of gluteus maximus weakness. Nine patients (three men, six women) showed mild muscle weakness and two of them complained of exercise intolerance and myalgia. Muscle strength evaluation and functional assessment were normal in the remaining nine patients (three men, six women). Serum CK was mildly elevated in six women and one man (III; Table 1). Twelve patients had increased resting blood lactate values. The mean degree of muscle 3243A>G mutation heteroplasmy was 73% ± 11%. RRFs were found in the muscles of 13 out of the 17 patients biopsied. Clinical examination showed the proximal muscles to be more
severely affected than the distal ones, which in turn were affected equally as much as the facial and mandibular muscles. The upper and lower limbs were equally affected.

5.3.2 Muscle computed tomography findings

The CT scans revealed abnormalities in the muscles of 13 patients (54%; 95% CI, 33–76%) (III; Table 2), but were normal in the remaining 11 patients. No changes in muscle density or cross-sectional area were detected in nine out of the 43 muscles among the 24 patients. A myopathic pattern was found most frequently in the pelvic muscles, with predominant involvement of the gluteus muscles (III; Table 2, Figures 1, 2). The most commonly affected muscle was the gluteus maximus, in 10 patients (42%). Muscle atrophy was common in the shoulder girdle and the legs, where the medial gastrocnemius was most commonly affected (seven patients, 29%), while the anterior tibial muscle was intact in all the patients. Marked atrophic changes could also be observed in the thighs (III; Figure 3) and the neck. Almost all the muscle findings were symmetric (III; Table 2) and no compensatory hypertrophy was observed. Variations in intramuscular density were seen in the gluteus maximus, gluteus medius and tensor fasciae lata muscles in consecutive pelvic CT scans of four patients (III; Figure 1). The six healthy controls showed homogeneous muscle density in their CT scans.

5.3.3 Electromyographic findings

Twelve patients underwent a needle EMG. Short-duration, low-amplitude, polyphasic motor unit potentials were abundant in some muscles of six patients, the most frequently affected being the gluteus maximus (three patients), trapezius (three patients), the short head of the biceps femoris (two patients) and the biceps brachialis (two patients). Fibrillation potentials were not detected, but complex repetitive discharges occurred in three myopathic muscles in patient 10. The EMGs of the four additional patients revealed myopathic findings in the biceps brachialis muscle in three cases. Two patients with myopathy (patients 10 and 11) had evidence of peripheral neuropathy in their clinical and neurophysiological examinations (III; Table 1).

5.3.4 Correlation of phenotype with muscle CT findings

A clear association was found between the presence of myopathic findings in muscle CT and low MRC scores (III; Table 3). On the other hand, the patients with myopathic findings in CT and those without did not differ in gender distribution, age, functional capacity, myopathic findings in EMG, the presence of RRFs, the presence of cardiomyopathy, the severity of hearing impairment or the degree of mutation heteroplasmy in muscle.
5.4 Variations in heteroplasmy in single muscle fibres of patients with 3243A>G (IV)

5.4.1 Variations in the proportion of 3243A>G mutation in various histological types of muscle fibres

Ten individuals (seven women, three men) with the 3243A>G mutation in mtDNA were investigated. Five of these had RRFs in their COX + SDH stain, and most of the RRFs were COX-positive. These patients also had COX-negative non-RRFs (IV; Table 1). The frequency of RRFs correlated with the clinical phenotype, the mean frequency being 0.5% in the moderately affected patients and 4.3% in the severely affected ones. None of the mildly affected patients had RRFs. The proportions of 3243A>G heteroplasmy, examined in 251 histologically normal fibres from all the patients (18–29 fibres per patient), were found to vary in the range 3–92% (mean 50%), and the distribution curve for heteroplasmy was normal in all the patients. The moderately affected patients harboured a higher proportion of 3243A>G mutations in their histologically normal muscle fibres (p < 0.01) than the mildly affected ones.

The RRFs had a significantly higher proportion of 3243A>G than the non-RRFs (p < 0.01), but no difference was found between the COX-negative RRFs (mean 90%, range 81–97%) and the COX-positive ones (mean 86%, range 58–97%). The distribution of the proportion of 3243A>G in RRFs was skewed to the left.

5.4.2 Proportion of 3243A>G mutations in consecutive longitudinal sections of single muscle fibres

Sections spanning a longitudinal length of 200 µm were obtained from the muscles fibres of the two severely affected men (patients 1 and 2), who had frequent RRFs. Both patients had cognitive decline, short stature and sensorineural hearing impairment. Patient 1 also had diabetes, epilepsy and lactic acidosis (IV; Table 1). The proportion of 3243A>G in the RRFs was higher than that in the adjacent histologically normal fibres (p < 0.001) (IV; Table 2). A correlation was found between mutation heteroplasmy in the RRF and that in the adjacent histologically normal fibres in the sections. The adjacent normal fibres in sections harbouring RRF with a mutation heteroplasmy 86%, had a lower proportion of mutations than those in the sections harbouring RRF with a mutation heteroplasmy > 86% (p = 0.05). A trend for a difference in histologically normal fibres between the longitudinal sections the was observed in patient 1 (p = 0.08 in sections adjacent to RRF1 and p = 0.06 in sections adjacent to RRF2; Table 2) but not in patient 2. The mutation load of RRF varied up to 30% in ten consecutive sections.
5.4.3 Variations in the proportion of 3243A>G mutations among single muscle fibres from a mother and her offspring

The case series included a mother and her six daughters. Only the mother had clinical myopathy. All the daughters harboured 3243A>G, but only two presented with any clinical features suggestive of a mitochondrial disease (IV; Table 1). The heteroplasmy of the 3243A>G mutation in histologically normal muscle fibres differed among the six daughters (p < 0.001), probably due to random genetic drift, but the mean heteroplasmy in the apparently normal muscle fibres was lower in the six siblings (42%) than in their mother (58%), and accordingly, five out of the six siblings had a lower mutation heteroplasmy than their mother (p < 0.01) (IV; Figure 1). A tendency towards a correlation was found between 3243A>G heteroplasmy in the muscles of the siblings and the age of the mother at gestation (Pearson correlation coefficient 0.589).
6 Discussion

6.1 Neuromuscular manifestations in patients harbouring 3243A>G in this population-based study

The frequency of patients with 3243A>G mtDNA presenting with abnormal clinical, histological and CT muscle findings was 50% and the frequency of peripheral neuropathy was 22%. The frequency of myopathy is similar to that previously reported in a meta-analysis of a large group of patients gathered by means of a computer search of the scientific literature, but the frequency of peripheral neuropathy is four times higher (Chinnery et al. 1997a). The individuals in this series were identified from a population-based cohort and examined systematically for clinical features of myopathy and peripheral neuropathy, and the work therefore represents the largest clinical study of the occurrence of myopathy and peripheral neuropathy among patients with the 3243A>G mtDNA mutation to be carried out to date and provides a more reliable estimate of the frequency of these neuromuscular manifestations and their phenotypic variation in patients with 3243A>G than has hitherto been achieved.

6.2 Peripheral neuropathy as a manifestation of mitochondrial disease

Peripheral neuropathy has frequently been described in mitochondrial disorders. Most case studies of peripheral polyneuropathy among patients with MELAS syndrome have reported axonal changes, suggesting that disturbed axonal transport is the major pathogenic mechanism. In metabolic neuropathies axon loss occurs rather more commonly than demyelination. The possible mechanism of axon degeneration in mitochondrial diseases is not fully known, but the primary involvement of the axons could be attributed to a reduction in axonal transport, as this mechanism is known to be responsible for the trafficking of organelles and macromolecules to the periphery of neurons (Droz & Leblond 1962).
Axonal transport makes use of a microtubule-associated molecular motor that is driven by ATP. Mitochondria are transported towards the nerve terminus along microtubules by kinesin and towards the cell centre by dynein. Inhibition of kinesin, by overexpression of the microtubule-associated protein tau has been shown to induce perinuclear clustering of mitochondria in Chinese hamster ovary cells (Ebneth et al. 1998). A similar defect in the melanosome transport complex has been observed in the melanocytes of patients with mitochondrial vitiligo (Karvonen et al. 1999). Melanocytes are intriguingly similar to alpha motor neurons, as both are post-mitotic cells and both serve a specific unit of other cells, via axons (neurons) or dendrites (melanocytes). A reduction in cellular energy may thus retard axonal transport and lead to depletion of essential macromolecules and organelles in the distal parts of the axons and dying-back axon degeneration.

Primary demyelination has been reported in cases of peripheral neuropathy associated with a mitochondrial disease (Goebel et al. 1986), whereas polyneuropathy associated with the MELAS syndrome is seldom uniformly demyelinating (Rusanen et al. 1995). The accumulation of mitochondria has been detected in capillary endothelial cells, in smooth muscle cells of the endoneurial and epineurial arterioles and in Schwann cells (Goebel et al. 1986, Schröder & Sommer 1991, Molnar et al. 1995), raising the possibility of a vascular injury to the peripheral nerve (Nardin & Johns 2001). These findings suggest that dysfunction of the myelin sheath may be the primary event in some cases of mitochondrial neuropathy, but the peripheral nerves may be affected by mitochondrial disturbance in more than one way. The frequency of carpal tunnel syndrome (CTS) in the general population, 2.7% (Atroshi et al. 1999), is lower than that found here among 3243A>G patients, 9.4%. Although this finding was not statistically significant, we suggest that patients with 3243A>G may have increased risk of CTS. Mitochondrial disease may increase the vulnerability of the peripheral nerve to compartment syndrome conditions in the same way as diabetes and hypothyroidism.

Mutant mtDNA usually constitutes a similar proportion of total mtDNA in the peripheral nerves as in other tissues such as muscle (Love et al. 1993, Chu et al. 1997, Fadic et al. 1997, Naumann et al. 1997), although cases with tissue mosaicism involving peripheral nerves have also been described, and in such patients the 3243A>G mutation found in muscle was not present in myelinated fibres (Huang et al. 1999). Although the degree of mutant heteroplasmy has been found to determine the clinical phenotype of diseases caused by mtDNA mutations (Ciafaloni et al. 1992), no correlation was found here between the presence of neuropathy and the degree of 3243A>G mutant heteroplasmy in muscle, in spite of the fact that the clinical disease was overall more severe in the patients with neuropathy. On the other hand, a high degree of mutant heteroplasmy has been found to increase the severity of sensorineural hearing impairment (Uimonen et al. 2001). Thus there are other factors which increase the susceptibility of patients with mitochondrial disease to neuropathy as well as the degree of mutant heteroplasmy in muscle.

The patients with neuropathy did not show any greater incidence of diabetes than those without neuropathy, but they were older. Age appeared to contribute to the development of neuropathy, and a similar effect of age on severity has been described among 3243A>G patients with sensorineural hearing loss (Uimonen et al. 2001). Neuropathy appeared to be more common among the men with 3243A>G in the present series than among the women, as reported earlier for another mitochondrial disease, Leber’s hereditary optic neuropathy (Oostra et al. 1994). The higher frequency of neuropathy among men...
observed here and the higher frequency of hearing impairment among men (Uimonen et al. 2001) suggest that phenotypic differences between the sexes may be a more universal phenomenon in mitochondrial diseases.

6.3 Myopathic features associated with 3243A>G

Characteristic clinical features consisted of symmetric mild or moderate proximal limb weakness and extraocular muscle weakness. Proximal involvement of muscles, especially the pelvic muscles, is a common finding in muscle computed tomography of 3243A>G patients. The upper and lower limbs were equally affected, but the proximal muscles were more severely affected than the distal ones. Extraocular muscle weakness is quite a typical feature, as observed earlier (Moraes et al. 1993), although it has been considered to be atypical of patients with 3243A>G. The myopathic patients had worse functional disability than the non-myopathic ones, but cardiomyopathy, stroke-like episodes or other severe features were no more common amongst them. These findings suggest that the functional disability was essentially a result of the myopathy itself.

Myopathic findings display a selective involvement of muscles in cases with the 3243A>G mutation. Selective distribution of muscle involvement has also been described in other myopathies, such as polymyositis (Vliet et al. 1988) and Emery-Dreifuss muscular dystrophy (Mercuri et al. 2002) and extremely in tibial muscular dystrophy (Udd et al. 1993). The gluteus maximus is one of the principal ambulatory muscles affecting the gait, by counteracting the tilting of the pelvis together with the tensor fasciae lata and making it active in an upright position, thus suggesting a high demand for aerobic metabolism. Posterior leg, thigh and neck muscle involvement has also been found in patients with the 3243A>G mutation who exhibit primary myopathies (Calo et al. 1986, Mahjneh et al. 1996) or peripheral neuropathies (Marconi et al. 2001).

The gracilis and sartorius muscles are characteristically of the fusiform, stretched-out type. We found myopathic changes in these muscles only in one patient with clinical myopathy. On the other hand, sparing of the gracilis and sartorius muscles has been reported in many myopathies, including dystrophies (O’Doherty et al. 1977, Hawley et al. 1984, Schwartz et al. 1988, Swash et al. 1995). This finding probably reflects biochemical and functional differences in these muscles (Schwartz et al. 1988). In the case of mitochondrial myopathies, a particularly prominent level of fatty infiltration has been detected by MRI in the gracilis and sartorius muscles of five out of six patients with CPEO (Fleckenstein et al. 1992).

As with neuropathy and hearing loss, we observed that the risk of myopathy increased with age. Mitochondrial diseases due to tRNA gene mutations are usually multisystem disorders with onset in infancy or adolescence, although the highest incidence of myopathy was recorded in the fifth decade of life among these patients with the 3243A>G mutation, suggesting that the decline in oxidative phosphorylation in the skeletal muscles had reached the threshold of clinical manifestation. Thus, myopathy is a late-onset clinical feature among patients with 3243A>G. A similar hypothesis involving a decline in phosphorylation capacity has been put forward for the age-related progression of mitochondrial diseases (Wallace 1992). Skeletal muscle undergoes changes with age, such as a
shift in the proportion of fibre types towards type I, neurogenic muscular atrophy and other structural and histochemical alterations, and furthermore, a clear decline has been reported in respiratory chain function in normal muscle (Boffoli et al. 1994). Ageing by itself does not appear to impair mitochondrial function in healthy elderly athletes, however (Brierley et al. 1997), and we found neither physical inactivity nor any other clinical variables to be associated with myopathy, although it has been suggested that altered physical activity may contribute to the decline (Brierley et al. 1996).

No correlation was found between mutation heteroplasmy and the presence of myopathy. In fact, the myopathic patients displayed slightly lower mean heteroplasmy, while a higher heteroplasmy was associated with CNS manifestations and diabetes. Patients with a higher 3243A>G mutation load in their muscle tissue may be afflicted with encephalopathy at a young age, whereas those with a lower mutation load tend to present with myopathy in their later years. The finding that the peak incidence of myopathy occurs in the fifth decade would support this assumption. An inverse correlation between mutation load and muscle symptoms has also been shown in a meta-analysis (Chinnery et al. 1997a).

Mitochondrial myopathy is usually associated with a high frequency of COX-negative fibres in muscle histochemistry (Chinnery et al. 1997a, Brierley et al. 1997, Larsson & Oldfors 2001), whereas patients with the 3243A>G mutation have a higher percentage of COX-positive RRFs than COX-negative ones (Goto et al. 1992). The more COX-negative fibres were present in muscle histochemistry, the more severe was the myopathy clinically, whereas the number of RRFs did not predict the severity of myopathy. This finding is probably due to the fact that the enzyme defect reflects the myopathic process more accurately than does mitochondrial proliferation. A progressive accumulation of COX-deficient muscle fibres has been proposed as one part of the natural history of mitochondrial myopathy (Weber et al. 1997) and a random accumulation of COX-negative muscle fibres has been documented as associated with the clinical progression of mitochondrial myopathy (Chinnery et al. 2003b).

Dystrophic features have been observed in the muscle histology of some patients with mitochondrial myopathy (Vissing et al. 1998), and we found radiological features here that were similar to those described in dystrophic muscle diseases (Hawley et al. 1984). We found radiological evidence of muscle atrophy in half of the patients with the 3243A>G mutation. The parenchymal changes in atrophic muscles were often spot-like, which in the absence of neuropathy in these patients suggest that the atrophy is myogenic in origin.

We found the most frequent CT changes in the antigravity muscle group, including the neck, back and hip extensors and plantar flexors. Muscle fibres are diverse in their contractile and energetic properties, and can be classified on the basis of the activity of mitochondrial enzymes, or on the basis of the expression of myosin heavy chain protein isoforms, into slow-twitch fibres (type I), and fast-twitch fibres, types IIA and IIX (previously designated as type IIB fibres). The distribution of fibre types varies from muscle to muscle, and even in different parts of the same muscle (Johnson et al. 1973). Muscles primarily involved in maintaining posture, e.g. the plantar flexors, have a higher proportion of type I fibres (Harridge et al. 1996). Anatomically, the type I and IIA fibres lie on a deeper plane, are situated closer to the trunk or limb axis and are responsible for the sustained strong activity required by postural functions. They are also more densely populated with mitochondria and possess a higher activity of cytochrome oxidase than type
IIX fibres (Howald et al. 1985). In consequence, a failure in energy production caused by a mtDNA mutation may lead to the pattern of clinical and radiological involvement observed in the proximal muscles of patients with the 3243A\(\rightarrow\)G mutation.

An increased number of mitochondria is usually a sign of a mitochondrial disease, and large subsarcolemmal aggregates of mitochondria are the most constant ultrastructural finding in cases of such diseases (Lindal et al. 1992). We observed an increased number of mitochondria and increased variation in their size and shape in the muscle of more than 90% of the patients in this series. Intramitochondrial crystals in the muscle were more common in the patients with limb weakness than in those without weakness. The presence of crystals points strongly to a mitochondrial dysfunction, although they have also been observed in other neuromuscular diseases such as muscular dystrophies (Fardeau et al. 1981) or peripheral neuropathy (Marbini et al. 1987). These crystals are composed mainly of the mitochondrial isoform of CK (Stadhouders et al. 1994), but the nature of the cellular injury causing mitochondria to accumulate and to crystallize excessive proteins is not fully known. The activity of mitochondrial CK in RRFs is significantly increased (Bouzini et al. 1996). Muscle cells require creatine and a lack of this compound has been shown to lead to crystal formation (Eppenberger-Eberhardt et al. 1991, Wyss & Wallmann 1994). Several mitochondrial and cytosolic proteins, including CK, interact with the voltage-dependent, anion-selective channel, which is the main pathway for metabolite diffusion across the mitochondrial outer membrane. Thus impairment of cytosolic formation of creatine phosphate, which is a major component of the muscle energy store, can cause metabolic overcompensation in mitochondrial creatine phosphate synthesis. This could result in an accumulation of mitochondrial CK and the formation of crystals.

The distribution of myopathic findings in the patients with the 3243A\(\rightarrow\)G mutation may also be explained by differences in the embryonic origin of the individual muscles. Even though the fate maps for the somitic origins of skeletal muscles are still incomplete, the gluteal muscles are thought to originate from the sacral and coccygeal somites, while the anterior thigh muscles, e.g. the sartorius, are derived from the extreme rostral lumbar somites. In addition it is known that some muscles receive myogenic precursor cells from as few as three contiguous somites, while others originate from as many as seven somites. Precursor cells may contain different proportions of mutant mtDNA as a consequence of the sampling effect in the mitochondrial bottleneck during embryonic development and as a consequence of subsequent mitotic segregation.

The extraocular muscles originate from preotic myotomes, which are thought to be derived from the three extreme rostral somitomeres (Hauschka 1994). The isolated involvement of extraocular muscles in chronic progressive external ophthalmoplegia (CPEO) may be partly explained by the embryonic origin of these muscles. On the other hand, they contain many mitochondria, presumably related to the high demand for energy due to almost constant eye movement. Thus embryonic factors may also play a role in the selective distribution of muscle involvement in mitochondrial myopathies.

Myofibre necrosis and membrane leakage are the major causes of serum CK elevation, and increased serum CK levels usually correlate well with the extent and severity of myopathy. We found that CK was mildly elevated only in some patients, although increased CK has been reported to correlate with limb weakness in patients with the 3243A\(\rightarrow\)G mutation (Sciacco et al. 2001). The present CK values did not correlate with the severity of myopathy, indicating that fibre necrosis, inflammatory changes and sar-
colemmal dispermeability are not common in patients with 3243A>G. We found associations between increased CK and diabetes, lactic acidosis and CNS manifestations. The significance of these associations remained unclear.

EMG abnormalities were more frequent in the proximal muscles, although they occurred less abundantly than changes in CT scans. In the lower limbs, EMG abnormalities were mainly found in the gluteal and hamstring muscles, where CT changes were also seen most often. This may indicate that this imaging modality is more sensitive for detecting muscular pathology. The EMG findings in the upper limbs were more disperse, although there, too, they were proximal, occurring most often in the trapezius muscle. Our EMG findings are compatible with the observation that many myopathic disorders, especially congenital and endocrine, affect the contractile properties of the muscle fibres without modifying their electric properties, and therefore a needle EMG does not produce detectable changes (Wilbourn 1993). Spontaneous fibrillation potentials may be the sole electrophysiological feature in mitochondrial myopathy. Fibrillation might arise from ectopic action potentials generated as a consequence of an unstable resting membrane potential due to metabolic impairment (Carvalho et al. 1993).

Among the 25 out of the 50 MELAS patients in this study who were identified as having myopathy on clinical grounds or by muscle imaging, the majority had clear mitochondrial abnormalities in their muscle biopsy. There were some, however, who had minimal or no muscle biopsy abnormalities suggestive of mitochondrial disease. In terms of clinical implications, these results indicate that a MELAS patient with isolated late onset mild proximal myopathy will have a significant risk of incorrect diagnosis despite thorough conventional neuromuscular evaluation. Genetic testing should be considered in such cases.

6.4 Muscle single fibre analysis in cases of MELAS

Muscle single fibre analysis among MELAS patients has been performed in previous studies from cross-sections of muscle, in order to show the degree of heteroplasmy in one fibre level (Sciacco et al. 1994, Tokunaga et al. 1994, Ozawa et al. 1998, Sciacco et al. 1998, Sue et al. 1999, Silvestri et al. 2000a, Silvestri et al. 2000b). We adopted a different approach and performed the analyses longitudinally along the fibres. The mutation load differed by 30% in 10 consecutive sections of ragged red fibres (RRFs), i.e. within a segment of 30 µm, and there was also a tendency for a difference to exist between histologically normal fibres. Histological studies on cross-sections of muscle from patients with mitochondrial myopathies have revealed a mosaic pattern of metabolic activity (Houshmand et al. 1996, Koga et al. 2000, Chinnery et al. 2003b), while the biochemical defect in longitudinal sections is often segmental (Matsuoka et al. 1991), and COX-negative segments, for example, may exceed 1 mm in length (Elson et al. 2002). We observed that most RRFs showed invariable positive COX activity that spanned at least 200 µm in length. This finding suggests that mitochondrial proliferation is widespread longitudinally, although some segments of the RRFs harbour a mutation load that is clearly below the threshold for biochemical dysfunction.
Interestingly, we found evidence of variation between longitudinal sections in histologically normal muscle fibres, suggesting segmental segregation in these as well. The mutation load in histologically normal fibres adjacent to a RRF seemed to depend on that in the RRF itself. The finding has no exact explanation and would need further studies in order to be addressed in more detail. Mitotic segregation during organogenesis may contribute to this finding, but perinuclear clustering of mitochondria has been shown to take place in cultured cells following inhibition of the molecular motor kinesin, which is driven by ATP (Ebneth et al. 1998). Segmental reduction in cellular energy could lead to clustering of the organelles, suggesting a basis for the non-random distribution of mutant and wild-type mtDNAs which causes segmental defects in all kinds of muscle fibres, i.e. in normal-looking fibres, COX-negative fibres and RRFs.

As major shifts in heteroplasmy can occur between generations in mitochondrial diseases (Hammans et al. 1995), we examined six siblings with variable proportions of 3243A>G mutations in their muscle, and interestingly, five of them had a lower degree of mutation heteroplasmy in histologically normal muscle fibres than their mother. This intergenerational variation may suggest that the oocytes of the mother with a high proportion of 3243A>G mutations were subject to negative selection, and thus the offspring carried a lower degree of mutation heteroplasmy. Selection against oocytes harbouring a high mutation heteroplasmy would also be compatible with the results of an epidemiological study showing that the average fertility of women with 3243A>G is not reduced (Moilanen & Majamaa 2001). On the other hand, the intergenerational variation could be due to mutation accumulation in post-mitotic tissues during ageing (Kovalenko et al. 1997), leading to a higher mutation load in the muscle of the mother. Such an age-related increase in the mutation load of the oocytes would explain the trendwise relationship that we observed between the degree of muscle 3243A>G heteroplasmy in the offspring and the age of the mother at gestation.

The fact that none of these patients had signs or clinical symptoms of myopathy suggests that either a small amount of wild-type mtDNA is sufficient to maintain oxidative phosphorylation in muscle or factors other than the mutation load have a greater impact on the development of myopathy. The only patient with myopathy in our series had a degree of mutation heteroplasmy that was below the median for the series, but she was clearly older than the other patients, suggesting that age may be a significant factor in the development of mitochondrial myopathy.
7 Conclusions

1. Peripheral neuropathy is common in patients with the 3243A>G mutation. The mitochondrial defect may cause neuropathy by axonal mechanisms, but also demyelinating pathogenetic mechanisms may be implicated. The predictive value of the degree of mutant heteroplasmy in muscle is poor, probably because other factors such as age and gender contribute to the neuropathy.

2. Myopathy is highly variable in patients with the 3243A>G mutation. Moderate proximal limb muscle weakness is the most common myopathic presentation, although mild or moderate external ophthalmoplegia can also be found. The fifth decade of life is the risk time for mitochondrial myopathy, and no other clinical variables were associated with an increased risk of myopathy apart from age. Histological and ultrastructural abnormalities were present in patients both with and without myopathy, but the presence of intramitochondrial crystals and COX-negative fibres and variations in the size and shape of the mitochondria were more common in the muscle of myopathic patients.

3. Computed tomography reveals frequent abnormal findings in the muscle of patients with the 3243A>G mutation. Imaging is a useful adjunct to clinical evaluation. Imaging was more sensitive than EMG to identify abnormality and can help to define the distribution of muscle involvement in mitochondrial myopathies.

4. Segregation of 3243A>G in individual muscle fibres is a complex process with random and non-random elements, which needs further scientific studies.
8 References


