The transmission and segregation of mitochondrial DNA mutations

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The transmission and segregation of mitochondrial DNA mutations

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Transmission and segregation of Mitochondrial DNA mutations

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ter verkrijging van de graad van doctor
Aan de universiteit van Maastricht,
op gezag van de Rector Magnificus,
Prof. Mr. G.P.M.F. Mols
volgens het besluit van het college van Decanen,
in het openbaar te verdedigen,
Op Donderdag 13 December om 12.00 uur

Door

Lorraine Johanna Arnolda Maria Jacobs

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Promotor

Prof.dr. J.P.M. Geraedts

Co-promotor

Dr. H.J.M. Smeets

Dr. I.F.M. de Coo

Beoordelingscommissie

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Dr. C.E.M. de Die-Smulders

Prof.dr. I. Liebaers (University Hospital Vrije Universiteit Brussel, België)

Prof.dr. J.S.H. Vles

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Het wonder van een nieuw leven
blijft ons verstand te boven gaan

Chapter 1

Introduction

Introduction

Mitochondrial encephalomyopathies are disorders associated with abnormalities of the terminal component of mitochondrial energy metabolism, i.e. oxidative phosphorylation (OXPHOS). Recent epidemiological studies have shown that ~1 in 8000 of the general population have an OXPHOS disorder (Chinnery, 2004). OXPHOS related diseases therefore cause significant morbidity and mortality. OXPHOS defects have a broad impact on public health, as they have been encountered in age-related neurodegenerative diseases, cardiovascular disease, diabetes, and cancer (Zeviani and Di Donato, 2004; Ritz and Berrut, 2005; Baysal, 2006). OXPHOS is carried out in the inner mitochondrial membrane by the four enzymatic complexes of the respiratory chain (complexes I-IV), plus the ATP-synthase complex (complex V) (Hatefi, 1985; Saraste, 1999). OXPHOS disease can be due to mutations in mitochondrial DNA (mtDNA) or nuclear DNA genes. This implicates a dual genetic control and intergenomic interactions. The nuclear component includes more than 70 genes encoding protein subunits of the OXPHOS complexes and hundreds of genes encoding factors controlling their expression, assembly, function and turnover (Thorburn *et al.*, 2004). An impressive and ever expanding number of pathogenic mutations in the mtDNA have been found in association with a wide spectrum of clinical presentations (Zeviani and Di Donato, 2004). Mutations in mtDNA include either large-scale rearrangements, which are in general sporadic, or point mutations, which are usually transmitted through the maternal lineage. While large-scale rearrangements are invariably heteroplasmic, point mutations may be either heteroplasmic or homoplasmic, and can affect structural genes or genes encoding the RNA apparatus (22 tRNA- and 2 rRNA-genes are encoded by the mtDNA) involved in mtDNA translation (Chinnery and Turnbull, 1999).

One of the intriguing features of OXPHOS disease is the extremely variable clinical presentation. It has been described that OXPHOS disease might affect any tissue and disease can manifest itself at any age. This truism is held up in clinical practice and patients may present in their first few hours after birth or in their 70's and 80's. Another important clinical issue relates to the potential involvement of a variety of different tissues. Thus, a patient with OXPHOS disease might present neurological features and subsequently develop diabetes or cardiomyopathy. These other clinical features may well be those, which are most life-threatening and important with regard to treatment. As far as heteroplasmic mtDNA mutations are concerned, some correlation between mutation load and clinical or biochemical manifestations has been established for few of them. However, the basis of the wide variability of clinical and biochemical features observed in OXPHOS disease remains largely unknown. Likewise, little is known about the mechanisms underlying the tissue specificity consistently reported for some mutations or, on the contrary, the widespread involvement of multiple organs, observed in other mutations, and the variations in the clinical presentations observed in different individuals carrying the same mutation. The extreme clinical, biochemical and genetic variability that characterises OXPHOS disorders creates major difficulties in clinical practice and laboratory diagnosis. This is of particular concern as

treatment is limited for patients with OXPHOS disease and common genetic approaches to prevent the transmission of OXPHOS disease are not applicable for the majority of patients with mtDNA defects. The unpredictable transmission of heteroplasmic mtDNA mutations, due to the genetic bottleneck [a restriction in the number of mtDNA molecules to be transmitted followed by an amplification of these founder molecules (Howell *et al.*, 1992)], the somatic variability in heteroplasmy levels between tissues and in time and the poor predictive value of heteroplasmy levels preclude conventional prenatal diagnosis as a reliable option for most carriers.

Aims and outline of thesis

The main topic of this thesis is to define the somatic segregation and familial transmission and, as well, the origin of heteroplasmic mtDNA mutations. This is related to their clinical expression and possible modifying factors. Our aims are:

- to develop reliable tools that can predict and/or prevent the transmission of mtDNA mutations, like prenatal diagnosis or preimplantation genetic diagnosis, in order to prevent the transmission of OXPHOS disease;
- to identify factors that influence the severity and nature of the phenotypic manifestations or distribution of the mtDNA load, in order to predict the effect of transmission of mtDNA mutations more accurately;
- to determine the presence and impact of *de novo* mtDNA mutations in oocytes

Chapter 2 is an introduction on the transmission of OXPHOS disease and methods to prevent this. The OXPHOS system is under dual genetic control, and mutations in both nuclear and mitochondrial genes can cause OXPHOS diseases. The expression and segregation of mitochondrial DNA (mtDNA) mutations is different from nuclear gene defects. The mtDNA mutations can be either homoplasmic or heteroplasmic and in the latter case disease becomes manifest when the mutation exceeds a tissue-specific threshold. This mutation load can vary between tissues and often an exact correlation between mutation load and phenotypic expression is lacking. The transmission of mtDNA mutations is exclusively maternal, but the mutation load between embryos can vary tremendously because of a segregational bottleneck. Diseases by nuclear gene mutations show a normal Mendelian inheritance pattern and often have a more constant clinical manifestation. Given the prevalence and severity of OXPHOS disorders and the lack of adequate therapy, existing and new methods for the prevention of transmission of OXPHOS disorders, like prenatal diagnosis (PND), preimplantation genetic diagnosis (PGD), cytoplasmic transfer (CT) and nuclear transfer (NT), are technically and ethically evaluated.

In chapter 3 and 4 we describe patients with rearrangements or a point mutation in the mtDNA, the former to identify clues to improve the predictive power of the genetic analysis and the latter to evaluate the possibility PND or PGD for this mutation. In chapter 3 we compare in detail the genetic defects and clinical outcome in 2 patients with Pearson

syndrome, an often fatal multisystem disease associated with mitochondrial DNA rearrangements, to identify factors, which might be involved in and can predict the severity of the disorder. Chapter 4 describes a family with three affected children with Leigh syndrome, a progressive neurodegenerative disorder. Mutation analysis revealed the T9176C mutation in the mtDNA ATPase 6 gene. The possibility of prenatal diagnosis was evaluated. The main problem was the lack of data on genotype-phenotype associations for the T9176C mutation and on variation of the mutation percentage in tissues and in time. Eventually, prenatal diagnosis was offered with understanding by the couple that there could be considerable uncertainty in the interpretation of the results. To get a better insight in the distribution of mtDNA mutations among single cells and the potential use for counselling carriers with respect to recurrence risks, we studied single lymphocytes and fibroblasts of several relatives with the m.9176T>C mutation (chapter 5). Possible differences in distribution may also reveal mechanisms involved in the segregation of mtDNA mutations, which could open up the possibility to manipulate variations in heteroplasmy levels. The basic mechanisms concerning the origin of *de novo* point mutations in the mtDNA were studied in human oocytes (chapter 6). As a large part of patients with OXPHOS defects carry *de novo* mutations in the mtDNA we determined the load of mutations and polymorphisms in the mitochondrial DNA (mtDNA) of human oocytes and we discussed the time frame in which these mutations can occur and the possible effect these mutations might have during life. Finally, in the general discussion (chapter 7) we evaluate strategies to influence heteroplasmy and to complement the existing genetic defect and we elaborate further on possible approaches to prevent the transmission of mtDNA caused OXPHOS disease, in case PND and PGD is not a reliable alternative.

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Chapter 2

The transmission of OXPHOS disease and methods to prevent this

LJAM Jacobs^{1,2}, G de Wert³, JPM Geraedts¹, IFM de Co⁴, HJM Smeets¹.

¹Department of Genetics and Cell Biology, ²Research Institute GROW, ³Department of Ethics and Philosophy, University Maastricht.

⁴Department of Child Neurology, Erasmus MC-University Medical Center Rotterdam.

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Abstract

Diseases due to defects of oxidative phosphorylation (OXPHOS) affect approximately 1 in 8,000 individuals. Clinical manifestations can be extremely variable and range from single affected tissues to multisystemic syndromes. In general tissues with a high energy demand, like brain, heart and muscle, are affected. The OXPHOS system is under dual genetic control and mutations in both nuclear and mitochondrial genes can cause OXPHOS diseases. The expression and segregation of mitochondrial DNA (mtDNA) mutations is different from nuclear gene defects. The mtDNA mutations can be either homoplasmic or heteroplasmic and in the latter case disease becomes manifest when the mutation exceeds a tissue-specific threshold. This mutation load can vary between tissues and often an exact correlation between mutation load and phenotypic expression is lacking. The transmission of mtDNA mutations is exclusively maternal, but the mutation load between embryos can vary tremendously due to a segregational bottleneck. Diseases by nuclear gene mutations show a normal Mendelian inheritance pattern and often have a more constant clinical manifestation. Given the prevalence and severity of OXPHOS disorders and the lack of adequate therapy, existing and new methods for the prevention of transmission of OXPHOS disorders, like prenatal diagnosis, preimplantation genetic diagnosis, cytoplasmic transfer and nuclear transfer, are technically and ethically evaluated.

Mitochondrial disorders

Mitochondrial disorders are a group of diseases and syndromes commonly defined by lack of energy due to defects in oxidative phosphorylation (OXPHOS) (Zeviani and Di Donato, 2004). They affect at least 1 in 8000 of the general population, making them the most common inherited metabolic diseases (Chinnery, 2004). Energy in the form of ATP is produced by the OXPHOS system, which consists of five multiprotein enzyme complexes that release the energy stored in the form of a proton gradient across the inner mitochondrial membrane (Saraste, 1999). Disease manifestations due to OXPHOS defects usually involve tissues with a high energy demand like brain, heart, liver and the renal and endocrine systems (Wallace, 1999). Clinical manifestations of OXPHOS diseases are extremely variable and range from a single affected tissue, like the loss of vision in Leber's hereditary optic neuropathy (LHON), to multisystemic syndromes like Leigh syndrome (subacute necrotizing encephalomyelopathy, LS), mitochondrial encephalopathy, lactic acidosis and stroke like episodes (MELAS), neuropathy, ataxia and retinitis pigmentosa (NARP) and myoclonic epilepsy with ragged red fibres (MERRF). Table I lists several syndromes and symptoms associated with OXPHOS disease. Involvement of the central nervous system, skeletal muscle or both is seen in many mitochondrial syndromes. A frequent symptom in paediatric patients is developmental delay and failure to thrive. Symptoms can present in just a single tissue or organ, but a multiorgan involvement in a patient or affected relatives is more common. When at least two organ systems unexplained by other diseases are involved in a single person or in affected (maternal) relatives, then an OXPHOS disease should be considered. Clinicians should be aware that apparently unrelated symptoms might have a common genetic cause.

Table 1: Common examples of OXPHOS syndromes.

Syndrome	Genetic cause	Symptoms (clinical phenotype, age of onset)
LHON (Leber's hereditary optic neuropathy)	Homoplasmic/heteroplasmic mtDNA mutation (the most common are the G11778A, G3460A and T14484C mutations)	Subacute bilateral loss of vision, sometime accompanied by dystonia and cardiac pre-excitation syndromes
MELAS (Mitochondrial encephalopathy, lactic acidosis and stroke like episodes)	Heteroplasmic mtDNA mutation (the most commonly reported is the A3243G mutation)	Median age of onset 2.7 to 3.4 years, with a range from 1 to 70 years Stroke like episodes with seizures and/or dementia and RRF and/or lactic acidosis, often accompanied by diabetes mellitus, cardiomyopathy, external ophthalmoplegia, cortical blindness, cerebellar ataxia and pigmentary retinopathy. Age of onset is variable and ranges from neonatal to 40 years of age.
MERRF (Myoclonus epilepsy with ragged red fibres)	Heteroplasmic mtDNA mutation (usually mutations in tRNA ^{lys} , most commonly the A8344G mutation)	Myoclonic seizures, cerebellar ataxia and myopathy, often accompanied by spasticity, dementia, hearing loss, optic atrophy, short stature and cardiomyopathy
NARP (Neuropathy, ataxia, and retinitis pigmentosa)	Heteroplasmic mtDNA mutation (the most commonly reported is the T8933G/C mutation)	Age of onset is variable, usually in late childhood to adulthood Neurogenic muscle weakness, ataxia, retinitis pigmentosa, seizures and mental retardation, often accompanied by hearing loss, dementia and developmental delay. Onset is usually during infancy or early childhood
Leigh syndrome	Leigh syndrome is caused by many mutations in mtDNA or nDNA genes (the most commonly reported mtDNA mutation is the T8933G/C mutation)	Recurrent attacks of psychomotor regression with seizures, dystonia and brainstem dysfunction, lactic acidosis and hypotonia, often accompanied by ataxia, respiratory disturbances, pigmentary retinopathy and spasticity. Typical ct or MRI abnormalities with bilateral symmetric signal alterations in the basal ganglia, thalamus, midbrain, and brainstem. Age of onset between 3 months and 2 years
PEO (Progressive external ophthalmoplegia)	Heteroplasmic mtDNA rearrangements and nuclear gene mutations	External ophthalmoplegia and bilateral ptosis often accompanied by proximal muscle weakness and exercise intolerance Age of onset is usually between 20 and 50 years
KSS (Kearns-Sayre syndrome)	Heteroplasmic mtDNA rearrangement	PEO onset before age of 20 with pigmentary retinopathy often accompanied by ataxia, neuropathy, cardiac conduction block and raised CSF protein Age of onset is before 20 years often during childhood
Pearson syndrome	Heteroplasmic mtDNA rearrangement	Sideroblastic anaemia with vacuolisation of marrow precursors, pancytopenia and exocrine pancreatic failure often accompanied by ophthalmoplegia, lactic acidosis and RRF
MNGIE (Mitochondrial neurogastrointestinal encephalomyopathy)	Nuclear gene mutations in the thymidine phosphorylase gene or the polymerase γ gene	Ptosis, progressive external ophthalmoplegia, gastrointestinal dysmotility (often pseudobstruction), diffuse leukoencephalopathy, thin body habitus, peripheral neuropathy and myopathy. Onset is usually in late childhood or adulthood
MDS (Mitochondrial depletion syndrome)	Nuclear gene mutations in genes coding for proteins involved in mtDNA integrity and maintenance	Individuals with the hepatocerebral form of mitochondrial DNA depletion syndrome have early progressive liver failure and neurologic abnormalities, hypoglycemia, and increased lactate in body fluids. Onset is within a few weeks after birth and patients die before 9 months of age

Mitochondrial DNA (mtDNA)

The first description of a circular DNA structure located in the mitochondria dates from 40 years ago (Nass, 1966). Several unique characteristics discriminate mitochondrial from nuclear DNA:

1. The mtDNA is a multicopy genome. A cell contains hundreds of mitochondria and each mitochondrion contains five to ten copies of mtDNA (Goto, 2001). Dependent on the tissue and energy demand each cell contains between 500-10 000 mtDNA molecules, except for mature oocytes which contain between 100 000 and 600 000 mtDNA molecules (Reynier *et al.*, 2001). Oocytes store mitochondria to deal with the lack of mtDNA replication during the first cleavage stages of the embryo (Schaefer *et al.*, 2001).
2. In a cell all mtDNA molecules can be identical (homoplasmy), or two types of mtDNA molecules, that differ in sequence, in the same cell, tissue or even in the same organelle can coexist (heteroplasmy, (Holt *et al.*, 1988; Lightowlers *et al.*, 1997).
3. The mtDNA is transmitted entirely through the maternal line.
4. The mtDNA is a double stranded circle (figure 1) of 16.569 bp with a genetic code different from the nuclear DNA (Fernandez-Silva *et al.*, 2003). The mtDNA encodes 37 genes, of which 13 genes encode OXPHOS subunits (complex I (7), III (1), IV (3) and V (2)) and 22 tRNA- and 2 rRNA-genes, required for mitochondrial translation (Clayton, 1991; Wallace *et al.*, 1995). Approximately 6% of the mtDNA is noncoding, located predominantly in the D-loop and involved in the replication and transcription of the mtDNA (Berdanier and Everts, 2001). The mtDNA is compact, it contains no introns, several overlapping genes and incomplete termination codons (Lightowlers *et al.*, 1997).

initiated; HSP: H-strand promoter; LSP: L-strand promoter.

Figure 1

Map of the human mtDNA presenting the protein coding genes for the seven subunits of complex I (ND1-ND6), the three subunits of cytochrome c oxidase (COI-COIII), cytochrome b (cytb) and the two subunits of ATP synthase (ATPase 6 and 8); the 12S and 16S ribosomal RNAs (rRNAs); and the 22 transfer RNAs (tRNAs) identified by the three letter code for the corresponding amino acids. Furthermore the location of the origin of replication of both the H (OH) and L (OL) strands are indicated as well as the location of the promoter site where transcription is

MtDNA molecules are packed in somatic cells as nucleoids in which 6 to 10 molecules form a group with several different proteins (Jacobs *et al.*, 2000; Iborra *et al.*, 2004; Legros *et al.*, 2004). These nucleoids are no static entities and mtDNA molecules exchange between nucleoids. The nucleoids are attached to the inner mitochondrial membrane near the OXPHOS system, where reactive oxygen species (ROS) are being produced (Richter *et al.*, 1988). Due to the lack of histones and other protective proteins and an ineffective repair mechanism the mtDNA mutates ten to sixteen times as frequent as the nuclear DNA and due to the lack of introns the mutations have a high probability of affecting genes and being pathogenic (Larsson and Clayton, 1995; Treem and Sokol, 1998).

Replication, transcription and translation of the mtDNA

Replication of the mtDNA is called relaxed, because it is not connected to the cell cycle, and there is a constant degradation and production of mtDNA (Chinnery and Samuels, 1999). Replication of mtDNA takes place in post-mitotic terminally differentiated cells. In most cell types two possible mechanisms for the replication of the mtDNA exist (Bowmaker *et al.*, 2003; Holt and Jacobs, 2003; Reyes *et al.*, 2005). The strand displacement mechanism involves unidirectional initiation from the origin of replication of the “heavy” H-strand (O_H) located in the D-loop region of the mtDNA molecule (figure 1). The replication of this leading strand initiates the synthesis of the lagging-strand from the light L-strand origin of replication (O_L) (Shadel and Clayton, 1997; Bogenhagen and Clayton, 2003). Alternatively, strand-coupled replication of the mtDNA implies initiation of lagging strand synthesis at multiple sites probably involving the synthesis of short Okazaki fragments (Holt *et al.*, 2000; Bogenhagen and Clayton, 2003). The original strand displacement mechanism is probably the main replication method in cells which are in a steady state level whereas the strand coupled model seems to be predominant in cells recovering after depletion and in cells in need of accelerating mtDNA synthesis (Holt *et al.*, 2000; Fish *et al.*, 2004). The mtDNA is synthesized by a mitochondrial specific polymerase, DNA polymerase gamma (POLG), which requires additional factors like Twinkle (a ring helicase, (Spelbrink *et al.*, 2001)) and mitochondrial topoisomerases I and II responsible for respectively the removal and introduction of supercoils in the mtDNA (Kosovsky and Soslau, 1993; Zhang *et al.*, 2001). The mechanism regulating mtDNA replication is still not completely understood. Tfam, a limiting factor, and the size of the nucleoside pool are known to play an important role in the regulation of the mtDNA copy number (Ekstrand *et al.*, 2004; Kanki *et al.*, 2004; Kang and Hamasaki, 2005) but other factors will exist (Kaukonen *et al.*, 2000; Brown and Clayton, 2002).

Transcription of the mtDNA requires mtRNA polymerase, mitochondrial transcription factor A (Tfam) and B1 or B2 (TFB1M or TFB2M) and several other trans-acting factors (Gaspari *et al.*, 2004; Kang and Hamasaki, 2005). L-strand transcription is initiated at the L-strand promoter (LSP) and results in a single polycistronic precursor RNA. The H-strand is transcribed by two overlapping units starting at two different initiation sites HSP₁ and HSP₂

(Fernandez-Silva *et al.*, 2003). Transcription can be regulated at the level of initiation, termination, by the mitochondrial transcription termination factor (mTERF) (Asin-Cayuela *et al.*, 2005), or both. Autonomous regulation of the mtDNA transcription occurs as in isolated mitochondria the transcription of mtDNA continues for several hours (Enriquez *et al.*, 1996). External signals, which play a role in the transcription regulation are for example ATP levels in the cells and thyroid hormones (Enriquez *et al.*, 1996; Weitzel *et al.*, 2003).

In humans mitochondrial translation occurs at the mitochondrial ribosomes (Sasarman *et al.*, 2002), composed of a small ribosomal subunit (the 12S rRNA subunit encoded by the mtDNA and 29 nuclear encoded proteins) and a large ribosomal subunit (the 16S rRNA subunit encoded by the mtDNA and 48 nuclear encoded proteins) (Koc, E.C. *et al.*, 2001; Koc, E. C. *et al.*, 2001). Additional factors are initiation factors (IF2 and IF3 (Ma and Spremulli, 1996; Koc and Spremulli, 2002)), elongation factors (EFTu (Ling *et al.*, 1997), EFTs (Xin *et al.*, 1995), EFG1 (Gao *et al.*, 2001) and EFG2 (Lochmuller *et al.*, 1999; Hammarsund *et al.*, 2001)) and release factors (RF1 (Zhang and Spremulli, 1998)).

Biochemical investigations in OXPHOS disease

In general lactate (cell redox state, normal <20) and alanine levels are increased. Histochemical studies of skeletal muscle with accumulation of abnormal mitochondria under the sarcolemmal membrane in muscle fibres [Ragged Red Fibres (RRF)] or cytochrome oxidase (COX) negative fibres confirm mitochondrial dysfunction. Electron microscopy may provide additional information. Biochemical studies carried out in skeletal muscle or cultured skin fibroblasts or in any other (preferably affected) available tissue can determine enzyme deficiencies in one or more of the OXPHOS enzyme complexes (van den Heuvel and Smeitink, 2001). Spectrophotometric methods or Blue Native polyacrylamide gel electrophoresis combined with histochemistry (BN-PAGE) can both be applied to determine the activity of the individual OXPHOS complexes or combinations of complexes (Munnich and Rustin, 2001). (Van Coster *et al.*, 2001). These biochemical measurements are preferably performed in fresh muscle specimens or other fresh tissues clinically expressing the disease, as frozen muscle or cultured fibroblasts do not always present the enzymatic deficiencies. Some difficulties are associated with the biochemical analysis. Normal variation in enzyme activity is high and therefore the frequently detected moderate decreases in activity remain inconclusive. Furthermore, substantial variation exists in normal activity range as determined by different centres, because of the use of different protocols and the lack of widely accepted diagnostic criteria (Thorburn *et al.*, 2004). A classification scheme has been developed by Bernier *et al.* including clinical features and enzyme activities found in several groups of patients (Bernier *et al.*, 2002).

Genetic causes of OXPHOS disease

OXPHOS diseases can be caused by mutations in the nuclear and mtDNA. Nuclear OXPHOS mutations can be classified as (i) gene defects altering the stability of mtDNA (ii) gene defects in structural components or assembly factors of the OXPHOS complexes (iii)

defects in non protein components of the respiratory chain, like CoQ10 or taffazzin and (iv) gene defects in proteins indirectly related to OXPHOS (Chinnery, 2003; Zeviani and Di Donato, 2004). OXPHOS diseases caused by nuclear gene mutations usually follow a Mendelian inheritance pattern. Disease causing mutations in the mtDNA can be either large rearrangements or point mutations or a reduced copy number (mtDNA depletion).

MtDNA rearrangements

Large-scale rearrangements are usually single deletions. Since 1988 (Holt *et al.*, 1988) over 200 different mtDNA deletions have been reported, associated with several, different OXPHOS diseases. Three main clinical phenotypes are Kearns-Sayre syndrome (KSS), chronic progressive external ophthalmoplegia (PEO) and Pearson syndrome (table I). The vast majority of deletions reported are flanked by short repeat sequences ranging from 3-14 bp in length (Mita *et al.*, 1990; Ota *et al.*, 1994). No minimal area of overlap exists between the different deletions, but always at least one tRNA is removed (Tang Y. *et al.*, 2000). The severity of the disease and the age of onset are partly dependent on the amount and tissue distribution of the mtDNA rearrangement and the presence of deletion dimers or partially duplicated mtDNA molecules (Poulton and Holt, 1994; Rotig *et al.*, 1995; Jacobs *et al.*, 2004).

MtDNA point mutations

Point mutations in the mtDNA can be pathogenic or neutral. Neutral polymorphisms are common and based on a combination of specific polymorphisms the mtDNA can be classified in haplogroups. Over 150 pathogenic point mutations in the mtDNA that affect protein coding genes or RNA genes have been reported since 1988 (Wallace *et al.*, 1988). Most pathogenic point mutations are heteroplasmic, but homoplasmic disease causing point mutations in the mtDNA have been described as well. The clinical phenotype of homoplasmic mutations (table II) is generally restricted to a single tissue. Penetrance is often incomplete and other factors like nuclear encoded proteins, epigenetic factors, environment or lifestyle (tobacco smoking (Tsao *et al.*, 1999)), mtDNA haplogroups (Brown *et al.*, 2002) are likely to be involved (Guan *et al.*, 2001).

Heteroplasmic point mutations in protein encoding and in RNA genes are more often pathogenic (table II). Many mutations are infrequent or even private, presenting in a single family. All mutations display clinical heterogeneity (Sparaco *et al.*, 2003), but this is most evident for the common m.3243A>G mutation (table II). This variable phenotypic expression can not be explained by the heteroplasmy level only and nuclear genes may be involved (Dunbar *et al.*, 1995; Jacobs and Holt, 2000; Torroni *et al.*, 2003). The threshold at which ATP production decreases is dependent on the tissue and mutation analysed. It appears to be lower in those tissues with a higher energy demand such as brain and muscle (Larsson and Clayton, 1995). The existence of such a threshold implies that in the normal situation there is an overcapacity of the OXPHOS system (Rossignol *et al.*, 2003), required to deal with an

increased energy demand. This can also be considered a protective mechanism against deleterious mutations, which inevitably will accumulate during life.

Table II: The most common mtDNA point mutations

Disease	mtDNA mutation	Homo/heteroplasmy	Reference
LHON	m.3460G>A	homoplasmic	(Huoponen <i>et al.</i> , 1991)
	m.11778G>A	homoplasmic	(Wallace <i>et al.</i> , 1988)
	m.14484T>C	homoplasmic	(Johns <i>et al.</i> , 1992)
Hypertrophic cardiomyopathy	m.4300A>C	homoplasmic	(Taylor <i>et al.</i> , 2003)
Aminoglycoside induced hearing loss	m.1555A>G	homoplasmic	(Usami <i>et al.</i> , 2000)
Leigh syndrome	m.1624C>T	homoplasmic	(McFarland <i>et al.</i> , 2004)
NARP/Leigh syndrome	m.8993T>G/C	heteroplasmic	(Holt <i>et al.</i> , 1990)
MERRF	m.8344A>G	heteroplasmic	(Shoffner <i>et al.</i> , 1990)
MELAS	m.3243A>G	heteroplasmic	(Goto <i>et al.</i> , 1990)
Maternally inherited diabetes and deafness (MIDD)	m.3243A>G	heteroplasmic	(van den Ouweland <i>et al.</i> , 1995; Akbari <i>et al.</i> , 2004; Maassen <i>et al.</i> , 2004)
Non-syndromic hearing loss (NSHL)	m.3243A>G	heteroplasmic	(Mancuso <i>et al.</i> , 2004)
KSS and CPEO	m.3243A>G	heteroplasmic	(Bosbach <i>et al.</i> , 2003)
Renal failure	m.3243A>G	heteroplasmic	(Jansen <i>et al.</i> , 1997)

Depletion and multiple mtDNA deletions

MtDNA depletion is a reduction in copy number of mtDNA molecules, which can be the consequence of a nuclear gene defect. Mitochondrial DNA depletion syndrome (MDS, table I) with or without multiple mtDNA deletions is a severe autosomal recessive genetic disease caused by a mutation in one of the genes involved in mtDNA synthesis or nucleotide metabolism. Other mutations are detected in genes involved in mtDNA replication and the maintenance of the mitochondrial dNTP pool. Defects in comparable genes (ANT1, Twinkle, and POLG1) are involved in multiple mtDNA deletions, which can present with or without depletion. Especially, mutations in the POLG gene have a clinically heterogeneous presentation and both autosomal dominant and autosomal recessive families have been reported.

Acquired mtDNA mutations

Inherited mtDNA mutations are usually present in all or most of the human tissues, but somatic mutations occur as well. Cytochrome *b* mutations have been described in muscle of patients only (Andreu *et al.*, 1999). Age-related ROS damage is the most common source of acquired somatic mtDNA mutations. Over 200 different deletions and several point mutations have been found in the mtDNA that accumulate during ageing, especially in ageing muscle (Cottrell and Turnbull, 2000; Wei and Lee, 2002), in humans, but also in other species like monkeys (Lec *et al.*, 1993; Schwarze *et al.*, 1995), mice (Tanhauser and Laipis, 1995; Khaidakov *et al.*, 2003) and nematodes (Melov *et al.*, 1995). The mutant load of these

individual mutations usually does not exceed 1%, but the total number of mtDNA mutations can be of such that the mitochondrial respiration and OXPHOS is significantly impaired (Hayakawa *et al.*, 1996; Liu *et al.*, 1998). A direct relation between acquired mtDNA mutations and ageing has been shown in a mice-model with a deficient polymerase gamma leading to deletions and point mutations in the mtDNA. These mtDNA-mutator mice show a reduced lifespan and premature onset of ageing problems like hair loss, osteoporosis anaemia and reduced fertility (Trifunovic *et al.*, 2004). Depletion of the mtDNA can also be acquired and for example pharmacologically induced by antiviral nucleoside analogs, as used in HIV therapy (Kakuda, 2000).

Treatment of OXPHOS disease

Despite extensive studies on use of various pharmacological agents and vitamin supplements there is still no cure for OXPHOS disease. Pharmacological therapy mainly relies on the administration of artificial electron acceptors, metabolites and cofactors or oxygen radical scavengers (Dimauro *et al.*, 2004). The administration of these factors can have a beneficial effect in some cases, but the effect is often transient. Novel strategies are being developed directed at manipulating the level of heteroplasmy in the cell (Chinnery and Turnbull, 2001; Chinnery, 2004). These techniques aim at lowering the level of mutant mtDNA by selectively inhibiting the replication of mutant mtDNA by sequence specific peptide nucleic acids (PNAs) or by the removal of mutated mtDNA by means of restriction enzymes. Alternative strategies attempt to treat the disease at the biochemical level by supplying cells with the normal mitochondrial proteins. Both these strategies encounter problems when executed in isolated organelle models with respect to the specificity and delivery of the product (Taylor *et al.*, 2001). Another novel strategy is to redesign mitochondrial genes for expression from the nucleus and import normal copies of the redesigned gene from the cytosol into the mitochondria. The same can be done with allotopic expression of tRNA's. For the allotopic expression of both mitochondrial proteins and tRNAs the correctly engineered genes must be delivered, recombined into the nucleus and expressed in a large number of cells to be a viable therapeutic approach (Smith *et al.*, 2004).

Physical exercise can also be important to prevent disease manifestations. Most patients with mitochondrial disease are inactive because of exercise intolerance or fear for muscle damage, in spite of the fact that aerobic training increases work and oxidation capacity in these patients (Taivassalo *et al.*, 2001; Taivassalo and Haller, 2004). Questions remain on the (long term) effect of exercise on the mutant load, which may rise during life (Chinnery, 2004). Until a definite cure is developed patients can only be given support and some limited therapy aimed at improving the quality of life. Palliative therapy is directed at preventing, for example, the complications of diabetes mellitus and cardiomyopathy and surgical correction of ptosis and cataracts (Dimauro *et al.*, 2004).

MtDNA segregation and transmission

The mtDNA is transmitted through the maternal line via the mitochondria contained in the ooplasm. Maternal transmission is also a hallmark of mtDNA related diseases. Mature human oocytes contain between 100 000 and 600 000 mitochondria and mtDNA copies (Reynier *et al.*, 2001; Poulton and Marchington, 2002). This is in contrast to sperm cells which have been reported to contain between 10 and 700 copies mtDNA (Hecht *et al.*, 1984; Shitara *et al.*, 2000; Diez-Sanchez *et al.*, 2003; May-Panloup *et al.*, 2003). The mtDNA content of the spermatozoon decreases 5- to 6 fold during the spermatogenesis, probably because of a down-regulation of the mitochondrial transcription factor A (Tfam) (Larsson *et al.*, 1997; Rantanen and Larsson, 2000; Diez-Sanchez *et al.*, 2003). During spermatid development ubiquitin binds to the mitochondria, which makes the sperm mitochondria prone to proteolysis (Sutovsky, 2003), resulting in the loss of paternal mtDNA molecules (Shitara *et al.*, 1998; Sutovsky *et al.*, 2003). In another study *t-tpis*, a testis specific translocator, belonging to the translocator of mitochondrial outer membrane (TOM) complex, has been identified as a sperm mitochondria specific factor, which incorporates an elimination factor present in the oocyte. The elimination factor is not yet identified but it probably activates an endonuclease system. The ubiquitination process is thought to follow the selective digestion of sperm mtDNA by endonucleases. Elimination of sperm mitochondria in mouse can be inhibited by treatment with anti-*tpis* and (Hayashida *et al.*, 2005). Recently, transmission of paternal mtDNA was detected in skeletal muscle of a patient (Schwartz and Vissing, 2002), but this is an infrequent phenomenon (Filosto *et al.*, 2003; Johns, 2003; Schwartz and Vissing, 2003; Taylor *et al.*, 2003; Schwartz and Vissing, 2004). Paternal transmission has also been studied in ICSI and IVF embryos and offspring. In these cases low amounts of paternal mtDNA were detected in 16 of the 32 abnormal polyploid embryos (St John *et al.*, 2000) but not in offspring and normal embryos (Danan *et al.*, 1999; Marchington *et al.*, 2002).

Correct functioning and intactness of the mitochondria is vital for sperm motility. OXPHOS inhibitors decrease sperm motility (Ruiz-Pesini *et al.*, 2000; St John *et al.*, 2005), which suggests that mutations affecting mitochondrial functioning could have an effect on sperm motility. The m.3243A>G mtDNA mutation shows a higher mutation level in semen fraction with a lower motility (Spiropoulos *et al.*, 2002) and analysis of semen from men with lower semen quality revealed a higher incidence of homoplasmic base changes in the mtDNA especially at two locations, nt 9055 and nt 11719 (Holyoake *et al.*, 2001). Kao *et al.* observed a higher incidence of especially the 4977 bp 'common' mtDNA-deletion, in semen with a lower motility (Kao *et al.*, 1998), but this was not confirmed by others (Cummins *et al.*, 1998; St John *et al.*, 2001). Multiple mtDNA deletions have been observed in both normozoospermic and oligozoospermic men but as the semen quality diminishes the number of multiple deletions. A deviant number of CAG repeats (normally 10) in the polymerase gamma gene has been associated with unexplained male infertility (Rovio *et al.*, 2001; Jensen *et al.*, 2004). This association was not confirmed by others (Krausz *et al.*, 2004; Akinin-Seifer

et al., 2005). Pathogenic POLG mutations have however been associated with hypofertility in both males and females (Ferrari *et al.*, 2005) and premature menopause has been found in a large number of females suffering from CPEO caused by POLG mutations (Luoma *et al.*, 2004), probably due to a link with steroid hormone genesis (Bose *et al.*, 2002). These data are confirmed by the mutator-mic, carrying a proofreading deficient polymerase gamma, which show reduced fertility of both the male and female mice (Trifunovic *et al.*, 2004).

Mitotic segregation and relaxed replication of mtDNA

During cell division, mitochondria are randomly divided (Rotig and Munnich, 2003) and in heteroplasmic cells this can lead to a shift in the proportion of mutant mtDNA in the daughter cells. A loss of mutations is observed in fast dividing tissues, probably due to a selection against cells containing high mutation loads. An example is the average decrease of 1% per year of the m.3243A>G mutation in blood of patients (Rahman *et al.*, 2001). Increased ROS production is a critical factor triggering mtDNA replication, but also increasing mtDNA damage, eventually leading to apoptosis. In post mitotic tissues accumulation of mtDNA deletions and point mutations has been observed (Larsson *et al.*, 1990; Weber *et al.*, 1997). This proliferation only takes place in cells containing high amounts of mutant mtDNA and because of this, heteroplasmy percentage in tissues as a whole increases and variation in mutation load between muscle fibres develops (Chinnery *et al.*, 2002). In case of deletions the replicative advantage of the smaller molecule also adds to the accumulation of the mutated mtDNA in tissues and cells (Diaz *et al.*, 2002).

Polymorphism and mutations in oocytes

Mutations in oocytes have been described as part of the transmission of pathogenic familial mutations and as *de novo* events. The first group is important for the recurrence risk of mtDNA disease in families and carriers (see next paragraph), the second could potentially explain the occurrence of new disease cases. Deletions in the mtDNA have been reported in 40-60% of unfertilized oocytes or oocytes that failed to develop into mature metaphase II oocytes, although usually in very low mutation percentages (Chen *et al.*, 1995; Keefe *et al.*, 1995; Brenner *et al.*, 1998; Reynier *et al.*, 1998; Barritt *et al.*, 1999; Hsieh *et al.*, 2002). Since none of the donating couples showed symptoms of mtDNA deletion syndromes these mutations probably arose in the oocyte. Recently, we screened the entire mtDNA in oocytes for predominantly heteroplasmic point mutations and found that over 25% of the oocytes contained point mutations. The mutation percentages varied from very low levels (<1%) to very high levels (>99%) with most oocytes containing low level mutation percentages (<30%) (Jacobs *et al.* in preparation). Therefore, at least 25% of the unfertilised oocytes contain a mtDNA mutation. Under the assumption that at least 10% of the point mutations in the mtDNA will be pathogenic this would mean that more than 5% of the oocytes harbour a possible pathogenic mutation in the mtDNA. Mostly, these mutations are present in very low levels. Some percentages were above the threshold of expression and these *de novo* mutations can have a direct phenotypic effect (De Coo *et al.*, 1996; Degoul *et al.*, 1997; Maassen *et al.*,

2002; Thorburn, 2004). The low level mutations can get lost by cell division, but also fixed during life by random genetic drift, which has been observed in rapidly dividing colonic crypt cells (Taylor *et al.*, 2003) and cancer cells (Carew and Huang, 2002). This also means that a very low level of mtDNA mutation in the oocyte can, because of relaxed replication, accumulate during life and might predispose for diseases, like Alzheimer and Parkinson's disease, which are associated with mtDNA mutations (Chinnery *et al.*, 2002; Coskun *et al.*, 2004).

It is unlikely that mutations in the oocyte in general influence the fertilisability as carriers of mtDNA mutation do not present with fertility problems and children with a high mutation load are being born (Moilanen and Majamaa, 2001). However, oocytes can accumulate mutations in an age dependant manner. The m.414T>G point mutation is present in 40% of the oocytes of women aged ≥ 37 years in contrast to 4% of the oocytes of women aged < 37 years. which could be associated with reproductive senescing (Barritt *et al.*, 2000). Reynier *et al.* has shown a lowered number of mitochondria in oocytes from patients with fertilisation failure due to unknown causes (Reynier *et al.*, 2001) and a lower number of mitochondria is found in ageing oocytes (de Bruin *et al.*, 2004). This means that the number of mitochondria in itself is important and not necessarily the ATP production by the OXPHOS system during embryo development. Therefore, acquired mtDNA mutations affecting mtDNA replication might affect the fertility.

Segregation of mtDNA diseases in families

The segregation of mtDNA disease in families is not straightforward and is highly dependent on the nature and amount of the mtDNA mutation. A woman carrying an mtDNA mutation will transmit a variable amount of this mutation to her offspring. The percentage heteroplasmy of point mutations in the offspring is related to the mutation percentage in the mother (Chinnery *et al.*, 1998) although extreme shifts in mutation percentages occur (White *et al.*, 1999; Carelli *et al.*, 2002). Only a few studies report on the inheritance of heteroplasmic mtDNA mutations (Chinnery *et al.*, 2000; Wong *et al.*, 2002) and it appears that mutations, like the m.8363G>A, m.3460G>A and m.8993T>C, are in general randomly transmitted to offspring although in some cases skewing in favour of the mutation can be observed (Larsson *et al.*, 1992; Chinnery *et al.*, 2000; Hurvitz *et al.*, 2002; Wong *et al.*, 2002). Transmission of the m.8344A>G, the m.3243A>G and m.8993T>G mutations is possibly not completely random, when comparing blood levels in mother and child (Chinnery *et al.*, 2000). The mutation percentage of the m.8344A>G mutation is lower in the offspring and of the m.3243A>G and m.8993T>G mutations higher than expected by random transmission only (White *et al.*, 1999; White *et al.*, 1999; Chinnery *et al.*, 2000; Wong *et al.*, 2002). However, the number of reported transmissions is small, a selection bias is likely, because analysis is performed after discovery of an offspring with clinical symptoms and the age of sampling differs between mother and child.

Analysis of 82 oocytes collected from a woman carrying the m.3243A>G mutation with a mutation load of 18% in muscle and 7% in leukocytes, revealed a binominal

distribution pattern. The mutation percentage in the oocytes ranged from 0% to 45% (mean 12.6%), which was a random segregation pattern (Brown *et al.*, 2001). Oocytes from a carrier of the m.8993T>G mutation demonstrated an extremely skewed segregation pattern in seven oocytes of a woman with a mutant load of 50% in blood. Six of the seven oocytes contained a mutant load >95% and the remaining oocyte showed no evidence of the mutation (Blok *et al.*, 1997). It is unclear whether this is a good representation of the entire pool of her oocytes and of other women carrying this mutation.

For most mutations a relation exists between maternal mutation load and the mutation load in offspring and therefore the chance of being affected. This has been extensively studied for the m.8344A>G, m.3243A>G and m.8993T>G/C mutation (Chinnery *et al.*, 1998). Carriers of the m.8344A>G mutation are at risk of affected offspring if the mutation load in blood is >40%. This risk ranges from 12% (mutation load 40-59%) to 78% (mutation load >80%). For the m.3243A>G mutation the chance of affected offspring ranges from 25% (mutation load <20% in blood) to 57% (mutation load 40-60%). The risk of affected offspring is therefore substantial even at low mutation levels in the carrier. Finally, for the m.8993T>G/C mutations the risk of affected offspring rises from 0% (mutation load <20%) to >75% (mutation load 61-80%) (White *et al.*, 1999).

The segregation of large single deletions is different and these deletions are in general de novo. Chinnery *et al.* collected data on 226 families in which a single mtDNA deletion was identified in the proband. Possible other mtDNA rearrangements like mtDNA duplications and deletion dimers, which may affect the transmission (Rotig *et al.*, 1992; Poulton *et al.*, 1993; Ballinger *et al.*, 1994; Shanske *et al.*, 2002), were not taken into account. The overall recurrence risk for disease caused by single mtDNA deletions was estimated at 4.11% (Chinnery *et al.*, 2004). Transmission of mtDNA deletions in the form of duplications has also been observed in mice strains containing a pathogenic 4696 bp deletion in the mtDNA. After introduction of the deletion, partially duplicated molecules were formed which were transmitted to offspring and caused deletion symptoms (Nakada *et al.*, 2001). MtDNA deletion disorders can also be caused by nuclear gene mutations and usually multiple deletions are observed which are transmitted in a Mendelian way (Kaukonen *et al.*, 2000; Spelbrink *et al.*, 2001; Van Goethem *et al.*, 2001).

Bottleneck location and size

In the 1980's a study on the segregation of mtDNA in Holstein cows revealed a rapid shift in the mtDNA genotype within two generational transitions (Hauswirth and Laipis, 1982). This shift has been confirmed several times in these cows (Ashley *et al.*, 1989; Koehler *et al.*, 1991), in other species like mice (heteroplasmic New Zealand Black/BINJ progeny (Meirelles and Smith, 1997) and in humans for the homopolymeric tract heteroplasmy located between nt 303 and 315 of the mtDNA (Lutz *et al.*, 2000). This has led to the identification of the 'mtDNA bottleneck' (figure 2) which is a restriction in the number of mtDNA molecules to be transmitted followed by an amplification of these founder molecules (Howell *et al.*, 1992). The exclusive maternal transmission of mtDNA, the high mutation rate and the

lack of a good repair mechanism and recombination would lead to decay of the mtDNA (Muller's ratchet (Muller, 1964; Hoekstra, 2000)). The stringent bottleneck has an evolutionary advantage as a sort of reset and acts to maintain a healthy mtDNA by filtering out mutations and minimizing heteroplasmy (Cummins, 1998; Cummins, 2001). Because this filtering happens very early during the development the chance to preserve age-related mutations in the early oocyte is small, although the low amount of mtDNA copies per mitochondria in the early developmental stages of the oocytes renders these oocytes vulnerable for mutational events (Keefe *et al.*, 1995).

Figure 2
Schematic drawing of the possible location and effect of the bottleneck on the transmission of a mtDNA mutation. The green colored mitochondria represent the normal mtDNA and the red colored mitochondria represent the mutated mtDNA.
PGC: Primordial germ cell

When the mitochondrial bottleneck exactly occurs during oocyte or embryo development and what the size is, is not yet clear (Poulton *et al.*, 1998). Early during the first developmental stages of oocytes the number of mitochondria and mtDNA molecules is reduced and the lowest number of mitochondria (<10) is found in the early primordial germ cells (PGCs) of a three weeks old embryo. The number of mitochondria is estimated from published electron micrographs of PGCs (Jansen and de Boer, 1998). It can not be excluded that in the embryonic germ cell line a week earlier an even lower number of mitochondria is present (Jansen, 2000). The mtDNA copy number is unknown in PGCs but in oocytes usually only one mtDNA molecule per mitochondrion is observed (Michaels *et al.*, 1982; Chen *et al.*, 1995). In mouse and frog there is no mtDNA synthesis during embryogenesis until the stage of gastrulation (Larsson *et al.*, 1998; Jansen, 2000) except for a small period of time during the 1- to 2-cell stage of mouse preimplantation development where there is some mtDNA turnover. The mtDNA content of the embryo does however not increase during this time (McConnell and Petrie, 2004; Thundathil *et al.*, 2005). In humans no mtDNA synthesis, measured by

BrdU incorporation, is observed until the late morulae and blastocyst stage (McConnell, personal communication). This suggests that in humans most mitochondria remain haploid during the first developmental stages (Jansen and de Boer, 1998). The mean number of mitochondria and mtDNA molecules increases from 10 in the PGC to about 200 in the oogonium and eventually to 100 000-600 000 in the mature oocyte (Jansen, 2000). Segregation of the mtDNA during embryogenesis has been studied in mouse models by Jenuth *et al.* in which BALB/c cytoplasm was introduced in NZB/BINJ oocytes. The mtDNA variants remain evenly distributed in the developing foetal tissues and no evidence is found for an additional bottleneck during embryogenesis (Jenuth *et al.*, 1996; Meirelles and Smith, 1997), although events during embryonal development still can influence the final heteroplasmy percentage (Meirelles *et al.*, 2001). From these studies it appears that the major component of the bottleneck occurs between the primordial germ cell and the primary oocyte stage.

The bottleneck has considerable implications for a carrier of mtDNA mutations and the mutation load can vary largely in both ways among her oocytes. The exact size of the bottleneck is hard to determine and may vary among individuals (Brown, 1997). Several studies have attempted to calculate the number of mtDNA units inherited through the bottleneck in cows, humans and mice (Howell *et al.*, 1992; Bendall *et al.*, 1996; Blok *et al.*, 1997; Jenuth *et al.*, 1997; Marchington *et al.*, 1998; Brown *et al.*, 2001). A repeated selection model, which attempts to take the number of cell divisions of oogenesis into account, and a single selection model which proposes the bottleneck as a one time sampling of mtDNA molecules from a large pool have been applied (Poulton *et al.*, 1998). The repeated selection model appears to represent the physiology more closely but assumes an identical sampling of mtDNA molecules every cell division, approximately 15, during oogenesis. The single selection model assumes that the bottleneck occurs only once, that replication is equal from all templates and that the levels of heteroplasmy relate to the proportions in oocytes (Bendall *et al.*, 1996). It has become clear that one common bottleneck size does not exist and that it will vary between meioses within and between different women. The bottleneck size using the single selection model is calculated to be 1-30 segregating units (one unit could represent one mtDNA molecule one nucleoid or one mitochondrion) in contrast to 20-200 units when using a repeated selection model (Bendall *et al.*, 1996; Poulton *et al.*, 1998).

Mouse models for OXPHOS disease

Animal models are essential for understanding the pathophysiological mechanisms of OXPHOS disease and for testing therapeutic interventions, but only few natural models exist (hearing loss in mice (Johnson *et al.*, 2001)). Over the last decade several mouse models have been developed for OXPHOS disease for both nuclear and mtDNA mutations. Only 2 mice models with mtDNA mutations exist, the CAP-resistant (CAP^R) mice with the m.2433T>C mutation in the 16SrRNA and the mtDNA deletion mice with a 4.696 bp deletion (Sligh *et al.*, 2000; Wallace, 2001). Disease symptoms were related to human OXPHOS disease, but for the mtDNA deletions most mice died of renal failure which is uncommon in human deletion

patients (Inoue *et al.*, 2000; Sligh *et al.*, 2000; Wallace, 2001). Both these transgenic animal lines demonstrated transmission of the mutated mtDNA to successive generations and can be used to study the inheritance and segregation of pathogenic mtDNA mutations. The CAP^R mice transmitted the heteroplasmic mtDNA mutation to some of their progeny in homoplasmic or heteroplasmic state. Progeny, born alive, exhibited growth retardation, myopathy and dilated cardiomyopathy. Most animals died either in utero or within the first day after birth, one animal survived 11 days (Sligh *et al.*, 2000). The mtDNA deletion mice transmitted the rearranged mtDNA through three successive generations with a tendency to increasing heteroplasmy percentage to a maximum of 90% in muscle of some animals, most likely due to the replication advantage of the smaller mtDNA molecule. A percentage above 90% has not been found and may be lethality in oocytes or embryos. Severe disease and COX negative fibres were only found in mouse with predominantly (>60%) deleted mtDNA (Inoue *et al.*, 2000).

Several mouse models showing an OXPHOS disease phenotype caused by nuclear mutations have been developed (Wallace, 2001; Zeviani, 2001; Biousse *et al.*, 2002). Mutations were introduced in genes associated with the OXPHOS system, like in protein complex genes, radical scavenger genes (Sod2 mutant mouse), transcription factors (Tfam-deficient mouse), adenine nucleotide translocator genes (Ant1-deficient mouse) (Li *et al.*, 1995; Lebovitz *et al.*, 1996; Graham *et al.*, 1997; Larsson *et al.*, 1998; Wang *et al.*, 1999). These mouse models show different OXPHOS related symptoms, but fertility is usually normal. This in contrast to the earlier mentioned mutator-mice with a proofreading deficient polymerase gamma. These mice show a premature onset of ageing and a reduced fertility, both males and females. Female reproductivity was nil after the age of 20 weeks and male fertility was severely reduced probably because of low sperm count and smaller testes size (Trifunovic *et al.*, 2004).

How to prevent transmission of mitochondrial disease

A definitive diagnosis of mitochondrial disease is needed for prognosis and genetic counselling of patients and their family (Thorburn and Dahl, 2001). As these disorders can not be cured, counselling is important to judge the recurrence risk of mitochondrial disease and the options to prevent the transmission of this disease. Refraining from children or adoption is the safest and most reliable method, but this is usually not the first choice. IVF enables prospective parents to opt for using donor oocytes. In some cases prenatal diagnosis (PND) or preimplantation genetic diagnosis (PGD) is possible, but other, more experimentally, methods are being developed as well (figure 3). The ethical aspects concerned with these techniques are discussed separately.

Figure 3

Scheme presenting the possible causes of OXPHOS disease and options for the prevention of transmission of these diseases.

PND: Prenatal diagnosis

PGD: Preimplantation genetic diagnosis

* Criteria developed by the European NeuroMuscular Consortium concerning prenatal options for carriers of mtDNA mutations

Prenatal diagnosis

Prenatal diagnosis (PND) of OXPHOS disease can be performed at the level of the enzyme or at the level of the DNA. Although the latter is preferable, the genetic defect is often not

known for patients with OXPHOS disease and the recurrence risk for these patients is hard to determine and based on family information only. If an enzyme deficiency is detectable in fibroblasts, then biochemical analysis of amniocytes might be an option, as fibroblasts, chorionic cells and amniocytes have the same embryonic origin (Graff *et al.*, 2002). Biochemical analysis of foetal samples is feasible although the methods used must be sufficiently sensitive given the low amount of foetal cells that can be obtained (table III). Another limitation is that only 50% of the patients express the enzymatic defect in fibroblasts and that knowledge on complex assembly and activity during embryonic development is lacking. OXPHOS diseases caused by nuclear gene mutations show a Mendelian mode of inheritance. For known DNA mutations, PND can be offered by direct mutation analysis of a chorionic villus sample and/or amniotic cells. If only the causing gene and location are known but not the exact mutation, intragenic or closely linked polymorphic markers are being used. DNA-diagnostics is more reliable than enzymatic analysis and should be used whenever possible.

PND for heteroplasmic point mutations in the mtDNA has its own complexity. Genotype-phenotype correlations are less straightforward and (time-dependent) differences may occur between the tested foetal tissue and the actual embryo. For point mutations in the mtDNA three criteria have been proposed to allow reliable PND (Poulton and Marchington, 2000; Poulton and Turnbull, 2000). (I) A close correlation between the mutant load and disease severity. (II) A uniform distribution of mutant mtDNA in all tissues. (III) No change in mutant load over time. Sufficient data are available for only 3 mutations (m.8993T>G/C, m.8344A>G, m.3243A>G) to judge these criteria properly. For the m.8993T>G/C and m.8344A>G mutation PND can be reliably performed, although for each of these a grey zone of inconclusive results exists. For example a mutant load of <20% for the m.8993T>G would predict healthy offspring, whereas a mutant load of 60% would give a 25% chance of disease (White *et al.* 1990). The number of data used to calculate these risks for the m.8993T>C mutation are so low that statistically even a mutant load of 0% does not preserve from a severe outcome. The amount of data required to reduce the confidence intervals of these percentages is for most mutations not available. For private mutations or mutations, which have only been reported a few times, PND should be carefully evaluated, based on genotype-phenotype correlations, available number of data and additional experiments (Jacobs *et al.*, 2005). Until now nine prenatal tests were reported, for the m.8993T>G and m.8993T>C, the m.3243A>G and the m.9176T>C mutation (table III). Also PND for mtDNA rearrangements is becoming an issue, as the recurrence risk for mtDNA deletion disorders appears to be around 5% (Chinnery *et al.*, 2004) and two PND have been performed (table III). MtDNA depletion syndromes are usually caused by nuclear gene defects, but if the causing mutation is not known PND remains a possibility. Amniocytes of children suffering from a mtDNA depletion disorder have been studied and were found to express the mtDNA depletion (Blake *et al.*, 1999). The exact timing of onset of mtDNA depletion during foetal development is still unknown. A second report describes two cases of mtDNA depletion presenting prenatally

with skin oedema and diminished foetal movements at 36 weeks pregnancy (Arnon *et al.*, 2002).

Preimplantation genetic diagnosis/ polar body analysis

Preimplantation genetic diagnosis (PGD) is an alternative to PND. Oocytes are fertilized in vitro and cells from the usually 8-cellular embryo are dissected and tested for the presence of a genetic defect. Unaffected embryos are transferred into the uterus (Handyside *et al.*, 1990). PGD avoids the dilemmatic choice of a pregnancy termination, which is an advantage compared to PND. PGD can be performed by sampling either polar bodies (Rechitsky *et al.*, 1999; Briggs *et al.*, 2000) or blastomeres (Holding and Monk, 1989; Handyside *et al.*, 1990). PGD is an option for mitochondrial disease due to nuclear gene defects, but it may also be a solution for mtDNA disease. The high copy number of mtDNA makes the analysis less prone to artefacts like amplification failure and allelic drop-out (Thorburn, 2004). PGD for mtDNA mutations is, however, not straightforward with respect to the interpretation of the data. In a heteroplasmic mouse model the distribution of both genotypes was identical between the ooplasm and polar body of a mature oocyte and between the blastomeres of 2-, 4-, and 6-8-cell embryos (Molnar and Shoubridge, 1999; Dean *et al.*, 2003). Both, polar bodies and blastomeres can be analysed, but the efficiency in diagnosing blastomeres is higher (Dean *et al.*, 2003). Analysis of polar bodies might be preferred by consenting couples with a strong reservation against embryo testing but the lower amount of mtDNA molecules in polar bodies may make the analysis susceptible to allelic drop-out and preferential amplification. The criteria for reliable PND also apply for PGD. PGD is especially suited for women with a high mutation load and a high risk of affected offspring (Poulton and Turnbull, 2000). Embryos transferred to the uterus should have a mutant load, which would guarantee a healthy outcome. For some women this could mean that they might need multiple PGD cycles before a suitable embryo can be identified. A disadvantage of PGD is the need of an in vitro fertilisation procedure as only 20%-25% of the IVF cycles results in a pregnancy (Broekmans and Klinkert, 2004). If the IVF/PGD procedure is unsuccessful the analysis of the embryos could still give valuable information for subsequent PGD cycles or other reproductive choices (Thorburn and Dahl, 2001).

Table III: PND of different OXPHOS diseases using biochemical or genetic methods

Disease	mutation	Method	result	reference
pyruvate carboxylase (PC) deficiency	unknown	direct measurement of PC activity in CVS	One elective termination and one healthy baby	(Van Coster <i>et al.</i> , 1998)
cytochrome <i>c</i> oxidase deficiency (Leigh syndrome)	unknown	Measurement of the substrate-stimulated ATP production and two-dimensional (Blue Native/SDS) polyacrylamide gel electrophoresis to analyse activity and composition of the OXPHOS complexes	An enzymatic defect was ruled out and two (twin) healthy children were born	(Houstek <i>et al.</i> , 1999)
NADH:ubiquinone oxidoreductase (complex I) deficiency	unknown	biochemical assays of complex I in fetal tissues (native and cultured CVS)	23 pregnancies analysed. 15 healthy babies born, 3 known to be affected children born and 5 provoked or spontaneous abortions	(Niers <i>et al.</i> , 2001)
carnitine palmitoyltransferase 2 (CPT2) deficiency	983A>G (D328G)	Direct measurement of CPT2 activity in CVS and molecular analysis of the mutation	One known to be affected baby born and one pregnancy termination	(Vekemans <i>et al.</i> , 2003)
Complex I defeiciency	E214K/IVS8+4A>C	Sequence analysis*	Elective termination of affected fetus	(Amiel <i>et al.</i> , 2001)
	NDUFV1 gene			
	A432P/ΔTC (989-990)			
	NDUFV1 gene			
	A524V/MIL			
	SDH- <i>Fp</i> gene			
NARP	P174L/ΔGA (363-364)	Direct mutation analysis using restriction enzyme digestion	Fetus was found heterozygous, pregnancy continued	(Harding <i>et al.</i> , 1992)
	SCO1 gene			
	G180E/IVS6-1G>C			
	SURF1 gene			
NARP	m.8993T>G		Fetus was found homozygous normal, pregnancy continued	(Bartley J. <i>et al.</i> , 1996)
	m.8993T>G		Two pregnancies analysed both revealed high heteroplasmy percentages (>80%) and were terminated	
Leigh syndrome	m.8993T>G	Direct mutation analysis using restriction	A near homoplasmic level was found in	(Ferlin <i>et al.</i> , 1997)

Disease	mutation	Method	result	reference
		enzyme digestion	fetus (both CVS and foetal cells) and the pregnancy was terminated.	
NARP and Leigh syndrome	m.8993T>G	Direct mutation analysis using restriction enzyme digestion	Two pregnancies analysed both did not contain the mtDNA mutation and were continued. Two healthy babies are born	(White <i>et al.</i> , 1999)
Leigh syndrome	m.8993T>C	Direct mutation analysis using restriction enzyme digestion	Both CVS and amniocytes were analysed and revealed no signs of the mutation. A healthy child was delivered at term	(Leshinsky-Silver <i>et al.</i> , 2003)
MELAS	m.3243A>G	Direct mutation analysis using restriction enzyme digestion	Amniotic fluid cells revealed a high mutation percentage comparable to that of the affected mother and sibling but at the age of four no signs of MELAS syndrome were detected	(Chou <i>et al.</i> , 2004)
Leigh syndrome	m.9176T>C	Direct mutation analysis using restriction enzyme digestion	CVS and amniotic fluid cells revealed a mutation percentage at risk. The pregnancy was however continued and an apparently healthy child was born	(Jacobs <i>et al.</i> , 2005)
PEO	5 kb mtDNA deletion (nt 9986- nt15042)	Southern blot analysis of the entire mtDNA	No Δ mtDNA was detected in CVS and an apparently healthy baby was born	(Graff <i>et al.</i> , 2000)
KSS	Δ mtDNA	Southern blot analysis of the entire mtDNA	No Δ mtDNA was detected in CVS and an apparently healthy baby was born	(Thorburn and Dahl, 2001)

* The nuclear gene defects are a small subset of what has been performed in clinical practice.

Donor oocytes

The use of donor oocytes with sperm of the partner is a reliable method to prevent the transmission of OXPHOS disease caused by mtDNA mutations. The use of donor oocytes of maternal relatives is not advisable since these may carry the same mtDNA mutations even though the mutation is undetectable in blood of the possible donor.

Cytoplasmic transfer

Cytoplasmic transfer (CT), an adaption from the ICSI technique ((Cohen *et al.*, 1997; Cohen *et al.*, 1998), has been tested on women experiencing repeated embryonic development failure, thought to be caused by depleted ATP levels in these oocytes (Van Blerkom *et al.*, 1995; Van Blerkom *et al.*, 2001). This resulted in 13 clinical pregnancies from which one spontaneous abortion and one selective abortion in a twin pair both because of Turner syndrome. With this technique 16 children have been born from which one developed pervasive development syndrome at the age of 18 months (Barritt *et al.*, 2001). This relatively high number of chromosomal abnormalities and birth defects when using CT may be caused by the disruption of the cytoskeleton with the introduction of the ooplasm. CT has been considered to dilute the mtDNA level to below the critical threshold for disease by the transfer of healthy mtDNA (Kagawa and Hayashi, 1997) and reports on the transmission of donor mtDNA in offspring after using CT exist (Brenner *et al.*, 2000; Barritt *et al.*, 2001). In these cases only small amounts (5%-15%) of donor cytoplasm were transferred to the recipient's oocyte. Donor mtDNA was demonstrated in about 50% of the embryos and placentas after CT. When analysing the mtDNA in one year old children born after CT, donor mtDNA is demonstrated in blood of only 2 out of 15 children (Brenner *et al.*, 2000; Barritt *et al.*, 2001). To prevent the transmission of mitochondrial disease a larger amount of donor cytoplasm needs to be used, up to 50% (Thorburn and Dahl, 2001) or purified mitochondria should be introduced. It still needs to be determined whether it is possible to introduce such a large amount of cytoplasm in the oocyte or to replace a large amount of the oocyte's cytoplasm. These questions make CT for the moment an inappropriate method to prevent the transmission of mitochondrial disease.

Nuclear transfer

The nuclear transfer (NT) technique involves the fusion of the nucleus of a somatic donor cell with an enucleated recipient oocyte (cloning), which is subsequently activated electrically, or biochemically. NT is being tested in animal models. Offspring is often liable to serious defects (Cibelli *et al.*, 2002) like for example placental oedema, respiratory problems and kidney/brain/liver malformations. In case of mtDNA mutations two strategies could be used. First, the nucleus of the mother's unfertilised oocyte is transferred into an enucleated donor oocyte which subsequently is fertilised using the partner's sperm (Roberts, 1999). Second the nucleus of a blastomere cell is transferred in the enucleated donor oocyte. With both methods a small amount of cytoplasm and mitochondria is transferred with the nucleus, which means that some mutant mtDNA may be present in the embryo. These perinuclear

mitochondria might have a replicative advantage over mitochondria further from the nucleus (Shadel and Clayton, 1997), although recent studies in mouse oocytes have demonstrated that these karyoplast mitochondria are homogeneously distributed throughout the entire cytoplasm before the oocyte has completed its maturation (Fulka, 2004).

One of the major setbacks with NT is the need for donor oocytes. Also, there are several questions concerning the technique, the compatibility between recipient oocyte and donor nucleus, the reaction of the nucleus and recipient oocyte as a consequence of the transfer, the disruption of the cytoskeleton because of the transfer and the defects seen in animal offspring after using this technique. Another possible problem is associated with possible imprinting problems due to the transfer of an embryo nucleus. This has been proven in animal models after somatic cell nuclear transfer (Jaenisch, 2004) and could affect the embryo cell nuclear transfer although these effects are probably minor since the major part of the imprinting takes place later during embryonic development.

Ethical considerations

Most of the methods to prevent the transmission of OXPHOS disease are relatively new or in a developmental phase and have considerable ethical implications. PND and PGD are controversial in itself, mainly because of the inherent selection of foetuses/embryos. Ethical questions imply the moral status of the embryo and the so called 'disability rights'. There is a strong consensus that selective abortion and selective transfer can be morally justified to prevent the birth of seriously affected children. This might be at odds with the interests of handicapped people in our society but the use of PND and PGD does not deny the dignity and equal rights of handicapped citizens nor necessarily undermines societal support for handicapped people and their families (Buchanan, 2000). Apart from these general ethical questions, some (more or less) specific moral issues may arise in the context of PND/PGD to prevent the transmission of OXPHOS disorders. A distinction exists between the optimal situation, when the disease is caused by a known nuclear gene mutation and the test result is reliable, and the suboptimal situation, when the causing mutation is unknown or located in the mtDNA and the test result can be somewhat unreliable. The risks of false-negative, false positive and inconclusive results need to be analysed and it should be determined whether or not the prenatal test can be performed accurately. From an ethical (and legal) perspective, it is crucial that prospective parents are adequately informed and counselled on the uncertainties and limitations of the various tests before the analysis is performed, in order to enable them to make a well-considered decision.

Most uncertainties are associated with PND and PGD for mtDNA mutations. If the criteria proposed to allow reliable PND are fulfilled, PND and/or PGD of the mtDNA mutation should be possible. For the so-called private mutations (representing in only one family) and mutations which have only been reported a few times, PND and PGD should be carefully evaluated. It is important to determine a safe margin for the mutation load and to discuss this margin and the consequence of an inconclusive mutation percentage or a mutation percentage above the determined threshold for disease with the parents. A question for further

debate is as to whether the criteria to evaluate the possibility for PND or PGD for mtDNA (Poulton and Turnbull, 2000) mutations are flexible guidelines or strict rules. If a well-informed couple, with a private or less suitable mutation, after adequate counselling decides to opt for PND or PGD to lower the risk of conceiving a severely affected child, knowing that the technique cannot give an absolute guarantee that the child will be healthy, should this be considered an option? Who determines which mutations are suitable? The possible use of PGD raises some additional issues (de Wert, 2002). There may arise a dilemma between the required mutant load and embryo quality (in terms of viability). Can the transfer of embryos which are more viable, but at higher genetic risk be morally justified? Who makes the final decision in case of conflicts: the reproductive physician or the prospective parents, especially the woman?

Obviously, prospective parents may consider the use of donor oocytes as an alternative option. The ethical concerns associated with the use of donor oocytes in the current context are the same as for the use of donor oocytes for other medical indications (Cohen and National Advisory Board on Ethics in Reproduction, 1996). The donation of oocytes is prohibited in some countries, for instance to prevent misuse of oocytes for women having reached the menopausal age, to protect the child from possible negative influences because of its conception by donor oocytes and because of possible large-scale commercialization of donor oocytes (Robertson, 2004). Relevant ethical concerns are especially related to the welfare of the donor and the offspring. The reproductive physician might be inclined to primarily focus on what is best for the recipient and to overlook the interests and needs of the donor (Kalfoglou and Geller, 2000; Kalfoglou, 2001). Guidelines should protect the autonomy, privacy and health of candidate egg donors. The major controversy with regard to the offspring conceived by use of donor gametes concerns their 'right to know', including both their right to be informed about the way they were conceived and the right to know to whom they are genetically related (ASRM., 2004). A growing number of countries acknowledge the moral and legal right of these children 'to know their origins'. A major practical problem regards the shortage of egg donors. The pros and cons of financial compensation for the donor need further debate (Steinbock, 2004).

Oocyte donation might also be used as a vehicle for other future reproductive options for carriers of mtDNA mutations and unknown OXPHOS disease causing mutations, namely CT and NT. These methods raise additional conceptual and ethical issues. A first conceptual issue, concerning both procedures, is whether or not they constitute a germ-line intervention. The answer probably should be affirmative, insofar as the germ-line of resulting children is modified, as shown for heteroplasmic mouse lines (Meirelles and Smith, 1997). Germ-line interventions are often considered as unjustified and are prohibited. But why should such interventions be categorically wrong if the purpose is therapeutic – and the procedure would be safe? In view of the therapeutic character of these techniques, one could well argue that these protect instead of damage the interests of the offspring (Robertson, 1999). With CT and NT, the genetic change only involves the mitochondrial DNA. Would it, therefore, be justified

to ethically differentiate this therapy from possible therapeutic germline interventions involving (the insertion or modification of) nuclear genes?

A second conceptual issue, relevant for NT only, is whether this procedure amounts to human cloning. It is important to discern the various possible sources of the nucleus to be transferred (de Wert, 2002). If the nucleus of an unfertilised egg is transferred, the procedure would definitely not involve cloning. If however the nucleus or nuclei of one or more blastomeres are transferred to an enucleated egg, however, this would involve embryo cloning – even if one would create only one additional embryo. The fact that the newly created embryo would have its own mitochondrial DNA would not make things different, as clones are usually defined as organisms having the same nuclear DNA. The transfer of identical embryos might result in the birth of genetically identical children – and might, therefore, constitute human reproductive embryo cloning. NT using a human embryonic source can be considered unjustified because the rest of the embryo would be destroyed. In fact, however, all available blastomeres of the embryo could be used, thereby avoiding any embryo loss. The question, then, becomes what is to be preferred from an ethical point of view: the loss of (the rest) of an embryo or the avoidance of reproductive embryo cloning?

Another ethical issue (regarding both CT and NT) concerns the splitting of the female genetic contribution to offspring into two parts, namely the (major) nuclear genetic source and the (minor) mtDNA source (Robertson, 1999). As a result, the child would have two genetic mothers which can be considered unnatural. This could have a socially, psychologically and/or legal effect especially if a major part or all of the oocytes mtDNA is replaced by donor mtDNA. Can we safely assume that the impact will be minimal, as the donor of the mtDNA has only a minimal genetic role, while the major genetic part, the nucleus, is provided by the woman who will be both the gestational and rearing mother? And last, but not least: how to weigh the health risks of these experimental techniques for future children. As a general rule, experimental reproductive technologies should only be introduced in the clinic after adequate preclinical safety studies have been performed. Clearly, the criteria to be used for defining adequate preclinical studies need further debate. No doubt, however, the clinical application of both CT and NT in order to prevent the transmission of OXPHOS disease is, for the time being, premature and, therefore, unjustified. Human embryo research may contribute to the clarification of (some of the) possible risks of these technologies. The ethics of this research is beyond the scope of this article.

In view of the severe impact of mitochondrial disorders, research into the development of new preventive strategies is important. At the same time, this review illustrates that the possible strategies to prevent the transmission of mitochondrial disorders raise lots of ethical issues, general and specific, conceptual and normative, and at the level of both clinical ethics and social ethics. Further proactive ethical analysis and interdisciplinary debate should contribute to the development of adequate guidance.

In conclusion, transmission of OXPHOS diseases is complex, due to the different and often unknown genetic causes. Reliable prenatal or preimplantation genetic diagnosis is largely limited to the group of patients with characterized mutations in nuclear genes, although certain mtDNA mutations are suitable as well. These methods are therefore neither sufficient nor satisfactory for the majority of mtDNA mutations carriers and new approaches are being developed. Mouse models for mtDNA mutation will be very helpful to study the mechanism of the transmission and segregation of mtDNA mutations, and the possibility of paternal mtDNA transmission. These and other animal models can be used to test for safety and consistency of new methods. When safe and ethically acceptable these will provide carriers the choice to prevent the transmission of OXPHOS disease to their children and will reduce the number of people affected by it.

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Chapter 3

Pearson syndrome and the role of deletion dimers and duplications in the mtDNA

L.J.A.M. Jacobs¹, R.J.E. Jongbloed¹, F.A. Wijburg², J.B.C. de Klerk³,
J.P.M. Geraedts¹, J.G. Nijland¹, H.R. Scholte⁴, I.F.M. de Co⁵, H.J.M. Smeets¹.

¹ *Department of Genetics and Cell Biology, University of Maastricht, Research Institute Growth and Development (GROW), Maastricht.* ² *Department of Paediatrics, Academic Medical Centre Amsterdam, Amsterdam.* ³ *Department of Paediatrics,* ⁴ *Biochemistry and* ⁵ *Neurology, Erasmus MC-University Medical Center Rotterdam*

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Abstract

Pearson syndrome is an often fatal multisystem disease associated with mitochondrial DNA rearrangements. Here we report a patient with a novel mtDNA deletion of 3.4 kb ranging from nucleotides 6097 to 9541 in combination with deletion dimers. The mutation percentage in different tissues (blood, muscle and liver) varied between 64% and 95%. After a remission period of about a year the patient suddenly died at the age of three years due to a severe lactic acidosis. A second patient with a previously reported deletion of 8 kb and a milder phenotype was found to have mitochondrial duplications and died at the age of ten years. From these data and data from previous reports we hypothesised that duplications might be beneficial in the clinical course of the disease and in life expectancy.

Introduction

Pearson syndrome (McKusick 557000) is a multisystem disease including refractory anaemia, vacuolisation of marrow precursors and pancreatic fibrosis (Pearson H.A., 1979). The disease starts during infancy and affects various tissues and organs. Most children diagnosed with Pearson syndrome die before the age of three years. It has been reported that children who survive eventually develop Kearns-Sayre syndrome (KSS) (Rotig *et al.*, 1995) (McKusick 530000) a progressive multisystem disease characterised by ophthalmoplegia, retinal pigmentary degeneration and at least one of the following symptoms: elevated cerebrospinal fluid protein, heart block sensorineural hearing loss, ataxia, small stature, ptosis, lactic acidosis and endocrine dysfunction (Kearns T.P., 1958; DiMauro, 1993). Age at onset of KSS usually occurs before the age of 20 years.

Pearson syndrome is caused by mtDNA deletions (Rotig *et al.*, 1990) and the most common deletion is a 4977 bp deletion located between nt 8488 and nt 13460. This deletion has been identified in more than 80% of the affected children. Since the first report in 1990 (Rotig *et al.*, 1990), many other deletions have been detected (MITOMAP, 2001). Here we describe a novel small mtDNA deletion in a young patient with Pearson syndrome. The phenotype and genotype of this patient have been compared to a previously described patient with Pearson syndrome (van den Ouweland *et al.*, 2000), who carried an 8 kb deletion. The presence and phenotypic affect of deletion dimers and duplications was investigated.

Patients

Patient 1: Patient 1, a girl, was born after a normal pregnancy to healthy unrelated parents who had no family history of mitochondrial myopathy and 2 healthy siblings. At 16 months of age the first symptoms became manifest and the girl was examined for a slight growth retardation and pancytopenia of unknown origin with anaemia. At 22 months of age, after a viral infection, the patient was hospitalised because of severe tachypnoea, lethargy and dyspnoea accompanied by fever. Biochemical analysis revealed a metabolic acidosis with a high lactate value in blood of 8.2 mM (normal 0.6-2.0 mM). These symptoms indicated Pearson syndrome, which was confirmed by DNA investigation. Biochemical tests of muscle revealed a respiratory chain defect. After one month the situation of the child improved

spontaneously and further hospitalisation was not necessary. One year later the condition of the child deteriorated rapidly due to an infection and she died at the age of 39 months of severe metabolic failure with a very high blood lactate level of 28 mM.

Patient 2: Patient 2 has been described previously (van den Ouweland et al., 2000). This patient carried an 8034 bp mtDNA deletion spanning from position 7934 to 15968 flanked by an imperfect 11 bp repeat. She showed a failure to thrive as the first sign of disease in her first year of life. At the age of three years this was followed by other signs of Pearson syndrome with anaemia, a severe Fanconi-like tubulopathy, lactic acidosis and diabetes mellitus type II, but no signs of exocrine pancreas dysfunction. After a period of about a year in which she seemed to recover, she developed a full blown Kearns-Sayre syndrome with ataxia, retinitis pigmentosa, external ophthalmoplegia, sensorineural deafness and growth hormone deficiency. The deletion was present in different percentages of mutated mtDNA in different tissues. The deletion could not be detected in the DNA from the patient's mother and the younger brother. Biochemical analysis of muscle and liver at age 2 y, 11 m and 4 y, 8 m indicated deficiency of complex IV (on wet weight basis: muscle 23 and 43% of controls, liver 21 and 12%), with highly increased citrate synthetase in muscle (204 and 305% of controls). The activity of complex I was 50 and 78% in muscle and 65% in liver, and that of complex II+III 23 and 43% and 21 and 12%. The oxidative phosphorylation in isolated muscle mitochondria was normal. The oxidation of pyruvate plus malate was normal on protein basis but decreased on basis of citrate synthetase to 41 and 51% of the average controls. She gradually developed more symptoms including renal disease until she died at the age of 10 years.

Methods

Long range PCR: Total DNA was extracted from peripheral blood, muscle and liver samples as described previously (Miller et al., 1988). A 16.2 kb amplified mtDNA fragment was generated by using the Expand Long PCR kit (Roche, Basel, Switzerland). Amplification was performed in a total volume of 50 µl, using dNTP's (Amersham Pharmacia Biotech AB, Uppsala, Sweden, 25 mM each), 5 µl Buffer 2 (provided with the Expand-Long PCR kit), 1.75 U DNA polymerase and 100 ng of each primers (forward primer (F): nt 15149-15175: 5'-TGAGGCCAAATATCATTCTGAGGGGC-3' and reverse primer (R): nt 14816-14790: 5'-TTTCATCATGCGGAGATGTTGGATGG-3'). The PCR reactions were carried out in a 9600 thermocycler (Perkin-Elmer) using the following conditions: 94°C 1 min; 14 cycles of 98°C for 10 s and 68°C for 15 min; 16 cycles of 98°C for 10 s and 68°C for 15 min plus 15 s per elongation step; and a final 10 min elongation at 72°C.

Short range PCR: Short range amplification has been performed in a total volume of 25 µl, using dNTPs (Amersham Pharmacia Biotech AB, Uppsala, Sweden, 4 mM each), 15 mM MgCl₂, 0.5 M KCl, 0.1 M Tris-HCl, 1 U Taq DNA polymerase (Invitrogen, Life Technologies, Breda, The Netherlands) and 100 ng of each primer. The PCR reactions were

carried out in a 9700 thermocycler (Perkin-Elmer) using the following conditions: 94°C for 3 min; 32 cycles of 92°C for 1 min, annealing temperature for 1 min and 72°C for 2 min; and a final 7 min elongation at 72°C

Sequence analysis: Sequencing reactions were performed in a total volume of 20 µl using 2 µl Big Dye termination solution according to the protocols of the manufacturer (Applied Biosystems, Foster City, USA) and 50 ng forward or reverse primer. Results were obtained on the ABI Prism 377 DNA automatic sequencer (Applied Biosystems, Foster City, USA) and analysed by using ABI software version 3.1.

Southern blot analysis: Total genomic DNA (5 µg) and PCR amplified mtDNA products were digested with different restriction endonucleases (Boehringer-Mannheim, Bayern, Germany). Subsequently DNA fragments were separated by electrophoresis on 0.9% agarose gels (MethaPhor; Biozym, Landgraaf, The Netherlands), transferred onto Hybond N⁺ membrane by Southern blotting (Sambrook, 1998) and hybridised with a, random primed

(Invitrogen, Life technologies, Breda, The Netherlands) ^{32}P dCTP-labelled amplified mtDNA fragment containing either the origin domain (forward primer Of 1330-1355 and reverse primer Or 778-753) or fragments located in the deleted region of the patients. Hybridisation and washing conditions are performed according to the standard conditions (Sambrook, 1998). Hybridisation signals were visualised on an X-ray film (Kodak Nederland B.V. Service Center Amsterdam, The Netherlands) by autoradiography and quantified using the Intelligent Quantifier software (B&L systems, Maarssen, The Netherlands).

Results

The amplified mtDNA fragment of patient 1 revealed a deletion of about 3.4 kb (figure 1). The fragment containing the deletion junction was sequenced and the deletion turned out to range from position 6097 to 9541. The deletion included several structural (COI partial, COII, ATPase8, ATPase6 and COIII partial) and tRNA (tRNA^{scr} , tRNA^{asp} and tRNA^{lys}) genes. At the junction site a short direct repeat sequence "AAT" was detected. Southern blot analysis of the PvuII digested DNA sample of patient 1 revealed a normal and a discrete 3.4 kb smaller mtDNA fragment (figure 2). The percentages of the deleted mtDNA were determined by measuring X-ray signal intensities of the normal mtDNA fragment and the deleted fragment from several tissues. The signal intensity of the deleted fragment was divided by the sum of the intensities of both the deleted and normal fragment, giving a molecular ratio. The proportion of deleted to total mtDNA was 74% in blood, 63% in muscle and 95% in liver. The deletion was undetectable in blood cells of the patient's mother analysed by Southern blot (results not shown).

It has been hypothesised (Poulton and Holt, 1994; Poulton *et al.*, 1994) that the presence of deletion dimers (a combination of two deleted fragments) and/or duplications (a combination of a normal and a deleted fragment) may affect the progression of the disease. Therefore, we investigated this in our patients. Deletion dimers and duplications were identified by cutting

the mtDNA with enzymes, which recognise restriction sites inside, or outside the deleted fragment and hybridising them with mtDNA probes inside or outside the deleted fragment. Digestions have been performed with PvuII, AspI or BamHI (Figure 1 and 3). PvuII cuts outside the deleted fragment of both patients, while the AspI and the BamHI enzyme cut inside the mitochondrial deletion of the first and second patient respectively. In the presence of deletion dimers or duplications, larger fragments than the wild-type mtDNA will appear on the Southern blot results, when using the origin probe and a restriction enzyme that cuts inside the deletion. These fragments include circular and nicked circular monomeric deletion fragments, dimeric fragments and circular dimeric fragments. However by using a probe located inside the deletion fragment a larger fragment will only be seen in case of a duplication. If a deletion dimer is present when using the deletion probe, only the normal mtDNA fragment will be visible on the southern blot.

Figure 3

Deletion dimers and duplications of normal and deleted mtDNA in two patients with Pearson Syndrome.

A: Patient 1 with a mtDNA deletion and a deletion dimer.

The mtDNA is digested with PvuII (lane 1) and AspI (lanes 2 and 3) and hybridised with the origin probe (lane 1 and 2) or a deletion-specific probe (lane 3). The normal mtDNA band of

16.5 kb is seen in all lanes, together with a 13.1 kb deletion fragment in lanes 1 and 2 (3.4 kb deletion) and a larger fragment of about 26 kb in lane 2 (indicated by an arrow) which is a deletion dimer. B: Patient 2 with a mtDNA deletion and a duplication

The mtDNA is digested with PvuII (lane 1) and BamHI (lanes 2 and 3) and hybridised with the origin probe (lane 1 and 2) or a deletion-specific probe. The normal fragment of 16.5 kb is seen in all lanes, together with an 8.5 kb deletion fragment in lane 1 (8 kb deletion) a circular deletion monomer and a duplication (arrow) in lane 2 and 3.

Fragment length is determined by using a λ -HindIII marker (sizes are indicated), restriction sites are as indicated in figure 1.

The first patient has a larger fragment after digestion using *AspI* as can be seen in lane 2 of figure 3A. This fragment could be a circular version of the deletion monomer but given the size and location of the monomeric deletion fragment, which is identical in lane 1 and 2, and of the larger fragment it is more likely to be a deletion dimer. The deletion is linearised by breaks in the DNA (results not shown) raised during DNA extraction or because of the age of the DNA. The deletion dimer is seen in all tissues tested but there are no signs of duplications (figure 3A lane 3). In the blood of the second patient a deletion fragment of 8.5 kb can be seen in lane 1 of figure 3B. In lane 2 after digestion using *BamHI* a slightly larger fragment is visible of about 11 kb, this is a circular version of the deletion fragment. A larger fragment of about 25 kb is visible in lanes 2 and 3. Because this fragment is also visible using a deletion probe we can conclude that it is a duplication.

Discussion

We report a novel mtDNA deletion in a child whom initially improved after being severely ill from Pearson syndrome but died a few months later. A short 3 bp repeat sequence (AAT) flanks the deletion. The vast majority of deletions reported are flanked by short repeat sequences as well, ranging from 3-14 bp in length (Tanaka *et al.*, 1989; Mita *et al.*, 1990; Ota *et al.*, 1994). It has been hypothesised that these deletions are the result of loop formations between these repeat sequences during mtDNA replication in the developing oocyte. No minimal area of overlap is found between the different deletions reported, either causing Pearson syndrome or any of the other deletion syndromes, so there is no minimal region in the mtDNA that has to be deleted to cause symptoms. It is however known that at least one tRNA gene has to be removed to cause one of the common deletion syndromes (KSS, CPEO or Pearson syndrome) (Tang Y. *et al.*, 2000). The 3.4 kb deletion in patient 1 was detected in all tissue samples tested including blood, muscle and liver. It belongs to the category of small deletions, although the clinical phenotype is severe and eventually fatal. Compared to other previously reported deletions located within the same area, no correlation can be found between the location and size of the deletion and the severity of the disease (Rotig *et al.*, 1995). In comparison with our patient 1 who has a 78% amount of a 3.4 kb deletion (position 6097-9541), Rötig *et al.* (1995) described a 5 year old Pearson patient having 80% of a 3.1 kb deletion (nt 6074-9179), this deletion is comparable to the one found in our patient (78% of a 3.4 kb deletion at position 6097-9541). But there patient suffered from primarily diarrhoea and anaemia and had no signs of pancreatic or hepatic involvement.

Patient 2 carried a deletion of 8 kb spanning from position 7934 to 15968 (van den Ouweland *et al.*, 2000). The differences between patient 1 and 2 involve the sizes of the deletions, the presence of dimers/duplications, the age at onset and the life expectancy. The deletion identified in the second patient was larger than the deletion in patient 1 and encompasses more genes (partial COII, ATPases 6 and 8, COIII, ND3, ND4L, ND4, ND5, ND6, cytb and 8 tRNAs (Lys, Gly, Arg, His, Ser, Leu, Glu, Thr and partial Pro)). However, the deletion percentages were comparable in 3 tissues tested in both patients. Despite the larger deletion,

patient 2 presents a less severe phenotype and died later than patient 1. This has been documented before in several deletion syndromes. Rotig *et al.* (1995) detected a 85% 6.7 kb deletion in blood of a 3 year old child with less severe symptoms and a better life expectancy than a child that died at the age of 13 months with more severe symptoms and a 55% 4.2 kb deletion in lymphoblastoid cells. This indicates that the size and percentage of the deletion are no good predictors of the clinical course and severity of the disease.

An alternative explanation for differences in life expectancy and phenotype could be the presence of deletion dimers or duplications in the mtDNA (Poulton and Holt, 1994; Rotig *et al.*, 1995). Both our patients displayed other mtDNA rearrangements in addition to the deletion in the form of dimerisations or duplications. The significance of these deletion-dimers and duplications is still unclear. Several investigations point towards little or no pathogenic significance of mtDNA duplications (Rotig *et al.*, 1995; Muraki *et al.*, 2001), although others find that mtDNA duplications might influence the severity of the deletion disease in a positive way (Poulton and Holt, 1994). MtDNA duplications may also be involved in the progression from Pearson syndrome to KSS, as duplications are a distinctive feature of KSS. Children with Pearson syndrome who progress to KSS, harbour duplications which could not be detected in children with only Pearson syndrome (Poulton and Holt, 1994; Poulton *et al.*, 1994; Poulton *et al.*, 1995). Increasing deletion dimers appear to be correlated with worsening of the disease symptoms (Poulton and Holt, 1994). Rötig *et al.* (1995) on the other hand found that most patients who develop KSS do not harbour duplications or deletion dimers and that there is no correlation between the rearrangements and the clinical course of the disease. As duplications may be missed by the method used it should be worthwhile to test these patients with a duplication specific PCR method (Muraki *et al.*, 2001).

Another aspect reported regularly for mitochondrial duplications but only once for deletions is that duplications can be maternally inherited (Rotig *et al.*, 1992; Dunbar *et al.*, 1993; Poulton *et al.*, 1993; Ballinger *et al.*, 1994; Manfredi G. *et al.*, 1997; Shanske *et al.*, 2002), suggesting that the recurrence risk for the mother of patient 2 may be increased. It is thought that deletions in oocytes could impair the fertilisation of the oocyte (Hsieh *et al.*, 2002) but this interference is neither known nor tested for duplications. This could be investigated by testing the mtDNA for deletions and duplications in oocytes of women with idiopathic infertility.

Here we describe two patients with Pearson like syndromes with a different clinical course. We have been looking for a relation between phenotypic differences and the presence of dimerisations and duplications. Our results confirm the earlier observations of Poulton (Poulton and Holt, 1994; Poulton *et al.*, 1995) that duplications, in contrast to dimerisations, may lead to a better life expectancy and a less severe phenotype with KSS-like symptoms.

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Chapter 4

Transmission and prenatal diagnosis of the T9176C mitochondrial DNA mutation

L.J.A.M. Jacobs¹, I.F.M. de Co², J.G. Nijland¹, R.J.H. Galjaard³, F.J. Los³, K. Schoonderwoerd⁴, M.F. Niermeijer⁵, J.P.M. Geraedts¹, H.R. Scholte⁴, H.J.M. Smeets¹.

¹Department of Genetics and Cell Biology, Research Institute GROW, University Maastricht.

Department of ²Child Neurology, ³Clinical Genetics and ⁴Biochemistry, Erasmus MC-University Medical Center Rotterdam, ⁵Department of Human Genetics, University Medical

Center Nijmegen, St Radboud, Nijmegen.

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Abstract

A family presented with 3 affected children with Leigh syndrome, a progressive neurodegenerative disorder. Analysis of the OXPHOS complexes in muscle of two of the affected patients showed an increase in activity of pyruvate dehydrogenase and a decrease of complex V activity. Mutation analysis revealed the T9176C mutation in the mt ATPase 6 gene (OMIM 516060) and the mutation load was above 90% in the patients. Unaffected maternal relatives were tested for carrier-ship and one of these with a mutation load of 55% in blood was pregnant of her first child. The possibility of prenatal diagnosis was evaluated. The main problem was the lack of data on genotype-phenotype correlations for the T9176C mutation and on variation of the mutation percentage in tissues and in time. Therefore multiple tissues of affected and unaffected carriers were analyzed. Eventually prenatal diagnosis was offered with understanding by the couple that there could be considerable uncertainty in the interpretation of the results. Prenatal diagnosis was carried out twice on cultured and uncultured chorion villi and amniotic fluid cells. The result was a mutation percentage just below the assumed threshold of expression (90%). The couple decided to continue the pregnancy and an apparently healthy child was born with an as yet unclear prognosis. This is the first prenatal diagnosis for a carrier of the T9176C mutation. Prenatal diagnosis for this mutation is technically reliable but the prognostic predictions are not straightforward. Key words: Leigh syndrome, mtDNA, prenatal diagnosis, preimplantation genetic diagnosis

Introduction

Leigh syndrome (OMIM 256000) or subacute necrotizing encephalomyopathy is a progressive neurodegenerative disorder with a poor prognosis and most of the patients die within a few years after age at onset (Rahman *et al.*, 1996). Symptoms, occurring in early infancy or childhood, are psychomotor developmental regression, optic atrophy, ophthalmoparesis, nystagmus, ataxia, dystonia, failure to thrive and respiratory abnormalities. Characteristics are bilateral necrotic lesions on MRI in basal ganglia and brainstem and lactic acidosis. Leigh syndrome is caused by functional or molecular defects in the enzyme complexes involved in the mitochondrial energy production, including pyruvate dehydrogenase and OXPHOS complexes I, II, IV and V (DiMauro and Tanji, 1997; Dahl, 1998; Tanji *et al.*, 2001). The inheritance of Leigh syndrome is either autosomal recessive, autosomal dominant, X-linked or maternal, and defects in nuclear genes or mitochondrial DNA (mtDNA) can cause this disease.

In this paper, we describe a family with Leigh disease caused by a mutation in the ATPase 6 gene (OMIM 516060) at position 9176 of the mtDNA (Thyagarajan *et al.*, 1993; Campos *et al.*, 1997; Dionisi-Vici *et al.*, 1998; Makino *et al.*, 1998; Makino *et al.*, 2000; Wilson *et al.*, 2000; Akagi *et al.*, 2002). The severe clinical manifestations of this disease occur at mutation percentages above 90% (Thyagarajan *et al.*, 1993; Campos *et al.*, 1997; Dionisi-Vici *et al.*, 1998; Makino *et al.*, 1998; Makino *et al.*, 2000; Wilson *et al.*, 2000; Akagi *et al.*, 2002). The mutation is maternally transmitted, but the percentage of this mutation may

vary widely between oocytes, making the transmission of the associated Leigh disease fairly unpredictable (Hauswirth and Laipis, 1982; Howell *et al.*, 2000). Mitochondria go through a genetic bottleneck in oogenesis or early embryogenesis, which involves both a reduction in number of mitochondria and of mtDNA molecules to only a very few, that subsequently repopulate the oocyte. For point mutations in the mtDNA three criteria have been proposed to allow reliable prenatal diagnosis (Poulton and Marchington, 2000; Poulton and Turnbull, 2000).

- (I) A close correlation between the proportion of mutant: wild-type mtDNA (mutation load) and disease severity.
- (II) A uniform distribution of mutant mtDNA in all tissues.
- (III) No change in mutant load over time.

For most mtDNA mutations this information is lacking. Only a few mutations meet these criteria like the T8993G/C (associated with another form of Leigh syndrome) and A8344G (associated with MERRF syndrome i.e. myoclonic epilepsy and ragged red fibers) mutation, but not the common A3243G (associated with the MELAS syndrome, i.e. mitochondrial encephalopathy with lactic acidosis and stroke like episodes). Here we describe the segregation of the T9176C mutation in a family with Leigh syndrome and the development and implementation of a prenatal test for this mutation.

Patients and Methods

Patients

Case reports from a family with maternal Leigh syndrome.

The index patient (III-2, fig 1) first presented at the age of 21 months, with a sub-acute onset of cerebellar ataxia and speech retardation. The pregnancy was complicated by toxicosis but delivery and development during the first year were unremarkable. She walked at 12 months. At the age of 3 years, after a period with fever, she had an attack with loss of tone of her right arm and leg that lasted for three days. Clinical examination showed a further increased cerebellar ataxia with signs of dystonic posturing of the right arm and leg. She was a happy child and was apparently not impeded by the handicap. She spoke a few words only but was able to express herself with gestures. At 4 years and 9 months of age she was admitted in the hospital because of the suspicion of a metabolic derangement, after four weeks of stomachache, dyspnoea, and increasing fatigue and weakness. A sick girl was seen with irregular breathing and deep set eyes. Apart from the cerebellar ataxia there were pyramidal tract signs with very easily elicited reflexes and extensor toe signs on both feet. Magnetic resonance imaging and computerized tomography of the brain showed extensive vaguely demarcated hypodensities in the cerebellar hemispheres, pons and mesencephalon. Supratentorially there were hypodense lesions in the caudate nuclei, the internal capsule and the basal ganglia. Peripheral and central CSF spaces were enlarged. Lesions could be compatible with infarctions in parts of the basilar and the internal carotid artery region. The clinical phenotype combined with lactic acidosis and CSF involvement made a mitochondrial

encephalopathy or Leigh-like syndrome likely. The child's condition worsened and in the following two weeks time she developed severe apnoeic and hypoventilation spells and became ventilator dependent. She died from progressive brainstem dysfunction. She underwent a muscle biopsy a few hours before death but there has been no post mortem examination.

Figure 1

Segregation of the mtDNA T9176C mutation in a large Dutch family with Leigh syndrome.

The percentage of the mutation is indicated below each individual. Solid symbols indicate affected and open symbols unaffected individuals. B: Blood, M: Muscle, F: Fibroblasts, H: Hair roots, Gb: Gall bladder, SI: Small intestine, CV: Uncultured Chorion villi, L: Liver, AC: Uncultured Amniotic fluid cells.

The younger brother (III-3) and sister (III-4) of the index patient (III-2) developed a cerebellar ataxia with an onset at age 2 years. Their language development was slightly delayed. Both received, from age 4 and 2 respectively, daily vitamin B complex containing 100 mg vit B1, 2, 3, 5, 6, 50 mg vitamin E and 3 dd 330 mg carnitine. From the age of 3, after viral illnesses, attacks of one to several days occurred with loss of tone resulting in paraparesis losing ambulation, sighing, dyspnoea and dystonic movements. At the age of 4 the diagnosis mitochondrial encephalomyopathy of the boy was confirmed by determination of the T9176C mutation in DNA from muscle and blood (van Den Bosch *et al.*, 2000). Subsequently the DNA analysis in the other family members took place. The boy (III-3) was still ambulant at age 8, with a cerebellar ataxia, generalized mild dystonic movement disorder, a discrete pyramidal syndrome and speaks only a few words. His total IQ is 55. The EEG showed a slight delay of the background pattern. The cerebellar hemispheres showed in the cortical and subcortical regions on MRI extensive abnormalities in the flair and T2 spin echo. A quadriceps muscle biopsy showed at electron microscopy an increase of lipid droplets and enlarged mitochondria. At the age of 6, the girl (III-4) had milder problems than her brother. She speaks in short, poorly articulated sentences. Her EEG showed a slight delay in

background pattern. The MRI showed very discrete abnormalities in the cerebellar hemispheres.

The mother of the index patient (II-2) has periodically stomach ache but no neurological abnormalities. None of the unaffected family members showed movement disorders or other neurological or psychiatric abnormalities. All numbered patients (fig 1) were clinically examined except the grandmother of the index patient (I-1). She died from a gastrointestinal tumor.

Methods

Muscle biochemistry

Muscle biopsies were obtained from the index patient (quadriceps) and brother under generalized anesthesia. Frozen muscle specimens were weighed and homogenised in 0.25 M sucrose, 10 mM Hepes and 1 mM EDTA, pH 7.1. The enzyme activities were assayed at 37°C, unless indicated otherwise. Complex I, NADH-coenzyme Q reductase, was measured as the rotenone sensitive oxidation of NADH with decylubiquinone as electron acceptor (De Vries et al., 1996). Complex II + III, succinate cytochrome c reductase, was measured as the antimycin sensitive reduction of cytochrome c in the presence of appropriate inhibitors at 25°C (Scholte et al., 1995). Complex II, succinate-coenzyme Q reductase, was determined as the theonyl trifluoroacetone (2 mM) sensitive reduction of 2,6-dichlorophenolindophenol (DCPIP) by succinate in the presence of decylubiquinone (Trounce et al., 1996). Complex III, ubiquinol cytochrome c reductase, was assayed as the antimycin sensitive reduction of cytochrome c by decylubiquinol at 25°C², in the presence of lauryl maltoside. Complex IV, cytochrome c oxidase, was measured as described (Cooperstein and Lazarow, 1951) at 25°C, or with the detergent (Mayr and Sperl, 2000) Tween-20, at 37°C. Complex V, ATP synthase, was determined as oligomycin sensitive uncoupler stimulated Mg-ATPase by assay of ADP with pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase and NADH (Rustin et al., 1994). This reaction was started by sonication (Mayr and Sperl, 2000). Pyruvate dehydrogenase was assayed as in Arts et al. (Arts et al., 1987) and citrate synthase according to Srere (Srere, 1969). The other methods were as in Scholte et al. (Scholte et al., 1995).

DNA extraction

DNA was extracted from blood, cultured fibroblast cells, hair roots, muscle tissue, chorionic villi cells (CVS) and amniotic fluid cells. DNA extraction from blood was done as described previously (Miller et al., 1988). DNA extraction from hair roots was performed using the Qiagen RNA/DNA minikit (Qiagen GmbH, Hilden, Germany). For the DNA extraction of the other samples the standard phenol/chloroform method was used (Sambrook, 1998).

Quantitative analysis of the T9176C mutation

The mtDNA was amplified using the polymerase chain reaction (PCR) with primers, corresponding to positions 09035-09055 of the L-strand and positions 09203-09177 of the H-

strand. The latter primer contains a mismatch at location 09184-09186 to create a restriction site for the restriction enzyme BstXI in case of the T9176C mutation. The PCR was performed using 4 mM for each dNTP (Amersham Pharmacia Biotech AB, Uppsala, Sweden, 25 mM each), 15 mM MgCl₂, 0.5 M NaCl, 0.1 M Tris-HCl, 50 ng per primer, 1 U Taq DNA polymerase (Life Technologies, Breda, Netherlands) in a 25 µl volume. PCR conditions were 94°C for 5 min, followed by 32 cycles of 92°C for 1 min, 52°C for 1 min and 72°C for 45 sec and a final step of 7 min at 72°C (9600 Thermocycler, Applied Biosystems, Foster City, USA). In the last cycle 50 fmol R6G labeled dUTP (Applied Biosystems, Foster City, USA) was added. The PCR product was purified with the Qiagen PCR purification kit (Qiagen GmbH, Hilden, Germany), digested with BstXI (Boehringer-Mannheim, Bayern, Germany) and separated by electrophoresis on a 4% non denaturing polyacrylamide gel at 40°C (ABI 377 Applied Biosystems, Foster City, USA). The wildtype fragment has a length of 169 bp and the mutated fragment is cleaved in fragments of 148 and 21 bp. The mutation percentage is determined by calculating the ratio between the area of the mutant peak (148 bp) and the total area of the mutant and normal peak (169 bp). The calculated mutation percentage is multiplied by 1.06 to correct for lower number of fluorescently labeled dUTPs that can be incorporated in the mutant peak. The experimental variation of this method is 3%.

Prenatal Diagnosis

Data from literature and of the family were used to determine if this mutation was suitable for prenatal diagnosis. CVS was obtained after 12 weeks of gestation and amniotic fluid after 17 weeks of gestation. Both samples were analyzed for the T9176C mutation immediately and after a culture period of two weeks.

Results

Clinical Biochemistry

In the index patient (III-2) blood lactate was 3.2 mmol/l (normal <1.8 mmol/l) and pyruvate was as in healthy (normal <160 µmol/l). Lactate 4.6 mmol/l (normal range 0.9-2.8 mmol/l) was also elevated in cerebrospinal fluid (CSF). CSF pyruvate was 80 µmol/l. A muscle biopsy of the quadriceps muscle of the index patient (III-2) appeared normal. Histochemical staining of cytochrome *c* oxidase and succinate hydrogenase yielded normal results. Electronmicroscopy showed some irregularly formed fibrils with thickening of the Z-bands. One concentric laminated body was seen possibly derived from mitochondria. A muscle biopsy from patient III-3 also showed a normal morphology by routine histology and histochemistry. Electronmicroscopy showed enlarged intermyofibrillar mitochondria and increased lipid droplets. One small aggregate was seen of elongated subsarcolemmal mitochondria. The brother (III-3) of the index patient showed a blood lactate of 2.2 mmol/l and a pyruvate of 140 µmol/l. CSF lactate was 3.2 mmol/l. Blood lactate in the sister (III-4) of the index patient was 2.3 mmol/l and pyruvate 150 µmol/l.

Muscle Biochemistry

Mitochondrial enzyme activities were strikingly abnormal with a severe decrease of complex V activity and an increase of pyruvate dehydrogenase activity. Complex V/ CS is 0.10 and 0.08 (normal 0.40) and pyruvate dehydrogenase/ CS is 0.10 and 0.09 (normal 0.03) for patient III-2 and III-3 respectively.

Mutation analysis and prenatal diagnosis

The identification of the T9176C mutation in muscle of the index patient was reported previously (van Den Bosch *et al.*, 2000). The internal variation of the method used to determine the mutation percentages is $\pm 3\%$. The mutation percentage in muscle was about 95% and in fibroblasts 90%. The same mutation was identified in a symptomatic brother and sister. The brother had a mutation percentage of about 93% in muscle and 94 % in hair roots. The sister had a mutation percentage of about 92% in blood and 93% in hair roots (pedigree figure 1). A number of maternal relatives were tested showing either the absence or presence of the mutation (figure 1). Mutation percentages appeared to be more constant among tissues in patients with the higher mutation ranges ($>90\%$). One of the female family members (III-1) was 6 weeks pregnant at the time of investigation. The mutation load in blood and hair roots was about 55% and 57%. She was at risk of having severely affected offspring, although the exact risk was unknown.

Figure 2

Scatter plots of literature data on the genotype-phenotype correlation of the mtDNA T9176C mutation and the detection methods use.

Asymptomatic carriers show a variation in the mutation percentage of 0% to 88%, and symptomatic carriers show a variation in the mutation percentage of 93% to 100%.

□ Method A: Last cycle addition of [α - 32 P]dATP.

Quantification by scanning in a Betascope 603 blot analyzer (Thyagarajan *et al.*, 1993; Campos *et al.*, 1997; Wilson *et al.*, 2000). Variance of the method is estimated to be $\pm 5\%$ heteroplasmy.

Δ Method B: Ethidium Bromide staining. Quantification using

UV detection (Dionisi-Vici *et al.*, 1998). Variance of the method is estimated to be $\pm 10\%$ heteroplasmy.

+ Method C: Last cycle addition of rhodamine-labeled forward primer. Quantification on an image analyzer

FMBIO II (Makino *et al.*, 1998; Makino *et al.*, 2000). Variance of the method is estimated at $\pm 5\%$ heteroplasmy.

We investigated whether this mutation would meet the criteria to allow reliable prenatal diagnosis (Poulton and Turnbull, 2000). Both data from literature (figure 2) and of the family (figure 1) were used. No symptomatic patients have been reported with a mutation

percentage below 90%, although the methods used vary in their precision. An estimate of the experimental variation is calculated, as it was not given in most references, and the number of patients is small (figure 2). Only one healthy individual had a percentage between 80% and 90% and two between 70% and 80%. All patients developed symptoms between 0 and 8 years except for one patient with a mutation percentage of 96% who developed symptoms at the age of 29. Obviously, data on the distribution of the mutation among different tissues was mainly limited to patients, all of whom had >90% mutation load in every tissue analyzed. Since no unaffected mutation carrier with less than 90% mutation has been followed during a longer period no firm conclusion is possible on either time related changes in the mutation percentage or the onset of symptoms in carriers with subcritical mutation percentages. Based on these limited data and on the data of the T9176G (Carrozzo *et al.*, 2001; Akagi *et al.*, 2002) (which has a somewhat more malignant disease course and also a lower mutation threshold for clinical expression of about 70%) and two other mutations in the ATPase6 gene (T89993G/C) (which have been studied in much more detail and for which reliable prenatal diagnosis is possible (Harding *et al.*, 1992; Bartley J. *et al.*, 1996; Ferlin *et al.*, 1997; White *et al.*, 1999; White *et al.*, 1999; Leshinsky-Silver *et al.*, 2003)), we decided to offer prenatal diagnosis. The prenatal sampling was undertaken with the strict understanding by the couple, written in an informed consent, that there could be considerable uncertainty in the interpretation of the results given the scarcity of data available. Somewhat arbitrary, a mutation percentage in fetal cells above 70% was considered a high risk of being affected, a mutant load between 50 and 70% would be inconclusive and a mutant load of less than 50% would mean a high chance of being unaffected.

Figure 3

Prenatal DNA-analysis for the T9176C mutation in the mtDNA.

The right-hand peak is the uncleaved PCR product and the left-hand peak the cleaved PCR product, due to the presence of the mutation. The mutation percentage is calculated by dividing the area of the mutant peak (148 bp) and the total area of the mutant and normal peak (148 bp + 169 bp) with a correction for the difference in number of fluorescently labeled nucleotides than can be incorporated.

A: Uncultured Chorionic villi 87% mutation.

B: Cultured Chorionic villi 85% mutation

C: Uncultured amniotic fluid cells 88% mutation

D: Cultured amniotic fluid cells 86% mutation.

To gather more information on tissue variation of the mutation and the time factor, we decided to analyze both a chorionic villus sample (12 weeks of gestation) and an amniotic fluid sample (17 weeks of gestation) directly and after a culturing period. The mutation percentage in CVS was about 87% and after culturing 85% (figure 3a and 3b) and in amniotic fluid cells 88% and after culturing 86% (figure 3c and 3d). The difference between cultured and uncultured cells is not statistically significant as the experimental variation was determined to be about 3%. Therefore the fetus was diagnosed as at risk of becoming severely affected, although the mutation percentage was just below the level of the other three affected children. Despite these results, uncertainties and associated risks the couple decided to continue the pregnancy. A healthy child was born at term after a normal pregnancy. The child did not show any abnormalities at the age of one year and for ethical reasons no further biochemical or genetic test will be performed as long as the child is healthy. It is important that the child remains under clinical control as the other affected children with a slightly higher mutation level did not show clinical signs of disease until the age of two.

Discussion

The segregation of point mutations in the mtDNA is not completely understood and the transmission of the mutation load is often unpredictable for heteroplasmic mutations. The family reported in this paper, with 3 affected children with Leigh disease and the T9176C mutation, is a clear illustration of this. Maternal relatives of the patients either do not carry the mutation or carry the mutation in varying percentages. They are all below the threshold of clinical expression, although in most cases no extensive neurological and biochemical examinations have been performed. In such families only DNA studies can provide evidence for the carrier status of an individual and can identify those at risk of transmitting the disease. The exact estimate of the risk of having an affected child is not possible due to the genetic bottleneck for mtDNA mutations and the limited number of data available for this mutation (Thyagarajan *et al.*, 1993; Campos *et al.*, 1997; Dionisi-Vici *et al.*, 1998; Makino *et al.*, 1998; Makino *et al.*, 2000; Wilson *et al.*, 2000). It has been proposed that oocyte sampling and testing could be an acceptable approach to determine the mutation load in individual oocytes and estimate the recurrence risk (Poulton and Marchington, 2002; Thorburn, 2004).

Because of these uncertainties prenatal diagnosis, based on DNA analysis of mtDNA mutations, is controversial. Until now seven prenatal tests were reported, for the T8993G and T8993C mutations (Harding *et al.*, 1992; Bartley J. *et al.*, 1996; Ferlin *et al.*, 1997; White *et al.*, 1999; White *et al.*, 1999; Leshinsky-Silver *et al.*, 2003). These mutations fulfill the criteria mentioned before and have well-established genotype-phenotype correlations, based on sufficient number of families tested. Recurrence risks have been calculated dependent on the mutation load of the mother and a safe margin for the mutant load in case of prenatal diagnosis is established. Disease caused by the T8993C mutation is clinically less severe than the T8993G mutation. The probability of having severe symptoms of the T8993C and the T8993G mutations are low if mutation loads are below 80% and 60% respectively.

The differences in recurrence risk for diseases caused by point mutations in the mtDNA and the potential pitfalls prompted a statement by a group of researchers, supported by the European NeuroMuscular Consortium, concerning prenatal options for carriers of mtDNA mutations (Poulton and Marchington, 2000; Poulton and Turnbull, 2000). Sufficient data are available for only 3 mutations today to judge these criteria properly. For the T8993G/C and A8344G mutation prenatal diagnosis can be reliably performed, but for the A3243G mutation this is not possible. For other mutations this is still unknown and the families involved can only be counselled in general terms. Whether prenatal diagnosis will be an option for these families will depend on the frequency of the mutation and the ethical discussions on acceptable risks.

The T9176C mutation has been described only a few times in literature (Thyagarajan *et al.*, 1993; Campos *et al.*, 1997; Dionisi-Vici *et al.*, 1998; Makino *et al.*, 1998; Makino *et al.*, 2000; Wilson *et al.*, 2000). As the methods used are often not comparable and as usually no information is provided on detection level and experimental variation, it is difficult to draw general conclusions on the mutation threshold for clinical expression. Until now severe symptoms were only reported for patients with mutation percentages above 90% in various tissues. As the T9176C mutation is located in the same gene as the T8993G/C mutation and shows some resemblance in clinical symptoms and progression of the disease, we considered this additional, though arguable, evidence that the T9176C mutation is also suitable for DNA-prenatal diagnosis. For the T9176C also a T9176G variant has been found with a somewhat more malignant progression similar to the T8993G mutation compared to the T8993C mutation (Carrozzo *et al.*, 2000; Carrozzo *et al.*, 2001; Akagi *et al.*, 2002). Given the high chance of an affected foetus or a borderline result it was advised not to offer prenatal diagnosis but consider oocyte donation or preimplantation genetic diagnosis (PGD) to carriers with a mutation percentage of more than 50% of the T8993G/C mutation (Poulton and Turnbull, 2000). Because the woman in our study was already pregnant it was not possible to discuss alternatives, but PGD will be offered for future pregnancies. The parents were informed about the uncertainties of borderline mutation percentages and the risk on an affected fetus and decided to continue with the prenatal diagnosis. The prenatal diagnosis revealed a percentage of 85%-88% with a variation of 3%. Although there was a high likelihood that the child would be affected the parents decided to continue the pregnancy. Assuming that the bottleneck occurs during oocyte development (Poulton, 1998), preimplantation genetic diagnosis (PGD) could be offered in case of mtDNA mutations as an alternative for conventional prenatal diagnosis, (Thorburn and Dahl, 2001). For PGD, one or two blastomeres are removed from an 8-cellular embryo, obtained by IVF procedures and intracytoplasmic sperm injection (ICSI), and these blastomeres are tested for the specific mutation. Healthy embryos are transferred to the uterus. PGD is technically easier for mtDNA mutations than for nuclear genes, as the copy number is much higher. Protocols have been optimized at the single cell level in our lab (Jacobs L.J. *et al.*, in preparation.). Data from heteroplasmic mice show that mutation percentages quantified in the biopsied cell are representative for the entire embryo (Dean *et al.*, 2003). The same criteria apply for prenatal

diagnosis as for PGD, but the main advantage of PGD is that no termination of pregnancy has to be considered in case of affected offspring. That these chances can be very high has been demonstrated in the oocytes of a carrier of the T8993G/C mutation (50% in blood), who had 6 oocytes with more than 95% mutation and 1 oocyte with no mutation at all (Blok *et al.*, 1997), yielding a chance of more than 85% for offspring being affected. PGD can prevent multiple terminations of pregnancy in those cases.

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Chapter 5

Skewed heteroplasmy levels in single fibroblasts and lymphocytes of carriers of the Leigh syndrome m.9176T>C mutation

LJAM Jacobs¹, M Drusedau¹, IFM de Co², P.J. Lindsey¹, HJM Smeets¹

¹ *Department of Genetics and Cell Biology, University of Maastricht, Research Institute Growth and Development (GROW), Maastricht.* ² *Department Neurology, Erasmus MC-University Medical Center Rotterdam*

In preparation

Abstract

Mitochondria are important organelles involved in energy production and necessary for a correct functioning of the cell. Mutations in the mitochondrial DNA (mtDNA) have a major impact on the function of a cell, illustrated by the long list of mtDNA related diseases. However, the segregation of heteroplasmic mtDNA from cell to daughter cells is a complex, only partly understood process. The distribution pattern of mutated mtDNA in different single cell types might help to clarify possible factors influencing the final mutation load in each cell. For this reason we studied the distribution of the mutant load of the m.9176T>C mutation (associated with a form of Leigh syndrome) in single lymphocytes and single fibroblast from carriers and patients. We observed a skewed distribution pattern of the mutation in both lymphocytes and fibroblast of the three carriers. The skewing is more extreme in 2 carriers and in fibroblasts and less in the third and in lymphocytes. This contrasts with the distribution pattern in lymphocytes and fibroblast of two patients, which is close to a normal Gaussian distribution pattern. If these differences in skewing would also occur in oocytes of these carriers, then this would imply a different recurrence risk based on the distribution pattern rather than on the mutation level. Further studies on distribution patterns of mutation load and data on the offspring are required to determine if there is a (genetically determined) factor that influences the distribution of the mutations and if these data can be used in the counseling process to determine the risk of affected offspring

Introduction

To date more than 200 point mutations and over 300 deletions have been reported in the mtDNA and new mutations are found at a constant pace (Brandon *et al.*, 2005). Heteroplasmy (the presence of two or more different types of mitochondrial DNA [mtDNA] molecules) is a common characteristic of pathogenic mtDNA mutations, although homoplasmic disease causing point mutations have been described as well. Heteroplasmic mutations become clinically manifest above a mutation-, tissue- and individual-specific threshold. This threshold is usually lower in tissues with a high energy demand such as brain and muscle (Larsson and Clayton, 1995). For many mutations differences have been observed in heteroplasmy level between tissues and in time during life, whereas other mutations appear to maintain more constant levels.

Mutations can also be studied at the single cell level. Every cell contains multiple (12- approx. 300.000) mitochondria. Although mitochondria are generally depicted as isolated kidney-shaped structures, they are in fact part of a larger cellular mitochondrial network. The mtDNA is organized into nucleoids, which are dynamic protein-rich structures that are able to divide and redistribute in the mitochondrial network and which are suggested to be the mitochondrial unit of inheritance. Cell can contain up to hundreds of nucleoids that contain 2-8 mtDNA-molecules each (Garrido *et al.* 2003; Legros *et al.* 2004). Several studies have analysed heteroplasmy levels in single cells or fibres, for the m.3243A>G, the m.8993T>G or m.8993T>C and the m.8344A>G mutations and demonstrated variation between single cells, meaning that the heteroplasmy percentage is not constant between cells of one person. These

studies also revealed different types of distribution patterns of the mutations when analyzing multiple single cells. These distribution patterns can vary per individual and per mutation type and often differ from a normal Gaussian distribution pattern (Anderson *et al.*, 1981; Mita *et al.*, 1998; Silvestri *et al.*, 2000; He *et al.*, 2002; Gigarel *et al.*, 2005). Substantial variation in single cells and a skewed distribution exist for mutations (like the m.8993T>G/C mutations) with a relative constant mutation load in different tissues. For the m.3243A>G mutation the largest variation between cells was observed in tissues with a high heteroplasmy value especially in single lymphocytes (Ozawa *et al.*, 1998; Saitoh *et al.*, 1999). Most cybrid clones carrying the A3243G pathogenic mutation, display two patterns: stable heteroplasmy, caused by correct replication, and heteroplasmy shifting to either wild-type or mutant. These processes are under genetic control (Enriquez *et al.*, 2003). A number of studies (Mita *et al.*, 1998; Moslemi *et al.*, 1998; Ozawa *et al.*, 1998; Koga *et al.*, 2000) have been performed on muscle fibres of affected patients and in these cases the largest variance is found in normal fibres whereas Cox-negative fibres usually display higher and more constant heteroplasmy levels. The variance is less as the tissue heteroplasmy level is at the extreme (highest or lowest 10%) of the scale (Mita *et al.*, 1998; Moslemi *et al.*, 1998; Koga *et al.*, 2000; Silvestri *et al.*, 2000; He *et al.*, 2002; Gigarel *et al.*, 2005). The question remains whether the distribution of mtDNA heteroplasmy among single cells is purely random or that selection occurs, which may depend on the nucleoid organization and replicative behaviour of the specific mutation, the tissue or the individual itself. The distribution pattern in oocytes is important for predicting the recurrence risk for carriers of point mutations in the mtDNA.

During the transmission of the mtDNA a so called “bottleneck” occurs in the early development of female oocytes. The currently prevailing hypothesis that the mtDNA bottleneck is generated by a small absolute mtDNA copy number in PGCs (Jansen and de Boer, 1998) has recently been questioned and data were presented that the mtDNA bottleneck in the germ line is created through a small effective number of segregating units (Cao *et al.*, 2007) leading to extreme shifts in mutation load in the offspring. Still, in case of random distribution of the mutated mtDNA molecules and a limited number of cell divisions an overall distribution around the mean would be expected, which has been shown for 82 primary oocytes of a woman harboring the m.3243A>G mutation. The level of mutant mtDNA appeared to be determined by random genetic drift leading to a binominal distribution in oocyte mutant load (Brown *et al.*, 2001). On the other hand, non-random distribution has been observed for the m.8993T>G mutation in oocytes and during embryonic development. The analysis of seven oocytes of a woman harboring the m.8993T>G mutation revealed a skewed segregation pattern with six oocytes showing a mutant load >95% and one oocyte with no evidence of mutation (Blok *et al.*, 1997). Three PGD embryos were analyzed for the m.8993T>G mutation and one revealed a 100% mutation load whereas in the remaining two no mutation was observed. This segregation pattern was also observed for a C tract polymorphism (between nucleotide 303 and 315) in embryos although at a slightly lesser extend (Steffann *et al.*, 2006). A distribution to the extremes in oocytes implies on the one hand a high risk of severely affected offspring, but on the other hand also the possibility to

have mutation free oocytes or embryos. This would also mean that prenatal diagnosis (PND) or, more likely, pre-implantation diagnosis (PGD) would allow selection of those healthy embryos. For this reason oocyte sampling could be useful for a female carrier to predict the chance of mitochondrial disease in a future pregnancy and to judge the possible success of the choice for PND or PGD (Poulton and Marchington, 2000). The aspiration of oocytes is however a difficult and painful procedure and it is of ethical concern, if it can be applied for counseling purposes only (Poulton and Marchington, 2000; Chinnery, 2004).

To get a better insight in the distribution of mtDNA mutation among single cells, we studied single lymphocytes and fibroblasts of five relatives from a family with the m.9176T>C mutation in the ATPase6 gene. Our aim is to determine if different cells of mutation carriers may differ with respect to the distribution of the mutations and if this can be related to the segregation pattern.

Materials and Methods

Leigh syndrome family with the m.9176T>C

Single cells were collected in a family with the m.9176T>C mutation from three carriers and two patients with Leigh syndrome. Both patients are the younger brother (patient one, age nine) and sister (patient two, age eight) of a patient who died of Leigh syndrome at the age of four. Carrier one is the mother of these children and carrier two is the sister of carrier one. Carrier three is the daughter of carrier two [Figure 1, (Jacobs *et al.*, 2005)].

Single cell collection

Human lymphocytes and fibroblasts were collected in Petri dishes (Greiner Bio-one B.V., Alphen a/d Rijn, The Netherlands) in 2 μ l Ca^{2+} and Mg^{2+} -free phosphate-buffered saline solution (PBS) with 1% polyvinylpyrrolidone (PVP) molecular weight 360 kDa (Sigma, Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 0.1 mg/ml Phenol Red (Sigma), with the help of a micromanipulator (ONO-121; Narishige, Paes Nederland BV) mounted on an inverted microscope (IX-70; Olympus, Zoeterwoude, The Netherlands). Cells were transferred in the PBS mixture to a 0.2 ml reaction tube using a mouth controlled glass micropipette. Cells were stored at -20°C until PCR was performed.

Quantitative analysis of the m.9176T>C mutation in single cells

Prior to PCR, the alkaline lysis buffer and the PCR mix without primers or *AmpliTaq Gold* DNA polymerase (Applied Biosystems, Foster City, USA), were decontaminated from DNA by UV-C irradiation for 1 h using an UV-C lamp type TUV 30W/G30T8 longlife (Philips, Eindhoven, The Netherlands). Blank samples were included in every PCR series to monitor DNA contamination. Cells were lysed by adding 2.5 μ l of alkaline lysis buffer [50 mmol/l dithiothreitol (DTT; Pharmacia Biotech, Benelux, Roosendaal, The Netherlands)/200 mmol/l NaOH] followed by 10 min of incubation at 65°C . PCR was performed with the GeneAmp[®] PCR System 9700 (Applied Biosystems, Foster City, USA) using primers (Applied Biosystems, Foster City, USA), corresponding to positions 09035-09055 of the L-

strand for the forward primer and positions 09203-09177 of the H-strand for the reverse primer. The latter primer contains a mismatch at location 09184-09186 to create a restriction site for the restriction enzyme BstXI in case of the T9176C mutation. PCR was performed using 1.5 mM for each dNTP (Amersham Pharmacia Biotech AB, Uppsala, Sweden, 25 mM each), 15 mM MgCl₂, 0.5 M NaCl, 0.1 M Tris-HCl, 50 ng per primer, 20 mmol/l Tricine pH 4.95 (Sigma, The Netherlands) and 1 U Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, USA) in a 25 µl volume. PCR conditions were 94°C for 7 min, followed by 45 cycles of 92°C for 1 min, 52°C for 1 min and 72°C for 45 sec and a final step of 7 min at 72°C. In the last cycle 10 pmol Fam-labeled Forward primer (Applied Biosystems, Foster City, USA) was added. The PCR product was digested with BstXI (Boehringer-Mannheim, Bayern, Germany) and purified with the Qiagen PCR purification kit (Qiagen GmbH, Hilden, Germany). 3 µl of this purified product was separated by capillary electrophoresis on an ABI3100 Genetic Analyzer (ABI PRISM Applied Biosystems, The Netherlands) using POP-6™ as a polymer. The results were analysed with GeneScan Analysis software version 3.7 provided by the manufacturer. The PCR fragment sizes were calculated using GeneScan™-500 TAMRA™ Size Standard. The wild-type fragment has a length of 169 bp and the mutated fragment is cleaved in fragments of 148 and 21 bp. The mutation percentage is determined by calculating the ratio between the area of the mutant peak (148 bp) and the total area of the mutant and normal peak (169 bp).

Statistical analysis

An independent-sample t-test is used to evaluate the significance of the difference observed between the single cell results of the two tested tissues, using SPSS. Grouped data were expressed as the mean ± standard deviation. A p-value of <0.05 was considered to have statistical significance. Non parametrical tests (Mann-Withey U test, Kolmogorov-Smirnov Z test and the Chi-Square test) were used to analyse a possible difference in single cell distribution.

Results

A family with 3 children with Leigh syndrome due to the m.9176T>C mutation, was studied for the mutation load in several tissues (figure 1) and single cells. This family has been described before (Jacobs *et al.*, 2005). The use of a labeled primer instead of labeled nucleotides has allowed a more accurate determination of the mutation load, which is in general higher than previously reported, especially in case of the carriers. The mutation load was determined in at least 25 single lymphocytes and fibroblasts of the same individual and the mean level of heteroplasmy and variation among the single cells was calculated (table I). Missing values were not included in the analysis since their number was negligible (7 out of 633 cells analyzed). The mean result from the combined single lymphocyte analyses deviates from the mean results acquired when analyzing the whole blood DNA samples, most likely because this contains also other cell types. The difference between the results however stays within one standard deviation obtained from the single lymphocytes sample. For fibroblasts this is not the case as cell cultures have been used.

Table I. Results of single cell analysis

Tissue/individual	Cell number	Mean $\pm$ stdev	minimal	maximum	P^*	$P^{\#}$
		heteroplasmy level				
Lymphocytes/ carrier 1	152	36.3% $\pm$ 38.2	0%	96.5%	0.143	0,007
Fibroblasts/ carrier 1	136	29.4% $\pm$ 41.5	0%	99.9%		
Lymphocytes/ carrier 2	26	54.4% $\pm$ 20.2	18.95%	83.53%	0.180	0,024
Fibroblasts/ carrier 2	84	45.5% $\pm$ 31.8	0%	94.5%		
Lymphocytes/ carrier 3	47	69.1% $\pm$ 27.5	0%	91.5%	0.124	0,015
Fibroblasts/ carrier 3	51	77.7% $\pm$ 27.1	0%	94.1%		
Lymphocytes/ patient 1	24	95.3% $\pm$ 8.9	59%	100%	0.449	0,191
Fibroblasts/ patient 1	48	93.2% $\pm$ 11.9	37.5%	100%		
Lymphocytes/ patient 2	31	88.1% $\pm$ 8.4	73.1%	100%	0.384	0,644
Fibroblasts/ patient 2	27	85.6% $\pm$ 11.5	59.0%	100%		

P^* : Statistical analysis of the proportion mutant mtDNA between lymphocytes and fibroblasts

$P^{\#}$: Statistical analysis of the distribution of the mutant mtDNA between single lymphocytes and single fibroblasts using the Kolmogorov-Smirnov Z-test

To determine whether the single cell variation has a normal Gaussian distribution, histograms were drawn using the percentages of single cell values divided over 10 categories, ranging from 0%-10% to 90%-100% (figure 2). The distribution is not normal (Gaussian) in both lymphocytes and fibroblasts of carriers and patients, although the two patients approach a normal distribution pattern. The distribution patterns in the carriers are different and show pronounced skewing. Striking is the difference between the distribution patterns of both cell types. The pattern in fibroblasts in all three carriers is more to the extreme heteroplasmy percentages than in lymphocytes. The heteroplasmy distribution of the lymphocytes in carrier

2 is closest to a normal pattern. The most skewed percentages are observed in carrier 1 showing an accumulation at the maximum and minimum heteroplasmy percentages in both cell types.

Figure 2: Single cell distribution of the m.9176T>C mutation in lymphocytes (left) and fibroblasts (right) of carriers (the first three graphs) and patients (bottom two graphs).

Statistical analysis

Using statistics the mean of the single cell heteroplasmy values between both cell types of one person was compared. This revealed no significant difference between lymphocytes and fibroblasts (table 1). The mean single cell heteroplasmy value obtained from lymphocytes was compared to the earlier determined heteroplasmy value in blood (figure 1). Also this test revealed no significant difference between the mean single cell value and the blood heteroplasmy value (results not shown). No statistical difference was observed between the values from single fibroblasts and entire cultures from carrier 1. Non-parametrical statistical tests were used to compare the distribution pattern (figure 2) between the two cell types from the carriers. These tests did not reveal a significant difference for all but carrier 1 based on the two-sample Kolmogorov-Smirnov test. A trend for more extreme heteroplasmy values was, however, demonstrated in the other carriers in the fibroblast samples as compared with the lymphocyte samples. Patients did not show a difference in distribution pattern of the single cell values. Comparing the distribution patterns (figure 2), only carrier 1 has a distribution pattern, which differs from the other carriers or patients. The likelihood of carrier 1 to receive three severely affected children based on the distribution pattern of the mutation in her fibroblast cells was estimated to be around 3.5%.

Discussion

The m.9176T>C mutation shows extensive variation in mutation levels in single cells of an individual. This variation is especially evident in the three carriers (Figure 1) who all show heteroplasmy levels between 0% and >90% in single cells. Despite this variation there is no statistical difference between the overall heteroplasmy value for blood and fibroblasts and the mean single cell value for lymphocytes and fibroblasts. This means that for this mutation the tissue heteroplasmy value is a good representation of the mean heteroplasmy value of single cells in this tissue, although it does not mean that the majority of cells have mutation percentages around the mean. Another striking observation is the significant difference in distribution pattern between lymphocytes and fibroblasts in the three carriers, but most prominent in carrier 1, based on 5% confidence. In all three carriers fibroblasts have more extreme heteroplasmy levels than lymphocytes. The distribution of the mutation levels in lymphocytes and fibroblasts is not normal and skewed to both extremes. The patients with mutation levels above 90% are closest to a normal distribution around the mean. Normal and abnormal distribution of single cell heteroplasmy values has been shown before in other investigations analyzing single cells or muscle fibers from mtDNA patients (Mita *et al.*, 1998; Ozawa *et al.*, 1998; Saitoh *et al.*, 1999; Silvestri *et al.*, 2000; He *et al.*, 2002; Gigarel *et al.*, 2005), but the differences were not as extreme as in our study. Studies addressing heteroplasmy levels of mtDNA point mutations, like the m.3243A>G and m.8344A>G, have analysed single muscle fibres, discriminating between COX-positive, COX-negative and normal fibres. The highest mutation load was present in the COX-negative fibres and the lowest in the normal fibres. The distribution within one type of fibre is in general a normal distribution around the mean value of the fibre type (Mita *et al.*, 1998; Moslemi *et al.*, 1998;

Koga *et al.*, 2000; Silvestri *et al.*, 2000). Other studies analysing an mtDNA deletion and the m.3243A>G mutation in muscle fibres show a distribution pattern with values reaching towards homoplasmy especially in patients with median mean heteroplasmy values (Ozawa *et al.*, 1998; He *et al.*, 2002).

Variation can be caused by cell division when the mitochondria are randomly divided between the daughter cells (Rotig and Munnich, 2003). This random division leads to slight differences between cells eventually leading to extreme variations and the situation of homoplasmy (Enriquez *et al.*, 2003). This process has elegantly been shown for fixation of new mtDNA mutations in colonic crypt cells (Taylor *et al.* 2003). The model used predicts that the random partitioning of mitochondrial genomes during crypt cell division, which occurs every 24 hour, will cause random genetic drift and lead to the clonal expansion of somatic mtDNA mutations during human life. The observed distribution of single cell heteroplasmy values in our and other studies could reflect a similar process, although the lesser number of cell divisions and the differences observed between individuals might indicate a more specific process. It is known that genetic factors may be involved (Enriquez, 2003). Also, in a mouse model a tissue specific selection for different mtDNA genotypes has revealed three quantitative-trait loci (QTLs) that could account for this tissue specificity ((Battersby *et al.*, 2003). Identification of the factors involved would open up strategies to induce a shift in the heteroplasmy percentage, which could be of major therapeutic importance (Smith *et al.*, 2004) (Chinnery *et al.*, 1999; Chinnery, 2002)..

The question remains whether processes involved in the somatic nucleoid segregation could affect for example disease progression or mutation transmission. We have only preliminary information from one family, but our data suggest that it is worthwhile to explore this further. The heteroplasmy percentage in blood of carrier 1 and 2, who are sisters, is comparable, respectively 43.5% and 47.8%, but the distribution pattern in fibroblasts differ and also the mutation load in their children (figure 1). The more random dispersion of the mutation in carrier 2 might reflect random segregation with a likelihood of mutation levels in her children around the mean, whereas the skewed distribution in fibroblasts of carrier 1 might indicate an increased chance of very high and very low mutation load. The mutation load observed in the children fits with this hypothesis. So it would be worthwhile to test mothers of children with mtDNA disease to see if the distribution pattern in fibroblasts is in line with the distribution of the mutation load in their children. If this can be confirmed then the distribution pattern of the mutation in the mother could be important for determining the recurrence risk and the success rate of PND or PGD.

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Chapter 6

MtDNA point mutations are present at various levels of heteroplasmy in human oocytes

Lorraine Jacobs^{1,2}, Mike Gerards¹, Patrick Chinnery³, John Dumoulin⁴, Ireneaus de Co⁵,
Joep Geraedts¹, Hubert Smeets¹

¹Departement of Genetics and Cell Biology and ²Research institute GROW, University of Maastricht. ³Mitochondrial Research Group, University of Newcastle upon Tyne, Newcastle Upon Tyne, UK. ⁴Department of IVF, Academic Hospital Maastricht. ⁵ Department of Neurology, Erasmus Medical Center Rotterdam

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Abstract

Little is known about the load of mutations and polymorphisms in the mtDNA of human oocytes and the possible effect these mutations may have during life. To investigate this, we optimised at the single cell level the recently developed method to screen the entire mtDNA for mainly heteroplasmic mutations by denaturing high performance liquid chromatography (DHPLC) analysis. This method is sensitive (~1% heteroplasmy detectable), specific and rapid. The entire mtDNA of 26 oocytes of 13 women was screened by this method. Ten different heteroplasmic mutations, of which only one was located in the D-loop and of which two were observed twice, were detected in seven oocytes with mutation loads ranging from less than 5% to 50%. From 8 women more than one oocyte was received and in four of them heteroplasmic differences between oocytes of the same woman were observed. In one of these four also two homoplasmic D-loop variants were detected. Additionally, four oocytes of a single woman were sequenced using the MitoChip[®] (which lacks the D-loop region), but all sequences were identical. It is concluded that heteroplasmic mtDNA mutations are common in oocytes and that depending on the position and mutation load they might increase the risk on developing OXPHOS disease early or later in life.

Introduction

A large number of mutations (more than 200) and polymorphisms has been reported for the mitochondrial DNA (mtDNA) (Brandon *et al.*, 2005). One of the reasons is that the mtDNA is located in the mitochondria near the OXPHOS system, which produces mutagenic reactive oxygen species (ROS) as part of the electron transport to produce energy in the form of ATP (Kuchino *et al.*, 1987). This high mutation rate in combination with a lack of good repair mechanisms and recombination and the exclusive maternal transmission of mtDNA would predict a decay of the mtDNA during evolution. This does not happen due to the 'mtDNA bottleneck' which is a restriction in the number of mtDNA molecules to be transmitted, followed by an amplification of these founder molecules in oocytes (Howell *et al.*, 1992). The bottleneck occurs during the early stages of oocyte development, in order to maintain homoplasmic mtDNA and minimize heteroplasmy (Cummins, 1998; Cummins, 2001). Because this happens very early during development the chance to preserve age-related mutations in the early oocyte is small, although the low amount of mtDNA copies per mitochondria in the early developmental stages of the oocytes renders these oocytes vulnerable for mutational events at this stage (Keefe *et al.*, 1995). As a result of the mtDNA bottleneck a preferentially healthy mtDNA population is maintained in oocytes and offspring. Several reports have shown that this bottleneck is no absolute safeguard (Poulton, 1995; Degoul *et al.*, 1997; Lutz *et al.*, 2000). There have been a number of investigations on the load of mtDNA deletions in oocytes that failed to develop into metaphase II oocytes (Chen *et al.*, 1995; Keefe *et al.*, 1995; Brenner *et al.*, 1998; Reynier *et al.*, 1998; Barritt *et al.*, 1999; Hsieh *et al.*, 2002) and on average 50% of the human oocytes contain mtDNA deletions although usually in extremely low heteroplasmy percentages.

Little is known about the presence of point mutations in the mtDNA of human oocytes. This is a major concern as, at least for children with mtDNA mutations, maternal inheritance is the exception rather than the norm and many children carry de novo mutations in the mtDNA (Thorburn, 2004). Therefore we screened the entire mtDNA of 26 oocytes for heteroplasmic mutations by denaturing high performance liquid chromatography (DHPLC) analysis (van Den Bosch *et al.*, 2000) and, additionally, 4 oocytes of 1 woman for homoplasmic and, less sensitive than by DHPLC, heteroplasmic variants by the resequencing MitoChip[®] (Maitra *et al.*, 2004). The presence and mutation load of point mutations in these oocytes might provide a clue for the chance of developing “spontaneous” severe OXPHOS disease or age-related complications, due to mtDNA defects. Furthermore, it may provide insight in the performance of the bottleneck.

Materials and Methods

Human oocytes

A total of 26 oocytes from 13 women were donated by consenting couples undergoing in vitro fertilisation (IVF) or intra cytoplasmic sperm injection (ICSI) because of male infertility problems, except for two women who had an obstruction of the fallopian tube. The oocytes used were apparently normal in morphological appearance, but immature in development although several oocytes developed into metaphase II oocytes prior to analysis. There was no enrichment for poor quality oocytes, in which an increase of mtDNA rearrangements has been previously reported (Barritt *et al.*, 1999). Oocytes were collected anonymously and no additional maternal material could be obtained. From the 26 oocytes four were germinal vesicles (GV), 13 were in metaphase I (MI) and nine were in the fully developed metaphase II (MII) stage. Donated oocytes were washed three times in sterile MQ in plastic dishes, and then transferred to a 0.2 ml tube in 1-2 µl volume. The oocytes were used immediately or frozen at -20°C. All pipetting was performed with a sterile, filtered mouth pipette.

Cell lysis

DNA was extracted from the oocytes using a proteinase K-based isolation method (Zhou *et al.*, 1997). Briefly, oocytes were thawed at room temperature and 10 µl of the DNA extraction solution (50 mM Tris-HCl, pH8.5, 1mM EDTA, and 0,5% Tween 20, containing 200 µg/ml proteinase K) was added. Samples were incubated at 55°C for two hours, followed by 10 minutes incubation at 95°C to inactivate the proteinase K.

PCR amplification of the mtDNA in single cells

Two rounds of nested PCR were performed. In the first round the entire mtDNA was amplified using three Expand Long PCR reactions (at positions I: 612-7165 II: 6642-12639 III: 11909-742 using primers IF: gaaaatgttagacgggctcac IR: cgccgatgaatatgatgtgaa IIF: tatcctaccaggcttcggaata IIR: atgatggaccatgtaacgaaca IIIF: aaccacgtctcctgatcaaat IIIR:

atcgtggtgatttagaggggtga). The reactions were performed using the Expand™ Long Template PCR System (Roche, Basel, Switzerland) containing 500 µM per dNTP (Amersham Pharmacia Biotech AB, Uppsala, Sweden, 25 mM each), 2 ng/µl of each primer, 5 µl 10x PCR buffer II, 3µl cell-lysate and 0.5 µl Expand Long polymerase (with proofreading capacity) in a 50 µl reaction volume. The reactions were carried out in a 9700 thermocycler (Applied Biosystems, Foster City, USA) as follows: 95°C 3 min; 10 cycles of 93°C for 10 s, 60°C for 30 s and 68°C for 8 min; 20 cycles of 93°C for 10 s, 60°C for 30 s and 68°C for 8 min plus 20 s per elongation step; and a final 7 min elongation at 68°C. In the second round the entire mtDNA was amplified in thirteen separate fragments (van Den Bosch *et al.*, 2000), using 3 µl of the Expand Long PCR product in a reaction volume of 50 µl containing 0.8 µM per dNTP, 0.4 µM of each primer, 1.5 mM MgSO₄, 5 µl Optimase® polymerase buffer and 2.5U Optimase® polymerase (Transgenomic, Omaha, USA). The reactions were carried out in a 9700 thermocycler (Perkin-Elmer) according to the Optimase ProtocolWriter™ as either a normal step PCR procedure or as a touchdown PCR if the melting temperature of both primers was separated by more than three degrees. Following PCR amplification the DNA samples were denatured at 95°C reannealed at 65°C and cooled to 4°C to form heteroduplexes. If necessary, smaller PCR fragments were amplified by using 1 µl of the expand long PCR product in a total reaction volume of 50 µl containing 0.8 µM per dNTP, 0.4 µM of each primer, 1.5 mM MgSO₄, 5 µl Optimase® polymerase buffer and 2.5U Optimase® polymerase (transgenomic).

DHPLC analysis of the mtDNA and fragment collection

Amplified second round products were cleaved with restriction enzymes to obtain smaller fragments of 90 to 560 basepairs in length as described before (van Den Bosch *et al.*, 2000). After purification with the QIAquick™ PCR purification kit (Qiagen, GmbH, Hilden, Germany), DHPLC analysis was performed on the Transgenomic WAVE® system. Low percentage heteroduplex peaks were reanalysed on the WAVE and the deviant peaks were collected using the FCX-200 fragment collector (Transgenomic, Omaha, USA). Fragment collection was performed in a two-step procedure. In the first DHPLC run the time window for the elution of the peaks of interest was determined. During a second injection DHPLC fragments were collected during the previously defined time interval.

Sequence analysis of PCR fragments

Sequencing reactions were performed in a total volume of 20 µl using 2 µl Big Dye termination solution according to the protocols of the manufacturer (Applied Biosystems, Foster City, USA) and 50 ng forward or reverse primer, using a ABI Prism 3100 DNA automatic sequencer (Applied Biosystems, Foster City, USA) and ABI analysis software version 3.1.

Table I: Heteroplasmic mtDNA changes in 26 oocytes

Oocyte	Developmental phase	Nucleotide substitutions	Gene location: Amino acid change	% heteroplamy	reference
1	MII				
2	MII				
3	MI				
4	MII				
5	MI				
6	MII				
7	MI				
8	MI				
9	MI	G15607A	CYTB: No	<10%#	(Povalko <i>et al.</i> , 2005)
10	GV				
11	MI				
12	MI				
13	MII				
14	GV				
15	GV				
16	GV				
17	MI	A16289G	Non coding: No	35%	(Horai <i>et al.</i> , 1993)
18	MI				
19	MI				
20	MI	A5390G	ND2: No	30%	(Povalko <i>et al.</i> , 2005)
		T6776C*	COI: No	<5%	(Marzuki <i>et al.</i> , 1991; Chinnery <i>et al.</i> , 2001)
		A6791G*	COI: No	<5%	(Marzuki <i>et al.</i> , 1991)
		T8542C*	ATPase 6 and 8: Phe→Leu (ATPase 6)	<5%	NR
21	MII	T14766C	CYTB: Thr → Ile	50%	(Marzuki <i>et al.</i> , 1991; Povalko <i>et al.</i> , 2005)
22	MII	A6629G*	COI: No	<5%	(Herrnstadt <i>et al.</i> , 2002)
23	MII				
24	MII	T6776C*	COI: No	<5%	(Marzuki <i>et al.</i> , 1991; Chinnery <i>et al.</i> , 2001)
		A6791G*	COI: No	<5%	(Marzuki <i>et al.</i> , 1991)
25	MI	CA514CACA	Non coding: No	15%	(Tikhomirov and Baranov, 2005)
26	MI	A6408G	COI: Ile→Val	15%	NR

#Determined using restriction fragment analysis

*Enriched by fragment collector

NR: Not reported before

GV: Germinal vesicle oocyte

MI: Metaphase I oocyte

MII: Metaphase II oocyte

Resequencing the mtDNA in single cells with the MitoChip[®]

The 3 initial long range PCR fragments were re-amplified and PCR products were purified from residual primers and nucleotides using QIAQuick PCR cleanup kit and resuspended in 30 µl of EB buffer (QIAGEN). The yield of each PCR product after purification was determined using a nanodrop ND-1000 spectrophotometer and equimolar amounts of the three fragments were pooled. After fragmentation and labelling, the products were hybridised on a pre-hybridised MitoChip[®] as described in the Affymetrix customSeq[™] Resequencing protocol (Affymetrix, Santa Clara, USA). Following hybridisation, the chips were washed and stained on the GeneChip fluidics station 400 (Affymetrix) using the pre-programmed CustomSeq Resequencing wash and stain protocol (DNA ARRAY-WS2). The MitoChips were scanned using the Affymetrix GeneChip scanner 3000 creating .CEL files for subsequent batch analysis. The Affymetrix GeneChip DNA Analysis Software (GDAS) version 3.0.1.3 beta was used for the analysis of the .CEL files.

Results

The entire mtDNA of 26 oocytes was screened by DHPLC analysis. Although these are single cell, it does not mean that this is a single template analysis. Oocytes contain about 100,000 mtDNA molecules and the number of templates is comparable to 50,000 cells for a nuclear gene. Three types of alterations were detected by DHPLC analysis: heteroduplex peaks, indicating heteroplasmic mutations, shifts of the entire homoduplex peak and the loss or gain of a restriction site. The last two alterations pointed towards homoplasmic changes and were only studied when differing between oocytes of a single woman. A total of 12 heteroduplex peaks and two homoduplex shifts (table 1 and 2) were identified and characterised by sequence analysis. All changes identified were A to G or T to C changes. For six samples, heteroplasmic peak fraction collection was required (table 1). Heteroplasmy levels were determined using restriction fragment analysis (oocyte 9) or estimated from the DHPLC and sequencing peak surfaces. In case fragment collection was required the heteroplasmy level was estimated to be below 5%, based on the detection level of DHPLC analysis.

Two homoplasmic changes were observed in the non-coding D-loop region between two oocytes of the same woman. As DHPLC analysis has a limited sensitivity to detect homoplasmic alterations, we do not know how many homoplasmic mutations might have been missed, not only in the D-loop, but especially in the remaining coding part of the mtDNA. Therefore, we used the MitoChip[®], which contains the entire coding part of mtDNA but unfortunately not the D-loop region, to analyze the 4 oocytes of woman 4. We did not detect any additional homoplasmic or heteroplasmic differences between the oocytes of this woman. The detection level of the MitoChip[®] appears to be mutation dependent and varies from 5%-30% heteroplasmy in our hands (van Eijsden *et al.*, 2006). This is less sensitive than reported by others (Jakupciak *et al.*, 2005).

Table II: Differences in the mtDNA of oocytes obtained from one woman

Woman	Oocyte	Nucleotide Differences	Gene location: Amino acid change	Difference
1	1+2	No		
3	4+5	No		
4	6+7+8+9	A15607G	CYTB: No	<10% heteroplasmy in oocyte 9 other oocytes have 100% polymorphism
6	11+12	No		
8	14+15+16	No		
11	19+20	A5390G	ND2: No	30% heteroplasmy in oocyte 20
		T6776C*	COI: No	<5% heteroplasmy in oocyte 20
		A6791G*	COI: No	<5% heteroplasmy in oocyte 20
		T8542C*	ATPase 6 and 8: Phe→Leu (ATPase 6)	<5% heteroplasmy in oocyte 20
		C150T	Non coding: No	100% polymorphism in oocyte 20
		A200G	Non coding: No	100% polymorphism in oocyte 20
12	21+22+23+24	C14766T	CYTB: Thr → Ile	50% heteroplasmy in oocyte 21
		A6629G*	COI: No	<5% heteroplasmy in oocyte 22
		T6776C*	COI: No	<5% heteroplasmy in oocyte 24
		A6791G*	COI: No	<5% heteroplasmy in oocyte 24
13	25+26	A6408G	COI: Ile→Val	15% heteroplasmy in oocyte 26

*Enriched by fragment collector

Discussion

DHPLC analysis has been frequently reported for the detection of mtDNA mutations (van Den Bosch *et al.*, 2000; Liu *et al.*, 2002; Conley *et al.*, 2003; Biggin *et al.*, 2005) and we adapted the method at the single cell level. DHPLC analysis is highly sensitive with a detection level of less than 5% and probably 1% heteroplasmy. Several different mutations were identified in oocytes with heteroplasmy percentages varying from less than 5% to 50%. It is unlikely that PCR artefacts or nuclear pseudogenes can explain the low level alterations, because the DNA polymerases have a proofreading capacity and the number of mtDNA templates is high (100,000 mtDNA molecules per oocyte) compared to the two copies of a nuclear template. Based on the number of templates and PCR cycles and length of fragments, the maximum level for PCR induced mutation could be between 0.05 and 0.1% for a specific mutation, which is below the detection limit of DHPLC analysis. The possibility of a contamination has also been excluded by the use of negative controls with no DNA in each step of the procedure. All changes identified were A to G or T to C changes. Most changes were known variants, except for two, m.6408A>G and m.8542T>C, which have not been described before. Two mutations, m.6776T>C and m.6791A>G, were identified twice, which indicates that hot spots may exist, although the majority of the heteroplasmic mutations is scattered across the mtDNA. Earlier observations using bioinformatics and perceived mutations indicated a prominent instability of the guanine nucleotide in the mitochondrial genome. This instability was however most pronounced at the 3' and/or 5' end of the protein coding genes (Tanaka and Ozawa, 1994; Samuels *et al.*, 2003). In contrast, the variants in the oocytes are predominant in the middle part of the coding genes and do not preferentially

involve guanine residues (Samuels *et al.*, 2003). Three of the observed changes were located in the D-loop, which is in line with literature that predicts that many point mutations may be contained in the mitochondrial control region (Chinnery *et al.*, 1999; Clayton, 2000; Fernandez-Silva *et al.*, 2003). This hypervariability of the D-loop has been observed in other tissues as well especially in relation to ageing and cancer (Cormio *et al.*, 2005), but a bias exists as often only the D-loop is studied and mutations in other areas can be missed (Lee *et al.*, 2005; Lievre *et al.*, 2005). Recent data suggest that also in cancer mutations are being distributed among the entire mtDNA including the D-loop (Maitra *et al.*, 2004; Jakupciak *et al.*, 2005). So despite a preference for the D-loop region mutations in the mtDNA can arise at any location in the oocytes.

The heteroplasmy level of the point mutations in the coding region of the mtDNA in oocytes ranged from <5% to about 50%. As the maternal sequence is unknown, the mutation load is given for the nucleotide, which is most likely the mutated one, although this can be ambiguous. In case of multiple oocytes of a single woman, the sequence which was shared by most of the oocytes was considered as the maternal sequence. Although we did not detect an evidently pathogenic mutation, it can be expected that pathogenic mutations will not be uncommon, as mutations are scattered throughout the mtDNA. On average 20 (between 15-30 locations) positions in a tRNA molecule and 25% of the amino acids in the proteins are evolutionary conserved, indicating that as an estimate about 5% of the mutations in the mtDNA can alter a conserved nucleotide and thus potentially be pathogenic. We found heteroplasmic changes in 26.9% of the oocytes, which means that probably about 1% of the oocytes will carry a pathogenic point mutation. We did not detect mutation levels above 50% (although for oocyte 17 we can not be sure, because we could not deduce the maternal reference sequence from other oocytes), which is under the threshold level for common point mutations, like m.8344A>G, m.8993T>G, m.8993T>C and m.9176T>C [thresholds of respectively >70%, >60%, >80% and >90% (Chinnery *et al.*, 1997; White *et al.*, 1999; Jacobs *et al.*, 2005)], although other point mutations, like the m.3243A>G mutation, can lead to clinical symptoms at heteroplasmy levels <30% (Thorburn and Dahl, 2001). Under the assumption that mutation levels above 50% may exist and have not been detected due to the small sample size, or that mutation levels in cells might increase in the first cell divisions, we predict that about 1% of the oocytes will have a mutation level reaching above the threshold of expression. Combined with the calculated number of pathogenic mutations in oocytes, this would account for about 1:10,000 de novo cases of mtDNA disease, which is in line with the observed prevalence of OXPHOs disease and the high frequency of de novo mutations (Chinnery and Turnbull, 2001; Thorburn, 2004) and which is supported by case reports on de novo mutations in severely affected patients (De Coo *et al.*, 1996; Degoul *et al.*, 1997; Maassen *et al.*, 2002; Thorburn, 2004).

In our study no deletions were detected after analysis of the first round PCR products, but we only systematically analysed the shortest fragment, as the other 2 fragments were not visible on agarose gel. Also the common deletion could not be detected by the nature of our PCR-assay. In previous reports mtDNA rearrangements have been observed in over 50% of

the oocytes analysed, especially in those at the germinal vesicle stage (Chen *et al.*, 1995; Brenner *et al.*, 1998; Barritt *et al.*, 1999; Hsieh *et al.*, 2002). Differences between these studies and ours were the number of PCR cycles [at least 65 (Chen *et al.*, 1995; Brenner *et al.*, 1998; Barritt *et al.*, 1999) compared to our first round of 30 cycles] and the size of the mtDNA fragment tested, which could explain why we did not detect these rearrangements in the mtDNA. In general the mutation levels of the point mutations are low and mutations can be lost during further cell divisions. This is also the case for the heteroplasmy level of the de novo rearrangements, which was about 0.1% of the mtDNA (Chen *et al.*, 1995). Low-level mutations can, however, ultimately have phenotypic consequences as they can get fixed in some occasions by random genetic drift. This has been observed in rapidly dividing colonic crypt cells (Taylor *et al.*, 2003) and cancer cells (Carew and Huang, 2002). This means that a low level of a mtDNA mutation in the oocyte may accumulate during life in specific tissues (like cytochrome *b* mutations in muscle (Andreu *et al.*, 1999). A deterioration of the OXPHOS system during life due to this accumulation may be a key factor in many age-related diseases (Cottrell and Turnbull, 2000; Wei and Lee, 2002).

The bottleneck preserves a homoplasmic mtDNA content early during oocyte formation. The lowest number of mitochondria (<10) is found in the early primordial germ cells (PGCs) of a three weeks old embryo (Jansen and de Boer, 1998), allowing most individuals to start with an entirely homoplasmic “clean” mtDNA (Cummins, 1998; Cummins, 2001). Because this process is taking place early the chance to preserve age-related mutations in the early oocyte is small. Based on our data this system works very well. The bottleneck is calculated to be between 1 and 30 mtDNA molecules (Bendall *et al.*, 1996; Poulton *et al.*, 1998), indicating that a mtDNA mutation leaking through with replication being proportional would lead to a heteroplasmy percentage between 3.5% and 100%. Two homoplasmic differences were detected between two oocytes of a single woman. A single mtDNA molecule as segregating unit can explain this, although we can not be sure as we could not analyse the maternal mtDNA. It does however indicate that the segregation can happen very fast, as has been shown for Holstein cattle (Koehler *et al.*, 1991) and pathogenic mutations in humans (De Coo *et al.*, 1996; Degoul *et al.*, 1997). Five of our mutations had a heteroplasmy percentage of about 15% to 50% and these mutations could have been leaking through the bottleneck or originated very early during oocyte development. At that stage the amount of mitochondria and mtDNA molecules in the oocyte is low, usually only one mtDNA molecule per mitochondrion (Michaels *et al.*, 1982; Chen *et al.*, 1995) and, if a mutation occurs, it affects a higher proportion of the mtDNA molecules. Since none of the mutations was observed in other oocytes of the same woman this indicates that the mutations probably originated early during oocyte development although the number of analyzed oocytes in total and per woman is still rather small. The levels of other mutations were very low (below 5%) and are, therefore, thought to have occurred de novo after the bottleneck and the earliest developmental stages of the oocyte and after migration of the primordial germ cells (PGC) to the ovary. If the mutated mtDNA does not specifically increase, then they must have occurred before the embryo reached the fetal stages because by then the number of mitochondria in the

oocyte is >1000 and mutations would not be detected anymore as single heteroplasmic peaks by DHPLC analysis.

Seven oocytes carried one or more point mutations in the mtDNA. Five of these contained only a single heteroplasmic mutation, whereas one oocyte contained two and the last one four heteroplasmic mutations. In case an oocyte carries more than one mutation the levels are generally low and can for this reason be considered as early embryonic, somatic mutations. Because of these small numbers, it is unclear if some oocytes are more susceptible for mutational events than others or that it is just a chance event. Several reasons can be considered for the existence of multiple mtDNA mutations in one oocyte. The first is possible environmental damage by, for example ROS, radiation or chemical substances. Another aspect that could play a role is defects in genes associated with mtDNA replication and repair. Recently it was shown that the mtDNA in mice can be subject to several forms of DNA repair (Larsen *et al.*, 2005). If this is also the case in human cells problems arising in one of these repair mechanisms could also influence the occurrence of mutations in oocytes. It is known from human patients and mice studies that a dysfunctional polymerase gamma leads to deletions and point mutations in the mtDNA (Trifunovic *et al.*, 2004). However, if there were genetic problems in the mtDNA repair mechanism one would expect to observe this susceptibility in more oocytes from the same woman, although this may be not that obvious as repair may depend on oocyte and embryo quality and this may differ in the same cohort.

From our study it can be concluded that the mtDNA bottleneck is in general an effective mechanism in preserving a homoplasmic state of the mtDNA. However, we observed 14 mutations in 8 oocytes (total 26), 10 of which were present in the coding part of the mtDNA. The distribution of these mutations appears to be random and as four of these mutations have substantial heteroplasmy levels (ranging from 15% to 50%), we hypothesize that these mutations, presumably occurring very early during oocyte development, may contribute to de novo mtDNA disease, which is a frequent cause of OXPHOS disease in children (Thorburn, 2004).

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Chapter 7

General discussion

Diseases due to defects of oxidative phosphorylation (OXPHOS), mitochondrial diseases, affect approximately 1 in 8,000 of the general population making them the most common inherited metabolic diseases (Chinnery, 2004). OXPHOS diseases are an important cause of morbidity and mortality and in general OXPHOS disorders cannot be cured. Many treatment modalities have been proposed but efficacy has been limited. Patients can be counselled as guidance for their future and can be informed about the transmission. Clinical monitoring and interventions, such as cardiac pacing, surgical correction of ptosis, cataract surgery and mechanical aids, can prevent known complications of mitochondrial disease. Antioxidants may be beneficial to patients as free radicals produced by the respiratory chain are considered important in pathogenesis of mitochondrial disorders. Coenzyme Q works as an electron transporter and free radical scavenger and has been reported to be beneficial in KSS and MELAS (Dimauro et al., 2004). Standard doses of vitamin C, E, K, riboflavin, thiamine, succinate, nicotinamide have been used to bypass blocks in respiratory chain (Dimauro et al., 2004). A substantial part of OXPHOS disease are due to heteroplasmic defects in the mtDNA, which has the additional problem that clinical expression can be extremely heterogeneous and prognosis unpredictable, partly due to variation in mutation load between tissues. Therapeutic strategies are being developed either directed at replacing the defective gene or at reducing mutation heteroplasmy levels. Furthermore, the transmission of these mutations can be highly unpredictable and common options for the prevention of transmission of inherited disease, like PND or PGD, are not applicable to the majority of mtDNA caused disease. This discussion will concentrate on strategies for treating mtDNA based disease, for example, by modulating the mutation load, and on options to prevent the transmission of the mtDNA caused disease, which were the central themes of this thesis.

Therapeutic approaches of mtDNA mutations

The increasing number of patients and disorders, in which OXPHOS defects are fundamental and the severity of the clinical symptoms involved stress the need for the development of new therapeutic interventions. A broad variety of strategies are being considered and tested.

Allotopic expression of mtDNA encoded proteins

A possible strategy to restore the function of a defective mtDNA encoded protein is the allotopic expression of mitochondrial genes by the introduction of a wild-type mitochondrial gene in the nucleus and adding a targeting signal for the introduction of the protein in the mitochondria. This approach was successful in yeast (Law *et al.*, 1988; Law *et al.*, 1990), but replication in mammalian cells turned out to be difficult. Critical factors are the transformation of the mitochondrial genetic code to the nuclear genetic code and the translocation and assembly of mitochondrial proteins translated in the cytosol, fused to an N-terminus targeting signal. Also, the delivery and prolongation of the production of the protein has to be optimised (Oca-Cossio *et al.*, 2003). An alternative is the delivery of a self-replicating plasmid into the mitochondria in the form of DQAsomes (liposome-like cationic

vesicles). DQAsomes can bind and transport DNA to the mitochondria to selectively release this DNA in the mitochondria, when the DNA is coupled to a Mitochondrial Leader Sequence (MLS) peptide. Initial data on this technique are successful although it is still unknown if mitochondrial genes delivered to the mitochondria using DQAsomes are expressed in the cells. Alternatively, DNA can be transported in the form of plasmids or oligonucleotides (D'Souza *et al.*, 2005).

Reduction of the mutation load

An alternative strategy is the reduction of the heteroplasmy level of mtDNA mutations. Heteroplasmy is the key factor in the manifestation of mtDNA caused disease. Even if it is not the only factor involved, it is evident that strategies to reduce the mutation load will be beneficial. Techniques are being developed to prevent the replication of mutated mtDNA. Tanaka and co-workers introduced gene constructs with the mitochondrial targeting sequence and restriction endonuclease activity for the m.8993T>G mutation (Tanaka *et al.*, 2002). The endonuclease SmaI cuts mtDNA molecules containing the m.8993T>G mutation which leads to a selective destruction of the mutated molecules. This strategy has been shown to be effective in cybrids and even *in vivo* in hamsters, when targeted against cytochrome c and causing a reduction in cytochrome c activity (Tanaka *et al.*, 2002). This technique is now being explored in heteroplasmic mice and is effective *in vivo* as well as in cell lines (Bayona-Bafaluy *et al.*, 2005). A major disadvantage is that this approach is only applicable to those mutations with new and unique restriction sites. Another option to reduce the heteroplasmy levels in cells is the selective inhibition of the replication of mutated mtDNA molecules. A promising approach is the use of peptide nucleic acid (PNA) oligomers. PNAs are nucleic acid analogues that selectively bind complementary DNA or RNA inhibiting replication and translation. Studies have shown that PNAs can be targeted to the mitochondria. However no reduction of replication has been observed so far (Muratovska *et al.*, 2001; Taylor *et al.*, 2001).

An alternative approach is not directed at the molecules containing the mtDNA mutation, but at specifically inducing the replication of the normal mtDNA molecule (Smith *et al.*, 2004). Segregation studies imply the existence of factors coded by nuclear genes which probably promote the replication of normal mtDNA over mutated mtDNA, but none of these factors has yet been characterised (Dunbar *et al.*, 1995; Jenuth *et al.*, 1997; Battersby *et al.*, 2003). The identification of genetic factors that modify the heteroplasmy levels would be of key importance. Experiments in mice have revealed tissue specific factors that influence the mtDNA type present (Jenuth *et al.*, 1996; Jenuth *et al.*, 1997). Observation on other (Mita *et al.*, 1998; Ozawa *et al.*, 1998; Saitoh *et al.*, 1999; Silvestri *et al.*, 2000; He *et al.*, 2002; Gigarel *et al.*, 2005) and our own patients (chapter 5) also imply that genetic or environmental factors exist in humans that influence the mutant load. The identification of these factors, could provide the basis for a therapeutic strategy for OXPHOS disease caused by mtDNA mutations. This would also be of importance for genetic counselling in case these genetic factors might influence the chance of having affected offspring.

Prevention of the transmission of mtDNA caused OXPHOS disease

Several methods have been applied to prevent the transmission of inherited OXPHOS disease. Two of these techniques, PND and PGD have also been developed for OXPHOS disease due to mtDNA mutations. Although this is technically feasible, interpretation remains problematic; this raises ethical issues (chapter 2 and 4). So far PND for mtDNA mutations has been performed several times (Harding *et al.*, 1992; White *et al.*, 1999; Leshinsky-Silver *et al.*, 2003; Chou *et al.*, 2004; Jacobs *et al.*, 2005; Bouchet *et al.*, 2006), with reliable results resulting in ongoing pregnancies with low or no detectable mutation load and in a few cases in pregnancy terminations, because of high mutation loads. Only one case has yet been described where PGD was used to prevent the transmission of the m.8993T>G mutation (Steffann *et al.*, 2006). As PND or PGD may not be sufficiently reliable for most mtDNA mutations, new approaches need to be developed which can be applied in a more general manner.

Strategies under development: cytoplasmic and nuclear transfer

Techniques like cytoplasmic transfer (CT) and nuclear transfer (NT) are potentially promising to prevent transmission of mtDNA mutations but are still at the developmental stage of technical feasibility. The use of animal models harbouring disease causing mtDNA mutations (Larsson *et al.*, 1998; Inoue *et al.*, 2000; Sligh *et al.*, 2000; Nakada *et al.*, 2001; Biousse *et al.*, 2002) will allow *in vivo* testing and provide the knowledge to determine the feasibility for the human situation.

Cytoplasmic transfer (CT)

CT is an adaptation from the ICSI technique ((Cohen *et al.*, 1997; Cohen *et al.*, 1998), where donor cytoplasm is injected into the oocytes. This technique has been tested on women experiencing repeated embryonic development failure, which was thought to be caused by a reduced ATP and mtDNA level in their oocytes (Van Blerkom *et al.*, 1995; Van Blerkom *et al.*, 2001). Although this was not supposed to be due to an mtDNA defect, CT could theoretically be extended to prevent the transmission of mtDNA disease by diluting the mtDNA level to below the critical threshold for disease by the transfer of healthy mtDNA (Kagawa and Hayashi, 1997). From earlier tests it appeared that CT is associated with a relatively high number of chromosomal abnormalities and birth defects (Barritt *et al.*, 2001). It still needs to be experimentally determined whether it is possible to introduce the required, large amount of cytoplasm in the oocyte or to replace a substantial amount of the oocyte's cytoplasm by cytoplasm containing healthy mitochondria. According to experiments in mice, due to restrictions in volume no more than approximately 30% heteroplasmy could be achieved using CT. This might be insufficient to recondition the oocytes in case of mtDNA mutations (Thorburn and Dahl, 2001). Alternatively, purified mitochondria from oocytes could be used. Experiments on pig oocytes have proven that oocytes can be supplemented with pure populations of mitochondria leading to more reproductive competent cells (El Shourbagy *et al.*, 2006).

Nuclear Transfer (NT)

Nuclear transfer (NT) involves the injection of a donor nucleus from an oocyte or early embryonic cell into an enucleated recipient oocyte. This technique is an adaptation from nuclear transfer techniques that are being used for reproductive cloning (Roberts, 1999). Because it is still unclear whether the differentiation process can be fully reversed the use of an undifferentiated nucleus (like the nucleus of an oocyte) would make nuclear transfer more safely applicable. A disadvantage of the use of oocytes as nuclear donors is the lack of a nuclear membrane, which makes the risk of chromosomal loss during transfer significant. This problem could be overcome by the transfer of the clearly visible germinal vesicle but the limited developmental possibility of this premature stage of the oocytes makes this method less feasible. Furthermore problems may occur with respect to the compatibility between recipient oocyte and donor nucleus, the reaction of the nucleus and recipient oocyte as a consequence of the transfer, the disruption of the cytoskeleton because of the transfer and the defects (like placental oedema and respiratory problems) seen in animal offspring after using this technique (Cibelli *et al.*, 2002). Also, the manipulations should not interfere with the fertilization of the oocyte. A better option would be the use of the male and female pronuclei for transfer. These are distinct structures in the fertilised embryo that could be transferred into an enucleated oocyte. This latter technique has been proven useful in reducing the mutant load in mice offspring (Sato *et al.*, 2005). Although in these mice, because of the presence of mutant mtDNA in the karyoplast, the mutant load in offspring was found to be higher than the expected mutant load on basis of the amount of mutated mtDNA in the karyoplast of the nuclear donor cell. This might mean that differences in mtDNA replication efficiency exist and that the karyoplast mtDNA is more frequently replicated. It was also a concern if the nucleus could be freed completely from possibly mutated mitochondria, especially as these perinuclear mitochondria might have a replicative advantage over mitochondria further from the nucleus (Shadel and Clayton, 1997). Recent studies using mouse oocytes demonstrated that karyoplast mitochondria are homogeneously distributed throughout the entire cytoplasm before the oocyte has completed its maturation (Fulka, 2004).

To experimentally validate NT and CT appropriate animal models are needed, but only few natural models exist (for example there is a mouse model with mtDNA caused hearing loss (Johnson *et al.*, 2001)). Over the last decade a number of mouse models have been developed for OXPHOS disease, both for nuclear and mtDNA mutations. Only 2 mouse models with mtDNA mutations exist, the CAP-resistant (CAP^R) mouse with the m.2433T>C mutation in the 16SrRNA and the mtDNA deletion mice with a 4.696 bp deletion (Sligh *et al.*, 2000; Wallace, 2001). Disease symptoms were related to human OXPHOS disease, caused by comparable mutations. Both transgenic animal lines demonstrated transmission of the mutated mtDNA to successive generations and can be used to study the inheritance and segregation of pathogenic mtDNA mutations. The CAP^R mice transmitted the heteroplasmic mtDNA mutation to some of their progeny in homoplasmic or heteroplasmic state but most animals died either in utero or within the first day after birth, one animal survived 11 days (Sligh *et*

al., 2000). The mtDNA deletion mice transmitted the rearranged mtDNA through three successive generations with a tendency to increasing heteroplasmy percentage to a maximum of 90% in muscle of some animals, most likely due to the replication advantage of the smaller mtDNA molecule (Inoue *et al.*, 2000). By using these animal models newly developed techniques to prevent transmission of mtDNA disease can be studied to determine whether they can be successful. An example is the study with respect to the efficiency of NT in these animal models with a deletion in the mtDNA (Sato *et al.*, 2005). The estimated heteroplasmy percentage after transfer was 2%. However after measuring the heteroplasmy percentages in offspring the results were significantly higher than expected (heteroplasmy percentages were 6-21%) and heteroplasmy percentages increased with the age of the mice. In the end the heteroplasmy percentages in transplanted mice were much lower than in the non-transplanted controls and no significant symptoms related to the deleted mtDNA were observed in the transplanted mice. So this study shows that these models can be used to study the safety and effect of these innovative treatment options. Furthermore they can be used to compare the efficacy of different technical approaches.

The transmission and segregation of mtDNA mutations in conclusion

The transmission and segregation of mtDNA mutations are complex and not readily understood or manipulated to treat or prevent the transmission of mtDNA caused disease. Not only the type of mtDNA mutation has an important role, but also the genetic background and most likely lifestyle of the person or family carrying this mutation. These factors influence the distribution pattern among tissues and in time and the transmission of disease to the offspring. This complexity also implies that there are several points where interventions might be possible and effective as a preventive or therapeutic approach. Some of these strategies will be directed at mtDNA replication to reduce the mutation load in cells, others can be used in reproductive options to prevent disease, like the application of PGD or NT to prevent the transmission of the mtDNA mutation. Whatever option is considered, it should be technically safe, ethically evaluated and effective, as it is important to realise that the ultimate goal is still to improve the life of people who are dealing with serious illness whether they experience it themselves or with someone they care for.

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Summary
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Summary

Mitochondrial encephalomyopathies are disorders associated with abnormalities of the terminal component of mitochondrial energy metabolism, i.e. oxidative phosphorylation (OXPHOS). Recent epidemiological studies have shown that ~1 in 8000 of the general population have an OXPHOS disorder. OXPHOS related diseases therefore cause significant morbidity and mortality. OXPHOS disease can be due to mutations in mitochondrial DNA (mtDNA) or nuclear DNA genes. The main topic of this thesis is to define the somatic segregation and familial transmission and, as well, the origin of heteroplasmic mutations in the mtDNA. This is related to their clinical expression and possible modifying factors. The aims were:

- to develop reliable tools that can predict and/or prevent the transmission of mtDNA mutations, like prenatal diagnosis or preimplantation genetic diagnosis;
- to identify factors that influence the severity and nature of the phenotypic manifestations or distribution of the mtDNA load, in order to predict the effect of transmission of mtDNA mutations more accurately;
- to determine the presence and impact of *de novo* mtDNA mutations in oocytes.

These aims have been accomplished by studying patients characteristics coupled to the load of the mtDNA mutations and their transmission as well as by analysing mtDNA mutations in the more unique state of the single cell. For the latter studies mtDNA mutations have been studied in lymphocytes, fibroblasts and oocytes.

Chapter 2 is an introduction to the transmission of OXPHOS disease and methods to prevent this. The expression and segregation of mtDNA mutations is different from nuclear gene defects and the most prominent characteristics of the mtDNA genes and mutations are highlighted. Diseases caused by nuclear gene mutations show a normal Mendelian inheritance pattern and often have more constant clinical manifestations as compared to diseases caused by mtDNA mutations. The heteroplasmic nature of many pathogenic mtDNA mutations is an important feature for disease manifestation, although homoplasmic mutations are known as well. In case of heteroplasmic mtDNA mutations disease usually becomes manifest when the mutation exceeds a tissue-specific threshold. This threshold can vary between tissues and between patients and an exact correlation between mutation load and phenotypic expression is often lacking. The transmission of mtDNA mutations is regarded to be exclusively maternal, although few possible paternal exceptions have been described. The extreme variability in mutation load between siblings, which can already be observed between embryos, is caused by a bottleneck of a limited number of segregating units during early oocyte development. Given the prevalence and severity of OXPHOS diseases and the lack of adequate therapy, existing and new methods for the prevention of transmission of OXPHOS disorders, like prenatal diagnosis (PND), preimplantation genetic diagnosis (PGD), cytoplasmic transfer (CT) and nuclear transfer (NT), are technically and ethically evaluated.

Chapter 3 gives a detailed comparison of the genetic defects and clinical outcome in 2 patients with Pearson syndrome, an often fatal multisystem disease associated with mitochondrial DNA rearrangements, to identify factors, which might be involved in and can predict the severity of the disorder. These rearrangements, which are most frequently deletions, are most likely the result of loop formations between repeat sequences in the mtDNA during mtDNA replication in the developing oocyte. A comparison is made between the presence of deletion dimers (a combination of two deleted fragments) and duplications (a combination of a normal and a deleted fragment) and the outcome of the disease. It is hypothesized that duplications might have a beneficial effect on disease severity. Because of the often *de novo* character of rearrangement the transmission risk is much lower than for the mtDNA point mutations.

Chapter 4 describes a family with three affected children with Leigh syndrome, a progressive neurodegenerative disorder. The disease was diagnosed by clinical features, lactic acidosis and a decrease of complex V activity. Mutation analysis revealed the m.9176T>C mutation in the mtDNA ATPase 6 gene. Because of the pregnancy of a maternal relative of the proband the possibility of prenatal diagnosis (PND), and for a future pregnancy also preimplantation genetic diagnosis (PGD), was evaluated. The main problem was the lack of data on genotype-phenotype correlations for the m.9176T>C mutation and on the variation of the mutation percentage in tissues and in time. Additional data were collected from different maternal relatives for different tissues. Eventually, prenatal diagnosis was offered, with understanding by the couple that there could be considerable uncertainty in the interpretation of the results. Prenatal diagnosis was carried out twice on cultured and uncultured chorion villi and amniotic fluid cells to evaluate tissue and time related effects on the mutation percentage. The fetal mutation percentage turned out to be within a grey area, just below the assumed threshold of expression (90%) and above the arbitrary safe percentage of 60%. The parents decided to continue the pregnancy and a healthy child was born at term after a normal pregnancy. It can be concluded that the challenge for PND in case of mtDNA mutations is not the technique, but the interpretation of the result.

To get a better insight in the distribution of mtDNA mutations among single cells and the potential use for counselling carriers with respect to recurrence risks, the m.9176T>C mutation at the single cell level was studied (chapter 5). The mutation load was determined in single lymphocytes and fibroblasts of several relatives, patients and carriers from the family with the m.9176T>C mutation. The results revealed a skewed distribution pattern of the mutation in both lymphocytes and fibroblasts of three carriers with moderate mutation levels, although the pattern seems more deviant in fibroblasts. In contrast the distribution pattern in lymphocytes and fibroblast of the two patients with very high mutation levels (90%) is close to a normal Gaussian distribution pattern. In one of the carriers an extremely skewed pattern could be observed in fibroblasts going either to 0% and 100% mutation load. Although heteroplasmy is known to migrate to homoplasmy of either the mutation or the wild type by

cell division, our data show that this differs between cell types and individuals and can occur in oocytes more rapidly than expected. This could imply that (genetic) factors exist that influence the distribution pattern and if the same factors would influence the mutation load in oocytes this may help predicting the recurrence risk. The carrier with the extremely skewed distribution has three children with very high mutation loads and severe disease symptoms, which fits with our hypotheses. More single cell studies in larger families are required to determine the predictive power of distribution patterns in single cells for the mutation load in offspring. The differences in distribution might also reveal yet unknown and possibly genetic mechanisms involved in the segregation of mtDNA mutations, which could open up the possibility to manipulate variations in heteroplasmy levels.

As a large part of patients with OXPHOS defects carry de novo mutations in the mtDNA the load of mutations and polymorphisms in the mitochondrial DNA (mtDNA) of healthy human oocytes, was determined, as these mutations might have originated in the oocyte (chapter 6). Two sensitive and accurate analytical systems to identify these mutations and polymorphisms were used. The mtDNA of 26 oocytes has been screened completely for heteroplasmic mutations using DHPLC analysis. Ten different heteroplasmic mutations, of which one was located in the D-loop and two were observed twice, were detected in seven oocytes with mutation loads ranging from less than 5% to 50%. Furthermore, the mtDNA (with exception of the D-loop region) of four oocytes of a single woman has been screened using a resequencing CHIP (Affymetrix MitoChip®), but no differences between the four oocytes were detected. It is concluded that heteroplasmic mtDNA mutations are common in oocytes and that depending on the position and mutation load they might increase the risk on developing OXPHOS disease early or later in life.

Finally, in the general discussion (chapter 7) the current strategies to improve the quality of life of patients or to prevent the transmission of mtDNA disease are discussed. Since no cure is known at the moment for mitochondrial diseases, only palliative therapies are applied. A number of therapeutic approaches are under development either to complement the genetic defect (for example by allotopic expression of mtDNA encoded proteins) or to reduce the mutation load of mtDNA mutations (for example by selective inhibition of the replication of mutant mtDNA or the stimulation of replication of normal mtDNA), which potentially can alleviate disease manifestations. Furthermore, possible approaches to prevent the transmission of mtDNA mutations, in case PND and PGD is not reliable, are being discussed. Techniques like nuclear transfer (NT) and cytoplasmic transfer (CT) are under development and are currently tested in animal models. Whatever option is considered, it should be technically safe, ethically evaluated and effective, as it is important to realise that the ultimate goal is still to improve the life of people who are dealing with serious illness whether they experience it themselves or with someone they care for.

Samenvatting

Mitochondriële Encephalomyopathieën zijn aandoeningen geassocieerd met deficiënties in het laatste deel van het mitochondriale energiemetabolisme, de zogenaamde oxidatieve fosforylering (OXPHOS). Recent epidemiologisch onderzoek heeft aangetoond dat ongeveer 1 op de 8000 mensen in de algemene populatie een OXPHOS-aandoening heeft. Daarom leiden OXPHOS-gerelateerde aandoeningen tot significante morbiditeit en mortaliteit. OXPHOS- aandoeningen kunnen worden veroorzaakt door mutaties in het mitochondriale DNA (mtDNA) of in nucleaire genen. Het voornaamste thema van dit proefschrift is het onderzoek naar enerzijds de somatische segregatie en familiale transmissie en anderzijds de oorsprong van heteroplasmische mutaties in het mtDNA. Deze worden onderzocht in relatie tot de klinische expressie en mogelijk modificerende factoren. Onze doelen zijn:

- het ontwikkelen van betrouwbare methodes voor het voorspellen en/of het voorkomen van de transmissie van mtDNA-mutaties, zoals prenatale diagnostiek (PND) en preïmplantatie genetische diagnostiek (PGD);
- het identificeren van factoren die ernst en fenotype of het mtDNA-mutatiepercentage beïnvloeden, om het effect van de overerving van mtDNA mutaties beter te kunnen voorspellen;
- het bepalen van de aanwezigheid en impact van de novo mtDNA-mutaties in eicellen.

Deze doelen zijn bereikt door het bestuderen van kenmerkende patiëntgegevens in samenhang met mtDNA-mutatiepercentage en -overerving en door het bestuderen van de mtDNA-mutaties in de meer unieke situatie van de individuele cel. De mtDNA-mutaties bestudeerd in individuele lymfocyten, fibroblasten en eicellen.

In het inleidende hoofdstuk 2 van dit proefschrift wordt ingegaan op de transmissie van OXPHOS-aandoeningen en methoden om overdracht te voorkomen. De expressie en segregatie van mtDNA-mutaties is afwijkend van die van nucleaire gendefecten en de meest karakteristieke kenmerken van de mitochondriale genen en mutaties worden belicht. Aandoeningen veroorzaakt door nucleaire genmutaties vertonen een normaal mendeliaans overervingspatroon en hebben vaak een meer constante klinische manifestatie dan aandoeningen veroorzaakt door mtDNA-mutaties. De heteroplasmische aard van veel pathogene mtDNA mutaties is een belangrijk kenmerk bij de manifestatie van de aandoening, alhoewel homoplasmische aandoeningen ook bestaan. In het geval van heteroplasmische mtDNA-mutaties worden de aandoeningen pas manifest als het mutatiepercentage een bepaalde weefsel specifieke drempelwaarde overschrijdt. Deze drempelwaarde kan variëren tussen weefsels en patiënten en het is daarom vaak niet mogelijk een exacte correlatie tussen het mutatiepercentage en het fenotypisch tot uiting komen van de aandoening te bepalen. Overerving van mtDNA-mutaties gaat bijna uitsluitend via de moederlijke lijn. Er zijn slechts enkele gevallen beschreven van overerving via de vaderlijke lijn. De (zeer) grote variaties in mutatiepercentage tussen broers en zussen, die al bestaat in de embryonale fase, wordt veroorzaakt door een genetische “flessenhals” (bottleneck) tijdens de vroege

eicelontwikkeling, waardoor slechts een beperkt aantal segregerende units de uiteindelijke samenstelling van het mtDNA in de eicel bepalen. Vanwege het ontbreken van een adequate therapie voor OXPHOS-aandoeningen wordt technisch en ethisch onderzocht of met bestaande en nieuwe methoden de transmissie van deze aandoeningen kan worden voorkomen. Potentiële benaderingen zijn prenataal onderzoek (PND), preïmplantatie genetische diagnostiek (PGD), cytoplasma transfer (CT) en kern transplantatie (NT).

In hoofdstuk 3 wordt het genetisch defect en de klinische gevolgen hiervan bij twee patiënten met Pearson-syndroom beschreven. Dit is een vaak fataal verlopende multisysteemaandoening die geassocieerd is met mtDNA-herschikkingen (o.a. deleties en duplicaties). Het doel was om factoren te identificeren die mogelijk betrokken zijn bij en de ernst kunnen voorspellen van de klinische manifestatie. Deze herschikkingen, voornamelijk deleties, zijn waarschijnlijk het gevolg van onjuiste paring tussen repeterende DNA-sequenties tijdens de mtDNA-replicatie in de zich ontwikkelende eicel. Een vergelijking wordt gemaakt tussen de aanwezigheid van deletiedimeren (een combinatie van twee gedeleteerde fragmenten) en duplicaties (een combinatie van een normaal en een gedeleteerd fragment) en de ernst van de aandoening. Duplicaties lijken een voordelig effect op de ernst van de ziekte te hebben. Door het voornamelijk *de novo* karakter van herschikkingen is de kans op transmissie vele malen kleiner dan voor mtDNA-puntmutaties.

Hoofdstuk 4 beschrijft een familie met drie kinderen met Leigh-syndroom, een progressieve neurodegeneratieve aandoening. De ziekte is gediagnosticeerd op grond van klinische kenmerken, lactaat acidose en een verlaging in de complex V-activiteit. De m.9176T>C mutatie in het ATPase6 gen van het mtDNA is ontdekt met mutatie-analyse. Omdat een maternaal familielid zwanger was, is de mogelijkheid van prenataal onderzoek, en voor eventuele volgende zwangerschappen preïmplantatie genetische diagnostiek, verder geëvalueerd. Het voornaamste probleem hierbij was het gebrek aan data m.b.t. de genotype-fenotype correlatie voor de m.9176T>C mutatie en de variatie van het mutatie-percentages in verschillende weefsels en in de tijd. Van een aantal maternale familieleden zijn aanvullende gegevens verzameld uit meerdere weefsels. Uiteindelijk is prenataal onderzoek aangeboden waarbij het paar bekend was met de bestaande onzekerheden ten aanzien van de interpretatie van de resultaten. Prenataal onderzoek is twee maal verricht op gekweekte en ongekweekte vlokken en vruchtwatercellen om weefsel- en tijds-gerelateerde effecten op het mutatiepercentage te kunnen evalueren. Het foetale mutatie-percentages was lager dan de mogelijke drempelwaarde van 90%, waarboven Leigh syndroom manifest wordt, maar hoger dan het enigszins arbitrair bepaalde veilige percentages van 60%. De ouders hebben besloten de zwangerschap te continueren en na een normale zwangerschap en zwangerschapstermijn is een gezond kindje geboren. Er kan geconcludeerd worden dat het probleem bij prenataal onderzoek van mtDNA-mutaties niet de techniek is, maar de interpretatie van de resultaten.

Om een beter inzicht te krijgen in de verdeling van mtDNA-mutaties tussen enkele cellen en het belang hiervan bij de counseling van dragers ten aanzien van het risico van herhaling, is de m.9176T>C mutatie op het individuele cel-niveau bestudeerd (hoofdstuk 5). Het mutatiepercentage is bepaald in individuele lymfocyten en fibroblasten van verschillende familieleden, patiënten en dragers, met de m.9176T>C mutatie. Onze resultaten tonen een van een normaal afwijkende verdeling in lymfocyten en fibroblasten van drie dragers met een matig mutatieniveau. Het patroon lijkt sterker afwijkend in de fibroblasten. Daarentegen lijkt het verdelingspatroon in lymfocyten en fibroblasten van de twee patiënten met zeer hoge mutatieniveaus (90%) erg op een normaal Gaussiaans verdelingspatroon. In één van de dragers wordt een extreem afwijkend patroon gevonden in de fibroblasten, waarbij percentages vooral richting 0% en 100% gaan. Alhoewel het bekend is dat heteroplasmie migreert naar homoplasmie van òf de mutatie òf het wild-type, tijdens celdeling, tonen onze gegevens aan dat dit verschilt tussen celtype en individuen en in eicellen sneller kan gebeuren dan verwacht. Dit zou kunnen betekenen dat er (genetische) factoren bestaan die het verdelingspatroon beïnvloeden. Als dezelfde factoren de mutatiebelasting in eicellen beïnvloeden, kan dit van belang zijn bij het bepalen van het herhalingsrisico. De drager met de extreem afwijkende verdeling heeft drie kinderen met erg hoge mutatie-niveaus en ernstige ziektesymptomen, wat past bij onze hypothese. Meerdere onderzoeken op het niveau van de individuele cel zijn nodig in grote families om de voorspellende waarde van het verdelingspatroon in individuele cellen voor het mutatieniveau bij nakomelingen te bepalen. De verdelingsverschillen kunnen mogelijk ook nog onbekende, mogelijk genetische, mechanismen onthullen die betrokken zijn bij de segregatie van mtDNA-mutaties. Dit kan ingangen opleveren om het heteroplasmieniveau te beïnvloeden.

Omdat een groot deel van de patiënten met OXPHOS-aandoeningen een *de novo*-mutatie in het mtDNA dragen en deze mutaties waarschijnlijk zijn ontstaan in de eicellen, zijn gezonde eicellen onderzocht op de aanwezigheid van heteroplasmische varianten in het mtDNA (hoofdstuk 6). Hiervoor werd gebruik gemaakt van twee gevoelige en nauwkeurige analysemethoden om varianten op te sporen. Het mtDNA van 26 eicellen is volledig gescreend op heteroplasmische varianten m.b.v. DHPLC-analyse. Tien verschillende heteroplasmische varianten, waarvan er één is gelokaliseerd in de D-loop en twee tweemaal zijn gevonden, zijn gedetecteerd in zeven eicellen waarbij het percentage varieerde van minder dan 5% tot 50%. Verder is het mtDNA (met uitzondering van de D-loop) van vier eicellen verkregen van één donor gescreend door gebruik te maken van een resequencing CHIP (Affymetrix MitoChip®), maar er zijn geen verschillen tussen de vier eicellen gedetecteerd. Er kan geconcludeerd worden dat heteroplasmische varianten in het mtDNA gangbaar zijn in eicellen en dat afhankelijk van de locatie in het mtDNA en het percentage dit het risico op een OXPHOS-aandoening in een vroeg of later levensstadium kan verhogen.

Uiteindelijk worden in de algemene discussie (hoofdstuk 7) de huidige strategieën bediscussieerd die worden gebruikt ter verbetering van de levenskwaliteit van patiënten of om

transmissie van mtDNA-aandoeningen te voorkomen. Omdat momenteel genezing niet mogelijk is bij mitochondriale aandoeningen, worden alleen palliatieve therapieën toegepast. Er zijn een aantal nieuwe therapeutische benaderingen in ontwikkeling die of het genetische defect aanvullen (bijvoorbeeld door de allotopische expressie van mtDNA gecodeerde eiwitten), of de mutatiebelasting van de mtDNA-mutatie verminderen (bijvoorbeeld door de selectieve remming van de replicatie van het gemuteerde mtDNA of de stimulatie van de replicatie van het normaal mtDNA). Deze therapieën kunnen mogelijk op termijn de ziekteverschijnselen verminderen. Verder worden er nog mogelijke benaderingen bediscussieerd, indien PND en PGD niet betrouwbaar kunnen worden uitgevoerd. Technieken zoals kerntransplantatie (NT) en cytoplasma-overdracht (CT), zijn in ontwikkeling en worden momenteel getest op dieren. Welke optie ook in beschouwing wordt genomen, wat belangrijk is, is dat nieuwe methoden met name uit technisch oogpunt veilig zijn, ethisch geëvalueerd en effectief. Het is van groot belang zich te blijven realiseren dat het uiteindelijke doel het verbeteren van de levenskwaliteit van mensen is, die te maken hebben met een ernstige aandoening, of ze deze nu zelf dragen of dat deze aanwezig is bij iemand voor wie ze zorgen.

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Curriculum Vitae

Lorraine Johanna Arnolda Maria Jacobs werd geboren op 26 mei 1975 te Bakel alwaar ze ook is opgegroeid en de lagere school heeft bezocht. Van 1987 to 1993 doorliep zij het voortgezet wetenschappelijk onderwijs (VWO) aan het Dr. Knippenberg college te Helmond. Alvorens aan haar opleiding Gezondheidswetenschappen aan de Universiteit van Maastricht te beginnen, heeft zij een jaar geneeskunde gestudeert aan de Universiteit van Antwerpen. Tijdens haar opleiding Gezondheidswetenschappen groeide de interesse in de genetica en heeft zij gekozen voor de afstudeer richting van de Biologische Gezondheidkunde welke in 1998 is afgerond met een genetische stage op een combinatie van de afdelingen Klinische Genetica en IVF van het academisch ziekenhuis van Maastricht. Aangezien het onderzoek haar erg goed beviel is zij na het behalen van haar doctoraal examen begonnen als assistent in opleiding (AIO) op de afdeling Genetica en Celbiologie, aan de faculteit der Geneeskunde van de Universiteit Maastricht. Het doel van haar onderzoek was Ontwikkeling van Pre-implantatie diagnostiek voor neuromusculaire aandoeningen veroorzaakt door mutaties in het mitochondriaal DNA. Gedurende deze periode als AIO heeft zij verschillende cursussen en trainingen gevolgd en certificaten behaald. Sinds 1 Februari 2006 is zij werkzaam als Study director voor Eurofins Medinet B.V..

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