

The transmission of OXPHOS disease and methods to prevent this

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Diseases owing to defects of oxidative phosphorylation (OXPHOS) affect approximately 1 in 8000 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 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.

Key words: mitochondria/OXPHOS disease/PGD/PND/transmission

Mitochondrial disorders

Mitochondrial disorders are a group of diseases and syndromes commonly defined by lack of energy owing 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 disease (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 because of 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 multi-organ 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.

Mitochondrial DNA

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 mitochondrial DNA (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 and

Table I. Common examples of oxidative phosphorylation (OXPHOS) syndromes

Syndrome	Genetic cause	Symptoms (clinical phenotype, age of onset)
Leber's hereditary optic neuropathy (LHON)	Homoplasmic/heteroplasmic mitochondrial DNA (mtDNA) mutation (the most common are the G11778A, G3460A and T14484C mutations) Direct mutation analysis using restriction enzyme digestion	Subacute bilateral loss of vision, sometime accompanied by dystonia and cardiac pre-excitation syndromes
Mitochondrial encephalopathy, lactic acidosis and stroke like episodes (MELAS)	Heteroplasmic mtDNA mutation (the most commonly reported is the A3243G mutation)	Stroke like episodes with seizures and/or dementia and ragged red fibres (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
Myoclonus epilepsy with RRF (MERRF)	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 Age of onset is variable, usually in late childhood to adulthood
Neuropathy, ataxia and retinitis pigmentosa (NARP)	Heteroplasmic mtDNA mutation (the most commonly reported is the T8933G/C mutation)	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 magnetic resonance imaging (MRI) abnormalities with bilateral symmetric signal alterations in the basal ganglia, thalamus, midbrain and brainstem Age of onset between 3 months and 2 years
Progressive external ophthalmoplegia (PEO)	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
Kearns–Sayre syndrome (KSS)	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 vacuolization of marrow precursors, pancytopenia and exocrine pancreatic failure often accompanied by ophthalmoplegia, lactic acidosis and RRF
Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)	Nuclear gene mutations in the thymidine phosphorylase gene or the polymerase γ gene	Ptosis, progressive external ophthalmoplegia, gastrointestinal dysmotility (often pseudoobstruction), diffuse leukoencephalopathy, thin body habitus, peripheral neuropathy and myopathy Onset is usually in late childhood or adulthood
Mitochondrial depletion syndrome (MDS)	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, hypoglycaemia and increased lactate in body fluids Onset is within a few weeks after birth and patients die before 9 months of age

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 transfer RNA (tRNA) and 2 ribosomal RNA (rRNA) genes required for mitochondrial translation (Clayton, 1991; Wallace *et al.*, 1995). Approximately 6% of the mtDNA is non-coding, 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).

MtDNA molecules are packed in somatic cells as nucleoids in which six to ten molecules form a group with several different proteins (Jacobs *et al.*, 2000; Iborra *et al.*, 2004; Legros *et al.*, 2004). These nucleoids are not static entities, and mtDNA molecules exchange between nucleoids. The nucleoids are attached to the inner mitochondrial membrane near the OXPHOS system,



Figure 1. Map of the human mitochondrial DNA (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 (O_H) and L (O_L) strands is indicated as well as the location of the promoter site where transcription is initiated; HSP, H-strand promoter; LSP, L-strand promoter.

where reactive oxygen species (ROS) are produced (Richter *et al.*, 1988). Because of the lack of histones and other protective proteins and an ineffective repair mechanism, the mtDNA mutates 10–16 times more frequently as the nuclear DNA, and because of 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 termed ‘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 the removal and introduction of supercoils in the mtDNA, respectively (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 also 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 transacting 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 include, e.g. 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 *et al.*, 2001a,b). 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 (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.* (2002) including clinical features and enzyme activities found in several groups of patients.

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 nonprotein 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 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 to 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 *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 into 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)] and 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 cannot be explained by the heteroplasmy level only, and so 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 mitochondrial DNA (mtDNA) point mutations

Disease	MtDNA mutation	Homo/heteroplasmy	Reference
Leber's hereditary optic neuropathy (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)
Neuropathy, ataxia and retinitis pigmentosa (NARP)/Leigh syndrome	m.8993T>G/C	Heteroplasmic	Holt <i>et al.</i> (1990)
Myoclonic epilepsy with ragged red fibres (MERRF)	m.8344A>G	Heteroplasmic	Shoffner <i>et al.</i> (1990)
Mitochondrial encephalopathy, lactic acidosis and stroke like episodes (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)
Kearns–Sayre syndrome (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. MtDNA-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, 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 (Lee *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 mouse 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, e.g. pharmacologically induced by antiviral nucleoside analogues, 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 tRNAs. 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, e.g., 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 five- to six-fold during the spermatogenesis, probably because of a down-regulation of the mitochondrial 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 the 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 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 affect 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.* (1998) observed a higher incidence of especially the 4977 bp 'common' mtDNA deletion, in semen with a lower motility, 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 accumulates. 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; Akin-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 many females suffering from CPEO caused by POLG mutations (Luoma *et al.*, 2004), probably because of a link with steroid hormone genesis (Bose *et al.*, 2002). These data are confirmed by the mutator mice, carrying a proofreading-deficient polymerase gamma, which show reduced fertility of both male and female mice (Trifunovic *et al.*, 2004).

Mitotic segregation 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 because of 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). Because 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 high levels (>50%) with most oocytes containing low level mutation percentages (<30%) (Jacobs *et al.*, in preparation). 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 can be 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 mutations 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 fertilizability as carriers of mtDNA mutation do not present with fertility problems, and children with a high mutation load are 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.* (2001) have shown a lowered number of mitochondria in oocytes from patients with fertilization failure owing to unknown causes, 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.*, 1999a,b; 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 binomial 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.* (2004) 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 mouse 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 1980s, 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, 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).

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 3-week-old embryo. The number of mitochondria is estimated from published electron micrographs of PGCs (Jansen and de Boer, 1998). It cannot 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

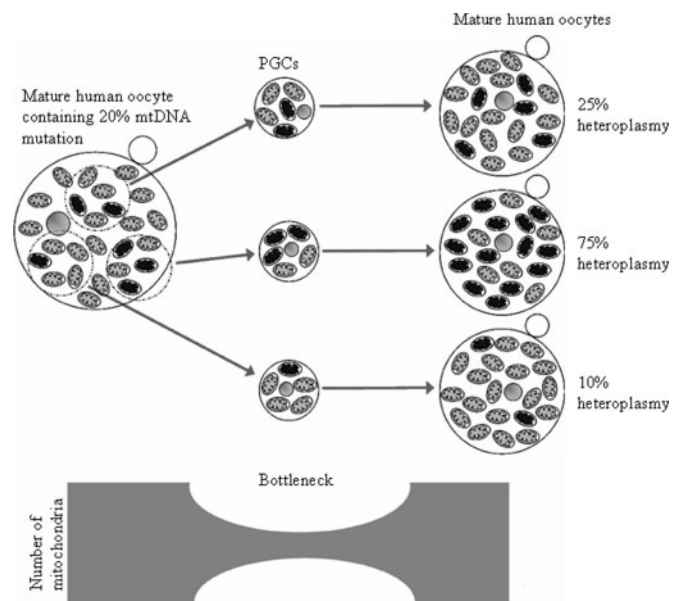


Figure 2. Schematic drawing of the possible location and effect of the bottleneck on the transmission of a mitochondrial DNA (mtDNA) mutation. The light coloured mitochondria represent the normal mtDNA, and the darker coloured mitochondria represent the mutated mtDNA. PGC, primordial germ cell.

observed (Michaels *et al.*, 1982; Chen *et al.*, 1995). In mice and frogs, there is no mtDNA synthesis during embryogenesis until the stage of gastrulation (Larsson *et al.*, 1998; Jansen, 2000) except for a small period during the one- to two-cell stage of mouse pre-implantation 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.* (1996), in which BALB/c cytoplasm was introduced in NZB/BINJ oocytes. The mtDNA variants remain evenly distributed in the developing fetal 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 PGC 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 two mouse 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 the 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 because of the replication advantage of the smaller mtDNA molecule. A percentage above 90% has not been found and may cause lethality in oocytes or embryos. Severe disease and COX-negative fibres were only found in mice 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, including protein complex genes, radical scavenger genes (Sod2 mutant mouse), transcription factors (Tfam-deficient mouse) and 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 families (Thorburn and Dahl, 2001). As these disorders cannot 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.

PND

PND of OXPHOS disease can be performed at the level of the enzyme or at the DNA level. Although the latter is preferable, the

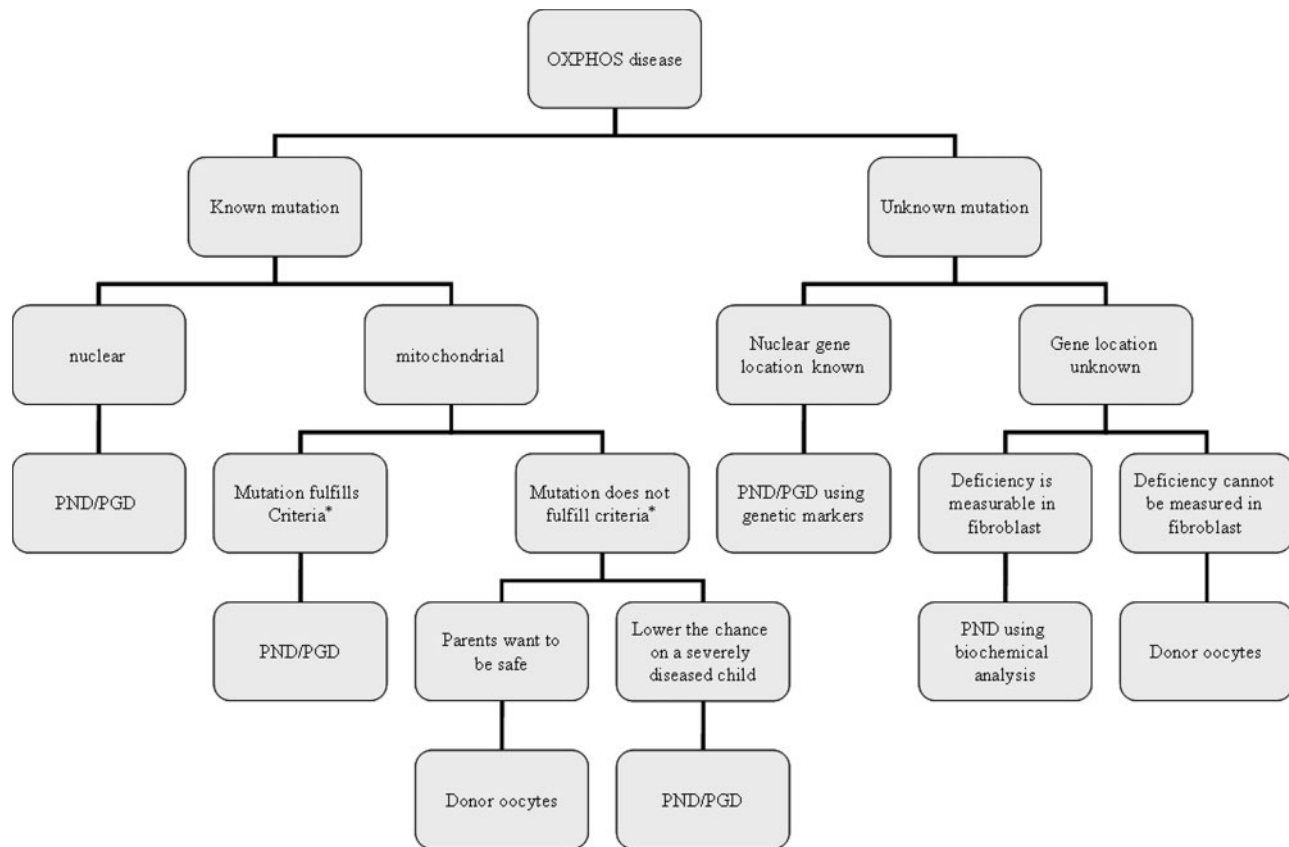


Figure 3. Scheme presenting the possible causes of oxidative phosphorylation (OXPHOS) disease and options for the prevention of transmission of these diseases. PGD, preimplantation genetic diagnosis; PND, prenatal diagnosis. *, criteria developed by the European NeuroMuscular Consortium concerning prenatal options for carriers of mitochondrial DNA (mtDNA) mutations.

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 fetal samples is feasible, although the methods used must be sufficiently sensitive given the low amount of fetal 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 sampling (CVS) and/or amniotic cells. Where only the causing gene and location are known, but not the exact mutation, intragenic or closely linked polymorphic markers are 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 fetal 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 three mutations (m.8993T>G/C, m.8344A>G and m.3243A>G) to judge these criteria properly. For the m.8993T>G/C and m.8344A>G mutations, 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 is 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 mutations (Table III). Also PND for mtDNA rearrangements is becoming an issue, as the recurrence risk for mtDNA-deletion disorders appears to be around 4% (Chinnery *et al.*, 2004), and two PND have been performed (Table II). MDSs 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

Table III. Prenatal diagnosis (PND) of different oxidative phosphorylation (OXPHOS) diseases using biochemical or genetic methods

Disease	Mutation	Method	Result	Reference
Pyruvate carboxylase (PC) deficiency	Unknown	Direct measurement of PC activity in chorionic villus sampling (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/sodium dodecyl sulphate (SDS)] polyacrylamide gel electrophoresis (PAGE) to analyse activity and composition of the OXPHOS complexes	An enzymatic defect was ruled out and two (twin) healthy children were born	Housteck <i>et al.</i> (1999)
NADH: ubiquinone oxidoreductase (complex I) deficiency	Unknown	Biochemical assays of complex I in fetal tissues (native and cultured CVS)	Twenty-three pregnancies analysed. Fifteen healthy babies born, three known to be affected children born and five 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 deficiency	E214K/IVS8+4A>C NDUFV1 gene A432P/ΔTC (989–990) NDUFV1 gene A524V/M1L SDH- <i>Fp</i> gene P174L/ΔGA (363–364) SCO1 gene G180E/IVS6–1G>C SURF1 gene	Sequence analysis*	Elective termination of affected fetus Fetus was heterozygous, pregnancy continued Fetus was homozygous normal, spontaneous abortion Fetus was heterozygous, pregnancy continued Fetus was homozygous normal, pregnancy continued	Amiel <i>et al.</i> (2001)
Neuropathy, ataxia and retinitis pigmentosa (NARP)	m.8993T>G	Direct mutation analysis using restriction enzyme digestion	Two pregnancies analysed both revealed high heteroplasmy percentages (>80%) and were terminated	Harding <i>et al.</i> (1992)
NARP	m.8993T>G	Direct mutation analysis using restriction enzyme digestion in amniocytes	Mutation percentages were between 64% and 68%. Pregnancy was continued and the baby is healthy at 4 months.	Bartley <i>et al.</i> (1996)
Leigh syndrome	m.8993T>G	Direct mutation analysis using restriction enzyme digestion	A near homoplasmic level was found in fetus (both CVS and fetal cells), and the pregnancy was terminated	Ferlin <i>et al.</i> (1997)
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)
Mitochondrial encephalopathy, lactic acidosis and stroke like episodes (MELAS)	m.3243A>G	Direct mutation analysis using restriction enzyme digestion	Amniotic fluid cells revealed a high mutation percentage comparable with that of the affected mother and sibling, but at the age of 4 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)
Progressive external ophthalmoplegia (PEO)	5 kb mtDNA deletion (nt 9986–nt 15042)	Southern blot analysis of the entire mtDNA	No ΔmtDNA was detected in CVS, and an apparently healthy baby was born	Graff <i>et al.</i> (2000)
Kearns–Sayre syndrome (KSS)	ΔmtDNA	Southern blot analysis of the entire mtDNA	No ΔmtDNA was detected in CVS, and an apparently healthy baby was born	Thorburn and Dahl (2001)

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

studied and were found to express the mtDNA depletion (Blake *et al.*, 1999). The exact timing of onset of mtDNA depletion during fetal development is still unknown. A second report describes two cases of mtDNA depletion presenting prenatally with skin oedema and diminished fetal movements at 36 weeks of pregnancy (Arnon *et al.*, 2002).

PGD/polar body analysis

PGD is an alternative to PND. Oocytes are fertilized *in vitro*, and cells from the usually eight-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 with 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 because of 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 dropout (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 two-, four-, and six- to eight-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 dropout 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 IVF 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).

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, because 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 of the ICSI technique (Cohen *et al.*, 1997, 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, 2001). This resulted in 13 clinical pregnancies from which one was spontaneously aborted and one selectively aborted in a twin pair, both because of Turner's 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-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 1-year-old children born after CT, donor mtDNA was demonstrated in blood of only 2 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 into 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 are often liable to serious defects (Cibelli *et al.*, 2002), like e.g. 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 unfertilized oocyte is transferred into an enucleated donor oocyte which subsequently is fertilized using the partner's sperm (Roberts, 1999). Second, the nucleus of a blastomere cell is transferred into 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 because of the transfer of an embryo nucleus. This has been proven in animal models after somatic cell NT (Jaenisch, 2004) and could affect the embryo cell

NT although these effects are probably minor because the major part of the imprinting takes place later during embryonic development.

Ethical considerations

Most of the methods to prevent the transmission of OXPPOS disease are relatively new or in a developmental phase and have considerable ethical implications. PND and PGD are controversial in themselves, mainly because of the inherent selection of fetuses/embryos. Ethical questions imply concern to 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. The criticism that PND and PGD are at odds with the interests of handicapped people in our society, is not convincing, as these techniques do not deny the dignity and equal rights of handicapped citizens nor necessarily undermine 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 OXPPOS 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, 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 possible implication of an inconclusive mutation percentage or a mutation percentage above the determined threshold for disease with the parents. A question for further debate is 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 (by 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 oocyte 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 (Ethics Committee of the American Society for Reproductive Medicine, 2004). A growing number of countries acknowledge the moral and legal right of these children 'to know their origins'. A major practical problem exists regarding the shortage of oocyte 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 OXPPOS 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 in most countries. 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 mtDNA. Would it, therefore, be justified to ethically differentiate this therapy from possible therapeutic germ-line 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 unfertilized oocyte 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, this would involve embryo cloning—even if one were to create only a single additional embryo. The fact that the newly created embryo would have its own mtDNA 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 which 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. Some people may object that this is unnatural. How to ethically evaluate the possible adverse social, psychological, and/or legal consequences, especially if a major part or all of the oocyte's 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, whereas the major genetic part, the nucleus, is provided by the woman who will be both the gestational and rearing mother? and last, but not the least: how to weigh up 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 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, owing 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 most mtDNA mutation 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 chance of preventing the transmission of OXPHOS disease to their children and will reduce the number of people affected by it.

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