MITOCHONDRIAL DNA VARIATION IN EXTREMELY SELECTED TRAITS: LONGEVITY AND ELITE ATHLETIC PERFORMANCE

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Abstract

Mitochondria contain a maternally inherited 16,568bp genome (mtDNA) that encodes for 13 out of more than 70 subunits of complexes of the respiratory chain that produce ATP by oxidative phosphorylation (OXPHOS). As a byproduct of OXPHOS, reactive oxygen species (ROS) are formed, which may play a role in ageing. MtDNA has accumulated numerous polymorphisms during evolution, leading to haplogroups characterized by ancient polymorphisms and defined by letters. MtDNA polymorphisms are thought to be neutral, but some may be slightly deleterious or even advantageous and may influence phenotypes of complex traits. Interestingly, several complex traits such as longevity and maximal aerobic power show maternal inheritance. Associations between mtDNA polymorphisms and longevity have been reported, but no systematic study has been made of the role of mtDNA in longevity. In addition, there are no previous reports on mtDNA haplogroups in elite athletic performance.

Associations are demonstrated here between mtDNA haplogroups J, K and U and longevity in Finns. Interestingly, subhaplogroup J2 and haplogroup K, which were found in increased frequency among the 225 very old subjects studied, were not found among the 52 endurance athletes but were present in 11% of the 89 sprint athletes Uncoupling of OXPHOS reduces ATP and ROS production. Thus, a mitochondrial genome with a higher level of uncoupling may promote longevity but may not be favourable in situations that require a high level of ATP production, such as elite endurance performance. A more detailed analysis also showed an association between a combination of three common mtDNA polymorphisms and longevity in both the Finns and the Japanese, providing the first epidemiological support for the assumption that the nature of a mutation is determined by interactions with other mutations in mtDNA. In addition, a systematic approach was applied to study the role of mtDNA in longevity. Association analyses of mtDNA allele combinations in longevity revealed that the mtDNA control region, the tRNA and rRNA genes and the nucleotide repeats in mtDNA may play a role in longevity, since the alleles and allele combinations that showed the strongest associations with longevity, either negative or positive, were among these genes. Differences in overall variation in mtDNA between the very old and their controls were also studied, revealing more differences at synonymous (silent) sites than at non-synonymous (amino acid altering) sites.

The findings support previous data suggesting that certain mtDNA haplogroups are associated with longevity. In addition, those haplogroups that increased in frequency among the very old Finns were not found among Finnish endurance athletes. Also, a novel systematic approach was applied to study mtDNA alleles, allele combinations and overall sequence variation in longevity, suggesting that there are interactions between various mtDNA positions and that the tRNA and rRNA genes and short tandem repeats in mtDNA may play a role in longevity.

Keywords: adaptive evolution, association, epistasis, haplogroup, phylogenetic network

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"Do what you believe in and believe in what you do. Everything else is a waste of time and energy" ~Nisargadatta~

With love and hugs,

Oulu, May 2005 Anna-Kaisa Niemi

Abbreviations

ADP	adenosine diphosphate
ACE	angiotensin-converting enzyme
ACTN	alpha-actinin gene
ApoE	apolipoprotein E
ATP	adenosine triphosphate
bp	base pair
CoA	coenzyme A
COX	cytochrome c oxidase
CPEO	chronic progressive external ophthalmoplegia
CRS	Cambridge reference sequence
CSB	conserved sequence block
CSGE	conformation-sensitive gel electrophoresis
D-loop	displacement loop
DNA	deoxyribonucleic acid
FADH	reduced flavin adenine dinucleotide
FMN	flavin mononucleotide
HGPS	Hutchinson-Gilford progeria syndrome
HSP	heavy strand promoter
HVS	hypervariable segment
KSS	Kearns-Sayre syndrome
LHON	Leber's hereditary optic neuropathy
LMNA	lamin A gene, mutated in Hutchinson-Gilford progeria syndrome
LNA	locked nucleic acid
LSP	light strand promoter
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonus epilepsy with ragged-red fibres
MRCA	most recent common ancestor
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NADH	reduced nicotine adenine dinucleotide
NARP	neurogenic weakness, ataxia and retinitis pigmentosa

nDNA	nuclear DNA
O_H	origin of replication of the heavy strand of mtDNA
O_L	origin of replication of the light strand of mtDNA
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PEO	progressive external ophthalmoplegia
POLG	human mtDNA polymerase gamma gene
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
RNA	ribonucleic acid
rRNA	ribosomal RNA
RRF	ragged red fibres
SDH	succinate dehydrogenase
SOD	superoxide dismutase
SNP	single nucleotide polymorphism
TFAM	mitochondrial transcription factor A
tRNA	transfer RNA
UCP	uncoupling protein
YBP	years before present
WRN	human RecQ helicase gene, mutated in Werner's progeria syndrome
WS	Werner's progeria syndrome

	alpha
	beta
?	gamma
	epsilon

List of original manuscripts

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

I Niemi AK, Hervonen A, Hurme M, Karhunen PJ, Jylhä M, Majamaa K (2003) Mitochondrial DNA polymorphisms associated with longevity in a Finnish population. Hum Genet 112:29-33.

II Niemi AK, Moilanen J, Tanaka M, Hervonen A, Hurme M, Lehtimäki T, Arai Y, Hirose N, Majamaa K (2005) A combination of three common inherited mitochondrial DNA polymorphisms promotes longevity in Finnish and Japanese subjects. Eur J Hum Genet 13: 166-170.

III Moilanen J, Niemi AK, Tanaka M, Fuku N, Nishigaki Y, Takeyasu T, Fujita Y, Oshida Y, Hamada N, Ito M, Arai Y, Hirose N, Hervonen A, Majamaa K. Systematic assessment of mtDNA sequence variation in longevity by combinatorial partitioning, permutation tests. Submitted for publication.

IV Niemi AK, Majamaa K. Mitochondrial DNA and ACTN3 genotypes in Finnish elite endurance and sprint athletes. Eur J Hum Genet. In press.

Contents

1 Introduction

Mitochondria are small cytoplasmic organelles that contain their own DNA (mitochondrial DNA, mtDNA), which is a maternally inherited circular genome of 16,568bp. Each cell contains thousands of mitochondria and each mitochondrion 2-10 copies of mtDNA, so that every one of our cells contains thousands of copies of mtDNA. MtDNA encodes for essential subunits of the respiratory chain, which is composed of five complexes (I-V), of which complexes I, III, IV and V have subunits encoded by mtDNA. The respiratory chain is responsible for the production of ATP by oxidative phosphorylation (OXPHOS), the main function of mitochondria, to meet cellular energetic needs. In addition, the mitochondria are important for thermogenesis, calcium homeostasis, apoptosis, iron homeostasis, various pathways of intermediary metabolism and cellular signalling. Mitochondria also produce reactive oxygen species (ROS) as a byproduct of OXPHOS.

The mitochondria are the major site of ROS production in cells. ROS have deleterious side effects, as they cause damage to nucleic acids, proteins and lipids, and the mitochondrial DNA and mitochondrial membranes are especially vulnerable since they are directly exposed to the ROS produced during cellular respiration. ROS are involved in various pathological processes such as carcinogenesis and neurodegeneration, and are thought to be important in the ageing process. The free radical theory of ageing states that "ageing and degenerative diseases associated with it are attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connective tissues" (Harman 1956). This theory is supported by the observations that variations in the lifespans of various animals are correlated with protective antioxidant capacity, and that cellular free radical damage increases with age. Also, oxidative damage to the mtDNA increases with age.

Uniparental inheritance and a high mutation rate have led to continent-specific mtDNA lineages (haplogroups), which are defined by ancient polymorphisms and characterized by considerable variation. During evolution mtDNA lineages have accumulated a great number of polymorphisms, which are thought to occur randomly and to be neutral, but there may also be polymorphisms in mtDNA that are slightly deleterious or advantageous, and are fixed in certain mtDNA lineages. These alleles may influence or determine the clinical phenotypes of many complex traits. Interestingly,

several complex traits show maternal inheritance, suggesting that mutations in mtDNA may contribute to their phenotypic expression. Examples of at least partly maternally inherited complex traits are longevity, type 2 diabetes, epilepsy and maximal aerobic power. The frequency of mtDNA haplogroups has been shown to differ between patients and controls, e.g. in cases of sperm dysmotility related to male infertility, migraine and Leber's hereditary optic neuropathy, indicating that the haplogroup background may have slightly deleterious polymorphisms and modify susceptibility to such diseases.

In addition to slightly deleterious polymorphisms, there may also be advantageous polymorphisms resulting from adaptive evolution, which may increase the chances of an advantageous phenotype occurring or reduce the risk of a deleterious phenotype. One example of an advantageous phenotype is longevity and an association has been suggested between this and haplogroup J in Italian subjects, haplogroups J and K in Irish ones and haplogroup D in Japanese ones. There may also be lineage-specific combinations of advantageous polymorphisms, which could explain why a beneficial effect is seen only in certain haplogroups.

OXPHOS is a major contributor to ATP synthesis in endurance exercise but not in sprint events, where the anaerobic breakdown of muscle glycogen to lactate produces a large proportion of ATP. Among the most common signs of diseases caused by defects in mtDNA are exercise intolerance, muscle weakness and production of lactate due to inefficient OXPHOS. Thus there may be genetic differences between sprinters and endurance athletes that determine aerobic performance, e.g. differences in mitochondrial DNA.

The aim here was to assess the role of mtDNA in two highly selected traits: longevity and elite athletic performance by comparing the frequencies of mtDNA haplogroups between Finnish nonagenarians and controls and between Finnish elite endurance and sprint athletes. MtDNA nucleotide variation was also studied in Finnish and Japanese very old and control populations and the significance of each nucleotide site, of allele combinations and of the overall nucleotide variation in mtDNA for longevity was evaluated.

2 Review of the literature

2.1 Mitochondria

Mitochondria are small cytoplasmic organelles that produce energy aerobically in the eukaryotic cells of animals and plants. According to the traditional endosymbiont theory, the incidents that started the evolution of mitochondria took place more than a billion years ago, when an aerobic bacterium entered a primordial eukaryotic cell and became a symbiont partner responsible for aerobic metabolism. A more recent theory, however, suggests a "big bang" in the formation of eukaryotes: an anaerobic bacterium fused with an aerobic respiration-competent proteobacterium to form a primitive eukaryote from which all present eukaryotic cells derive (Gray *et al.* 1999, Vellai & Vida 1999).

The first descriptions of mitochondria in cells were produced in the 19th century, when granular elements and inclusions were noticed in the cytoplasm of the cells. These were called *sarcosomes* at first, since they were thought to occur only in muscle cells, where they are indeed most numerous. Filamentous structures, called *fila,* were found in the cytoplasm of many cells in the 1890's, and the name *mitochondrion,* derived from the Greek words *mitos* (thread) and *chondros* (grain), was first introduced by Benda at the end of the century. Other names used for mitochondria included blepharoblasts, chondriosomes, mitogel, parabasal bodies, plasmasomes, chondriokonts and vermicules. Mitochondria were recognized as a site for aerobic respiration in the 1950's, and DNA was found inside them in the 1960's. The complete sequence of a mammalian mitochondrial DNA, the genes encoded by it and the first molecular cause of mitochondrial diseases were identified in the 1980's (Scheffler 2000, Nisoli *et al.* 2004).

Mitochondria are unique cellular organelles because they contain their own DNA (mitochondrial DNA, mtDNA). This is maternally inherited and encodes for essential subunits of the respiratory chain that are responsible for aerobic energy production. Each eukaryotic cell contains several hundreds to thousands of mitochondria and each mitochondrion 2-10 copies of mtDNA. Therefore, every one of our cells contains thousands of copies of mtDNA, as compared with only two copies of each gene, one from the mother and one from the father, in the nucleus.

The main function of the mitochondria is to produce energy, in the form of ATP, from glucose and fatty acids by oxidative phosphorylation (OXPHOS). The OXPHOS system also produces heat, by compromising ATP production. Mitochondria are also involved in

many other cellular functions, such as calcium homeostasis, apoptosis (programmed cell death), iron homeostasis, various pathways of intermediary metabolism and cellular signalling. Mitochondria produce reactive oxygen species (ROS), which have deleterious side effects and are thought to contribute to cellular ageing.

2.1.1 Structure

Mitochondria have traditionally been regarded as bean-shaped, bacteria-sized (1-2 µm by 0.5-1.0 µm) organelles, but it is now known that they are dynamic organelles, which undergo continuous fusion and fission and that they vary greatly in size and shape, even forming complex branched networks (Shaw & Nunnari 2002, Karbowski & Youle 2003). Mitochondria are most numerous in the cells of tissues that have high aerobic energetic demand, such as neurons, skeletal muscle, heart muscle, the liver and the cochlea. There may be more than 1000 mitochondria in a single liver cell, for example. Also, the number of mitochondria within a cell can changes in response to various environmental stimuli such as exercise training, which increases the number in muscle cells (Hood 2001, Hoppeler & Flück 2003). Mitochondria are most abundant in type I muscle fibres (slow-twitch, high aerobic and low glycolytic capacity) and in type IIa (fast twitch, high aerobic and high glycolytic capacity) than in type IIb (fast twitch, low aerobic, high glycolytic capacity) (Sjöström *et al.* 1982, Philippi & Sillau 1994).

Mitochondria contain two membranes, an inner (IM) and an outer (OM) membrane, separated by a space called the intermembrane space (IMS). The IM surrounds a space called the matrix, while the OM completely encloses the mitochondrion, serving as its outer boundary. The OM contains porins, integral membrane proteins that form large, non-selective membrane channels, and it is especially permeable and allows large molecules to pass freely into the IMS. The IM lies beneath the OM but has deep folds or invaginations called cristae, which greatly increase the amount of surface available to the proteins responsible for aerobic respiration. The IM is highly impermeable, and therefore virtually all molecules and ions require special transporters. The IM contains the enzyme complexes of oxidative phosphorylation for the synthesis of ATP. The internal structure of mitochondria is traditionally depicted as a “baffle model” in which the cristae of the IM form folds similar to the bellows of an accordion (Palade 1952, Sjöstrand 1956). Electron microscopic (EM) tomography studies have shown, however, that the cristae are pleomorphic, form lamellar and tubular structures and are connected to the IM by crista junctions (Frey & Mannella 2000).

The matrix, the inner aqueous compartment of mitochondria, contains a variety of enzymes of intermediary metabolism, including those of the citric acid cycle and fatty acid oxidation that provide substrates for the OXPHOS system. It also contains various other proteins, ribosomes, tRNAs and mitochondrial DNA.

2.1.2 Function

2.1.2.1 Production of ATP

The main function of mitochondria is to provide energy for cellular processes by forming ATP in the process of oxidative phosphorylation (OXPHOS). The respiratory chain, composed of five multimeric enzyme complexes (I-V), is located in the IM and couples the reduction of oxygen to water with synthesis of ATP (Figure 1). Reducing equivalents (electrons) are carried along the respiratory chain, and complexes I, III and IV pump protons from the matrix into the intermembrane space, creating a chemical proton gradient and an electrical membrane potential across the inner membrane. Complex V uses this electrochemical gradient to synthesize ATP from ADP and inorganic phosphate.

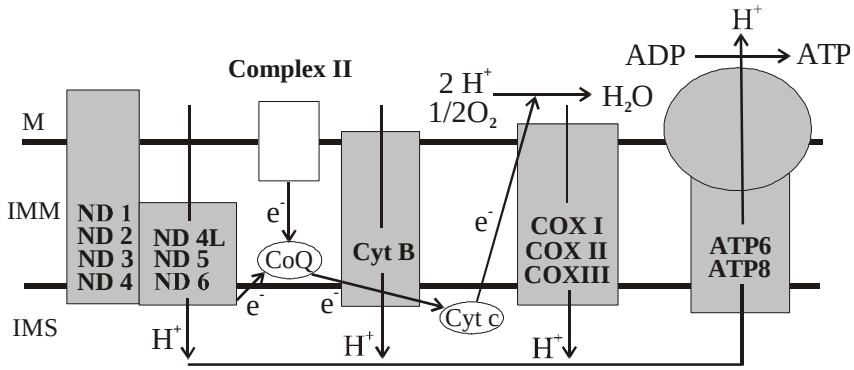
Complex I (NADH dehydrogenase) is composed of more than 40 protein subunits, of which seven, ND1, ND2, ND3, ND4, ND4L, ND5 and ND6, are encoded by mtDNA, and it also includes a flavin mononucleotide (FMN) and six iron-sulphur (Fe-S) centres. It pumps protons across the IM by the oxidation of NADH to NAD^+ and the reduction of ubiquinone into ubiquinol.

Complex II (succinate dehydrogenase) is composed of four subunits, all encoded by nuclear DNA. It contains flavin-adenine dinucleotide (FAD), three Fe-S centres and cytochrome b. It is not involved in proton pumping but transfers electrons from succinate to ubiquinone, reducing the latter to ubiquinole.

Complex III (cytochrome bc1 complex) is composed of eleven subunits, of which one, cytochrome b is encoded by mtDNA (MTCYTB gene). It also contains a Rieske Fe-S centre and cytochrome c1, which are involved in electron transfer, the rest of the protein subunits being responsible for binding ubiquinone. Complex III pumps protons across the IM and oxidates ubiquinole back to ubiquinone in a reaction that reduces cytochrome c, which transfers electrons from complex III to complex IV.

Complex IV of the respiratory chain (cytochrome c oxidase) is composed of thirteen subunits, of which three, COX1, COX2, COX3, are encoded by mtDNA, and it also contains cytochrome $a+a_3$, CuA and CuB, which are involved in electron transfer to molecular oxygen, reducing it to water. Complex IV also pumps protons across the IM.

Complex V (ATP synthase) uses the proton gradient formed by complexes I, III and IV to produce ATP, the most usable form of energy in living cells. It has fourteen subunits, of which two, ATP6 and ATP8, are encoded by mtDNA.



	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA encoded subunits	7	0	1	3	2
nDNA encoded subunits	~40	4	10	10	~14

Fig. 1. Mitochondrial respiratory chain complexes. Complexes I, III, IV and V (grey) contain subunits encoded by mtDNA. These are depicted in bold font in the respective complexes. M = mitochondrial matrix. IMM = inner mitochondrial membrane. IMS = intermembrane space.

The protons that respiratory chain complexes translocate across the IM are derived from the hydrogen atoms of carbohydrates and fatty acids, which are the main sources of energy in our food. Fatty acids are translocated across the inner mitochondrial membrane into the matrix as acyl-carnitines and transformed into acyl-CoAs, the substrates of -oxidation, which produces acetyl-CoA. Glucose, from carbohydrates, is transformed into pyruvate in glycolysis and the pyruvate is then translocated into the mitochondrial matrix, where it is transformed to acetyl-CoA. The acetyl-CoA is then taken up by the citric acid cycle (Krebs cycle), which is the final common pathway for the hydrogen-containing foodstuffs in our diet. The citric acid cycle produces NADH (reduced nicotine adenine dinucleotide) and $FADH_2$ (reduced flavin adenine dinucleotide) for the use of complex I and complex II of the respiratory chain. (Figure 2)

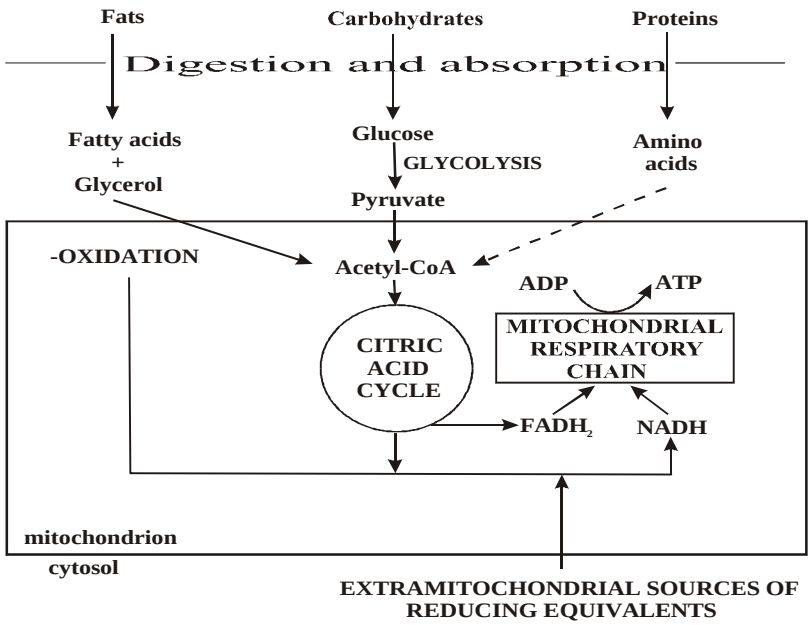


Fig. 2. Oxidative generation of ATP from carbohydrates, lipids and proteins. Oxidation of the major foodstuffs generates reducing equivalents (NADH, $FADH_2$), which are collected by respiratory chain complexes to generate ATP by oxidative phosphorylation.

2.1.2.2 Thermogenesis

The mitochondria also produce heat. The electrochemical gradient across the IM formed in proton pumping is normally coupled to oxidative phosphorylation, and thus to ATP production. The membrane potential can also be discharged, however, by allowing the protons to leak back into the matrix without the involvement of ATP synthase. In thermogenesis, therefore, the energy contained in our food in the form of hydrogen (protons) is used for the production of heat but not ATP, making these two mutually exclusive events. Thermogenesis can be achieved by either extrinsic or intrinsic uncoupling of oxidative phosphorylation (Skulachev 1998, Kadenbach 2003). The artificial extrinsic uncouplers include arsenate, 2,4-dinitrophenol (DNP), carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), valinomycin, and gramicidin D, while the natural extrinsic uncouplers are the fatty acids, calcium, thyroid hormones and uncoupling proteins (thermogenins, UCP), for example. Intrinsic uncoupling means that the proton pumping enzymes of the respiratory complexes (I, III, IV and V) allow protons to “slip” through the IM without coupling it to oxidative phosphorylation (Kadenbach 2003). Uncouplers reduce the electrochemical gradient required for ATP synthesis by making the IM permeable to protons.

The capability for heat production is important in cold environments, the tissue specialized in additional heat production in mammals under cold conditions being the brown fat. The function of brown adipose tissue is to convert energy from food into heat, and the brown fat is essential for both the classical non-shivering thermogenesis and cold acclimation-recruited norepinephrine-induced thermogenesis. Extra heat is needed during neonatal period, for example, and upon entry into a febrile state or arousal from hibernation in animals. Brown fat also allows for an active life even in cold surroundings, by "burning" large amounts of lipids and glucose. The development of brown adipose tissue with its characteristic protein, uncoupling protein-1 (UCP1), was probably vital for the evolutionary success of mammals (Cannon & Nedergaard 2004), and it has also been suggested that the evolution of the mitochondrial genome was influenced by climate (Ruiz-Pesini *et al.* 2004).

Some pathological states can be ascribed to uncoupling and subsequent lack of ATP. A classical example is Luft's disease (Luft *et al.* 1962), which is characterized by muscle weakness, severe hypermetabolism and defective phosphorylation capacity in the mitochondrial respiratory chain. Other states of pathologically increased uncoupling are hyperthyroidism (Luvisetto 1997), malignant hyperthermia (Cheah *et al.* 1989) and Reye syndrome (Asimakis & Aprille 1977). Hypothyroidism, on the other hand, can be regarded as a pathological reduction in uncoupling and heat production (Silva 2001).

2.1.2.3 Production of reactive oxygen species

Reactive oxygen species (ROS) are highly reactive derivatives of the oxygen molecule which contain an unpaired electron in the outermost shell of electrons. This is an extremely unstable configuration, and ROS quickly react with other molecules or radicals to achieve a stable configuration. ROS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH). Mitochondria are the major site of ROS production in cells, and the respiratory chain produces ROS at complex I and complex III. ROS can damage other molecules and cell structures, and mitochondrial DNA and mitochondrial membranes are especially vulnerable since they are directly exposed to ROS produced during cellular respiration. (Fleury *et al.* 2002)

ROS are involved in various pathological processes such as carcinogenesis (Cejas *et al.* 2004) and neurodegeneration (Wei 1998, Enns 2003) and in the ageing process (Wei 1998, Droge 2003), but the ROS produced by mitochondria have an important physiological role as well, as they affect gene expression and protein phosphorylation by functioning as signalling molecules under subtoxic conditions (Suzuki *et al.* 1997, Brookes *et al.* 2002). ROS are also important in cell death transduction pathways, and therefore in apoptosis (Skulachev 1996, Fleury *et al.* 2002), and they may also induce "mitoptosis", death of individual mitochondria in a cell, and therefore be involved in mitochondrial selection by removing mitochondria that overproduce ROS (Skulachev 1996).

2.1.2.4 Apoptosis

Apoptosis, “cell suicide”, is an important physiological mechanism whereby individual cells are destroyed in order to remove them from an organism. Apoptosis is important in embryogenesis, immune cell maturation and response and cellular responses to injury, for example, and its absence may cause uncontrolled cell division and subsequently cancer. Apoptosis may also be involved in cell selection by eliminating ROS-producing cells. Mitochondria play an important role in apoptosis by releasing pro-apoptotic proteins from the IMS when the outer mitochondrial membrane (OM) becomes permeable to them. The permeability of the OM increases when permeability transition pores (PTP) are opened in the inner mitochondrial membrane. The most important of the “pro-apoptotic” proteins is cytochrome c, which activates pro-caspases and leads to the formation of apoptosomes and the inhibition of anti-apoptotic cytosolic proteins. This activates a cascade that induces apoptosis. Another mechanism for the release of these proteins that induce apoptosis is by osmotic disequilibrium, which leads to an expansion of the matrix space and, since the folded crista surface area of the IM is far greater than that of the OM, to subsequent rupture of the OM and release of cytochrome c and other factors (Green & Reed 1998). Some of the mitochondrial pro-apoptotic proteins then enter the nucleus and induce chromosomal condensation and DNA fragmentation, hallmarks of apoptosis (Skulachev 1996, Green & Reed 1998, Gulbins *et al.* 2003, Orrenius 2004).

2.1.2.5 Calcium homeostasis

Mitochondria are involved in the sequestration of intracellular calcium (Ca^{2+}), which has various physiological functions. First, Ca^{2+} is involved in the stimulation and control of oxidative phosphorylation, as it activates pyruvate dehydrogenase and two enzymes of the citric acid cycle, thereby controlling the rate of production of NADH, which provides reducing equivalents for the respiratory chain. Ca^{2+} also activates ATP synthase (complex V), and OXPHOS is stimulated by Ca^{2+} pulses. Only a small amount of Ca^{2+} is necessary to activate ATP production. Second, high concentrations of intramitochondrial Ca^{2+} will open a permeability transition pore (PTP), which leads to swelling of the mitochondria and rupture of the OM, and subsequently to apoptosis, eliminating damaged cells or, alternatively, to mitoptosis, eliminating damaged mitochondria from a cell. Third, the mitochondria modulate cytosolic Ca^{2+} levels both spatially and temporally. Mitochondria are often located in areas of the cell that are also involved in Ca^{2+} regulation, such as near the endoplasmic reticulum (ER), the major site of Ca^{2+} sequestration in cells, and near the nuclear membrane, where they may play a role both in supplying ATP for nuclear functions and in regulating Ca^{2+}-mediated signal transduction and the expression of nuclear genes (Carafoli 2003, Ganitkevich 2003, Gunter et al. 2004).

A defect in mitochondrial Ca^{2+}-metabolism has been proposed as the cause of Huntington's disease (HD) (Jones *et al.* 1997), which results from an expansion of exonic CAG triplet (polyglutamine) repeats in the gene encoding huntingtin (Htt). Mitochondria from patients with HD have a lower membrane potential and depolarize at lower calcium

loads than do those from controls. In addition, mutant Htt has been identified on neuronal mitochondrial membranes and the mitochondrial calcium defect has been reproduced in normal mitochondria by means of a protein containing an abnormally long polyglutamine repeat (Panov *et al.* 2002).

2.1.2.6 Iron metabolism

The mitochondria play an important role in the iron metabolism of the cell, since they provide the site for the synthesis of heme and for the maturation of various iron-sulphur (Fe-S) proteins which are involved in electron transfer processes and various enzymatic reactions. Most of known Fe-S proteins are located within the mitochondria, including aconitase and respiratory chain complexes I, II and III (Lill & Kispal 2000). The mitochondrial Fe-S protein maturation apparatus not only assembles mitochondrial Fe-S proteins but also initiates the formation of extramitochondrial Fe-S proteins, which are subsequently exported into the cytosol via a transporter protein (Lill et al. 1999, Lill & Kispal 2000, Lill & Kispal 2001). Frataxin, a protein deficient in Friedreich's ataxia, is a mitochondrial protein involved in the metabolism of Fe-S clusters. In Friedreich's ataxia, there is a generalized deficiency of proteins containing the Fe-S cluster, and yeast cells deficient in frataxin homologue are unable to carry out OXPHOS, lose mitochondrial DNA, accumulate iron in their mitochondria and show increased sensitivity to oxidative stress (Pandolfo 2002). Other diseases involving defective mitochondrial Fe-S cluster metabolism include an iron storage disease (Lill et al. 1999), X-linked sideroblastic anaemia and cerebellar ataxia (Lill & Kispal 2001).

Heme, the major functional form of iron, is synthesized in mitochondria, and disturbed heme metabolism causes mitochondrial decay, iron accumulation and oxidative stress and may be involved in ageing and age-related disorders (Atamna 2004). Additionally, GRACILE syndrome, an autosomal recessive metabolic disorder belonging to the Finnish disease heritage, results from a deficit in the mitochondrial inner membrane protein BCS1L, which acts as a chaperone in the assembly of the Rieske Fe-S protein of complex III. BCS1L may also be involved in mitochondrial iron import. GRACILE leads to mitochondrial failure, with severe lactic acidosis, iron overload (liver haemosiderosis, hyperferritinaemia, hypotransferrinaemia and increased plasma free iron) and early death (Fellman 2002, Pandolfo 2002, Visapää et al. 2002). Another disease with iron accumulation in mitochondria is X-linked sideroblastic anaemia and ataxia (XLSA/A), which is caused by mutations in the ABC7 gene encoding a mitochondrial iron transport protein (Allikmets et al.1999).

2.1.2.7 Other functions of mitochondria

Mitochondria are also involved in cellular signalling networks and signal transduction (Brookes et al. 2002) and in various metabolic pathways in the cell. Enzymes of the citric

acid cycle and beta-oxidation of fatty acids are located in the mitochondrial matrix, and mitochondria play an important role in many reactions of intermediary metabolism and contain enzymes for steroid synthesis, heme synthesis, uridine synthesis and carnitine metabolism and enzymes of the urea cycle.

2.2 Mitochondrial DNA

The mitochondrial genome has several unique features relative to the nuclear genome. The organization of mtDNA is different from that of nuclear DNA (nDNA), the genetic code of mtDNA is different (see section 2.2.1), replication of mtDNA is independent of the cell cycle (see section 2.2.2), mtDNA is maternally inherited, the mtDNA pool of a cell is randomly segregated to daughter cells (see section 2.2.3), and a cell or tissue can be heteroplasmic, i.e. it can contain both wild-type (normal) and mutant mtDNA molecules. Also, a certain level of mutant DNA is required before it affects the function of a cell or tissue. This is called threshold effect (see section 2.2.4). The mutation rate of mtDNA is higher than that of nDNA (see section 2.2.5), and each cell harbours a high number of copies of mtDNA molecules, in contrast to only two copies of each allele of nDNA. Additionally, mitochondrial genes have sometimes been translocated to the nucleus during evolution, although they are not transcribed to mRNAs there but remain untranscribed as silent genes called pseudogenes (see section 2.2.6). Furthermore, mitochondrial DNA has been thought to be a non-recombining genome, although there is evidence that some recombination may occur (see section 2.3).

2.2.1 Organization and genetic code

Human mitochondrial DNA is a closed, circular, double-stranded molecule of 16,568 base pairs (Anderson *et al.* 1981, Andrews *et al.* 1999) located in the mitochondrial matrix and present in up to thousands of copies per cell. It has been completely sequenced and all 37 genes encoded by it have been identified.

Human mtDNA encodes for 13 polypeptides of the respiratory chain, 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) (Figure 3). All 13 polypeptides are essential components for the assembly and function of the respiratory chain. Seven of them are subunits of complex I (encoded by MTND1, MTND2, MTND3, MTND4, MTND4L, MTND5 and MTND6), one of complex III (encoded by MTCYB), three of complex IV (encoded by MTCO1, MTCO2 and MTCO3) and two of complex V (encoded by MTATP6 and MTATP8). The 22 tRNAs and two rRNAs (12sRNA and 16sRNA) encoded by mtDNA make up part of the machinery necessary for its translation (DiMauro & Schon 2003).

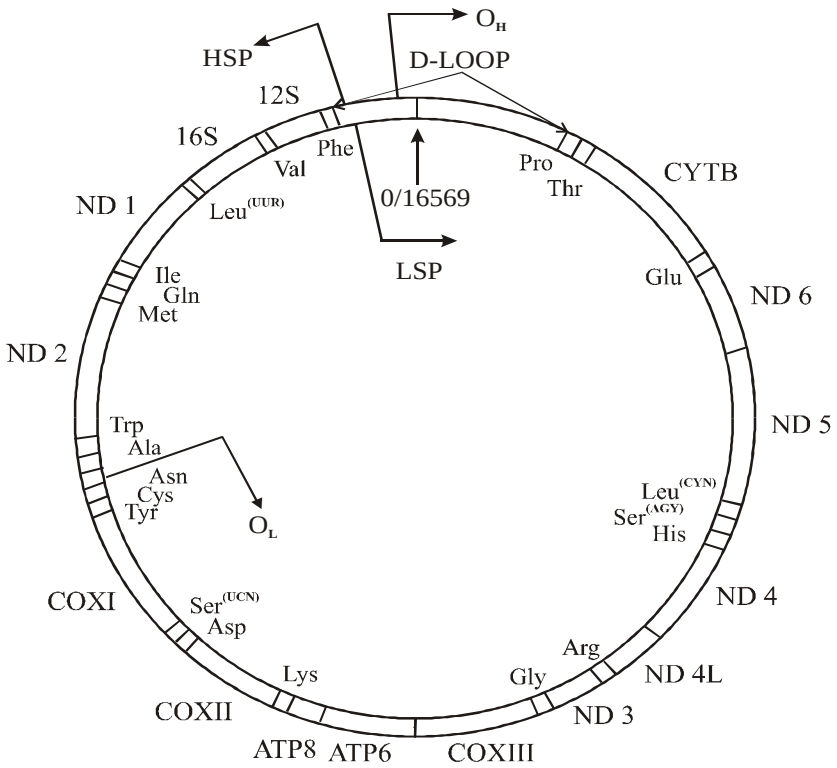


Fig. 3. Human mitochondrial DNA. The ND6 subunit and tRNAs for Gln, Ala, Asn, Cys, Tyr, Ser (UCN), Glu and Pro are encoded by the light (L) strand. The protein-coding genes and rRNA genes lie outside the circle and tRNA and genes are inside the circle. The cross-line within the D-loop depicts the position of the first and last nucleotides of mtDNA. O_H = replication origin of the H-strand. O_L = replication origin of the L-strand. HSP = heavy strand promoter. LSP = light strand promoter.

The two complementary strands of mtDNA can be distinguished by means of their base content, which results in different densities for each strand, the heavy strand (H-strand) being rich in guanine bases and the light strand (L-strand) rich in cytosine (Kasamatsu & Vinograd 1974, Fernández-Silva *et al.* 2003). The H-strand encodes for most of the information, i.e. 12 polypeptides, 14tRNAs and both rRNAs, whereas the L-strand encodes for only one polypeptide, the MTND6 gene, and eight tRNAs.

MtDNA contains two non-coding regions, the first of which is a triple-stranded 1.1kb structure called the displacement loop (D-loop), also known as the control region. This is located between the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$ and contains the main regulatory sites for the replication and transcription of mtDNA. The D-loop is the most variable region of mtDNA, but also contains conserved sequence blocks (CSBs). It contains the replication origin of the H-strand (O_H) and the promoter positions for the transcription of both the H-strand (heavy strand promoter, HSP) and L-strand (light strand promoter, LSP). Binding sites for mitochondrial transcription factor A (TFAM) are similarly located in the D-loop. The second non-coding region in mtDNA is only about 30bp long,

encompassing nucleotides 5744-5763, and is located within a tRNA cluster and contains the origin of replication of the L-strand (O_L) (Shadel & Clayton 1997, Fernández-Silva *et al.* 2003).

MtDNA is tightly packed and economically organized, since it contains no introns, but instead the protein-coding genes are separated by tRNAs, which punctuate the genome. Furthermore, some of the protein-coding genes overlap and some of the termination codons are not encoded but generated post-transcriptionally by polyadenylation of the mRNAs. The overlapping genes are MTATP8 (nucleotides 8366-8572) and MTATP6 (nts 8627-9207), which overlap by 46 nucleotides, and ND4L (nts 10470-10766) and ND4 (nts 10760-12137), which overlap by six nucleotides (MITOMAP, Fernández-Silva *et al.* 2003). MtDNA is transcribed as a single large polycistronic RNA, which is processed to yield separate mature mRNAs. The tRNA genes play an important role in the processing of the polycistronic RNA, since they are interspaced between the protein-coding genes and their typical secondary cloverleaf structure acts as a signal for the processing enzymes. This is called "tRNA punctuation" (Ojala *et al.* 1981, Fernández-Silva *et al.* 2003).

The genetic code of mtDNA differs from the universal genetic code (Anderson *et al.* 1981, Wallace 1982, Knight *et al.* 2001). UGA codes tryptophan (Trp) instead of a termination codon, and AGA and AGG are signals for termination instead of arginine (Arg). In addition, AUA codes for methionine (Met) instead of isoleucine (Ile), and both AUA and AUU may be used as initiation codons instead of AUG. Due to these differences in the genetic code, the protein-coding genes are not intelligible to the nucleocytosolic transcription-translation system.

2.2.2 Replication

Nuclear DNA replicates only once during each cell cycle, but the replication of mtDNA is independent of the cell cycle phase and nDNA replication and is therefore relaxed (Bogenhagen & Clayton 1977), although still under nuclear genetic control. Replication takes place in the mitochondrial matrix, the generally accepted model being known as the asynchronous displacement mechanism, with two unidirectional origins (Shadel & Clayton 1997, Taanman 1999, Fernández-Silva *et al.* 2003). Asynchronous means that initiation of the replication of the H-strand and L-strand involves two separate events, that of L-strand taking place after two-thirds of the H-strand has been replicated.

Replication of mtDNA starts at O_H, the primary origin suggested being at nt 191, with secondary origins at nts 146-151 (Zhang *et al.* 2003). O_H is located downstream from LSP, and the initiation of H-strand replication requires the presence of short RNA primers, which are processed from the LSP RNA transcripts (Chang & Clayton 1985, Shadel & Clayton 1997). Replication of mtDNA is therefore linked with mitochondrial transcription (Clayton 1992), and it is not clear which mechanism decides between elongating the RNA transcript and forming RNA primers for the initiation of DNA synthesis. These might be trans-acting factors or cis-acting elements involved in

transcription initiation from the LSP, or CSBs located in the vicinity of O_H (Fernández-Silva *et al.* 2003).

After replication of the H-strand has started, there are two possibilities. In the majority of the cases the replication ceases at termination-associated sequences (TAS), creating the triplex DNA strand structure called the D-loop. The other possibility is that the entire length of the mtDNA will be replicated. Then replication will proceed until O_L is reached, which is about two-thirds of the way down the genome, and the initiation of L-strand replication will start in the second (30nts-long) non-coding region of the mtDNA. Once exposed as a single strand, this region will adopt a distinctive stem-loop structure, which serves as the recognition structure for a DNA primase enzyme that provides a short RNA primer required for DNA synthesis. After this, replication proceeds over the entire length (Shadel & Clayton 1997, Taanman 1999). The whole process of mtDNA replication is rather slow and is estimated to take approximately one hour (~270nt/min) (Graves *et al.* 1998).

The machinery for the replication of mammalian mtDNA is encoded by nDNA, and several factors specific for mtDNA have been identified (Fernández-Silva *et al.* 2003): RNA-processing endoribonuclease (RNase MRP) for RNA primer formation (Shadel & Clayton 1997), DNA polymerase (POLG) for DNA synthesis (Graves *et al.* 1998), DNA helicase, called Twinkle in humans, for unwinding DNA (Spelbrink *et al.* 2001), topoisomerases (TOPOImt and TOPOIImt) to remove and introduce supercoils (Castora *et al.* 1985, Kosovsky & Soslau 1993), single-strand binding proteins (mtSSB) to maintain the integrity of replicative intermediates of mtDNA and to increase the fidelity of POLG (Farr *et al.* 1999), DNA primase for the initiation of L-strand replication (Wong & Clayton 1986), a ligase to join the separate DNA strands at the end of the replication (Tomkinson & Mackey 1998, Farr *et al.* 1999), and transcription factor A (TFAM), which has transcription initiation as its main role (Shadel & Clayton 1997).

TFAM also plays a role in mtDNA maintenance (Fernández-Silva *et al.* 2003) and a direct correlation between TFAM and mtDNA levels has been shown (Ekstrand *et al.* 2004). In addition, heterozygous TFAM knockout mice show reduced mtDNA levels and homozygous knockouts lack mtDNA and die (Larsson *et al.* 1998).

In addition to the traditional asynchronous strand displacement model of mtDNA replication, another mechanism has also been proposed (Holt *et al.* 2000). Two-dimensional (2D) agarose gel electrophoresis has revealed two classes of replication intermediates, one with properties related to the orthodox, strand-asynchronous mode of mtDNA replication and another with mobility properties of coupled leading and lagging-strand replication products. In addition, intermediates of coupled, unidirectional mtDNA replication have been found in mouse and human tissue samples. These findings indicate that two modes of mtDNA replication operate in mammalian cells (Holt *et al.* 2000).

2.2.3 Maternal inheritance and genetic bottleneck

All the mitochondria for the zygote are derived from the oocyte at fertilization in mammals, and thus mitochondrial DNA is maternally inherited. New mutations thus

segregate along maternal lineages, so that a mother carrying an mtDNA point mutation will transmit it to her children, both females and males, but only her daughters will pass it on to their children (Shoubridge 2000). An oocyte contains more than 100,000 mitochondria, and these will establish the mtDNA pool that will segregate to the daughter cells of the developing embryo (Chen *et al.* 1995). Despite the fact that sperm mitochondria play an important role in spermatozoa, because of the high ATP demand of these cells, the number of mtDNA copies in sperm cells does not exceed 120-1500 (Diez-Sanchez 2003). Some sperm mitochondria enter the oocyte, but the paternal mtDNA molecules are actively and selectively eliminated by a ubiquitin-dependent mechanism The sperm mitochondria are tagged with ubiquitin during spermatogenesis, which ensures their execution after encountering the oocyte's cytoplasmic destruction machinery (Sutovsky *et al.* 1999, Sutovsky & Schatten 2000).

Despite the universal rule that mtDNA is maternally inherited, there is also evidence of paternal transmission of mtDNA in a patient with myopathic symptoms (Schwartz & Vissing 2002), but even here the paternal mtDNA was restricted to the skeletal muscle (Schwartz & Vissing 2002).

MtDNA sequence variants segregate rapidly between generations. This means that the level of mutant genome in the offspring of a mother can vary greatly between generations and between siblings, and therefore a mother carrying a heteroplasmic mtDNA mutation may have children with a range of clinical phenotypes from healthy to a severe or fatal neurological disorder (Poulton *et al.* 1998b, Chinnery *et al.* 2000a, Poulton & Marchington 2002). These rapid changes in phenotypes are explained by a phenomenon called “genetic bottleneck”. The high number of mtDNA molecules in the oocyte (~100,000) which form the mtDNA pool of the developing embryo drops dramatically in the primordial germ cells of the embryo to the level of about 100. This is the “bottleneck”. The small pool of mtDNA molecules then undergoes rapid expansion during oogenesis and later forms the mtDNA pool of the mature oocytes of a female. There may be unequal partitioning of different mtDNA molecules between primordial germ cells, and therefore the level of mutant DNA can change rapidly within a generation. The genotypes of the offspring are distributed evenly above and below the mean level, indicating that this process of mtDNA segregation into daughter cells is determined by random genetic drift, both in mice (Jenuth *et al.* 1996) and in humans (Brown *et al.* 2001). This random genetic drift also occurs in mitotic segregation, which can cause great variation in the level of mutant mtDNA between tissues in an individual, also causing great variation in clinical phenotypes. There is also evidence, however, that the tissue distribution of mutant mtDNA is non-random in the case of a pathogenic mutation (Chinnery *et al.* 1999).

2.2.4 Heteroplasmy and the threshold effect

Normally, all the mtDNAs of an individual are identical. This condition is known as homoplasmy. When a mutation arises, however, both normal (wild-type) and mutant mtDNA coexist, a condition known as heteroplasmy. This can occur at the level of single

mitochondria, since each mitochondrion contains several mtDNA molecules, or at the cell, tissue or organism level. A very low level of heteroplasmy does not impair the respiratory chain function of a cell or tissue, but once the level of mutant mtDNA exceeds a certain level, dysfunction of oxidative phosphorylation occurs and symptoms arise. This is known as the threshold effect (DiMauro 2001, DiMauro & Schon 2003).

The threshold for a pathogenic mutation to cause symptoms varies between tissues, being lower in those tissues that are post-mitotic, highly metabolically active and dependent on oxidative phosphorylation for energy production, such as the brain, skeletal muscle, heart muscle, cochlea and retina. Thus these are also the tissues that are most commonly affected in diseases caused by defects in mtDNA. Usually the mutant DNA has to reach a level of 60-80% before symptoms appear (Lightowlers *et al.* 1997, Leonard & Schapira 2000a). Most pathogenic mutations are heteroplasmic, since if they were homoplasmic they would often be lethal. The threshold level not only varies between tissues but also between different types of mutation, so that a threshold level of 60% has been demonstrated for mtDNA deletions, for example (Hayashi *et al.* 1991), and a level of about 90% for tRNA mutations (Chomyn *et al.* 1992).

A correlation between the percentage of mutant mitochondrial DNA and clinical severity has been shown (White *et al.* 1999, Chinnery *et al.* 2000b, Chinnery *et al.* 2001, Uimonen *et al.* 2001, Carelli *et al.* 2002). There is an increase in mutant load with increasing severity of symptoms both in mutation 8993T>C and in 8993T>G, but the probability of developing severe symptoms with a certain percentage of mutant DNA is greater for 8993T>G (White *et al.* 1999). There is also a relationship between the mutation load of a mother and her offspring (White *et al.* 1999). In addition, the severity of hearing impairment has been shown to correlate with the load of the 3243A>G mutation in skeletal muscle (Chinnery *et al.* 2000b, Uimonen *et al.* 2001).

2.2.5 High mutation rate

Mitochondrial DNA accumulates mutations about 10-20 times more rapidly than nuclear DNA (Brown *et al.* 1979, Wallace *et al.* 1987), which accounts for the faster evolution of the mitochondrial genome and for the accumulation of somatic mutations in post-mitotic tissues with age. Also, the hypervariable segments (HVS) of the control region of mtDNA evolve more rapidly than the coding region (Howell 1996, Parsons 1997). Both its maternal inheritance and its high mutation rate have made mtDNA an interesting target for population genetics and evolutionary studies (Stoneking *et al.* 1994).

The higher mutation rate of mtDNA has been traditionally explained as having three causes. First, mitochondrial DNA is thought to be “naked” and therefore vulnerable to damage due to the lack of protective histone proteins. Second, there is a lack of DNA repair mechanisms in mitochondria, and third, mtDNA is physically close to the inner membrane, where damaging reactive oxygen species are constantly being generated. More recent research, however, has shown that mammal mtDNA is organized in discrete protein complexes called nucleoids (Bogenhagen *et al.* 2003, Garrido *et al.* 2003). These are composed of several proteins, the most important and abundant being TFAM (Garrido

et al. 2003, Kanki *et al.* 2004), and they may play a role similar to that of the protective histones around nuclear DNA (Kanki *et al.* 2004). There is also strong evidence that mitochondria contain DNA repair mechanisms (Croteau *et al.* 1999, Dianov *et al.* 2001, Bohr *et al.* 2002, Mason *et al.* 2003). They contain all the enzymes necessary for the base excision repair (BER) type of DNA damage repair (Mandavilli *et al.* 2002), which is the main pathway for repairing oxidative DNA damage (Bohr *et al.* 2002). Oxidative damage accumulates in mtDNA during ageing (Golden & Melov 2001, Van Remmen & Richardson 2001, Mandavilli *et al.* 2002), and interestingly, mitochondrial repair of 8-oxoguanine (8-oxoG) is deficient in Cockayne syndrome B, which is a segmental premature ageing syndrome (Stevnsner *et al.* 2002).

The constant production of ROS by the electron transport chain and the close proximity of mtDNA to ROS generation may be the cause of the high accumulation of somatic mutations during ageing, especially in post-mitotic tissues such as brain and muscle (Wei 1998, Golden & Melov 2001, Van Remmen & Richardson 2001). Point mutations and deletions are found to increase in various tissues of aged individuals (Cortopassi *et al.* 1990, Zhang *et al.* 1992, Lee *et al.* 1994, Fahn *et al.* 1996, Tanaka *et al.* 1996), a higher burden of somatic mutations has been found in the brain of elderly subjects and Alzheimer's disease patients than in younger subjects, and an increasing mutation load has been seen to correlate negatively with cytochrome oxidase activity (Lin *et al.* 2002, Coskun *et al.* 2004).

Since mtDNA contains no introns, oxidative DNA damage is more likely to affect important genes than it is in nuclear DNA and thereby affect the functioning of the respiratory chain by reducing the efficiency of ATP production and/or increasing the amount of ROS generated. Indeed, respiratory chain function, especially that of cytochrome c oxidase (complex IV), has been shown to decline with age (Boffoli *et al.* 1994, Sharma *et al.* 1998, Ojaimi *et al.* 1999, Cottrell *et al.* 2001). There is also an increase in COX-negative muscle fibres in ageing (Muller-Hocker 1990, Muller-Hocker *et al.* 1992), and cybrid cells (rho^0 cells lacking mtDNA combined with enucleated mitochondria-containing cells from a different individual) show decreasing respiratory chain function with increasing age of the donor (Laderman *et al.* 1996).

2.2.6 Pseudogenes

A "pseudogene" is a non-functional copy of a normal gene that has been slightly altered so that it is no longer expressed. During the evolution of mitochondria, most of its genes have been gradually transferred to the nucleus and are now expressed by the nuclear DNA (nDNA). By this progressive transfer of its genes to its host eukaryote nucleus, the prokaryote genome has attained a high level of compactness. There are also nuclear mitochondrial pseudogenes (numts), which are the result of a transfer of parts of mtDNA to nDNA. Numts are similar, but not identical, to their mitochondrial counterparts; they vary in size and are randomly distributed along chromosomes, and are not expressed. Numts are typically located in the non-coding regions of nDNA, but a de novo transfer of 72 bp of mtDNA into exon 14 of the GLI3 gene has been demonstrated in a patient with

Pallister-Hall syndrome (Turner *et al.* 2003). This insertion was detected because it renders the gene non-functional (Turner *et al.* 2003). Numts can be sometimes erroneously amplified as mtDNA, and it can lead to misinterpretations (Davis *et al.* 1997, Wallace *et al.* 1997, Davis *et al.* 1998). All the portions of mtDNA are found as numts in nDNA, and a phylogenetic study indicates that the integration of mtDNA into the nucleus is an ongoing process. Numts are highly heterogeneous, some of them being substantially modified, possibly indicating more ancient integration, while others are almost perfectly conserved, indicating that they have been integrated into the nucleus relatively recently. This heterogeneity also supports the hypothesis that gene transfer is an ongoing process. In addition, several copies of a certain numt can be found in nDNA, which may be either a sign of secondary transfer events between chromosomes or a sign of numerous transfer events required for nuclear expression of genes previously expressed within the endosymbiont. (Tourmen *et al.* 2002, Woischnik & Moraes 2002)

2.3 Mitochondrial diseases

Although the classical mitochondrial diseases are caused by defects in the respiratory chain, mitochondrial diseases as a whole cover a broad spectrum related to defects in other functions of mitochondria such as -oxidation, carnitine metabolism, the urea cycle and mitochondrial iron metabolism. The discussion here will be restricted to the classical mitochondrial disorders, which will be referred to simply as mitochondrial diseases.

Mitochondrial diseases can be caused by a defect in either mitochondrial DNA or nuclear DNA. MtDNA defects are maternally inherited mutations in tRNA, rRNA or protein-coding genes, and cause defects in the respiratory chain. Nuclear DNA mutations causing mitochondrial respiratory chain dysfunction show Mendelian inheritance, and can be mutations in genes encoding either subunits of the respiratory chain or proteins responsible for assembly of the respiratory chain or maintenance of mtDNA. (Leonard & Shapira 2000a, Leonard & Shapira 2000b, Shanske *et al.* 2001, Smeitink *et al.* 2001, von Kleist-Retzow *et al.* 2003)

Due to the ubiquitous nature of mitochondria and mtDNA, random segregation, heteroplasmy, the threshold phenomenon and the importance of energy production in cells, mitochondrial disorders are extremely heterogeneous, ranging from single-organ involvement to a severe multisystem disease. They can manifest themselves at any age and in any organ, although neuromuscular symptoms are the most common. Any combination of symptomscan be a sign of mitochondrial disease, especially if there are similarly affected maternal relatives. The most common neuromuscular symptoms are migraine, seizures, ataxia, myoclonus, psychomotor retardation, premature exercise-induced fatigue, cardiomyopathy, ophthalmoplegia, and ptosis. Common non-neurological symptoms include diabetes mellitus, sensorineural hearing loss, pigmentary retinopathy, short stature and sideroblastic anaemia.

Laboratory findings indicative of a mitochondrial disorder are elevated lactate, elevated pyruvate, and an increased lactate/pyruvate ratio in serum and/or cerebrospinal fluid (CSF). Other laboratory findings include elevated serum alanine, anaemia and

elevated creatine kinase (CK). Biochemical tests for measuring the activities of respiratory chain complexes are useful for diagnostic purposes. Abnormal activity of a specific subunit of the respiratory chain suggests a mutation in a subunit encoding that complex, and a generalized defect may suggest a mutation in a tRNA gene. Enzyme activity measurements are not useful for monitoring disease severity, since repeated muscle biopsies are required and the predictive value of these measurements is not known.

Molecular genetic studies can detect specific mtDNA mutations when a certain syndrome is suspected or when known mtDNA mutations are screened in a sample from a patient. Molecular genetic analysis can also detect deletions and rearrangements in mtDNA, and the heteroplasmic state of the mutation can be studied as well. Blood (leukocytes) is the most common material used for molecular genetic analysis, but due to random segregation, a genetic defect may only be detected in the tissue affected, and therefore a negative result in a blood mtDNA analysis does not exclude OXPHOS disease caused by a defect in mtDNA. Other commonly used tissues are buccal mucous membrane, hair follicle, skin biopsy or muscle. PCR and RFLP analyses are used to detect known mutations, Southern blot analysis and long-template PCR to detect mtDNA rearrangements and direct sequencing of the mtDNA to detect new mutations.

A muscle biopsy is usually diagnostic, since mitochondrial diseases commonly affect the muscles. Both histological and ultrastructural changes typical of mtDNA diseases can be seen. Histological analysis employing Gomori trichrome stain typically reveals ragged red fibres (RRF), reflecting mitochondrial proliferation and their subsarcolemmal accumulation. These fibres stain strongly for succinate dehydrogenase (SDH, complex II), which is encoded by nDNA, and when the defect is in the mtDNA they often stain negatively for cytochrome c oxidase (COX, complex IV). Not all patients show RRF and SDH-positive/COX-negative fibres, however, and these findings can sometimes be seen in inflammatory myopathies as well, but completely COX-negative fibres are characteristic of mitochondrial disease. Ultrastructural findings at muscle biopsy show enlarged mitochondria, absence of cristae and intramitochondrial paracrystalline inclusions, the changes being most prominent in RRFs. (Leonard & Shapira 2000a, Taylor *et al.* 2004)

Imaging studies of brain and muscle tissue may show typical findings. Brain magnetic resonance imaging (MRI) or computed tomography (CT) may reveal basal ganglia calcifications, parieto-occipital stroke-like lesions and cerebral and/or cerebellar atrophy. Symmetrical lesions in the basal ganglia and brainstem are seen in patients with Leigh syndrome. Muscle CT is a useful addition to clinical evaluation of MELAS syndrome at least (Kärppä *et al.* 2004), while proton magnetic resonance spectroscopy (MRS) can sensitively detect an abnormal accumulation of lactate in the brain parenchyma and CSF (Lin *et al.* 2003), and phosphorus-MRS can reveal an impaired rate of resynthesis of high-energy phosphate compounds following exercise (Argov *et al.* 2000).

2.3.1 Diseases caused by defects in mitochondrial DNA

2.3.1.1 Large-scale rearrangements of mtDNA

Large-scale rearrangements of mtDNA are either deletions or duplications. Duplications are dimers of deleted and wild-type mtDNA molecules, while deletions of mtDNA often remove multiple tRNA genes as well as protein-coding genes. The most common deletion is a 4,977kb deletion that has been associated with a number of clinical phenotypes such as Kearns-Sayre syndrome (KSS), the Pearson marrow-pancreas syndrome (PS) and chronic progressive external ophthalmoplegia (CPEO). Each patient usually harbours only a single type of deletion and shows heteroplasmy. MtDNA rearrangements are almost always sporadic, suggesting that they arise de novo in embryogenesis or in the ovum, but duplication has been associated with maternally inherited diabetes mellitus (Dunbar *et al.* 1993), indicating that some rearrangements can be maternally inherited. Unlike nuclear chromosomal rearrangements, the incidence of mtDNA deletion disorders does not increase with maternal age (Chinnery *et al.* 2004). It has been thought previously that affected women have a negligible chance of having clinically affected offspring, but the actual risk of recurrence of deletions is about one in 24 births on average (Chinnery *et al.* 2004).

Kearns-Sayre syndrome was first described by Kearns and Sayre in 1958. The onset is usually in infancy or in adolescence, and the characteristic features are ptosis, external ophthalmoplegia and pigmentary retinopathy. A muscle biopsy typically shows ragged red fibres. Other manifestations include cerebellar ataxia, deafness, cardiomyopathy, cardiac conduction defects and diabetes. Most KSS patients harbour clonally expanded mtDNA rearrangements, and mtDNA deletions vary from 1.3 to 8.0kb (Shoffner 1996, Park *et al.* 2004). Pearson's bone marrow/pancreas syndrome is a rare, often fatal, disorder of early infancy that is characterized by impaired bone marrow (sideroblastic anemia, pancytopenia), exocrine pancreatic, hepatic and renal function. If they survive through infancy, patients with PS may later develop KSS. Large-scale mtDNA deletions are present in the blood (Shoffner 1996, Mayes *et al.* 2001). Chronic progressive external ophthalmoplegia (CPEO) is a heterogenous group of disorders characterized by a chronic, progressive, bilateral and usually symmetrical ocular motility deficit and ptosis. Proximal limb muscle myopathy may also be present (Shoffner 1996).

2.3.1.2 Point mutations in tRNA or rRNA genes

The most common class of mtDNA point mutations in humans are mutations in tRNA genes. Neuromuscular or cardiac phenotypes are the most common. The best-characterized syndromes caused by mutations in tRNA genes of mtDNA are MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy with ragged red fibres). When tRNA genes are affected, the translation of all subunits of the respiratory chain is compromised, which may reduce

their activities. Because of the high variability in the phenotypes caused by mtDNA mutations, it is hard to estimate the frequency of these disorders. MELAS syndrome is typically caused by the A to G transition in mtDNA nucleotide position 3243, which is located in $tRNA^{Leu(UUR)}$ gene, and the prevalence of this mutation is estimated to be as high as 16/100 000 in the general adult population, making this one of the most common mutations causing neurogenetic disorders (Majamaa *et al.* 1998).

In addition to the traditional encephalomyopathic phenotype, patients harbouring 3243A>G may present with a wide variety of clinical symptoms, including diabetes mellitus, occipital brain infarct, epilepsy, sensorineural hearing impairment, ophthalmoplegia, hypertrophic cardiomyopathy, cognitive decline, short stature and ataxia. Some carriers may even be asymptomatic, at least if the mutation is found through screening and no symptoms have yet appeared. The level of heteroplasmy is significantly higher in the patients with the traditional MELAS syndrome than in their oligosymptomatic or asymptomatic relatives, however. Also, the mutation load is usually significantly lower in the blood than in the muscle or other affected organ. (Liou *et al.* 1994, Shoffner 1996, Majamaa *et al.* 1998)

MERRF syndrome is caused in about 80% of cases by an A to G transition at position 8344 in mtDNA, which alters a conserved nucleotide in the $tRNA^{Lys}$ gene. Some patients harbour 8356T>C or 8363A>G in the same $tRNA^{Lys}$ gene. The clinical features of MERRF are epilepsy (myoclonic epilepsy, generalized or focal seizures), myopathy with ragged red fibres and cerebellar ataxia. Other findings may be dementia, corticospinal tract deficits, deafness, cardiomyopathy, peripheral neuropathy and elevated alanine and lactate in the urine. The threshold for symptoms to appear in the presence of 8344A>G is about 80-90%. (Shoffner 1996, Schmiedel *et al.* 2003)

There are several reports of other tRNA point mutations that have been associated with a variety of clinical presentations. More than two-thirds of the over 150 mutations in mtDNA that have been associated with human diseases are indeed found in the tRNA genes (Levinger *et al.* 2004), which may be due to their central function in mitochondrial protein synthesis, or due to the fact that research has previously been focused mainly on these genes, overlooking the role of the protein-coding genes.

Mutations in rRNA genes have rarely been reported as pathogenic, although an A to G transition at position 1555 in the 12S rRNA gene is known to be associated with non-syndromic sensorineural hearing loss, which is often induced by aminoglycoside (Estivill *et al.* 1998, Matsunaga *et al.* 2004, Noguchi *et al.* 2004). In addition, a heteroplasmic T to C transition at position 1095 in 12S rRNA has been associated with maternally inherited sensorineural deafness, levodopa-responsive Parkinsonism and neuropathy. Respiratory chain enzyme analysis also shows a significant reduction in cytochrome c oxidase activity in cells with 1095T>C (Thyagarajan *et al.* 2000).

2.3.1.3 Point mutations in protein-coding genes

Mutations in protein-coding genes of mtDNA affect subunits of the respiratory chain complexes I, III, IV and V. The severity of the mutation depends on the location of the

aminoacid change in the structure of the affected subunit and on the character of that change.

Complex I (NADH dehydrogenase). Mutations in the mtDNA-encoded complex I subunits ND1, ND2, ND3, ND4, ND4L and ND6 have been associated with Leber's hereditary optic neuropathy (LHON), which is a maternally inherited type of bilateral blindness caused by degeneration of the optic nerve. The onset is acute or subacute, and males are more often affected (Mroczek-Tonska *et al.* 2003). All known mtDNA mutations causing LHON affect protein-coding genes, almost exclusively complex I genes. LHON mutations can be classified as primary disease-causing mutations or as secondary mutations that contribute to LHON by increasing the probability of expressing the clinical phenotype. The four most common LHON primary mutations all reside in genes of complex I subunits and are, in order of increasing severity, 14484T>C in the ND6 subunit, 3460G>A in the ND1 subunit, 11778G>A in the ND4 subunit and 14459 G>A in the ND6 subunit (Torroni *et al.* 1997, Wallace *et al.* 1999). The activity of complex I has been found to be decreased in patients with Parkinson's disease (PD) (Schapira *et al.* 1990), and mutations in mtDNA genes encoding subunits of complex I are thought to increase the risk of contracting this disease (Kosel *et al.* 1998, Autere *et al.* 2004).

Complex III (cytochrome bc1). Mutations in mtDNA-encoded subunits of complex III have not often been associated with clinical phenotypes, although patients with a sporadic form of mitochondrial myopathy with progressive exercise intolerance, proximal limb weakness and in some cases attacks of myoglobinuria have been described as harbouring several somatic mutations in cyt b. No maternal inheritance has been shown and there have been no mutations in tissues other than muscle (Andreu *et al.* 1999a). A patient with progressive exercise intolerance and lactic acidosis has also been described as harbouring a muscle-restricted mutation in the cyt b gene of mtDNA (Bruno *et al.* 2003).

Complex IV (cytochrome c oxidase, COX). There are no syndromes or clinical phenotypes definitely associated with a particular mutation in genes encoding COX subunits, but several reports associate missense mutations in these genes with various phenotypes, such as a sporadic 5920G>A in the MTCO1 gene with recurrent myoglobinuria (Karadimas *et al.* 2000), 6489C>A in MTCO1 with epilepsia partialis continua (Varlamov *et al.* 2002), 6930G>A in MTCO1 with a mitochondrial multisystem disorder (Bruno *et al.* 1999), 7671T>A in MTCO2 with proximal myopathy and lactic acidosis (Rahman *et al.* 1999), 7706G>A in MTCO2 with Alpers-Huttenlocher-like disease (Uusimaa *et al.* 2003), and a frameshift mutation, the insertion of a C at nucleotide position 9537, in MTCO3 with a Leigh-like syndrome (Tiranti *et al.* 2000).

Complex V (ATP synthase, ATPase). NARP (neurogenic weakness, ataxia and retinitis pigmentosa) and MILS (maternally inherited Leigh syndrome) are known to be associated with ATPase mutations such as 8993T>C and 8993T>G in the ATP6 gene, the latter nucleotide change causing a more serious clinical picture. Also associated with MILS is 9176T>G in the ATP6 gene (Carrozzo *et al.* 2000b), which has been shown to inhibit ATP synthesis (Carrozzo *et al.* 2000a). Unlike NARP, which is an adult-onset syndrome, the onset of MILS occurs in infancy and it causes serious neurodevelopmental delay and is rapidly progressive. MILS is typically manifested when the level of heteroplasmy exceeds 90%, but NARP can be found when the heteroplasmy of the same mutation remains below that threshold (Shanske *et al.* 2001). 8993T>G causes increased

apoptosis in fibroblasts, probably through enhanced free radical production, and respiration and ATP synthesis can be partially restored with antioxidants in these cells (Mattiazzi *et al.* 2004).

2.3.2 Diseases caused by defects in nuclear DNA

Mitochondrial OXPHOS is under dual genetic control, since both mtDNA and nuclear DNA (nDNA) encode for structural subunits of the respiratory chain complexes, and, in addition, nDNA encodes for several proteins responsible for assembly of the respiratory chain and the maintenance of mtDNA, protein import into mtDNA and homeostasis of the internal mitochondrial environment. This dual genetic control makes the inheritance of mitochondrial disorders more complex, involving both Mendelian and maternal features (Leonard & Shapira 2000a, Smeitink *et al.* 2001, Zeviani *et al.* 2003, von Kleist-Retzow *et al.* 2003).

2.3.2.1 Genes encoding respiratory chain subunits

There are more than 80 structural subunits in respiratory chain complexes, of which 13 are encoded by mtDNA and the rest by nDNA, including all the subunits of complex II., The majority of OXPHOS deficiencies may therefore be caused by mutations in nDNA, although only a minority of the pathogenic mutations found are in nDNA.

Mutations in subunits of complex I have been associated with Leigh syndrome, in which the nucleotide changes affect the iron-sulphur (Fe-S) subunit genes NDUFS7 (Triepels *et al.* 1999) and NDUFS8 (Loeffen *et al.* 1998). A variety of clinical phenotypes have also been associated with mutations in other Fe-S subunit genes of complex I, such as those in the NDUFS1 (Benit *et al.* 2001), NDUFS2 (Loeffen *et al.* 2001) and NDUFS4 genes (van den Heuvel *et al.* 1998). Mutations in flavoprotein genes of complex I have been shown to cause a clinical disease, e.g. mutations in the NDUFV1 gene in patients with myoclonic epilepsy and leukodystrophy (Benit *et al.* 2001).

Complex II deficiency, caused by a homozygous mutation in the SDHA subunit gene, has been demonstrated in Leigh syndrome (Bourgeron *et al.* 1995), and heterozygous mutations in the SDHB, SDHC and SDHD subunit genes are known to cause familial pheochromocytoma and familial paraganglioma (Astuti *et al.* 2001a, Astuti *et al.* 2001b, Baysal et al. 2002).

No mutations in nDNA-encoded subunits of complex IV have been found to cause a clinical disease.

2.3.2.2 Defects in respiratory chain assembly

Mutations in assembly factors of OXPHOS complexes associated with a clinical phenotype have been reported only for complex III and complex IV. Mutations in the gene encoding the BCS1 assembly protein are reported to cause isolated complex III deficiency (de Lonlay *et al.* 2001), and absence of the SURF1 protein causes the accumulation of early intermediates and a drastic reduction in fully assembled complex IV, leading to a deficiency in COX activity and clinical Leigh syndrome (Tiranti *et al.* 1999). SCO1 and SCO2 are probably responsible for the insertion of copper into the COX holoenzyme, and mutations in SCO2 have been shown to cause a fatal infantile cardioencephalomyopathy (Papadopoulou *et al.* 1999) and a clinical phenotype mimicking Werdnig-Hoffmann disease, a form of spinal muscular atrophy (Salviati *et al.* 2003). The COX10 gene encodes heme A:farnesyltransferase, an assembly factor of complex IV, and mutations have been shown to cause cytochrome c oxidase deficiency in a consanguineous family (Valnot *et al.* 2000).

2.3.2.3 Defects in the maintenance of mitochondrial DNA

Nuclear genes are essential for the maintenance of the mtDNA. A variety of clinical phenotypes show impaired mtDNA stability, which can cause multiple deletions or even depletion of mtDNA, and these disorder are inherited in an autosomal recessive or dominant manner. Polymerase (POLG) is a unique DNA polymerase which is essential for the replication of mtDNA (Kaguni 2004). Mutations in POLG have been shown to cause autosomal dominant and recessive progressive external ophthalmoplegia (adPEO, arPEO), Parkinsonism and premature menopause in humans (van Goethem *et al.* 2001, Luoma *et al.* 2004) and recessive sensory ataxia combined with various other symptoms (Van Goethem *et al.* 2004) and a premature ageing phenotype in mice (Trifunovic *et al.* 2004). Mutations in the adenine nucleotide translocator (ANT1) and in a mitochondrial DNA helicase (Twinkle) have both been associated with multiple mtDNA deletions and a PEO phenotype (Kaukonen *et al.* 2000, Spelbrink *et al.* 2001). Interestingly, an age-dependent enhanced accumulation of point mutations and deletions in humans, specifically in the mtDNA control region, has been pointed out in association with mutations in Twinkle and POLG, possibly related to replication errors (Wanrooij *et al.* 2001).

Mutations in the gene encoding a mitochondrial deoxyguanosine kinase (DGUOK) responsible for the maintenance of balanced mitochondrial dNTP pools have been shown to cause a mitochondrial hepatocerebral syndrome (Mandel *et al.* 2000). Mutations in another enzyme responsible for deoxyribonucleotide metabolism, thymidine kinase 2 (TK2), cause a myopathic mitochondrial depletion syndrome (Saada *et al.* 2001).

Diminished thymidine phosphorylase (TP) activity, resulting in elevated concentrations of thymidine in the blood, is the results of mutations in the TP gene found in patients with multiple mtDNA deletions and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (Nishino *et al.* 1999, Kocaefe *et al.* 2003).

2.3.2.4 Other defects in nDNA affecting mitochondrial function

There are also numerous other proteins involved in the function of the respiratory chain and in the stability of the internal milieu of the mitochondria, mutations in which may cause a defect in any of the numerous mitochondrial functions (see functions in section 2.1.2).

All mitochondrially targeted proteins have to be imported into the mitochondria, and a human deafness dystonia syndrome, termed Mohr-Tranebjaerg syndrome, is caused by mutations in a gene encoding DDP protein involved in this mitochondrial protein import system (Koehler *et al.* 1999, Jin *et al.* 1996). Paraplegin possesses chaperone functions in mitochondria, and mutations in the paraplegin gene cause hereditary spastic paraplegia (Casari *et al.* 1998). Genes involved in mitochondrial membrane integrity and components include OPA1 and Taffazin, and mutations in these genes cause autosomal dominant optic atrophy (Delettre *et al.* 2000) and Barth syndrome (Bione *et al.* 1996, Vreken *et al.* 2000), respectively. Nuclear genes involved in mitochondrial iron homeostasis, such as frataxin, which is associated with Friedreich's ataxia, and ABC7, which shows a mutation in X-linked sideroblastic anaemia, are described in section 2.1.2.6. In addition, OXPHOS defects have been found in patients with Huntington's disease (Gu *et al.* 1996) and Wilson's disease (Gu *et al.* 2000).

In addition to mutations in succinate dehydrogenase (SDH) (see section 2.3.2.1), mutations in fumarate hydratase (FH, fumarase), which catalyses the next step in the Krebs cycle after SDH, also predispose the individual to neoplasia (Tomlinson *et al.* 2002, Eng *et al.* 2003). Inherited heterozygous mutations in the FH gene are associated with a predisposition to papillary renal-cell carcinoma and leiomyomatosis, whereas homozygous FH mutations cause severe neurological impairment (Eng *et al.* 2003).

2.4 Mitochondrial DNA variation

Uniparental inheritance and a high mutation rate have led to continent-specific mtDNA lineages (haplogroups), which are defined by ancient polymorphisms and characterized by considerable variation. Any two individuals will differ from each other by several nucleotides and the mean pairwise sequence difference among humans is about 60 nucleotides (Ingman *et al.* 2000). MtDNA has been a valuable tool in studying human evolution and in developing the "Out of Africa" hypothesis for the origin of modern humans (Johnson *et al.* 1983, Cann *et al.* 1987, Horai *et al.* 1995, Quintana-Murci *et al.* 1999, Ingman *et al.* 2000, Caramelli *et al.* 2003).

The use of mtDNA as a tool in phylogenetic and evolutionary studies has largely been based on the assumption that there is no recombination in mtDNA. If recombination did happen frequently, many inferences about human evolution would have to be reconsidered. A number of investigators have challenged this paradigm, and there has been evidence both for and against recombination (Awadalla *et al.* 1999, Elson *et al.* 2001, Innan & Nordborg 2002, Piganeau & Eyre-Walker 2003, Kraytzberg *et al.* 2004, Ladoukakis & Eyre-Walker 2004, Piganeau *et al.* 2004). Recent studies seem to suggest,

however, that recombination does indeed occur. An example of this is the presence of recombined mtDNA molecules in an individual with paternal mtDNA in muscle tissue (Scwartz and Vissing 2002, Kraytzberg *et al.* 2004). The frequency of recombination in mtDNA is nevertheless extremely low, and therefore the validity of phylogenetic and evolutionary studies is unlikely to be endangered (Elson *et al.* 2001, Ladoukakis & Eyre-Walker 2004).

During evolution mtDNA lineages have accumulated a great number of polymorphisms, which have been thought to occur randomly and be neutral. In addition to traditional pathogenic mutations that occur in different mtDNA lineages, however, there may also be slightly deleterious or even advantageous polymorphisms in mtDNA that are fixed in a certain haplogroup. These alleles may influence or determine clinical phenotypes, and it has been shown that the evolution of mtDNA is lineage-specific (Mishmar *et al.* 2003, Moilanen *et al.* 2003).

2.4.1 Mitochondrial DNA haplogroups

Mitochondrial DNA can be divided into haplogroups according to the ancient polymorphims present in its coding region and D-loop. A haplogroup is a group of haplotypes that share some sequence variations. The main haplogroups are designated with capital letters, and subhaplogroups within the main ones are denoted with additional numbers and letters. All human mtDNA haplogroups have emerged from two ancient macrohaplogroups M and N that originated in Africa. The Europeans emerged from macrohaplogroup N, and the Asians and native Americans from both macrohaplogroups. The European population is almost exclusively distributed among the nine haplogroups designated as H, I, J, K, T, U, V, W and X, whereas haplogroups A, B, C, D, F and G and certain subclusters of macrohaplogroups M and N such as M7 and N9 are characteristic of Asian populations. Haplogroups A, B, C and D characterize Native Americans and haplogroups L1, L2 and L3 African populations. (Torroni *et al.* 1996, Wallace *et al.* 1999, Finnilä *et al.* 2001, Herrnstadt *et al.* 2002, Silva *et al.* 2002, Kong *et al.* 2003)

2.4.1.1 The origin of mitochondrial DNA in Africa

African populations show the greatest extent of mtDNA variation in intrapopulation sequence divergence comparisons, the mean pairwise sequence difference being about 77 nucleotides, whereas that of non-Africans is only about 38 nucleotides (Ingman *et al.* 2000). This, too, supports the “Out of Africa” hypothesis, that Africans represent the most ancient human group and that all modern humans have a common African origin. There is a single mtDNA phylogenetic tree with its deepest root in Africa, and the “mitochondrial Eve” is estimated to have lived about 100,000-200,000 years before present (YBP) (Cann *et al.* 1987, Chen *et al.* 1995, Ingman *et al.* 2000).

The African haplogroup L comprises most of the African mtDNA and is estimated to have originated about 98,000-130,000 YBP. It is divided into L1, L2 and L3, of which L1 and L2 are more distant from the European and Asian haplogroups. The African L3, which is most prevalent in Eastern Africa, is considered the most recent common ancestor from which most of the European and Asian haplogroups emerged (Wallace *et al.* 1999, Herrnstadt *et al.* 2002). The "Out of Africa" expansion into Europe and Asia is estimated to have taken place some 60,000 YBP (Quintana-Murci *et al.* 1999, Wallace *et al.* 1999, Ingman *et al.* 2000). Interestingly, the oldest European haplogroup, U, is estimated to have emerged about 50,000-60,000 YBP, coinciding with the timing of the migration of our ancestors out of Africa. In addition, haplogroup U is the only one that Europeans share with Africans (Torroni *et al.* 1996, Wallace *et al.* 1999).

2.4.1.2 Mitochondrial DNA haplogroups in different continents

The frequencies of the European haplogroups vary slightly between European populations and even within populations, but haplogroup H is the most common among the Europeans, accounting for 40-50% of the population. The next most prevalent haplogroups in general are U, T, J and K, each with a frequency of approximately 5-10%, leaving haplogroups I, V, W and X, each with a frequency of approximately 0-5%, to represent a minority of Europeans (Torroni *et al.* 1996, Wallace *et al.* 1999, Meinilä *et al.* 2001, Herrnstadt *et al.* 2002).

The frequencies of the Asian haplogroups A, B, C, D, E, F, G and Z and the subhaplogroups of M and N vary greatly between populations, some comprising virtually 100% of certain populations, such as haplogroup B in some Pacific island populations, and being extremely rare in others, such as the absence of haplogroup B in Siberians (Torroni *et al.* 1993b, Wallace *et al.* 1999). The Asian haplogroups A, B, C and D are the progenitors of virtually all native American mtDNAs (Torroni *et al.* 1993a). In addition, the rare European haplogroup X, which is absent among Asians, is found among native Americans (Brown *et al.* 1998). An interesting link between the Asian and European mtDNA gene pools is the presence of an Asian-specific haplogroup Z in the Finnish and Saami populations (Finnilä *et al.* 2001, Meinilä *et al.* 2001).

2.4.1.3 Finnish mitochondrial DNA haplogroups

The Finns represent a genetically unique population because of their geographical and genetic isolation, which has shaped the gene pool (Norio *et al.* 1973). They exhibit an Asian pattern of Y chromosomal markers (Zerjal *et al.* 1997), but mtDNA analysis has revealed a Western Eurasian ancestry for them (Vilkki *et al.* 1998, Torroni *et al.* 1996). The Finnish mtDNA pool comprises all nine European haplogroups, with H the most frequent (Torroni *et al.* 1996, Meinilä *et al.* 2001). The frequency of haplogroup U varies considerably among the Finns, however, being about 28% in central and northern Finland

(Meinilä *et al.* 2001) compared with a frequency of about 16% reported elsewhere (Torroni *et al.* 1996), the latter material probably representing the southern parts of the country (Meinilä *et al.* 2001). Subcluster U5 is unusually common in northern Finland, comprising 92% of haplogroup U (Meinilä *et al.* 2001). But in addition to haplogroups H and U, all the other European haplogroups, I, J, K, T, V, W and X, are present among the Finns, and, as mentioned in section 2.4.1.2, the Asian-specific haplogroup Z is found at a low frequency among the Finns, suggesting an Asian genetic contribution to the mtDNA gene pool (Finnilä *et al.* 2001, Meinilä *et al.* 2001).

2.4.2 Mitochondrial DNA mutations

2.4.2.1 Pathogenic mutations

Because of the large number of polymorphisms in mtDNA, it may be hard to determine the pathogenic role of a certain mutation associated with a disease. However, if a mutation meets most of the following seven criteria, its pathogenicity is highly likely. First, the mutation should not have been described previously as a neutral polymorphism. Second, it should not be present in an unaffected, matched control group. Third, the mutation should change a nucleotide position which is highly conserved in evolution. This will increase the functional importance of the site in a tRNA, an rRNA or a protein molecule. Fourth, the mutation should be heteroplasmic, indicating that the state of homoplasmy is not tolerated, and is thus lethal. This is not always a requirement, since 11778G>A, causing LHON, is usually homoplasmic. Fifth, the degree of heteroplasmy in different family members should reflect the severity of the clinical phenotype. Sixth, the degree of heteroplasmy should be higher in the affected tissues than in unaffected or mildly affected ones. Seventh, the amount of mutant DNA should correlate with the biochemical phenotype in a single cell. This can be studied in muscle tissue by single fibre PCR. The mutation load should be higher in COX-negative muscle cells, for example, than in COX-positive ones. Examples of known pathogenic mutations are 3243A>G, causing MELAS syndrome, 8344A>G, causing MERFF, and 8993T>G/C, causing NARP/Leigh syndrome (DiMauro & Schon 2003, Taylor et al. 2004).

2.4.2.2 Slightly deleterious mutations

Analyses of mutations in mtDNA have revealed deviations from the neutral theory (Ballard & Kreitman 1994, Nielsen & Weinreich 1999, Gerber *et al.* 2001, Moilanen & Majamaa 2003). Slightly deleterious mutations in mtDNA can be regarded as mutations that affect moderately conserved nucleotides and thus functionally important but not crucial sites. Additionally, such a mutation may not be sufficient to cause a disease by itself but would require additional genetic or environmental factors to produce a clinical phenotype. The effect of a slightly deleterious mutation may also not be evident until

later in life, therefore being manifested as a late-onset disease. These slightly deleterious mutations could also be referred as “risk mutations”, since they increase the risk of a certain phenotype only when additional factors are present. In a phylogenetic tree, a deleterious mutation is expected to be located in the periphery of the tree, in the terminal branches, because they have arisen more recently and have not been eliminated by purifying selection (Moilanen & Majamaa 2003, Ruiz-Pesini *et al.* 2004).

The genetic factors that influence the deleterious effect of an mtDNA mutation can be other mtDNA point mutations, a combination of mtDNA point mutations present in a certain mtDNA lineage (haplogroup-specific effect), mutations in nuclear genes, or a combination of any of these. The frequency of mtDNA haplogroup H has been shown to be higher in patients with Lewy body dementia (Chinnery *et al.* 2000c), while haplogroup T is more prevalent in those white men with infertility problems who have reduced sperm motility than in those with normal sperm motility (Ruiz-Pesini *et al.* 2000). Additionally, haplogroup U is overrepresented in patients with migraine-associated occipital stroke (Finnilä *et al.* 2001) and in males with Alzheimer’s disease (van der Walt et al. 2004), and haplogroup J in LHON patients (Torroni *et al.* 1997). These observations indicate that the haplogroup background modifies susceptibility to diseases. Mitochondrial DNA point mutations have also been associated with an increased risk of developing dilated cardiomyopathy (Khogali *et al.* 2001), bipolar disorder (Kato *et al.* 2000), type 2 diabetes (Casteels *et al.* 1999), and insulin resistance (Poulton *et al.* 1998a). Nuclear-mitochondrial interactions have been suspected as one of the factors explaining the great clinical variability in the 3243A>G MELAS mutation (Jacobs & Holt 2000, Silvestri *et al.* 2000) and in the manifestation of the non-syndromic deafness associated with the mtDNA 1555A>G mutation (Bykhovskaya *et al.* 1998, Guan *et al.* 2001). Also, ApoE alleles may modify the effect of mtDNA 4336T>C polymorphism and the risk of Alzheimer’s disease (Edland *et al.* 2002).

Environmental factors that influence the deleterious effect of an mtDNA mutation can be unavoidable or avoidable on the part of an individual. Unavoidable factors include age and sex. The risk of developing visual loss in the presence of LHON mutations, for example, is much greater in males (Riordan-Eva *et al.* 1995). Avoidable factors influencing the pathogenicity of a mtDNA mutation include alcohol and smoking, which increase the risk of visual failure in those carrying LHON mutations (Charlmers & Harding 1996), while exposure to aminoglycoside antibiotics increases the risk of deafness in carriers of the 1555A>G mutation (Prezant *et al.* 1993, el-Schahawi *et al.* 1997, Fischel-Ghodsian *et al.* 1997).

2.4.2.3 Neutral polymorphisms

A large number of population-specific or haplogroup-specific mtDNA polymorphisms have been reported (Finnilä *et al.* 2001, Herrnstadt *et al.* 2002, Silva *et al.* 2002, Kong *et al.* 2003), and most of the variation has been considered neutral, since deleterious mutations are expected to have been eliminated by purifying selection during evolution. Neutral polymorphisms should be uniformly distributed between populations and

haplogroups, as they have arisen multiple times in the evolution of lineages and are thus homoplasmic (Ruiz-Pesini *et al.* 2004). Synonymous substitutions in protein-coding genes have been considered to be neutral, as have polymorphisms detected in the non-coding control region.

2.4.2.4 Advantageous polymorphisms

Since there are deviations from neutrality in the evolution of mtDNA (Ballard & Kreitman 1994, Nielsen & Weinreich 1999, Gerber et al. 2001, Moilanen & Majamaa 2003) in addition to slightly deleterious mutations, there may also be advantageous polymorphisms occurring via adaptive evolution (Rand et al. 1994, Mishmar et al. 2003, Ruiz-Pesini et al. 2004). These would increase the chance of an advantageous phenotype or reduce the risk, or delay the incidence, of a deleterious phenotype. These polymorphisms could exert their effect either independently or by interacting with environmental or other genetic factors. There may also be lineage-specific combinations of advantageous polymorphisms, which could explain why the risk of a certain disease phenotype is lower in some mtDNA haplogroups. The possibility of lineage-specific combinations of advantageous polymorphisms has not so far been assessed, however.

An example of an advantageous phenotype is longevity. MtDNA haplogroups J (de Benedictis *et al.* 1999, Ross *et al.* 2001), the 9055G>A polymorphism defining haplogroup K (Ivanova *et al.* 1998b, Ross *et al.* 2001) and the 5178C>A polymorphism defining haplogroup D (Tanaka *et al.* 1998) have been found to be more common among centenarians than among controls, suggesting that there are advantageous polymorphisms in these mtDNA lineages. MtDNA has also been associated with another advantageous phenotype, improved aerobic physical performance (Dionne *et al.* 1991, Murakami *et al.* 2002, Tanaka *et al.* 2004).

MtDNA polymorphisms or haplogroups have also been found to be associated with a decreased risk of certain clinical phenotypes. The 10398G allele (van der Walt *et al.* 2003) and the D4b2 haplotype (Tanaka *et al.* 2004), for example, have been associated with a decreased risk of Parkinson's disease, the 5178A allele with an anti-atherogenic effect (Matsunaga *et al.* 2001), the 15497G allele with a decreased risk of obesity (Okura *et al.* 2003), suggesting that these polymorphisms independently, or in association with others, are advantageous. Likewise, mtDNA haplogroups K and U seem to counteract the harmful effect of the APOE 4 allele in developing Alzheimer's disease (Carrieri *et al.* 2001) and haplogroup U has been associated with a decreased risk of Alzheimer's disease in females independent of the ApoE genotype (van der Walt et al. 2004). In addition, haplogroup H has been associated with a decreased risk of abnormal sperm motility (Ruiz-Pesini *et al.* 2000)

2.5 Neutral theory and adaptive evolution

According to the neutral theory of molecular evolution, most of the observed DNA variation is selectively neutral or nearly neutral (Kimura 1968), since purifying selection quickly removes deleterious alleles from the population. MtDNA has been used as a tool for population genetic and phylogenetic studies on the assumptions that its nucleotide variation is neutral and that it evolves in a clock-like manner, accumulating mutations at a constant rate without selection pressures (Gerber *et al.* 2001). There are many studies, however, that indicate that molecular variation and the evolution of mtDNA are influenced by selective forces, suggesting that mtDNA is not a completely "neutral" molecular marker (Ballard & Kreitman 1994, Rand & Kann 1996, Nachman 1998, Rand & Kann 1998, Gerber *et al.* 2001). The neutral model predicts that the ratio of non-synonymous to synonymous polymorphisms within a species will be the same as the ratio of non-synonymous to synonymous fixed differences between species. In practice, however, there is an excess of non-synonymous replacement mutations within species over those between species, suggesting the presence of mildly deleterious mutations that have not yet been purified by selection (Rand & Kann 1998, Gerber *et al.* 2001). Alternatively, the excess of non-synonymous mutations within species can be explained by adaptive fixation of advantageous mutations (McDonald & Kreitman 1991, Mishmar *et al.* 2003, Ruiz-Pesini *et al.* 2004). There is also evidence to suggest that a large proportion of protein divergence is adaptive and driven by positive Darwinian selection (Fay *et al.* 2001, Fay *et al.* 2002, Smith & Eyre-Walker 2002). To track adaptive evolution one can study any phenomenon that varies between the genes in a genome, since, unlike genome-wide phenomena, which can have multiple alternative explanations, differences between genes of the same genome are more likely to be due to selection (Liberles & Wayne 2002). Interestingly, mitochondrial DNA haplogroup J has been shown to differ from the other haplogroups in particular regions (Moilanen *et al.* 2003). Differences in performance among mtDNA haplotypes and the quantification of shifts in haplotype frequencies can be used to study the neutrality of nucleotide sequence variation (Gerber *et al.* 2001).

2.6 Complex traits

2.6.1 What is a complex trait?

Most of the variable traits, such as longevity, weight and height are determined by multiple environmental and/or genetic factors, and are therefore known as complex traits (Nadeau 2001, Glazier *et al.* 2002, Wright *et al.* 2003). Almost all common diseases with a genetic component, such as asthma, diabetes, cancer, Alzheimer’s disease and schizophrenia, are similarly complex traits. The genes that contribute to complex traits are also known as quantitative trait loci (QTL). In traditional monogenic Mendelian traits there is a strong relationship between genotype and phenotype, and therefore the

discovery of genes is easier and requires fewer affected individuals (Glazier *et al.* 2002). In complex traits however, multiple genetic and environmental components may interact with each other in many ways and alter the penetrance of the trait (Figure 4), which makes the search for the determining genes much more difficult. However, the genetic background may modify the expression of most Mendelian traits as well, making them genetically complex traits, too (Nadeau 2001).

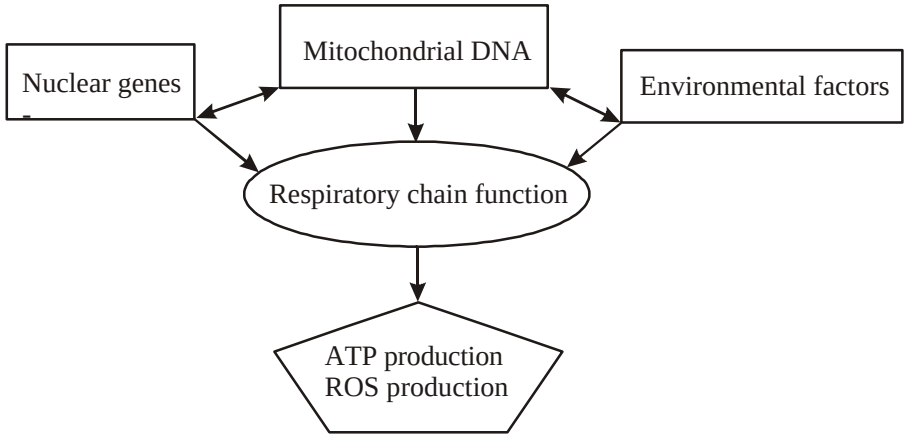


Fig. 4. Complex traits. Mitochondrial respiratory chain function is an example of a complex trait, as it is affected by mitochondrial DNA polymorphisms, nuclear genetic factors and various environmental factors.

2.6.2 Complex traits that show maternal inheritance

Due to the many roles of mitochondria in cellular functions (see section 2.1.2) and the heterogeneous phenotype of diseases caused by defects in mtDNA (see section 2.3.1), the involvement of mtDNA in complex traits is intriguing. Interestingly, several complex traits show maternal inheritance, suggesting that mutations in mtDNA may contribute to their phenotypic expression and penetrance. Examples of at least partly maternally inherited complex traits include longevity (Abbott *et al.* 1978, Brand *et al.* 1992, Sont & Vandenbroucke 1993, Korpelainen 1999), type 2 diabetes (Alcolado & Alcolado 1991, Thomas *et al.* 1994, Young *et al.* 1995), sperm motility (Moore & Reijo-Pera 2000), epilepsy (Ottman *et al.*1988, Greenberg *et al.*2000, Doose *et al.* 2001), bipolar disorder (McMahon *et al.* 1995, Kirk *et al.* 1999), Alzheimer's disease (Edland *et al.* 1996), Parkinson's disease (Wooten *et al.* 1997) and maximal aerobic power (Lesage *et al.* 1985, Perusse *et al.* 2001).

2.7 Longevity

2.7.1 Genes and environmental factors in determining longevity

Ageing and longevity are complex phenomena influenced by multiple genetic and environmental factors as well as various interactions between genes and between genes and the environment (Franceschi *et al.* 2000b, Barzilai & Shuldiner 2001, Hamet & Tremblay 2003, Perls & Terry 2003). Genes or the environment can promote longevity either by protecting individuals from age-related illnesses or by slowing down the ageing process (Barzilai & Shuldiner 2001, Martin 2002, Perls *et al.* 2002). Environmental factors that may have a strong influence on the lifespan include smoking (Basavaraj 1993, Taylor *et al.* 2002), diet (Seccareccia *et al.* 2003, Trichopoulou *et al.* 2003), physical activity (Sarna *et al.* 1997, Kujala *et al.* 1998, Lam *et al.* 2004), and also caloric restriction in animals (Heilbronn & Ravussin 2003, Koubova & Guarente 2003).

A strong relationship between genetics and longevity is suggested by studies of life expectancies of subjects who have reached a very old age and their offspring (Abbott *et al.* 1978, Gudmundsson *et al.* 2000, Mitchell *et al.* 2001, Perls *et al.* 2002), and it has also been noted that the lifespan of adopted children is greatly influenced by that of their biological parents (Sorensen *et al.* 1988). Genetic influences on longevity are likely to be greatest among the oldest individuals in a population and their siblings, and therefore extreme familial longevity is the phenotype best suited for identifying longevity assurance genes (Barzilai & Shuldiner 2001). Siblings do share some environmental and behavioural factors that may influence longevity, but their environmental characteristics are likely to diverge as they grow older, so that there should be a decline in survival advantage with age. The relative survival probabilities of the siblings of centenarians, on the other hand, show an increasing survival advantage over their lifetime, suggesting that the advantage is due more to genetic than to environmental factors (Perls *et al.* 2002).

2.7.2 The many theories of ageing

Since ageing is a complex phenomenon, several theories have emerged to explain both it and longevity (Knight 2000, Weinert & Timiras 2003). These can be classified into evolutionary (Rose & Charlesworth 1980, Gavrilov & Gavrilova 2002), molecular, cellular and system-based theories of ageing (Weinert & Timiras 2003).

The evolutionary theories include the mutation accumulation theory, the disposable soma theory and the antagonistic pleiotrophy theory. The mutation accumulation theory suggests that there are purely deleterious mutations that have no effect earlier in life but exert their effects later. These mutations would tend to accumulate in the population because of their minimal effects on fitness. According to the disposable soma theory the somatic organism is maintained only for the purpose of reproductive success and is disposable afterwards. The antagonistic pleiotropy theory suggests that there are genes with beneficial early-life effects but deleterious late-life effects. An evolutionary “trade-

off" is an essential concept in these theories: the balance of resources invested in longevity vs. reproductive fitness determines the lifespan (Weinert & Timiras 2003).

Molecular theories of ageing involve changes in the expression of genes regulating development and ageing (gene regulation theory), decline in the fidelity of gene expression, causing the production of abnormal proteins (error catastrophe theory) and the accumulation of somatic mutations with increased lifespan (somatic mutation theory). System-based theories of ageing explain it in terms of alterations in neuroendocrine functions controlling homeostasis, which result in physiological changes (neuroendocrine theory), by increased susceptibility to infectious and autoimmune diseases due to a decline in immune function (immunological theory), or by assuming a fixed metabolic potential for living organisms (rate of living theory) (Weinert & Timiras 2003).

The most popular theories of ageing are cellular ones, including the cellular senescence-telomere and free radical theories. Cellular senescence has been described as limiting cell divisions in cell cultures. This has been ascribed to the loss of telomeres from the chromosome endings or the loss of telomerase function (Masutomi *et al.* 2003, Martin-Ruiz *et al.* 2004, Zou *et al.* 2004). The free radical theory, which is one of the best-known, suggests that ageing is the result of cumulative effects of oxidative damage to various cellular structures such as DNA, proteins and lipids (Beckman & Ames 1998, Wickens 2001, Weinert & Timiras 2003).

2.7.3 Oxygen free radical theory of ageing

A free radical theory of ageing was first suggested by Harman (1956), who stated that "ageing and degenerative diseases associated with it are attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connective tissues". This theory is supported by the observations that variations in the lifespan of various animals are correlated with protective antioxidant capacity, that artificially enhanced expression of antioxidant enzymes increases the lifespan in experimental animals and that cellular free radical damage increases with age (Wickens 2001). Oxidative stress causes an accumulation of damage in cellular macromolecules and thereby detracts from their function. Mitochondria are both an important source of ROS and targets for them, and may be important in the general ageing process (Wei *et al.* 2001, Wickens 2001, Sastre *et al.* 2003). Oxidative damage to mtDNA increases with age (Ames *et al.* 1993) and mitochondrial function also decreases with increasing age (Wickens 2001, Singh 2004). Mitochondrial free radical generation is lower in long-lived than in short-lived species (Barja & Herrero 2000), and the rate of oxidative damage to mitochondrial DNA in mammals is directly related to the metabolic rate and inversely related to maximum life span (Hemnani & Parihar 1998, Barja & Herrero 2000).

A free radical is an atom, molecule or compound that contains one or more unpaired electrons, which makes these molecules highly reactive and capable of damaging other molecules (Knight 2000, Wickens 2001). Any free radical that contains oxygen can be regarded as a reactive oxygen species (ROS). ROS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH). Harman suggested in 1956

that the most likely source of these radicals in the animal cell would be the respiratory enzymes involved in direct utilization of molecular oxygen (Harman 1956), and the mitochondria are indeed the major site of ROS production in cells (Wickens 2001, Turrens 2003).

2.7.3.1 Sources of reactive oxygen species

There are numerous intracellular sources of reactive oxygen species, the four best known being mitochondrial respiration, peroxisomal -oxidation of fatty acids, mitochondrial cytochrome P-450 enzymes and phagocytic cells (Beckman & Ames 1998). In addition to intrinsic ROS production, there are various natural and synthetic extrinsic sources of ROS, including heavy metals, herbicides, pesticides, bleomycin, tobacco smoke, UV light and ionizing radiation (Vallyathan & Shi 1997, Churg 2003, Abdollahi *et al.* 2004).

Mitochondrial respiration, which uses about 90% of the oxygen taken up by the human body, is the major cellular source of ROS. Approximately 1-2% of total electron flow in the OXPHOS system results in ROS production. ROS are generated due to imperfect electron transport, which causes a one-electron reduction of O_2 to the superoxide anion (O_2^-), which is spontaneously and enzymatically dismutated to hydrogen peroxide (H_2O_2). Since almost all mitochondrial H_2O_2 orginates as O_2^-, and since most cellular H_2O_2 comes from mitochondria, O_2^- from mitochondrial respiration is probably the most important source of oxidants in the cell. (Beckmand & Ames 1998)

Several metals have a strong catalytic power enabling them to generate highly reactive oxygen species, such as hydroxyl radicals (OH). The formation of OH from H_2O_2 is catalyzed by iron, in what is known as Fenton's reaction, in which Fe^{++} is converted to Fe^{+++}. Since mitochondrial respiratory chain enzyme complexes are rich in iron, and since most of the cellular H_2O_2 comes from mitochondria, highly reactive hydroxyl radicals are constantly formed in the mitochondria, which can damage mtDNA and other mitochondrial macromolecules (Giulivi et al. 1995, Liochev 1996).

2.7.3.2 Adverse effects of reactive oxygen species

All the main biological macromolecules of the cell, nucleic acids, proteins and lipids, are targets of ROS attacks and are damaged by them. Due to the proximity of mitochodrial DNA and other mitochondrial structures to the sources of ROS production, mitochondria are the most vulnerable to these adverse effects. Thus mtDNA suffers more oxidative damage than nuclear DNA (Barja & Herrero 2000).

ROS attack DNA and other nucleic acids, causing base and sugar adducts, breaks in the DNA strands and breaks in cross-links to other molecules. There are age-dependent increases in 8-hydroxy-2-deoxyguanosine (OH8dG), a marker of oxidative damage to DNA, in human muscle tissue (Mecocci *et al.* 1999) and increased H_2O_2-induced DNA damage in the lymphocytes of elderly subjects relative to younger subjects (Mutlu-

Turkoglu *et al.* 2003). MtDNA seems to be preferentially affected by DNA oxidation, since although the amount of OH8dG increases progressively with normal ageing in both nDNA and mtDNA, the rate of increase with age is 10-15-fold greater in mtDNA (Mecocci *et al.* 1993).

There are increases in the levels of malondialdehyde (MDA), a marker of lipid peroxidation, with ageing in human muscle (Mecocci *et al.* 1999) and in plasma (Mutlu-Turkoglu *et al.* 2003), and the breakdown products of lipid peroxidation are thought to contribute to the formation of lipofuscin, a pigment that accumulates with age and is thought to be a universal correlate of animal senescence. Lipofuscin is particularly abundant in ageing post-mitotic cells (Wickens 2001).

Protein molecules are also affected by ROS and show oxidative damage to amino acid residues close to metal-binding sites, hydroxylation of aromatic groups and aliphatic amino acid side chains, protein-protein crosslinking and peptide fragmentation (Beckmand & Ames 1998, Stadtman & Levine 2003). Functional groups of proteins can react with oxidation products of polyunsaturated fatty acids and with carbohydrate derivatives and produce inactive derivatives (Stadtman & Levine 2003). In addition, levels of protein carbonyl (PC), a marker of protein oxidation, are positively correlated with ageing in human muscle (Mecocci *et al.* 1999) and plasma (Mutlu-Turkoglu *et al.* 2003). Oxidized proteins bearing carbonyl groups are generally dysfunctional (Levine & Stadtman 2001).

2.7.3.3 Antioxidant defences

A variety of natural mechanisms exist to protect cellular structures from ROS damage, either by preventing the formation of ROS or by neutralizing them. The deleterious effects of ROS and the importance of antioxidant defences are supported by the facts that the presence of some type of antioxidant defence is universal and that some antioxidants are very highly conserved (Beckman & Ames 1998). Natural antioxidant defences can be grouped into antioxidant enzymes, free radical scavengers and metal-binding proteins (Beckman & Ames 1998, Knight 2000).

The antioxidant enzymes include catalase, superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase and thioredoxin reductase. The SODs are metalloenzymes that catalytically scavenge the superoxide radical. There are three forms of SOD in humans, which exist in different cellular compartments: cytosolic copper-zinc SOD (CuZn-SOD), mitochondrial manganese SOD (Mn-SOD) and extracellular SOD (Bannister *et al.* 1987, Zelko *et al.* 2002). SODs are also highly conserved between species (Beckman & Ames 1998, Fink & Scandalios 2002). The free radical scavengers can be hydrophilic, such as vitamin C, urate and glutathione, or lipophilic, such as vitamin A, vitamin E, carotenoids, flavonoids, lipoic acid, melatonin and ubiquinol (Beckman & Ames 1998, Knight 2000). The metal-binding proteins, which reduce ROS production by transition metals, include ferritin, lactoferrin, transferrin, ceruloplasmin and myoglobin (Knight 2000).

2.7.3.4 Repair of oxidative damage

Cells also possess some degree of repair mechanism when oxidative damage by ROS occurs. For example, they contain enzymes that hydrolyse oxidised dNTPs and repair damaged DNA. These mechanisms include base excision repair (BER), which is the major pathway for the repair of oxidative DNA damage, transcription-coupled repair (TCR), mismatch repair (MMR) and homologous recombination (HR) systems, and also DNA glycosylases that initiate BER (Bohr & Dianov 1999, Slupphaug *et al.* 2003). Oxidized proteins are degraded by a proteasomal system, which is the main intracellular proteolytic pathway involved in protein turnover and the elimination of damaged proteins, and some of the oxidative damage to amino acid residues can be repaired by various enzymatic systems (Friguet *et al.* 2000, Mary *et al.* 2004). There is a decline in the proteasomal clearance of oxidative proteins upon ageing, however (Friguet *et al.* 2000), which may increase the accumulation of damaged proteins and compromise their function.

2.7.4 Telomeres and telomerase in cellular ageing

Telomeres are specialized nucleoprotein complexes that cap the ends of linear chromosomes and are composed of short tandem DNA repeats and numerous associated proteins. Telomeres are important for the protection, replication and stabilization of the chromosome ends, e.g. by preventing unwanted end-to-end fusion of chromosomes. Telomeres contain tandemly repeated DNA sequences that are highly conserved; all vertebrates have the same sequence repeat, a 6bp TTAGGG sequence (McEachern *et al.* 2000). DNA polymerases that replicate chromosomal DNA during cell division fail to replicate linear chromosome 3’ ends completely, leading to a loss of about 50-100 basepairs of telomeric DNA in each cell division. After the telomeres have been shortened below a critical length, the cell stops dividing and enters a state called replicative senescence, in which it is otherwise metabolically active but has lost its capacity for division (Wong & Collins 2003). Cultured cells in vitro can undergo only a limited number of cell divisions, known as the Hayflick limit (Hayflick & Moorhead 1961, Van Zant & de Haan 1999), which is thought to be caused by gradual erosion of the telomeric ends of the chromosomes. Telomeres are thus related to cell longevity and telomere shortening has been proposed as one of the factors that control the lifespan (Counter 1996, Weinert & Timiras 2003).

Telomerase is a ribonucleoprotein enzyme capable of stabilizing telomere length and thus maintaining the capacity for continued cell divisions. It does this by adding telomeric TTAGGG repeats onto the chromosomal ends thus preventing their shortening. Telomerase is composed of an RNA component (hTERC), which includes the template for telomeric DNA synthesis, and a cellular reverse transcriptase (hTERT), which is homologous to the viral reverse transcriptases. There are additional molecules in the telomerase complex, but these two components, hTERC and hTERT, are both necessary and sufficient for telomerase activity (Keith *et al.* 2002).

Most normal adult human cells do not express telomerase other than for activated lymphocytes, stem cells and male germ cells, but most human cancers do express telomerase, the activity being present in 90% of primary human tumours, suggesting a role for telomerase in providing cancer cells with their proliferative capacity (Preston 1997, Keith *et al.* 2002). Telomerase and its regulation is also an attractive target for the development of new anti-cancer therapeutics (Keith *et al.* 2002, Wong & Collins 2003).

The shortening of telomeres has also been shown to be important in human Werner's syndrome, a premature ageing syndrome (Chang *et al.* 2004) (see section 2.7.8.2), and fibroblasts from individuals with Werner's syndrome that undergo premature senescence can be rescued by enforced expression of telomerase (Wyllie *et al.* 2000). Interestingly, oxidative stress plays a role in telomere shortening, as mild hyperoxia shortens the telomeres and inhibits the proliferation of human fibroblasts (von Zglinicki *et al.* 1995, von Zglinicki 2002, Martin-Ruiz *et al.* 2004). Mitochondrial dysfunction can also lead to increased telomere loss (Liu *et al.* 2002).

2.7.5 Dietary restriction and maximum lifespan

The mean and maximum lifespans of various laboratory animals and organisms such as rats, mice, flies, worms and yeast can be extended by dietary restriction (DR) (Weindruch & Sohal 1997, Heilbronn & Ravussin 2003). This effect was first demonstrated in the 1930's, when DR was found to significantly extend the lifespan of rodents (McCay *et al.* 1935). Limiting the ad libitum dietary intake of mice by 40% increased their lifespan by 40% (Sohal *et al.*1994). DR increases lifespan probably by reducing oxidative stress and increasing antioxidative defences, which is thought to be the underlying anti-ageing action of DR (Yu 1996, Weindruch & Sohal 1997). DR mice have less accumulation of protein carbonyls, and their mitochondrial superoxide and hydrogen peroxide generation is lower (Sohal *et al.* 1994). Also, caloric restriction reduces mitochondrial free radical generation at complex I in the respiratory chain and lowers oxidative damage to mitochondrial DNA in the rat heart (Gredilla *et al.* 2001). Whether DR has similar lifespan-increasing effects in humans is not clear, and this would be hard to study due to the high genetic heterogeneity among humans and the high variability of diet, environmental exposures and physical activity compared with laboratory animals (Barzilai & Shuldiner 2001). Both undernutrition and overnutrition are known to shorten the human lifespan, however (Gardner & Halweil 2000).

2.7.6 Genes that affect the ageing process in animals

Genes that affect the lifespan may regulate various pathways in an organism, but in general they are often involved in oxidative and antioxidative processes, DNA repair and maintenance, insulin/IGF-like growth factor and growth hormone (GH) signalling and cell division (Butler *et al.* 2003, Hamet & Tremblay 2003). Most of the experimental

studies on the genetic basis of longevity in multicellular organisms have been conducted on the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* and mice (Hamet & Tremblay 2003). Interestingly long-lived mutant organisms of insulin signalling pathways also show increased resistance to oxidative stress and reduced oxidative damage to macromolecules (Barbieri *et al.* 2003), suggesting that ROS and oxidative damage may play an important role in ageing.

How the results of experimental studies can be extrapolated to humans is not clear. Decreased insulin-like signalling increases the lifespan in invertebrates, for example, but it may lead to insulin resistance and increased mortality in humans (Hamet & Tremblay 2003, Longo & Finch 2003). Many of the biological pathways are similar and relatively conserved in all living organisms, however, and flies, worms, mice and humans share many diseases or their equivalents and the susceptibility to them, including neurodegeneration, cancer and infectious diseases (Hamet & Tremblay 2003). Therefore, in addition to studies on human centenarians, experimental studies can give valuable clues in our search of human longevity genes.

2.7.6.1 Genes that increase the lifespan in fruit flies

Mutations in several genes, causing either a reduction or an increase in their expression, have been shown to increase longevity in *Drosophila melanogaster*, including Methuselah, Indy, chico, insulin-like receptor, Cu/Zn-superoxide dismutase, catalase and heat shock protein 70 (Aigaki *et al.* 2002).

Reduced expression of the mth (Methuselah) gene increases the lifespan of fruit flies by 35% and enhances resistance to various forms of stress, including heat, starvation, and oxidative stress. Mth is homologous to the G-protein-coupled transmembrane receptor, suggesting that this pathway modulates stress response and lifespan (Lin *et al.* 1998). Mutations in the Indy gene, an abbreviation for “I'm not dead yet”, doubled the lifespan of *Drosophila* and extended the reproductive period of the females by 40% (Rogina *et al.* 2000). Indy encodes a membrane protein that transports Krebs cycle intermediates, so that mutants may have altered energy metabolism and display a metabolic state that mimics caloric restriction (Rogina *et al.* 2000). A homozygous null allele of a chico gene, which encodes an insulin receptor substrate that functions in an insulin/IGF signalling pathway, extends the lifespan of female fruit flies by up to 48% and increases SOD levels. This does not happen in homozygous males, however, which show a decreased lifespan, but heterozygous mutants of both sexes live longer (Clancy *et al.* 2001). A hypomorphic genotype of the mutant InR gene (insulin-like receptor), which is homologous to mammalian insulin receptors, provides female fruit flies with an increase of up to 85% in longevity and males with reduced late age-specific mortality (Tatar *et al.* 2001).

Overexpression of copper-zinc superoxide dismutase (Cu/Zn-SOD), an important free radical scavenger, extends the longevity of *Drosophila* by 48% (Sun & Tower 1999), and simultaneous overexpression of Cu/Zn-SOD and catalase, the only enzyme involved in the elimination of H_2O_2 in *Drosophila*, increases the lifespan by one-third and lowers the

amount of protein oxidative damage (Orr & Sohal 1994). These findings, too, point to the importance of ROS and antioxidant systems in ageing and longevity. There is a positive correlation between the expression of Hsp70, a chaperone protein, and survival in *Drosophila*, suggesting that heat shock proteins provide protection from damage that causes ageing (Tatar *et al.* 1997). The importance of the nervous system in determining the longevity of fruit flies was shown by overexpression of DPOSH (Drosophila Plenty of SH3), which is a scaffolding protein, in the post-mitotic neurons only. This increased longevity by 14% (Seong *et al.* 2001). Meanwhile, overexpression of Cu/Zn-SOD in the motoneurons only elevated resistance to oxidative stress and extended the lifespan by 40% (Parkes *et al.* 1998).

2.7.6.2 Genes that increase the lifespan in nematodes

The nematode *Caenorhabditis elegans*, although evolutionarily far removed from the mammals, has been a widely used tool for studying the genetics of ageing and longevity, because it grows easily, develops rapidly and has a short lifespan, and because its genome has been sequenced and can easily be manipulated (Johnson 2003). In fact, the first single gene mutation shown to extend the lifespan in any organism was a “reduction-of-function” mutant age-1 (also known as daf-23) in *C. elegans*, which resulted in an increase of 40-60% in mean lifespan and 60-110% in maximal life in females and also extended life spans in males (Friedman & Johnson 1988).

The age-1 gene is involved in the insulin/IGF-1 signalling pathway, which is downregulated in mutants, and other “reduction-of-function” mutants that attenuate the same pathway, such as daf-2, which is an insulin receptor-like gene (Kimura *et al.* 1997), have been shown to double the lifespan of *C. elegans* (Kenyon *et al.* 1993). Normal alleles of daf-2 and age-1, which acts downstream from daf-2, activate a signalling cascade which eventually inactivates daf-16. This shortens the lifespan, as the activity of daf-16 is required for increased lifespan (Murakami & Johnson 1996, Guarente & Kenyon 2000). These long-living mutants show increased resistance to UV light (Murakami & Johnson 1996) and all long-living mutants of *C. elegans* show increased resistance to oxidative stress (Guarente & Kenyon 2000). Interestingly, expression of the sod-3 gene, which encodes an antioxidant enzyme Mn-SOD, increases in long-living daf-2 mutants (Honda & Honda 1999).

Furthermore, a mev-1 mutant of *C. elegans* has been found to be hypersensitive to raised oxygen concentrations, showing dramatic decreases in lifespan as oxygen concentrations are increased. Mev-1 mutants also accumulate age-related damage faster. Mev-1 encodes a subunit of succinate dehydrogenase cytochrome b, a component of complex II of the respiratory chain. The inability of complex II to catalyse electron transport from succinate to ubiquinone in mev-1 mutants may cause an increase in ROS levels, which would lead to premature ageing (Ishii *et al.* 1998).

2.7.6.3 Genes that increase the lifespan in mice

Mice provide a good model for ageing and longevity lying between the invertebrates and humans because of their more complex physiology, which resembles that of humans. At least eight genetic mouse models with increased lifespan have been described (Migliaccio *et al.* 1999, Coschigano *et al.* 2000, Bartke *et al.* 2001, Flurkey *et al.* 2001, Blüher *et al.* 2003, Holzenberger *et al.* 2003, Tirosh *et al.* 2004). Several mutant mice with an extended lifespan possess defects in the growth hormone/insulin-like growth factor 1 (GH/IGF-1) pathway and display dwarfism as well as an increased capacity to resist oxidative damage.

There are two mouse mutants, named Snell and Ames dwarf mice, that are severely growth-retarded, do not produce growth hormone (GH), thyroid-stimulating hormone (TSH) or prolactin (PRL) (Cheng *et al.* 1983) and have extended longevity (Bartke *et al.* 2001, Flurkey *et al.* 2001). The Snell dwarf mice have a loss-of-function mutation in the pit1 (pituitary specific trancsription factor 1) gene and show a 40% increase in longevity (Flurkey *et al.* 2001) and a decrease in the insulin/IGF-1 signalling pathway (Hsieh *et al.* 2002), while the Ames dwarf mice have a mutation in the Prop-1 gene, which affects the same pathway of pituitary function as pit-1 (Andersen *et al.* 1995), and have a 49-68% increase in lifespan (Bartke *et al.* 2001). Interestingly, both these long-lived dwarf mouse strains also show increased resistance to oxidative stress (Bartke *et al.* 2001, Murakami *et al.* 2003).

A mutation in the growth hormone-releasing hormone receptor (GHRHR) in mice lowers plasma GH levels but does not affect TSH or PRL levels. It also causes growth retardation and increases the lifespan by about 25% (Flurkey *et al.* 2001). A knockout mouse for the growth hormone receptor/binding protein (GHR/BP) gene shows a 40-50% increase in mean lifespan (Coschigano *et al.* 2000). These mice are also smaller and have lower levels of IGF-I (Coschigano *et al.* 2000). Mice with a fat tissue-specific insulin receptor knockout (FIRKO) have reduced fat mass, are protected against age-related metabolic abnormalities and have an increase of 18% in mean and maximum lifespan (Blüher *et al.* 2003)

Inactivation of the insulin-like growth factor receptor (IGF-1R) gene in mice increases the lifespan by 26% but does not affect growth (Holzenberger *et al.* 2003). These mice also show greater resistance to oxidative stress (Holzenberger *et al.* 2003). Interestingly, the IGF-1R gene is a homologue to the InR gene in *Drosophila* and the daf-2 gene in *C.elegans*, which increase the lifespan of these invertebrates (see sections 2.7.6.1 and 2.7.6.2, respectively)

A knockout mutation in the mouse p66shc gene, which encodes a cytoplasmic signal transducer involved in the transmission of mitogenic signals, prolongs lifespan by 30% (Migliaccio *et al.* 1999). These mice have increased resistance to various oxidants (Migliaccio *et al.* 1999) and reduced oxidative damage to nDNA and mtDNA (Trinei *et al.* 2002). Mice that overproduce the urokinase-type plasminogen activator (uPA), a protease involved in fibrinolysis and extracellular proteolysis, in many brain sites have a 20% longer lifespan than controls (Miskin & Masos 1997). These MUPA mice also show a reduced level of serum IGF-1 and a reduced incidence of spontaneous or induced

tumors in several tissues together with an increased mitochondrion-mediated apoptotic capacity (Tirosh *et al.* 2004).

2.7.7 Genes associated with longevity in humans

Since it is not possible to conduct experimental genetic studies in humans, information on genes that may promote their longevity have been obtained from analyses of either nonagenarians/centenarians or human premature ageing syndromes. Genes associated with premature ageing and decreased lifespan in humans are discussed in section 2.7.8.2. Genes that have been reported to be associated with longevity in humans may be either ones that reduce the risk of age-related illnesses such as Alzheimer's disease, cancer and cardiovascular diseases, and thereby reduce the risk of premature death, or alternatively, ones that have only been associated with longevity but not (yet) with age-related illness. The latter can be called longevity genes. Since allelic variants of some genes, such as the apolipoprotein E gene and the HLA genes, have been regarded as both longevity genes and "risk-reduction genes" in different studies, and since centenarians either do not have the genetic variants that predispose subjects to age-related diseases that can reduce their lifespan, or alternatively possess other, advantageous variants, it is best to discuss these two groups of longevity-promoting genes together.

The genes that have been most frequently associated with longevity in humans are mitochondrial DNA variants, apolipoprotein E (ApoE) alleles and human leucocyte antigen (HLA) variants. Mitochondrial DNA variants are important for respiratory chain function and the production of free radicals, ApoE alleles in lipid metabolism, and HLA variants in immune function. It may therefore be assumed that variations in these functions, due either to genes or to the environment, may be important in ageing and longevity. I will consider first the information available on ApoE variants and other apolipoproteins, then that on HLA variants, and finally that on other genes reported to be associated with longevity in humans. For mitochondrial DNA and longevity in humans, see section 2.7.9.

Frequencies of apolipoprotein E epsilon alleles have been shown to differ significantly between nonagenarians and controls in numerous studies, which report either a decreased frequency of the ApoE epsilon 4 allele or an increased frequency of the ApoE epsilon 2 allele, or both, among the long-lived (Kervinen *et al.* 1994, Schachter *et al.* 1994, Gerdes *et al.* 2000, Blanche *et al.* 2001, Frisoni *et al.* 2001, Rea *et al.* 2001, Wang *et al.* 2001), suggesting that the epsilon 4-allele is a deleterious one and the epsilon 2 allele an advantageous one. The ApoE epsilon 4 allele has also been closely associated with significantly increased risks of Alzheimer's disease (Kamboh 2004, Raber *et al.* 2004), ischaemic cerebrovascular disease (CVD; stroke or TIA) (McCarron *et al.* 1999) and cardiovascular diseases (Song *et al.* 2004), while the epsilon 2 allele increases lifespan and reduces the incidence of dementia in patients with Down's syndrome, who are particularly prone to Alzheimer's disease (Royston *et al.* 1994). Other apolipoproteins that have been reported to be associated with increased lifespan in humans are ApoA-IV (Merched *et al.*1998), ApoA1 (Garasto *et al.* 2003), VNTR polymorphisms in the ApoB

gene (de Benedictis *et al.*1997, de Benedictis *et al.* 1998, Varcasia *et al.* 2001, Garasto *et al.* 2004) and ApoC-III (Anisimov *et al.* 2001).

There are changes in immunological functions associated with ageing, such as a decrease in the ability to cope with stress, an increase in pro-inflammatory status (Franceschi *et al.* 2000a) and in susceptibility to infections and a decrease in immunoresponsiveness (Ginaldi *et al.* 1999). Many studies have shown associations between HLA alleles and longevity, e.g. in the case of HLA-DR alleles (Takata *et al.* 1987, Lagaay *et al.* 1991, Akisaka *et al.* 1997, Ivanova *et al.* 1998a, Henon *et al.* 1999, Franceschi *et al.* 2000b), HLA-DQ alleles (Akisaka *et al.* 1997, Ricci *et al.* 1998) and other HLA alleles (Ricci *et al.* 1998, Lio *et al.* 2002), suggesting that immune function is important in determining lifespan.

BRCA1 gene variants increase the risk of breast cancer (Arai *et al.* 2004, Nkondjock & Gharidian 2004), but other variants of this gene are enriched in nonagenarians relative to controls (Barzilai & Shuldiner 2001, Vijg *et al.* 2001). Differences in polymorphisms of p53, another gene in which mutations are frequently observed in cancer cases, have also been found between centenarians and controls (Gaspari *et al.* 2003).

An association has been found between longevity and allele variants of some genes responsible for xenobiotic metabolism, such as the CYP1B (Pesch *et al.* 2004) and glutathione-S-transferase genes (Gaspari *et al.* 2003, Pesch *et al.* 2004), suggesting that these polymorphic genes may be important in toxic defence mechanisms during ageing. A certain genotype of human sirtuin 3 (SIRT3), a mitochondrial NAD-dependent deacetylase which is evolutionary conserved, is more frequent in very elderly males (Rose *et al.* 2003).

Interestingly, two genes of circulating plasma proteins show increased frequencies of the same alleles among centenarians that have been shown to be associated with cardiovascular diseases. The first one is paraoxonase (PON1), an arylesterase, which protects low-density lipoprotein (LDL) against oxidative damage and has been found to differ in allele frequencies between very elderly populations and controls in both Italy and Ireland (Bonafe *et al.* 2002, Rea *et al.* 2004), and the other is plasminogen activator inhibitor 1 (PAI-1), which is involved in haemostasis. An allele, which is associated with impaired fibrinolysis and atherothrombotic events is also more prevalent among centenarians (Mannucci *et al.* 1997).

An A allele of IGF-1R has been found to be more frequent among the long-lived, indicating that the insulin/IGF-1 pathway is also important for human longevity, as has been shown for fruit flies, nematodes and mice (Bonafe *et al.* 2003). A variant of another gene, which has been shown to promote longevity in lower species has also been associated with longevity in humans. This is p66SHC1, which increases lifespan and resistance to oxidative stress in mice (Mooijaart *et al.* 2004).

Other genes found to have been associated with human longevity are the tyrosine hydroxylase (TH) (Tan *et al.* 2002) and heat shock protein 70 –1 (HSP70-1) genes (Altomare *et al.* 2003), while the frequency of the D allele of angiotensin I converting enzyme (ACE) has been liked with longevity (Schachter *et al.* 1994) but this has not been confirmed in other studies (Blanche *et al.* 2001, Panza *et al.* 2003). Interestingly, the ACE gene has also been linked with elite athletic performance, the D allele being less frequent among endurance athletes and the I allele more frequent (see section 2.8.2.2).

2.7.8 Premature ageing

2.7.8.1 Premature ageing in mice

Mutations in at least nine genes have been described as leading to premature ageing and decreased lifespan in mice (Katz *et al.* 1995, Rudolph *et al.* 1999, Vogel *et al.* 1999, De Boer *et al.* 2002, Nabeshima *et al.* 2002, Tyner *et al.* 2002, Mounkes *et al.* 2003, Baker *et al.* 2004, Trifunovich *et al.* 2004). Five of these are involved in the metabolism of either mitochondrial or nuclear DNA (Rudolph *et al.* 1999, Vogel *et al.* 1999, De Boer *et al.* 2002, Baker *et al.* 2004, Trifunovich *et al.* 2004).

Mice deficient in GLUT4 (GLUT4-null), an insulin-sensitive glucose transporter, which is the most abundant glucose transporter in muscle and adipose tissue, are growth-retarded, have cardiac hypertrophy, reduced adipose tissue, postprandial hyperinsulinaemia and shortened lifespan (Katz *et al.* 1995), suggesting that the GLUT4 protein is important for growth, glucose and fat metabolism and normal lifespan (Katz *et al.* 1995). Mutation in a gene named klotho causes shortened lifespan and abnormalities in calcium metabolism. Klotho is involved in calcium and phosphorus homeostasis by downregulating the synthesis of active vitamin D, and mice with reduced expression show kidney, lung and heart cell degradation by calcium-dependent proteolysis (Nabeshima 2002).

Mice with an autosomal recessive mutation in the lamin A gene (LMNA), which encodes for A-type lamins, major components of the nuclear lamina, show early signs of ageing and a markedly shortened lifespan (Mounkes *et al.* 2003) These mice have delayed growth, age-related bone, muscle and skin pathologies and nuclear morphology defects in fibroblasts (Mounkes *et al.* 2003)**.** These findings are similar to those reported in Hutchinson-Gilford progeria syndrome (HGPS), a human premature ageing syndrome in which the lamin A gene is also disrupted (Eriksson *et al.* 2003).

A mutation in the tumour suppressor gene p53, a transcription factor that undergoes mutation in most human cancers, shortens the lifespan of mice by 20% (Tyner *et al.* 2002). The mice also showed earlier onset of a variety of ageing phenotypes, such as delayed wound healing, early osteoporosis and organ atrophy, but also increased cancer resistance (Tyner *et al.* 2002). The mutation in these prematurely ageing mice causes an increase in p53 activity, and the age-associated loss of organ cellularity and delayed wound healing may be signs of a loss of stem cell proliferative capacity (Dumble *et al.* 2004).

Telomerase (mTR-/-) knockout mice showed age-dependent telomere shortening and genetic instability together with a shortened lifespan and reduced capacity to respond to stress (Rudolph *et al.* 1999). These mice also had an increased incidence of spontaneous malignancies (Rudolph *et al.* 1999). Ku86 is a protein involved in the repair of double-strand breaks in DNA formed after exposure to UV radiation, for example (Vogel *et al.* 1999), and Ku86-mutant mice show early changes characteristic of ageing, including osteopenia and atrophic skin, and a shortened lifespan, mostly due to cancer and sepsis (Vogel *et al.* 1999). In addition, mice with a mutation in the XPD gene, which encodes a DNA helicase that is important for both the repair and transcription of DNA and undergoes mutation in the human segmental ageing syndrome disorder

trichothiodystrophy (TTD), show many symptoms of premature ageing, including osteoporosis and kyphosis, early greying, cachexia and infertility. These mice also have a reduced lifespan (De Boer *et al.* 2002). If these TTD mice have an additional mutation in the XPA gene, ageing is further accelerated and there is increased sensitivity to oxidative DNA damage (De Boer *et al.* 2002).

Mutant mice with low levels of BubR1 protein, which is involved in the spindle assembly checkpoint during mitosis, show many premature features of ageing such as loss of subcutaneous fat, infertility, lordokyphosis, cataracts and impaired wound healing, and have a markedly reduced lifespan of about 6 months compared with that of 15 months in the control mice. The fibroblasts of these BubR1-deficient mice show progressively increased aneuploidy and senescence (Baker *et al.* 2004).

It is well known that point mutations and deletions of mtDNA accumulate in a variety of tissues with ageing, especially in post-mitotic tissues, but the relevance of these changes to ageing and longevity has been unclear. Interestingly, homozygous knock-in mice expressing a proof-reading-deficient version of PolgA, a catalytic subunit of mtDNA polymerase encoded by nDNA, develop three to five times higher levels of point mutations in mtDNA, and also higher levels of deleted mtDNA (Trifunovic *et al.* 2004). These mice also show an early onset of age-related changes, including osteoporosis, kyphosis, alopecia, weight loss, reduced subcutaneous fat, anaemia, reduced fertility and markedly reduced lifespan (Trifunovic *et al.* 2004). Also, cardiomyopathic changes such as an enlarged heart and a mosaic pattern of cytochrome c oxidase deficiency in cardiomyocytes, similar to that seen in ageing human hearts, were found (Trifunovic *et al.* 2004). The accumulation of random mutations in mtDNA in these mice may compromise the function of mtDNA-encoded respiratory subunits and lead to proton leak and increased ROS production (Trifunovic *et al.* 2004)

2.7.8.2 Premature ageing in humans

Several human genes have been identified in which mutations may lead to accelerated ageing (Martin & Oshima 2000, Hasty *et al.* 2003). The disorders caused by these mutations are termed segmental progeroid syndromes, because they display some but not all features of normal human ageing. The two best-known of these syndromes are Hutchinson-Gilford progeria (Hutchinson 1886, Gilford 1897, Sarkar & Shinton 2001, Eriksson *et al.* 2003, Hegele 2003) and Werner syndrome (Werner 1904, Yu *et al.* 1996, Mohaghegh *et al.* 2002, Hegele 2003), while others that mimic features of senescence include Rothmund-Thomson syndrome (Wang *et al.* 2001, Mohaghegh *et al.* 2002), Cockayne syndrome (Ozdirim *et al.* 1996, van der Horst *et al.* 1997, Stevnsner *et al.* 2002), trichothiodystrophy (Bergmann & Egly 2001), ataxia teleangiectasia (Concannon & Gatti 1997, Lavin *et al.* 2004, McKinnon 2004), Down syndrome (Pueschel 1990, Druzhyna *et al.* 1998, Raji & Rao 1998), Bloom syndrome (Ellis & German 1996, Mohaghegh *et al.* 2002, Kaneko & Kondo 2004), Nijmegen breakage syndrome (van der Burgt *et al.* 1996) and neonatal progeroid (Wiedemann-Rautensrauch) syndrome (Pivnick *et al.* 2000).

Hutchinson-Gilford progeria syndrome (HGPS), a childhood progeric syndrome, is caused by de novo point mutations in the lamin A gene (Eriksson *et al.* 2003). Its incidence is 1:8 000 000 live births, males being more often affected (1.5:1). Patients appear normal at birth, but the first symptoms appear by the age of 1-2 years and the mean age at death is 12 years. These children have short stature, low weight, lack of sexual maturation, loss of subcutaneous fat, wrinkled skin, alopecia, prominent scalp veins, “plucked bird” appearance, abnormal dentition, abnormal gait, hip dislocation, stiff joints, dystrophic nails and a high pitched voice. They have premature atherosclerosis and usually die of cardiac or cerebrovascular diseases. Despite all these features of normal ageing, HPGS children are not prone to cancer and do not develop cataracts or presbyacusis, features often seen in human ageing, and their intelligence is and remains normal (Martin & Oshima 2000, Sarkar & Shinton 2001, Hegele 2003).

Werner’s syndrome (WS), an adulthood progeric syndrome, is caused by homozygosity for null mutations in the WRN gene, which encodes for a RecQ family DNA helicase responsible for unwinding the DNA molecule during replication (Yu *et al.* 1996, Shen & Loeb 2001). The onset is in young adulthood and the median age at death, due to myocardial infarction or cancer, is 47-48 years (Martin & Oshima 2000, Hegele 2003). The key distinction between HGPS and WS is the age at onset. Also, WS patients develop bilateral cataracts and a variety of benign and malignant neoplasms, sarcomas of mesenchymal origin being more common than in the general ageing population (Martin & Oshima 2000). The vulnerability of WS patients to neoplasms can be explained by the abnormal DNA metabolism and genomic instability due to the defect in DNA helicase. Their cells have high somatic mutation rates and are particularly prone to deletions (Fukuchi *et al.* 1989). In addition to unwinding double-stranded DNA, WRN helicase has exonuclease activity (Oshima 2000) and resolves aberrant DNA structures (Shen & Loeb 2001), so that loss of these functions may contribute to the ageing phenotype associated with WS. The pathogenesis and appearance of this phenotype is related to telomere shortening (Chang *et al.* 2004), but no differences in allelic variants of the WRN gene were found between centenarians and controls (Castro *et al.* 1999).

Some of the other, milder segmental progeroid syndromes are also caused by a defective DNA maintenance system, e.g. Bloom syndrome (BLM helicase) (Kaneko & Kondo 2004), Rothmund-Thomson syndrome (helicase), Cockayne syndrome type B (transcription-coupled DNA repair), trichothiodystrophy (DNA repair and basal transcription), and ataxia telangiectasia (protein kinase that responds to DNA damage) (Martin & Oshima 2000, Hasty *et al.* 2003). Twinkle (mitochondrial DNA helicase) and POLG defects are also known to enhance the age-dependent accumulation of mutations in the control region of mtDNA in humans (Wanrooij *et al.* 2004).

The phenotype reminiscent of normal ageing observed in these segmental progeroid syndromes displaying genomic instability implies that the maintaining of genome integrity may be a major factor in cell viability, and thus in longevity (Hasty *et al.* 2003).

2.7.9 Mitochondrial DNA and longevity

The role of mtDNA in ageing and longevity is supported by observations that longevity is more often maternally inherited than paternally (see section 2.6.2) and that the respiratory chain is the major intracellular source of ROS, which play a role in ageing (see section 2.7.3.2). Associations between mtDNA and longevity have also been reported in human populations.

2.7.9.1 MtDNA polymorphisms associated with longevity

Longevity has been found to be associated with mtDNA coding region polymorphisms such as 5178A (characterizing haplogroup D) in the Japanese (Tanaka *et al.* 1998) and 9055A (characterizing haplogroup K) in the French (Ivanova *et al.* 1998b) and Irish (Ross *et al.* 2001), and with haplogroup J in the Italians (de Benedictis *et al.* 1999). Furthermore, the 150T polymorphism within a 1.1 kb non-coding control region of mtDNA has been reported to be more prevalent in centenarians than in controls (Zhang *et al.* 2003). These studies have focused on single loci, however, and have not taken into account associated nucleotide variation elsewhere in the mtDNA lineage.

In addition, mtDNA deletions that are found in aged organisms are flanked by sections of DNA sequence repeats, and there is a relationship between the lifespan of a species and the number of longer direct repeats in its mitochondrial genome. Long-lived species do not have as many of the longer direct repeats in their mtDNA as do shorter-lived species (Samuels 2004).

2.8 Extreme physical performance

2.8.1 Interaction of genes with the environment

Elite athletic performance is a complex trait, and in addition to nutritional and environmental factors, a number of genes influence physical performance and physical activity either independently or in interaction with other genes (Maia *et al.* 2002, Perusse *et al.* 2003, Rupert 2003, Yang *et al.* 2003, Heck *et al.* 2004, Rankinen *et al.* 2004).

2.8.2 Genes associated with elite athletic performance

The candidate genes for studies on the genetic component of elite athletic performance are those classes involved in fatty acid or carbohydrate metabolism, genes for skeletal

muscle structural proteins and metabolism, genes of the cardiovascular-pulmonary system and mitochondrial genes encoded either by nDNA or mtDNA, which are involved in aerobic respiration (Rupert 2003, Perusse *et al.* 2003, Heck *et al.* 2004, Rankinen *et al.* 2004). Most studies on genes in physical performance have been conducted on non-athletes and the measured outcomes have been related to physiological or anatomical changes in the body such as maximum oxygen uptake, changes in blood pressure, muscle strength, bone mineral density, serum cholesterol levels, heart rate and left ventricular mass (Perusse *et al.* 2003). There are also some studies conducted specifically on selected groups of athletes, however, which will be reviewed briefly in the following sections. Mitochondrial DNA in physical and elite athletic performance will be discussed in section 2.8.3.

2.8.2.1 Alpha-actinin-3 genotypes

The alpha-actinins are actin-binding proteins present in skeletal muscle cells, which are structural components of Z-lines, where they anchor actin-containing thin filaments (Mills *et al.* 2001). -actinin-2 (ACTN2), which is expressed in all human muscle fibres, and -actinin-3 (ACTN3), which is expressed only in fast-twitch (type 2B) muscle fibres, are the -actinin genes present in humans. ACTN3 protein is absent from the muscle tissue of approximately 16% of the world's population without causing any clinical phenotype (North *et al.* 1999). This absence is caused by a C to T transition at position 1747 in exon 16 of the gene, which converts an arginine to a stop codon (R577X) (North *et al.* 1999). The 577X allele is most frequent in Eurasia and least frequent in African Bantu, African Americans and Aboriginal Australians (Mills *et al.* 2001), and complete absence of ACTN3 protein (the 577XX genotype) varies from 25% in Asians to <1% in the African Bantu population (Yang *et al.* 2003). The ACTN3 gene is highly conserved, however, its nucleotide substitution rate at non-synonymous sites being lower than average, implying that it has provided some selective advantage during evolution (Mills *et al.* 2001). Since ACTN3 is expressed only in type 2B fast muscle fibres, it may be important for the efficiency of muscle contraction, which may have been crucial to human survival in the past (Mills *et al.* 2001).

If ACTN3 deficiency were to impair the efficiency of muscle contraction, its deficiency would reduce performance in sprint/power events, and thus the 577XX genotype should be less frequent in sprint athletes. Interestingly, this seems to be the case, since Australian sprinters were shown to have a lower frequency of the 577XX genotype (6%) than controls (18%) (Yang *et al.* 2003). Also, no Olympic sprinters had 577XX, but sprinters had a higher frequency of the 577RR genotype (Yang *et al.* 2003). Furthermore, endurance athletes had a higher frequency of the 577XX genotype (24%) than did the controls (18%). This suggests that sprint and endurance performance are partly genetically determined, and that there seems to be a "trade-off" between the relevant traits (Yang *et al.* 2003).

2.8.2.2 Other genes

Most studies of elite athletic performance and genes have been conducted on angiotensin converting enzyme (ACE) insertion (ACE I allele) and deletion (ACE D allele) polymorphisms. The D allele is associated with increased serum ACE activity, enhanced conversion of angiotensinogen I (AngI) to angiotensinogen II (Ang II), cardiovascular morbidity and decreased bradykinin activity (Brown *et al.* 1998, Payne & Montgomery 2003), whereas the I allele is associated with lower ACE activities and higher bradykinin activities. Ang II stimulates AT receptors, which mediate a response that increases blood pressure via salt and water retention and vasoconstriction. Stimulation of heart AT receptors leads to an increase in left ventricular mass. Bradykinin has a vasodilating effect, and thus its degradation enhances the hypertensive effect of AT receptor stimulation. Interestingly, bradykinin also alters glycogen and fatty acid metabolism in muscle and reduces lactate levels (Payne and Montgomery 2003).

The I allele is in general associated with endurance performance and the D allele with greater strength gain and power/sprinting-oriented performance (Jones *et al.* 2002). A higher frequency of the ACE I allele or the ACE II genotype has been reported among high altitude mountaineers and British Army recruits (Montgomery *et al.* 1998), Australian national rowers (Gayagay *et al.* 1998), British Olympic-standard runners, the frequency of the I allele increasing with the distance run (Myerson *et al.* 1999), Spanish professional athletes (Alvarez *et al.* 2000), Russian athletes in middle duration (1-20min) events (Nazarov *et al.* 2001) and South-African triathlonists (Collins *et al.* 2004). A higher frequency of the D allele has been reported in Russian athletes in short duration events (<1min) (Nazarov *et al.* 2001) and in Caucasian short-distance swimmers (Woods *et al.* 2001). Studies with heterogeneous cohorts of athletes from mixed sporting disciplines have found no association between ACE alleles and athletic performance (Rankinen *et al.* 2000, Sonna *et al.* 2001), showing the importance of selecting the group for study.

Interestingly, a bradykinin receptor genetic variant, B(2)R-9, conferring high kinin receptor activity, has been shown to be associated with elite athletic performance requiring endurance, especially in combination with the ACE II genotype (Williams *et al.* 2004). Other genetic variants reported to be associated with elite athletic performance are an alpha-2A-adrenoceptor (ADRA2A) gene variant that shows a weak association with endurance performance (Wolfarth *et al.* 2000) and a beta-2-adrenoceptor (ADRB2) gene variant associated with elite endurance performance in older women (Moore *et al.* 2001).

2.8.3 Mitochondrial DNA and physical performance

Mitochondria produce ATP aerobically by oxidative phosphorylation. It has been generally accepted that the use of oxygen is a major contributor to ATP synthesis in endurance exercise but not in short-duration sprint events. During high-intensity short-duration exercise such as sprinting, creatine-phosphate degradation and the anaerobic breakdown of muscle glycogen to lactate produce most of the ATP, and mitochondrial

oxidative phosphorylation is not as important a source of ATP as it is in endurance events. Also, some of the most common signs of diseases caused by defects in mtDNA are exercise intolerance, muscle weakness and lactate production due to inefficient OXPHOS (DiMauro & Andreu 2001, Taivassalo *et al.* 2003, Taylor *et al.* 2004). There may be differences in the genes determining aerobic performance, e.g. those in mitochondrial DNA, between sprinters and endurance athletes.

2.8.3.1 Oxidative phosphorylation and anaerobic glycolysis

Aerobic organisms have two main routes for synthesizing ATP, via anaerobic glycolysis in the cytosol and via oxidative phosphorylation, which takes place on the inner mitochondrial membrane in eukaryotes and yields about 17 times more ATP than glycolysis when glucose is used as a substrate (Kadenbach 2003).

Muscle metabolism and force production in trained sprinters, trained endurance runners and untrained subjects has been studied using phosphorus magnetic resonance spectroscopy (^{31}P-MRS) (Johansen & Quistorff 2003). The subjects performed four maximal isometric contractions of 30-sec duration with a 60-sec recovery. During the first contraction only the sprinters and the untrained subjects showed a significant drop in pH, indicating that glycolytic proton production was larger. In another study of metabolic enzyme activity patterns in muscle biopsies from athletes of different kinds, oxidative activity was highest in the endurance athletes and glycolytic activity in the sprinters (Boros-Hatfaludy *et al.* 1986).

In the past, an efficient energy system capable of either sustaining high intensity, e.g. to escape from predators, or maintaining prolonged physical activity, e.g. to travel long distances by foot, must have been crucial for human survival. Anaerobic glycolysis can allow short bursts of intense physical activity, whereas aerobic oxidative phosphorylation, can sustain physical activity for many hours (de Feo *et al.* 2003). Since both good sprinting and good endurance performance are in part genetically determined, and since aerobic performance shows a maternal rather than paternal inheritance (Lesage *et al.* 1985, Perusse *et al.* 2001), a role can be suggested for mtDNA in aerobic performance.

2.8.3.2 Exercise intolerance and lactic acidosis in mtDNA defects

Exercise intolerance, often accompanied by lactic acidosis, is a fundamental consequence of impaired OXPHOS function due to pathogenic mutations in muscle mtDNA. The degree of exercise intolerance has been found to correlate with the severity of impaired muscle OXPHOS, and the muscle mutation load has been found to determine exercise capacity (Jeppesen *et al.* 2003, Taivassalo *et al.* 2003). These mutations in the mtDNA of muscle tissue can be maternally inherited or sporadic, they can occur in many tissues or be restricted to muscle, and exercise intolerance can be the only symptom or part of a multisystemic syndrome (Taivassalo *et al.* 2003, Taylor *et al.* 2004). Several mutations in

mtDNA have been described as causes of exercise intolerance (Perusse *et al.* 2003, Taivassalo *et al.* 2003).

Myopathic symptoms such as exercise intolerance, myalgia and weakness, in combination with lactic acidosis and/or myoglobinuria, have been described in connection with mutations in various mitochondrial genes, such as the MTCYTB gene (Andreu *et al.* 1998, Andreu *et al.* 1999a, Bruno *et al.* 2003, Mancuso *et al.* 2003), the MTND1 gene (Musumeci *et al.* 2000), the MTND4 gene (Andreu *et al.* 1999b), the COX genes (Hanna *et al.* 1998, Karadimas *et al.* 2000) and the transfer RNA genes (Vissing *et al.* 1998, Pulkes *et al.* 2000, Vives-Bauza *et al.* 2001, Karadimas *et al.* 2002, Taylor *et al.* 2002, Grafakou *et al.* 2003).

2.8.3.3 MtDNA polymorphisms and athletic performance

Hardly any research has been published on the association of mtDNA with elite athletic performance. The MTATP6 and MTATP8 genes were sequenced in ten Japanese long-distance runners, and five of them were found to carry the 8794C>T transition in MTATP6 which defines the Asian haplogroup A (Tanaka *et al.* 2004). Three restriction fragment length polymorphisms (RFLPs) in the MTND5 gene were studied in 125 Caucasian male athletes and 65 controls, but no significant associations with endurance status were revealed (Rivera *et al.* 1998).

There are two studies in which endurance capacity, trainability and maximal oxygen uptake have been assessed in sedentary males in relation to certain mtDNA variants (Dionne *et al.* 1991, Murakami *et al.* 2002). One of these, which focused on five mtDNA nucleotide variants, suggested an association between mtDNA control region polymorphisms and endurance capacity (Murakami *et al.* 2002), and the other, in which 22 RFLPs were studied, suggested an association between one variant of MTND5 and maximum oxygen uptake (Dionne *et al.* 1991).

Despite the central role of mtDNA in aerobic energy production, the role of mtDNA has not previously been studied in a large cohort that expresses a trait that can be considered the main function of mitochondria, highly efficient ATP production. Similarly, differences in mtDNA between population groups that differ in their main ATP production pathways, such as sprinters and endurance runners, have not previously been studied.

3 Aims of the research

The purpose of this work was to study variations in mitochondrial DNA in population groups that represent two highly selected complex traits, longevity and elite athletic performance.

The specific aims were:

1. to determine differences in the frequencies of mitochondrial DNA haplogroups between a very old Finnish population and controls, and to study whether the average number of polymorphisms in mtDNA haplogroups differs among the haplogroups detected in these populations,
2. to determine the role of the 150C>T control region mutation in longevity and to further characterize sequence variations in the subhaplogroups of the Finnish and Japanese populations that harbour 150C>T,
3. to systematically assess the role of mitochondrial DNA alleles and allele combinations and overall variation in mtDNA in longevity in the Finnish and Japanese populations, and
4. to define the role of mitochondrial DNA and -actinin-3 in elite endurance and sprint athletic performance.

4 Subjects and methods

4.1 Subjects and controls

4.1.1 Subjects (I-IV)

4.1.1.1 Very old subjects (I-III)

The group of very old Finns (I-III) included 225 persons aged 90 or 91 years living in the city of Tampere, Finland, comprising 46 men (20.4%) and 179 women (79.6%), 162 of whom lived independently at home and 63 were institutionalized. The subjects were unrelated with the exception of one pair of twins and one pair of siblings. All those who belonged to haplogroup J (n=17) and a random sample of 35 subjects belonging to haplogroup H were included in paper III.

These subjects had been examined clinically, and information from hospital records regarding coronary artery disease, heart failure, peripheral artery arteriosclerosis, stroke, heart infarction, pulmonary embolism, cancer, diabetes, dementia and Parkinson's disease was used to evaluate the role of mtDNA haplogroups as risk factors for disease.

The group of Japanese centenarians (II, III) consisted of 96 persons aged 100-104 years living in the cities of Gifu and Tokyo, Japan.

4.1.1.2 Athletes (IV)

The subjects consisted of 141 Finnish elite track and field athletes (52 endurance athletes, 89 sprinters) and included three pairs of twins and five other pairs of siblings. Athletes were included if they had participated in the national track and field championships and/or in a national-level cross-country race. Their best achievements were requested, and they were considered top athletes, if they had represented Finland in the European or World Track and Field Championships (20 endurance athletes, 23 sprinters). Endurance athletes were runners whose main event was between 800 m and the marathon, including

three walkers, and the sprinters were athletes whose main event was a race between 100 m and 400 m, long jump, high jump, pole vault, heptathlon or decathlon. All the athletes participated in the mtDNA analysis and 108 (77%) of them, 40 endurance athletes (77%) and 68 sprinters (76%), participated in the ACTN3 analysis.

The birthplace of the maternal grandmother of the athletes was determined. Among the endurance athletes, 77% of the maternal grandmothers had been born south from the 64. latitude, and among the sprint athletes the corresponding figure was 82%. Furthermore, 81% of the controls had been born in this part of the country. These figures suggest that the groups were homogenous in their geographical background.

4.1.2 Controls (I-IV)

Five groups of population controls were used. The first (age 18-65 years; mean 40.5 years) consisted of 400 healthy blood donors, samples from whom were obtained anonymously from the Finnish Red Cross Office in Tampere. These were used in paper I, and 393 of them, due to a lack of some DNA samples, were used in paper II.

The second control group consisted of 257 infants (age 2-12 months; mean 6.5 months) born at Tampere University Hospital during a 10-month period, suggesting that the probability of siblings among them was low. These were used in paper I.

The third control group (age range 19-65 years) consisted of 201 healthy blood donors, samples from whom were obtained anonymously from the Finnish Red Cross Office in Tampere. It was required that the maternal grandmother of the donor was born in the province of Pirkanmaa. A set of 41 haplogroup H samples were selected at random from the 98 samples belonging to this haplogroup (paper III) and examined alongside the set of all 15 samples belonging to haplogroup J. A set of 40 randomly selected samples belonging to various haplogroups was used in paper IV.

The fourth control group consisted of 80 healthy blood donors, samples from whom were obtained at the Finnish Red Cross offices in Northern Ostrobothnia (40 samples) and Central Ostrobothnia (40 samples) (paper IV). The donors in the third and fourth control groups and their mothers were required to be healthy with respect to diabetes mellitus, sensorineural hearing impairment and neurological ailments.

The fifth control group consisted of 192 young Japanese males aged 18-25 years. 96 of these were used in paper II and all of them in paper III. These controls were from the same geographical region as the very old Japanese subjects.

In addition, data on mtDNA haplogroup frequencies of previously published 403 Finnish subjects (Meinilä et al. 2001) as well as the mtDNA haplogroup frequencies of 657 controls of paper I were included in the mtDNA haplogroup analyses of paper IV.

4.2 Molecular methods

4.2.1 DNA extraction (I-IV)

Total DNA was isolated from blood using a QIAamp Blood Kit (Qiagen, Hilden, Germany), or in the case of infants, by a non-enzymatic salt precipitation method (Lahiri & Nurnberger 1991).

4.2.2 Polymerase chain reaction (I-IV)

The primers for the polymerase chain reaction (PCR) were designed and numbered according to the Cambridge reference sequence (Anderson *et al.* 1981, Andrews *et al.* 1999), the mean primer length being 22 nts. Template DNA from blood was amplified in a PCR reaction and the amplified fragments used for restriction fragment length polymorphism (RFLP) analysis, conformation-sensitive gel electrophoresis (CSGE) or sequencing. In the PCR, the samples were denatured at 94°C for 1 min, annealed at a primer-specific temperature (50°C to 63°C) and extended at 72°C for 30 cycles.

4.2.3 Analysis of mtDNA haplogroups and subhaplogroups (I-IV)

MtDNA haplogroups (I, III, IV) and subhaplogroups (II, IV) of the subjects and controls were determined by RFLP (Torroni *et al.* 1996, Finnilä *et al.* 2000). The nucleotide sites defining the subhaplogroups were selected on the basis of sequence variation in the European population (Finnilä *et al.* 2001, Herrnstadt *et al.* 2002). (Table 1)

Table 1. Polymorphisms used to determine mtDNA haplogroups and subhaplogroups.

Haplogroup/subhaplogroup	Nucleotide variant	Restriction enzyme
H	7028C	-AluI
H1	3010G>A	-Hpy188III
H2	4769A	+AluI
H3	6776T>C	n.a.
V	4580G>A	-Hsp92II
V1	5263C>T	-HaeIII
U	12308A>G	+DdeI[a]
U2	3720A>G	+BseNI
U5	9477G>A	+Tsp509I
U8	9698T>C	n.a.
K	12308A>G	+DdeI[a]
	10398A>G	+DdeI
K1*	9093A>G	n.a.
T	15607A>G	+AluI
T1	12633C>A	-AvaII
T2	11812A>G	n.a.
J	13708G>A	-MvaI
J1	3010G>A	-Hpy188III
J2	7476C>T	-AluI
W	8251G>A	+AvaII
W1	12669C>T	+NdeII
W2	9612G>A	n.a.
I	1719G>A	-DdeI
	8251G>A	+AvaII
I1	6734G>A	-EcoRV
I2	15758A>G	+DdeI
X	1719G>A	-DdeI
Z	10400C>T	+AluI

The haplogroups and subhaplogroups were determined by restriction fragment analysis, or by direct sequencing (H3, T2, U8, W2, a subhaplogroup of K). Subhaplogroup K1 is defined by 1189T>C (Herrnstadt et al. 2002) and a subset of this subhaplogroup defined by 9093A>G appears to be frequent among the Finns (Finnilä et al. 2001). Allele status at a variable site is reported relative to rCRS. + = gain of a restriction site, - = loss of a restriction site, n.a. = restriction site not available. [a]= restriction site created by means of a mismatched oligonucleotide (Finnilä et al. 2000).

The amplified fragments were digested overnight and then electrophoresed through 1.5% agarose or 2-3% MetaPhor® Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) gel as appropriate. The variant nucleotides determined and the restriction enzymes used in the digestion of the amplified fragments are shown in Table 1. When a restriction site was not available (subhaplogroups H3, a subgroup of K1, T2, U8 and W2), the variant nucleotide was determined by direct sequencing (paper IV). A mismatched forward primer L12279 (5´-AAC AGC TAT CCA TTG GTC TTA GGC CCT

AA-3´), which creates a restriction site when 12308A>G is present, was used to determine haplogroups U and K.

4.2.4 Conformation-sensitive gel electrophoresis (I, III-IV)

CSGE is a highly sensitive and specific method for detecting single nucleotide polymorphisms (Körkkö *et al.* 1998, Finnilä *et al.* 2000), by visualizing differences in mobility in denaturing polyacrylamide gel electrophoresis in the case of DNA fragments that differ from a known control sequence. The coding region of mtDNA (nts 577-16023) was amplified in sixty-three fragments spanning nts 523-16090, the primers being designed in such a way that the mean size of the fragments was 354bp and neighbouring fragments overlapped by at least 80bp (Finnilä *et al.* 2000). PCR was carried out as described (see section 4.2.2).

To assess the quality of the PCR product, the amplified fragments were visualized on a 1.5% agarose gel and a suitable amount of the PCR product, usually 4-8 l, was taken for heteroduplex formation. Each amplified fragment was then mixed with a control sample with a known sequence that had been amplified with the same primers. The fragments were denatured at 95°C for 5 min and heteroduplexes allowed to anneal at 68°C for 30 min. These were then electrophoresed through a 15% polyacrylamide gel overnight at a constant voltage of 400V at room temperature after a 30 min pre-electrophoresis of the gel. The gel was then stained on a glass plate in a 150 g/l ethidium bromide solution for 5 min and destained in water. The fragments were visualized with an UV transilluminator and photographed (Grab-IT Annotating Grabber 2.04.7;UVP Inc, Upland, CA). The heteroduplexes that showed differential mobility on the polyacrylamide gel were selected for sequencing to identify the specific polymorphism.

4.2.5 Sequencing (II-IV)

The amplified PCR products were first treated with exonuclease I and shrimp alkaline phosphatase (Werle *et al.* 1994). The primers used for sequencing of the coding region of mtDNA were the same as for the amplification reactions for CSGE. The control region (paper II) was amplified in one fragment spanning nts 15975-725 and the sequence was determined between nts 16024 and 576 using three sequencing primers. The nucleotide sequences were analysed by automatic sequencing (ABI PRISM 377 Sequencer with Dye Terminator Cycle Sequencing Ready Kit, Perkin Elmer, Foster City, CA).

4.2.6 Detection of 150C>T polymorphism (II)

The allele status at nt 150 was determined by allele-specific amplification. DNA was amplified in the presence of an oligonucleotide containing an allele-specific Locked Nucleic Acid® (LNA) (Proligo LLC, Paris, France). Samples harbouring 150C could be amplified in the presence of a forward primer 5'-CTGTCTTTGATTCCTGCCTCATC (LNA underlined), and samples harbouring 150T in the presence of a forward primer 5'-CTGTCTTTGATTCCTGCCTCATT. The samples in both reactions were amplified with 5'-CTGTTAAAAGTGCATACCGCCAA as the reverse primer. The amplified 302-bp fragment was visualized by agarose gel electrophoresis. Sequencing (see section 4.2.5) of selected samples was used to verify the reliability of the results obtained by allele-specific amplification.

4.2.7 ACTN3 genotype analysis (IV)

ACTN3 genotypes were determined from exon 16 of ACTN3 (Mills *et al.* 2001). Alleles 577R and 577X (codons CGA=arginine and TGA=stop codon, respectively) were distinguished by the presence (577X) or absence (577R) of a DdeI restriction site. Digested PCR fragments of the 577X allele have 108, 97 and 86 bp, whereas digested PCR fragments of the 577R allele have 205 and 85bp. The digested PCR fragments were separated out by gel electrophoresis on 2% Metaphor Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA).

4.3 Phylogenetic analyses (II, III)

A phylogenetic tree depicting the origin and evolution of the mtDNA subhaplogroups harbouring 150C>T (II) was constructed from a data set that included a total of 322 control region sequences, of which 65 were from the Finnish nonagenarians, 65 from the Finnish controls, 96 from the Japanese centenarians and 96 from the Japanese controls.

Phylogenetic networks of the coding region (III) in Finnish and Japanese subjects were constructed by means of a reduced-median algorithm (Bandelt *et al.* 1995), as implemented in the Network program (version 4.1 for Windows**)** (available at http://www.fluxus-engineering.com/sharenet.htm). The weights of the nucleotide positions were equal. The data set included a total of 404 coding region sequences: 52 from the very old Finns, 56 from the Finnish controls, 96 from the Japanese centenarians and 192 from the Japanese controls.

4.4 Statistical analyses (I-IV)

The differences in the frequencies of mtDNA haplogroups and haplogroup clusters between the populations were evaluated using the exact test of population differentiation (Rousset et al. 1995) as implemented in ARLEQUIN 2.0 (Schneider et al. 2000), or, if applicable, by the ^{2} test or Fisher's exact test (paper I).

The one-tailed Fisher's exact test was used to assess the hypotheses that the frequency of 150T and the frequencies of haplogroups harbouring 150T would be higher among the very old subjects than among the controls. The frequencies among the very old subjects and the controls in each haplogroup were presented in 2 2 tables, and heterogeneity among these was evaluated using RelRisk 2.33 (Ott 1999). Associations between alleles at polymorphic sites and longevity were assessed using the chi-square statistic ^{2}. An estimate of the significance of the highest observed chi-square value ($^2_{max}$) was obtained in a randomization test in which the labels "case" and "control" were permuted 100,000 times and the global P-value was estimated by the proportion of permutation samples with $^2_{max}$ equal to or higher than that in the observed data (paper II).

Differences in the frequencies of mtDNA subhaplogroups between the very old Finns and controls were evaluated using the chi-square statistic ^{2} and Fisher's exact test. To assess how closely mtDNA coding region alleles in the Finns and Japanese and complete genome alleles in the Japanese were associated with longevity, association analysis was performed by the combinatorial partitioning method. The sequences were first aligned manually and then invariant columns were excluded, columns with equivalent content were identified and combined, and finally the resulting character matrix was analysed by a combinatorial partitioning method. Partitions in which the number of internal or external sequences was <5 were excluded due to the low statistical power of detecting true associations in such cases. Then the alleles and their combinations were evaluated systematically by Fisher's exact test. Since the rate of type I error may be high with multiple testing of this kind, the P-values obtained using Fisher's test were corrected by a resampling procedure (Wesfall & Young 1989) to obtain globally adjusted P-values (FEW, family-wise error rate). This permutation-based method takes into account the structure of the phylogeny and the distribution of alleles in a sample, but it is also very conservative and may lead to false exclusion of truly significant associations. Consequently, the multiple testing problem was also assessed by methods that estimate the false detection rate (FDR), which represents the expected proportion of false positives at a given level of significance. The combinatorial partitioning method considers all alleles and allele combinations and ensures that no important partitions will be missed, but the search for all possible partitions becomes computationally impossible for multiallele combinations, and therefore partitioning with stochastic optimization was performed to find the partitions that best separated the very old subjects from the controls. This method searches heuristically for the partitions with the lowest P-value. Combinatorial partitioning, permutation tests and stochastic optimization were carried out using custom software developed by the first author of manuscript III.(paper III).

Differences in the overall nucleotide diversity of non-synonymous and synonymous mtDNA sites between the very old and control subjects were evaluated by performing permutation tests and sliding-window analyses. Nucleotide diversity was characterized in

terms of the average number of pairwise differences (*k*), the number of polymorphic sites in the sample (*S*) and the number of singleton mutations in the sample ($?_s$). The sliding-window analyses (Moilanen *et al.* 2003), which allow comparisons between different areas of the genome, were performed with a window of 250 adjacent nucleotides that was moved in 40-bp steps. Unlike the association analyses, where only alleles or allele combinations with the number of internal or external branches was 5 were considered, all alleles were taken into account in sliding-window analyses (paper III).

Exact test of population differentiation was used to assess differences in the frequencies of mtDNA haplogroups between the endurance and sprint athletes, and to analyse differences in mtDNA haplogroup frequencies among the three ACTN3 genotypes (paper IV).

5 Results

5.1 MtDNA haplogroups differ significantly between very old subjects and controls (I)

5.1.1 MtDNA haplogroup and cluster frequencies

MtDNA haplogroups were determined in 882 samples, representing 225 very old subjects, 400 healthy middle-aged controls and 257 infant controls. The haplogroup frequencies of the very old subjects differed from those of the middle-aged controls ($P = 0.01$), that of haplogroup H being lower ($P = 0.001$), as was also the case with the closely related haplogroup V, while the frequency of haplogroup U was higher ($P = 0.03$) as was that of the related haplogroup K. To substantiate the observed differences, we analysed a group of 257 infants, and again found significant differences in haplogroup frequencies between the very old subjects and the infants ($P = 0.00005$), but not between the middle-aged controls and the infants ($P = 0.22$). Haplogroup J was present at a higher frequency among the very old subjects than in either of the control groups, although the frequency of subcluster J1 was 4.0% in the very old subjects and 4.5% in the controls, so that the difference was entirely due to subcluster J2, with frequencies of 4.4% in the very old subjects and 1.8% in the middle-aged controls (Table 1 in paper I).

We then analysed the frequencies of the clusters of phylogenetically related haplogroups HV, UK, TJ and WIX. Again the very old subjects and the middle-aged controls differed ($P = 0.002$), the frequency of cluster HV being lower in the former ($P = 0.001$), whereas that of cluster UK was higher ($P = 0.014$). The number of samples belonging to cluster WIX was almost twice as high in the very old subjects as in the middle-aged controls ($P = 0.078$). The mtDNA cluster frequencies also differed between the very old subjects and the infants ($P = 0.00001$), the frequency of cluster HV being significantly lower in the former ($P = 0.001$), while those of clusters WIX ($P = 0.048$) and UK ($P = 0.05$) were higher and that of cluster TJ significantly higher ($P = 0.004$). Again the middle-aged controls and infants did not differ significantly from each other (Table 2 in paper I).

5.1.2 Association between mtDNA haplogroups and clinical phenotypes

The genotype-phenotype association was studied in the very old subjects after stratification according to the presence or absence of various clinical phenotypes. An association was found between mtDNA haplogroups and diabetes mellitus (P = 0.044), the frequency of cluster UK being twice as high in patients with diabetes mellitus. In fact, 60% of the patients with diabetes mellitus (15/25) belonged to cluster UK, while only 32% of the non-diabetic subjects did, and furthermore, 28% (7/25) of the diabetics belonged to haplogroup K, the frequency of which was only 5% in the subjects without diabetes.

5.1.3 Average number of polymorphisms in haplogroups

The association between longevity and mtDNA haplogroups D in the Japanese and J in the Finns suggests either advantageous polymorphisms within these haplogroups or mildly deleterious polymorphisms within the remaining haplogroups. In order to test the hypothesis that new polymorphisms are part of an adaptive process of evolution, we calculated average substitution frequencies for the mtDNA haplogroups using data on 192 complete mtDNA sequences from Finns (Finnilä *et al.* 2001). Surprisingly, the average frequency of substitutions was higher in those haplogroups that were found more frequently among the very old subjects, suggesting that some substitutions can be advantageous and supporting the hypothesis of adaptive evolution (Figure I in paper I).

5.2 150C>T is associated with longevity in the Finns and Japanese (II)

5.2.1 150C>T and other control region polymorphisms in longevity

We found a significantly higher frequency of 150T in the very old Finnish and Japanese subjects than among their controls (Table 1 in paper II), this polymorphism being present in subhaplogroups J2, T2 and U5 among the Finns and in subhaplogroups D5, M7b and N9a among the Japanese. Sequencing of the control region in the samples from all the 150T carriers revealed that almost all of those belonging to subhaplogroup J2 harboured at least four mutations close to the origins of replication of mtDNA (Zhang *et al.* 2003), whereas no corresponding pattern was found for the other subhaplogroups. Polymorphisms near the origin of heavy strand replication could thus explain the association between longevity and subhaplogroup J2, but not that between longevity and D5 or M7b, also found at higher frequencies among the very old subjects.

Our further analyses revealed that the closest association with longevity among the control region sequences of 150T carriers occurred in the case of 489C (^{2} = 14.099; permutation test: P = 0.00165), to the extent that no other control region variants were

significantly associated with longevity among the 150T carriers when the position 489 was excluded ($^{2}_{max}$ = 7.075; permutation test: *P* = 0.126). A phylogenetic analysis then revealed that both 150T and 489C were present in subhaplogroups J2, D5 and M7b, whereas only 150T was present in T2, U5 and N9a. Furthermore, the coding region polymorphism 10398G was found to co-segregate with 489C in subhaplogroups J2, D5 and M7b.

A contingency table analysis of the frequencies of very old subjects and controls in subhaplogroups carrying 150T revealed a significant heterogeneity (^{2} = 17.604, df = 5, *P* = 0.0035). Subhaplogroups J2 (P = 0.015), D5 (P = 0.032) and M7b (P = 0.032) were more common among the very old, and the combined frequency of these subhaplogroups was significantly higher among the very old subjects (6.9%) than among the controls (1.0%) (P = 7.8×10^{-6}).

5.2.2 Evidence of epistatic interactions promoting longevity

150C>T has emerged separately early in the evolution of the European subhaplogroups J2, T2 and U5 and of the Asian subhaplogroups D5, M7b and N9a, but has only occasionally been noted elsewhere in the mtDNA phylogeny (Figure 1 in paper II). Subhaplogroups D5 and M7b in the Japanese belong to macrohaplogroup M, whereas N9a in the Japanese and J2, T2 and U5 in the Finns belong to macrohaplogroup N (see section 2.4.1). Most of the haplogroups in macrohaplogroup N harbour an ancient 10398G>A mutation, but haplogroup J has experienced a back-mutation at this site and now harbours an allele in common with subhaplogroups of macrohaplogroup M such as D5 and M7b. Furthermore, haplogroup J harbours 489T>C, which is also present in macrohaplogroup M. 150T is associated with longevity in subhaplogroups J2, D5 and M7b, which harbour 10398G and 489C, but not in subhaplogroups T2, U5 and N9a, which lack the latter two polymorphisms.

5.3 Systematic assessment of mtDNA variation in longevity in Finnish and Japanese populations (III)

5.3.1 Association analysis of mtDNA alleles and allele combinations

A two-dimensional (2D) representation (Figure 1 in paper III) was constructed to visualize the overall strengths of associations of loci and combinations of loci. The distribution of significant associations showed the closest associations to be located in the control region and between the MTCO2 and MTND4L genes (nucleotide positions 7500-10700) when complete genome was analysed, while the regions of the MTRNR2 and MTCO1 genes were devoid of significant associations.

The allele association analysis with combinatorial partitioning revealed that the most significant association in the coding region was for the combination of 14605A and 15257G. This association was negative, indicating that an individual carrying these alleles has markedly lower chances of belonging to the very old sector of the population (OR = 0.15, P = 0.0000617), and defined all other samples except for those belonging to subhaplogroups D4b2b and J2. The closest positive association for any single allele was found for 15257A (OR = 7.96, P = 0.003), a non-synonymous change in the MTCYB gene which defines subhaplogroup J2, and another close positive association was found for 7476T (OR = 7.03, P = 0.007) in the MTTS1 gene, which defines subhalogroup J2 (Table 1 in paper III).

Analysis of the complete genome revealed evidence for an association in the case of the lack of expansions in two short tandem repeats at positions 573 and 16193 in the mtDNA control region. All of the very old samples lacked these expansions, whereas only 79.7% of the controls did so (OR = 49.66, P = 0.0000001). The lack of an expansion at position 16193 alone also showed a positive association with longevity (OR = 26.76, P = 0.0000958) In addition, positive association with longevity was also shown for 194T (OR = 3.37, P = 0.006), 199C (OR = 2.54, P = 0.009) and for 204C (OR = 2.73, P = 0.057). (Table 1 in paper III).

Partitioning with stochastic optimization of the mtDNA coding region alleles revealed a complex set of 36 alleles that were present in 97% of the very old cases and in 78% of the controls (OR 10.26, $P = 1.1 \times 10^{-8}$). A high proportion of the alleles in this combination could be assumed to have some functional significance, such as 14990G, which is adjacent to a heme ligand in cytochrome b, and also several alleles in tRNA genes. The analysis of complete mtDNA sequences revealed a 12-allele combination that was present in all of the 96 very old subjects and only 71% of the controls (OR 25.62, $P = 6.514 \times 10^{-12}$) and included four expansions of tandem nucleotide repeats of cytocines (at positions 573, 965, 5899 and 16193) (Table 2 in paper III).

In both association analysis with combinatorial partitioning and partitioning with stochastic optimization, the alleles or combinations showing the closest associations with longevity included a high number of alleles in tRNA and rRNA genes (Tables 1 and 2 in paper III).

5.3.2 Phylogenetic network for Finnish and Japanese very old people and controls

The Finnish and Japanese samples included a total of 148 very old subjects (52 Finns and 96 Japanese) and 248 controls (56 Finns and 192 Japanese). The very old subjects showed clustering into certain mtDNA subhaplogroups: subhaplogroups J2 and H1 in the Finns and subhaplogroups D4b2, M7b and D5 in the Japanese (Figures 2 and 3 in paper III). The frequencies of various subhaplogroups differed between the very old Finns and controls, but these differences were not significant. The frequency of subhaplogroup J2 was higher among the very old subjects (15.4%) than among the controls (3.6%), and was 47.1% among the very old subjects belonging to haplogroup J, but only 13.3% among the

controls belonging to that subhaplogroup. At the same time, the frequency of subhaplogroup H1 was higher among the very old (34.6%) than among the controls (26.8%), whereas that of subhaplogroup H2 was lower (5.8% vs 12.5%).

Among the Japanese, the frequency of subhaplogroup D4b2 was higher among the very old subjects (17.7%) than among the controls (6.8%), as was that of subhaplogroup M7b (7.3% vs 3.6%), and that of D5 (8.3% vs. 2.6%). The subhaplogroup D4g was less frequent among the Japanese very old than among the controls (2.1% vs. 5.2%).

Many of the tRNA and rRNA changes and expansions of short tandem repeats with significant associations with longevity showed clustering into subhaplogroups that were more frequent among the very old than among the controls, such as subhaplogroups D4b2 and J2, or less frequent, such as subhaplogroup D4g (Figures 2 and 3 in paper III). Subhaplogroup D4b2 harbours a change at position 1382 in an rRNA gene and is otherwise devoid of tRNA or rRNA mutations. Subhaplogroup J2 harbours a substitution at position 7476 in a tRNA gene and a subsequent change at position 1850 in an rRNA gene. Subhaplogroup D4g harbours a mutation at position 4343 in a tRNA gene (MTTQ) and a CC duplication at position 573 in a tRNA gene (MTTF).

5.3.3 Overall sequence diversity of the mtDNA coding region in very old subjects and controls

In the analysis of both non-synonymous (non-degenerate) and synonymous (four-fold degenerate) sites, the average number of pairwise differences (*k*), the number of polymorphic (segregating) sites in the sample (*S*) and the number of singleton mutations in the sample ($?_s$) were first estimated among the very old subjects, after which empirical probability distributions (expected values) were calculated for these statistics by taking 10,000 random permutations with an equal number of sequences to the cases each time and shuffling the case-control labels at random in each permutation.

The analysis of non-synonymous sites revealed that these three measures among the very old subjects were not significantly different from the expected values, nor were any marked local differences revealed in the sliding-window analyses. The very old subjects nevertheless had slightly less polymorphic sites and singleton alleles between nt positions 9000 and 10000 than did the controls (Figure 4.A in paper III).

The average number of pairwise differences at synonymous sites among the very old subjects was higher than expected, but did not reach significance (P = 0.068), the number of polymorphic sites did not differ from that expected, and the number of singleton alleles was again higher than expected but not significantly so. The sliding-window analyses showed that the higher number of pairwise differences at synonymous sites in the very old subjects resulted from a generally greater incidence of pairwise differences in almost all regions of the genome and was not confined to any particular mtDNA region. The sliding-window analysis of singleton alleles, however, revealed that the very old subjects had a slight excess of private synonymous mutations between nt positions 8000 and 11000 in mtDNA, the highest estimate being obtained when the window spanned nt

positions 8808-10292 (Figure 4.B in paper III). This area encompasses the MTCO3 gene and regions of the MTATP6 and MTND3 genes.

5.4 MtDNA haplogroups and ACTN3 genotypes in Finnish elite athletes (IV)

5.4.1 MtDNA haplogroup frequencies among endurance and sprint athletes

The frequencies of mtDNA haplogroups differed between the endurance and sprint athletes ($P < 0.039$), haplogroups J and K being more common among the sprinters. Only one endurance athlete belonged to haplogroup J (1.9%), whereas its frequency among the sprinters was 6.7%. Two of the sprinters belonged to subhaplogroup J2, while the remaining four sprinters and the endurance athlete belonged to subhaplogroup J1. None of the endurance athletes belonged to haplogroup K, whereas the frequency of this haplogroup was 9.0% among the sprinters. The combined frequency of subhaplogroup J2 and haplogroup K among the sprinters was 11.2%. In addition, four endurance athletes (6.6%) belonged to haplogroup I, whereas none of the sprinters belonged to this haplogroup (Table 2 in paper IV). None of the Finnish athletes harboured 8794C>T in MTATP6, a mutation reported previously in Japanese endurance runners (Tanaka et al. 2004).

5.4.2 ACTN3 genotypes in athletes and controls

We found the 577XX genotype of ACTN3 to be less frequent among the sprinters than among the endurance runners and the 577RR genotype more frequent. None of the top sprinters belonged to genotype 577XX. When both the sprinters and endurance athletes were stratified into two groups on the basis of their top achievements, there was an inverse correlation between the frequency of 577XX and success in sprinting events (Figure 1 in paper IV). Furthermore, ACTN3 genotypes did not explain the differences in mtDNA genotypes between the two groups of athletes.

6 Discussion

6.1 MtDNA haplogroups in longevity and elite athletic performance

There were significant differences in mtDNA haplogroup frequencies between the very old subjects and controls and between the endurance athletes and sprinters in the Finnish population. Haplogroup H was less frequent among the very old than among controls, whereas haplogroups J, U and K were more frequent. The frequencies of related haplogroups also had similar tendencies to increase or decrease in most cases, and in consequence, the frequency of haplogroup cluster HV was significantly lower among the very old subjects and those of clusters UK and TJ higher.

The very old subjects were living in the city of Tampere and the controls were anonymous blood donors from the Finnish Red Cross Office in Tampere. It was required that the maternal grandmother of every donor should have been born in the province of Pirkanmaa and that the donors and their mother were healthy (see section 2.6.1) allowing that the mtDNA sequence information of the very old can be compared to that of the controls that are assumed to represent a general healthy population. The question of whether the results of the study on very old subjects from Tampere, Finland, can be extrapolated to Finnish population, and to other populations, is essential. The frequencies of mtDNA haplogroups vary slightly in different parts of the country and, therefore, the controls must be obtained from the same region as the cases. However, since mtDNA haplogroups in Finland are the same as those in Europe, and since the proportions of mtDNA haplogroups is quite similar in different parts of Finland (Meinilä et al. 2001) and in Europe (Torroni et al. 1996), the results obtained in this study most likely can be extrapolated to at least Finnish and European populations. Interestingly, an association between haplogroup J and longevity has also been reported among Italians (De Benedictis *et al.* 1999), and differences in the frequencies of subclusters comparable to our observation that subcluster J2 was more frequent among the very old subjects have also been reported in Irish centenarians and controls (Ross *et al.* 2001), although the assignment to subclusters in that case was based on the nucleotide sequence in HVS-I, which does not allow comparison with our data. The mutation 9055G>A, defining haplogroup K, has been found in increased frequency among French centenarians (Ivanova *et al.* 1998), and this haplogroup was also more frequent among the very old Finns (7.6%) than among either the midde-aged controls (6.0%) or the infants (4.7%).

Interestingly, we found that none of the Finnish endurance athletes belonged to either subhaplogroup J2 or haplogroup K, those found at a higher frequency among the very old, whereas the combined frequency of these two haplogroups among the sprinters was 11.2%. The frequency of subhaplogroup J2 in the Finnish population in general is at least 1.5% and that of haplogroup K 3.0%, giving a combined frequency among the Finns of 4.5% (Meinilä *et al.* 2001).

These data suggest associations between mtDNA haplogroups and both longevity and elite athletic performance, and in addition point to a pattern in which haplogroups or subhaplogroups that are increased in frequency among the very old, i.e. appear to promote longevity, seem not to be beneficial in terms of aerobic physical performance, since none of the endurance athletes belonged to either subhaplogroup J2 or haplogroup K.

6.2 Does uncoupling play a role in longevity and elite athletic performance?

The main function of the mitochondria is to produce ATP by OXPHOS, and while the uncoupling of OXPHOS generates heat, it concomitantly reduces the production of ATP, due to decreased proton translocation across the mitochondrial inner membrane or due to proton leak via ATP synthase (Kadenbach 2003). Uncoupling of OXPHOS also lowers the production of reactive oxygen species (ROS), which are obligatory by-products of OXPHOS (Skulachev 1998, Kadenbach 2003, Miwa *et al.* 2003) and are thought to play a role in the process of ageing (Barja *et al.* 2004, Huang and Manton 2004). Genes that increase the lifespan of fruit flies (see section 2.7.6.1), nematodes (see section 2.7.6.2) and mice (see section 2.7.6.3), often increase the capacity to resist oxidative stress. Interestingly, mice with enhanced uncoupling live longer (Speakman et al. 2004), and mice with less uncoupling produce more ROS (Hagen and Vidal-Puig 2002). MtDNA polymorphisms that increase uncoupling and concomitant heat production may have been important for climatic adaptation and may therefore have been subject to positive selection (Coskun *et al.* 2003, Ruiz-Pesini *et al.* 2004). Interestingly, differences in mtDNA variations have been found between populations living in a tropical climate and populations living in the arctic (Coskun *et al.* 2003, Ruiz-Pesini *et al.* 2004).

Endurance athletes should be a highly selected group in terms of the efficiency of ATP production, and they should therefore have a low level of mitochondrial uncoupling. On the other hand, an “uncoupling genotype”leading to less efficient OXPHOS and lower ATP production would produce less ROS as well (Coskun *et al*. 2003) and probably promote longevity. Interestingly, we found that the mtDNA haplogroups, which increased in frequency among the very old were not found among the endurance athletes. It may therefore be suggested that subhaplogroup J2 and haplogroup K are “uncoupling genomes” that encode respiratory chain components that produce less ROS and promote longevity (Ivanova *et al.* 1998, de Benedictis *et al.* 1999, Ross *et al*. 2001, Niemi *et al.* 2003). Due to this uncoupling, such genomes are not favourable in situations where highly efficient ATP production is required, such as endurance athletic performance.

Endurance athletes have a longer life expectancy than the general population, however (Sarna *et al*. 1993), and their mortality from all causes is lower than that of power athletes (Kujala *et al*. 2001). Longevity is a complex trait in which several genetic loci and exposures to various environmental factors play a role (Perls 2002). Endurance athletes smoke less and drink less alcohol than the general population, and athletes in general have healthier habits and maintain a higher level of physical activity even after their competitive years (Fogelholm *et al.* 1994). In addition, endurance athletes mostly have slow-twitch muscle fibres, which have been found to correlate with higher HDL cholesterol and lower triglyceride levels (Tikkanen *et al.* 1991, Tikkanen *et al.* 1996). These factors may overcome the possible deleterious ROS effects of the genetically highly efficient OXPHOS required for endurance performance.

The only genotype-phenotype association found in the very old subjects was between diabetes mellitus and haplogroup K, an "uncoupling genome", with 28% of the diabetics belonging to this haplogroup but only 5% of the non-diabetics. Insulin secretion is controlled by an ATP-sensitive K+ channel (MacDonald and Wheeler 2003), and inefficient ATP production may compromise insulin secretion and lead to diabetes (Maechler and Wollheim 2001, Maassen *et al.* 2004). Uncoupling proteins (UCP) play a role in insulin secretion and in the production of ATP and ROS (Hagen and Vidal-Puig 2002, Rousset *et al*. 2004). Over-expression of UCP-2 reduces both ATP production and insulin secretion (Langin 2003), whereas UCP-2 knockout mice have enhanced insulin secretory capacity (Joseph *et al.* 2002). These data support our hypothesis that haplogroup K is an "uncoupling genome" that may not be favourable in situations where efficient ATP production is required, such as insulin secretion.

6.3 Novel approaches to assessing the role of mtDNA in a trait

Analyses of overall variation in mtDNA have been attempted previously for sensorineural hearing impairment (SNHI) (Lehtonen et al. 2003) and for Parkinson's disease (PD) (Autere et al. 2004), the resuls suggesting that patients harbour slightly deleterious mutations in mtDNA that increase the risk of one or other of these diseases. There are no previous studies, however, that have adopted a systematic approach to assessing mtDNA alleles, allele combinations and overall sequence diversity in mtDNA by comparing cases and controls with respect to a given trait. This was done here in the systematic evaluation of mtDNA alleles and allele combinations in relation to longevity (papers II and III). The association analysis employing the combinatorial partitioning method described in paper III was performed in order to assess how closely mtDNA alleles in the Finns and Japanese are associated with longevity. Since multiple allele testing of this kind may result in a high rate of type I error, the P-values obtained from Fisher's exact test were corrected by two methods, a permutation-based resampling procedure (Westfall and Young 1989) that gives a globally adjusted P-value (FWE) but is also very conservative, and a method that estimates the false detection rate (FDR). Despite the very conservative nature of the former method, significant FWE values were obtained for certain alleles and allele combinations. In addition, stochastic optimization detected multi-allele

combinations that differed highly significantly between the very old subjects and the controls.

Since association analyses of single alleles or allele combinations are unlikely to detect possible differences in overall nucleotide variations at non-synonymous and synonymous sites in mtDNA between very old subjects and controls, the possibility that increased variation in itself, either along the entire genome or in some particular regions of mtDNA, may play a role in determining longevity was assessed by systematically evaluating differences in these sites between the two groups by sliding-window analysis (Moilanen *et al.* 2003) and by a similar permutation-based approach to that was in the allele association analyses. Nucleotide sites in mtDNA are subject to different selective constraints due to the different consequences of the mutations at those sites. Non-synonymous sites, in which a nucleotide change always leads to an amino acid change, are the most conserved, and nucleotide variation at such sites can reveal the role of accumulated amino acid variation in a trait, whereas variations at synonymous sites, in which a nucleotide change never changes an amino acid, can reveal differences in the mutation rate (Li *et al.* 1985, Gerber *et al.* 2001, Yang 2002). These sites were therefore evaluated, and the results revealed that variation at non-synonymous sites did not differ between the very old subject and the controls, whereas that at synonymous sites did, suggesting that the latter may play a role in longevity.

Furthermore, combining allele association analysis in a large number of samples with the construction of a phylogenetic network for these same samples provided an opportunity for more detailed analyses. Allele association analysis together with the information revealed in the phylogenetic network allowed clustering of significant alleles into certain branches of network to be detected, for example. It also pointed to other events that show signs of consistency, such as the occurrence of certain types of nucleotide changes in succession. This led to the observation that certain mtDNA lineages that showed either a higher or a lower frequency in the very old harboured successive changes in tRNA and rRNA genes, in control region of mtDNA and/or expansions of tandem nucleotide repeats.

6.4 MtDNA alleles and allele combinations in longevity

Longevity is a complex trait influenced by various genes, interactions between several genetic loci and environmental exposures during life (Perls *et al.* 2002, Hamet & Tremblay 2003, Perls & Terry 2003). Previous reports on associations between mtDNA variation and longevity have focused on single polymorphisms, such as 9055A (Ivanova *et al.* 1998b), 5178A (Tanaka *et al.* 1998) and 150T (Zhang et al. 2003), but nucleotide variation elsewhere in the mtDNA has not been taken into account. If several genetic loci determine a given trait rather than a single locus, a combination of alleles should show a closer association with that trait than a single locus. It was evident in paper II that not only was the frequency of 150T higher among the very old Finns and Japanese but this association was present only in subhaplogroups J2, D5 and M7b. Interestingly, all three subhaplogroups harbour 489C and 10398G alleles in addition to 150T, suggesting that

longevity is partly determined by epistatic effects between these three loci in mtDNA. It was also shown in paper III that there are certain allele combinations having either a positive or a negative association with longevity. In addition, the stochastic optimization method revealed a complex set of 12 alleles that were present in all of the very old subjects but in only 71% of the controls, and a set of 36 alleles that were present in 97% of the very old subjects and 78% of the controls. The associations of the combinations of mtDNA variants identified in papers II and III with longevity provide the first epidemiological support for the assumption that the nature of an allele is influenced by other loci in mtDNA (Moilanen *et al.* 2003). Due to the apparent possibility of epistasis, attempts to assess the role of mtDNA variation in complex traits by single-loci association analyses may overlook loci whose contribution is revealed only when considered in combination with others. 5178A, defining haplogroup D, has previously been associated with longevity (Tanaka *et al.* 1998), but it may have been a surrogate marker, since clustering of centenarians was evident only in subhaplogroup D4b2 in the phylogenetic network presented in paper III. The polymorphisms in the root of this haplogroup, such as 5178C>A may play some role in longevity, but they may require the presence of some additional alleles that are present in subhaplogroup D4b2, for example.

The combinatorial partitioning association analysis and stochastic optimization analysis of paper III revealed close associations with longevity in the case of short tandem repeats, tRNA and rRNA genes and alleles of the mtDNA control region close to the origin of H-strand replication. The phylogenetic network showed that these changes occurred in succession and, interestingly, in those branches of the network in which the frequency of very old people was either substantially higher or lower, or, as in some cases, marked by a complete absence of very old people. Subhaplogroup J2, for example, showed the closest association with longevity and harboured a 7476C>T mutation in the MTTS1 gene followed immediately by 1850C>T in the MTRNR2 gene. The function of RNA molecules depends on complex epistatic interactions between sites, so that a compensatory second-site substitution can suppress the deleterious effect of a mutation (Kern & Kondrashov 2004). It has been reported that a pathogenic mutation and its compensating substitution are fixed in an mtDNA lineage in rapid succession and that perhaps as many as 50% of all nucleotide substitutions in mammalian tRNAs participate in such interactions, indicating that the evolution of tRNAs is highly dependent on epistasis (Kern & Kondrashov 2004). This may also be the case with rRNA genes and between tRNA and rRNA genes, since they are closely involved in translation events. A compensating substitution may also “over-correct” the deleterious effect of a previous mutation, giving a rise to an unusually advantageous combination that may promote longevity, for example. This can be hypothesized in the case of subhaplogroup J2, since the 7476C>T substitution in the MTTS1 gene and the 1850C>T substitution in the MTRNR2 gene have been rapidly fixed in succession and together define a branch that is highly positively correlated with longevity.

An association between mtDNA control region allele 150T, located close to the secondary origin of heavy strand replication, and longevity has been reported (Zhang et al. 2003). In paper II we found, too, that 150T is associated with longevity both in the Finns and the Japanese In addition, paper III revealed positive associations between longevity and three alleles close to the primary origin of heavy strand replication. These alleles might play a role in the regulation of mtDNA replication and, thus, the mtDNA

copy number. Acceleration of mtDNA replication might compensate for functional deterioration of mitochondrial function that occurs in old age (Zhang et al. 2003) and, therefore, these and other alleles in the control region of mtDNA may play a role in longevity.

In addition to alleles in tRNA and rRNA genes and alleles in certain regions of control region, short tandem repeats in mtDNA seem to play a role in longevity, since none of the very old subjects harboured extra cytosines at positions 573 or 16193. In addition, expansions of short tandem repeats seem to be related to tRNA mutations, since subhaplogroup D4g, for example, which has been found to be negatively associated with longevity, harboured a tRNA mutation at position 4343 and a cytosine duplication at position 573. Among the 11 samples of subhaplogroup D4g, there was only one from a very old subject, and interestingly, it was the only sample that did not harbour these extra cytosines at position 573. It has been suggested that repeat sequences in mtDNA may constrain the lifespan of mammals (Samuels 2004), and interestingly, that the less direct repeats there are in a genome, the longer the lifespan of that mammalian species is, which is in line with our observations that the very old are lacking in expansions of tandem repeats. Our work provides the first epidemiological support for the assumption that mtDNA repeats play a role in ageing and in limiting the lifespan of a mammalian species.

Variation in repeat sequences has been shown to be important in many pathological conditions. Huntington's disease, for example, is caused by an expansion of CAG triplet repeats in the huntingtin gene (Jones *et al.*1997), the Fragile X (FRAXA) syndrome by unstable expansions of CGG trinucleotide repeats of the FMR (Fragile X Mental Retardation) gene (Mandel & Biancalana 2004), Friedreich's ataxia by an expansion of a GAA trinucleotide repeat in intron 1 of the frataxin gene (Delatycki *et al.* 2000), and myotonic dystrophy (DM) by either a CTG or CCTG expansion in the DMPK (dystrophia myotonica-protein kinase) gene (Ranum & Day 2004a, Ranum & Day 2004b). Interestingly, it has been suggested that the CTG and CCTG expansions of in DM are pathogenic at the RNA level, in that they alter cellular function, including alternative splicing of various genes (Ranum & Day 2004a, Ranum & Day 2004b). In addition, VNTR (variable number of tandem repeats) polymorphisms of the ApoB gene have been associated with longevity (de Benedictis *et al.*1997, de Benedictis *et al.* 1998, Varcasia *et al.* 2001, Garasto *et al.* 2004). Furthermore, given that telomeres (see section 2.7.4) are composed of short tandem DNA repeats (TTAGGG)n that are highly conserved, with all vertebrates having the same sequence repeat (McEachern *et al.* 2000), and that a gradual shortening of telomeres places a limit on cell divisions in a culture (Hayflick & Moorhead 1961, Van Zant & de Haan 1999), it may be concluded that telomeric DNA repeats are related to cell longevity and telomere shortening may control lifespan (Counter 1996, Weinert & Timiras 2003).

The previous evidence on the importance of expansions of tandem repeats in various pathological conditions, the correlation of the length of mtDNA repeats in a mammalian species with lifespan (Samuels 2004) and the present findings together suggest that repeat sequences in mtDNA may play a greater role in determining longevity than has previously been thought.

6.5 Overall sequence variation of mtDNA in longevity

Paper I showed that the average frequency of nucleotide substitutions (non-synonymous changes in protein-coding genes and changes in tRNA or rRNA genes) was higher in the haplogroups that were found more frequently among the very old subjects, suggesting the presence of advantageous alleles, but no overall difference in non-synonymous mtDNA sites was observed between the very old subjects and controls in paper III. The difference in the results may be due to the approaches adopted. First, the average number of nucleotide changes was calculated in paper I from 192 sequences obtained from healthy Finns (Finnilä *et al*. 2001) and the substitution rates between various haplogroups were compared, whereas in paper III the nucleotide variation was compared between sequences for the very old subjects and controls. Second, all the non-synonymous changes were considered in paper I, whereas only the non-synonymous sites were studied in paper III. Third, the variation in tRNA genes and rRNA genes was also included in paper I, and fourth, paper I presented mean values for a population, whereas in paper III all the alleles were considered separately. However, since the allele association analyses in paper III revealed a large number of substitutions in tRNA and rRNA genes having close negative or positive associations with longevity, the results of paper III may be said to be in line with those of paper I.

Although there were no significant differences in overall sequence diversity at non-synonymous sites between the very old subjects and controls, the variation at synonymous sites did show some differences. The average number of pairwise differences and the number of singleton alleles were both higher than expected in the very old subjects, and the sliding-window analysis showed that they had an excess of private synonymous alleles in the region of the MTATP6, MTCO3 and MTND3 genes. These data indicate that even synonymous mtDNA sites may have more important functional roles than has previously been thought, and therefore they, too, may be subject to selection. The role of other coding region alleles in longevity may nevertheless be more important than was indicated here, since only non-synonymous sites were considered, which does not take into account all non-synonymous nucleotide changes, as it ignores those at two-fold degenerate sites, for example.

6.6 Nature of mitochondrial DNA mutations

The neutral theory of molecular evolution states that most of the variation observed in DNA is selectively neutral or nearly neutral (Kimura 1968). Numerous population-specific or haplogroup-specific mtDNA polymorphisms have been reported (Finnilä *et al.* 2001, Herrnstadt *et al.* 2002, Silva *et al.* 2002, Kong *et al.* 2003), and most of the variation has been considered neutral. Analyses of mtDNA mutations have revealed deviations from the neutral theory, however (Ballard & Kreitman 1994, Nielsen & Weinreich 1999, Gerber *et al.* 2001, Moilanen & Majamaa 2003), and many studies suggest that variation and evolution in mtDNA may be influenced by selective forces, indicating that mtDNA is not a completely "neutral" molecular marker (Ballard &

Kreitman 1994, Rand & Kann 1996, Nachman 1998, Rand & Kann 1998, Gerber *et al.* 2001). Variation in mtDNA may therefore be neutral, slightly deleterious (Rand & Kann 1998, Gerber *et al.* 2001), or even advantageous (McDonald & Kreitman 1991, Mishmar *et al.* 2003, Ruiz-Pesini *et al.* 2004) (Figure 5).

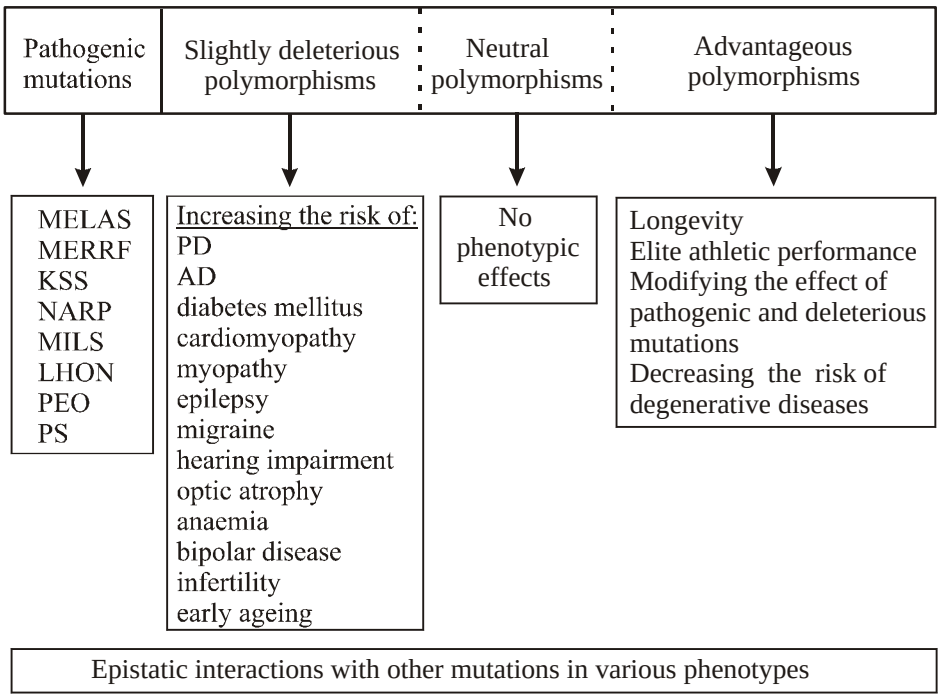


Fig. 5. Correlations between the nature of mitochondrial DNA mutations and polymorphisms and their phenotypic effects. PS = Pearson's syndrome. PD = Parkinson's disease. AD = Alzheimer's disease.

There may be alleles or combinations of alleles in mtDNA that play a role in determining complex traits. Phenotypes involving individuals who may harbour advantageous alleles include highly selected traits such as longevity and elite athletic performance, and examination of these traits may reveal advantageous alleles that may not only promote that particular trait but also modify the risk of a deleterious one. Information on alleles in highly selected traits can be of help when assessing the pathogenicity of a new polymorphism in a patient with a condition thought to be caused by an mtDNA mutation. If a given polymorphism is present in a healthy very old person, it can be considered unlikely to cause a condition in a patient, especially one who belongs to the same mtDNA lineage. The present findings suggest that haplogroups J2 and K may harbour alleles that favour longevity, as a result of a higher level of uncoupling and lower level of ROS production, but alleles that may be advantageous in one trait may not be advantageous in another. For example, these "uncoupling genomes" would not be favourable for endurance performance, and may even be deleterious by increasing the risk of diabetes, where ATP production should be high and the level of uncoupling low (see section 6.2).

It has been demonstrated here that certain allele combinations are present only in certain subhaplogroups associated with longevity, and that certain regions of mtDNA show closer associations with longevity than others. This may be a sign of positive selection, since neutral polymorphisms should be uniformly distributed between haplogroups, indicating that they have arisen multiple times in the evolution of different lineages (Ruiz-Pesini *et al.* 2004), and since differences between genes of the same genome can be explained by selection (Liberles & Wayne 2002).

The nature of an advantageous allele may be such that it is not crucial to survival and that its absence does not cause a clinical pathology, but such that without the allele the probability of expressing a highly selected trait such as longevity or elite athletic performance is low. The XX genotype of the ACTN3 gene, for example, causes complete absence of -actinin-3 in fast-twitch (type 2B) muscle fibres (North *et al.* 1999). Approximately 16% of the world's population harbours this XX genotype without any evidence of clinical pathology (North *et al.* 1999). Since ACTN3 is expressed only in type 2B fast muscle fibres, it may be important for the efficiency of muscle contraction (Mills *et al.* 2001). Interestingly, the frequency of the XX genotype has been shown to be lower among Australian sprinters than among controls, and none of the top sprinters harbour XX, whereas the frequency of the RR genotype is higher among sprinters (Yang *et al.* 2003). The present work also showed that the frequency of the RR genotype was higher among Finnish sprinters and that of XX lower, and again, none of the top sprinters were XX. These data suggest that the ACTN3 R allele is an advantageous allele without which an individual cannot achieve top sprinting performance.

A similar situation may prevail in the case of alleles or allele combinations of mtDNA found among the very old. The combination of 150T, 489C and 10398G, for example, or the allele combinations found here in paper III, may act in a similar way to the R allele of the ACTN3 gene. The absence of these advantageous alleles does not cause any evident clinical disease, but it also indicates an extremely low probability of reaching a very advanced age.

These findings support the hypothesis presented in this chapter that an advantageous allele is not a necessity for life but it may be necessary for the expression of a highly selected trait such as longevity or elite athletic performance.

6.7 Mitochondrial DNA and theories of ageing

The maternal inheritance of longevity (Abbott *et al.* 1978, Brand *et al.* 1992, Sont & Vandenbroucke 1993, Korpelainen 1999), the oxygen free radical theory of ageing (see section 2.7.3) with mitochondria being the most important source of ROS in cells (Beckman & Ames 1998), and the epidemiological evidence provided in previous studies (Ivanova et al. 1998b, Tanaka et al. 1998, de Benedictis et al. 1999, Ross et al. 2001, Zhang et al. 2003), together with the present onservations of associations between certain mtDNA variants and longevity, suggest that mitochondria and mtDNA do play a role in ageing and in determining longevity. There are several theories of ageing (Knight 2000, Weinert and Timiras 2003) (see section 2.7.2), but most evidence supports the oxygen

free radical theory (see section 2.7.3) and the telomerase theory (see section 2.7.4). There is also evidence that DNA maintenance is important, since many of the premature ageing syndromes in mice (see section 2.7.8.1) and humans (see section 2.7.8.2) are caused by defects in genes involved in DNA metabolism. Could mitochondria and mtDNA be involved in all these processes and therefore be a central factor in ageing and in regulation of the length of the lifespan? Could the different theories of ageing all be just different aspects of the same network of processes?

The mitochondrial respiratory chain produces most of the cellular ROS, which may damage macromolecules such as DNA and proteins, the signs of this damage increasing with ageing (see section 2.7.3.2). ROS play a role in telomere shortening (von Zglinicki *et al.* 1995, von Zglinicki 2002, Martin-Ruiz *et al.* 2004), and mitochondrial dysfunction leads to increased telomere loss (Liu *et al.* 2002), suggesting that mitochondrial production of reactive oxygen species may be important for cellular ageing. Advantageous alleles in mtDNA lineages, such as those in subhapogroups J2 and K, may lead to reduced production of ROS, by increasing the level of uncoupling, for example. This may help in preventing the loss of telomeres and in maintaining the replicative capacity of a cell (Figure 6). Furthermore, a role for IGF-signalling pathway in ageing has been suggested (see sections 2.7.6.2 and 2.7.6.3). In general, down-regulation of this pathway extends the lifespan of an organism (Holzenberger 2004). Interestingly, down-regulation of this pathway also increases resistance to oxidative stress in *C.elegans* (Guarente & Kenyon 2000) and in mice (Holzenberger *et al.* 2003, Holzenberger 2004).

The maintenance of mtDNA stability and integrity also seems to be important in ageing, since a proofreading-deficient version of mitochondrial DNA polymerase (POLG) causes premature ageing in mice, with increased numbers of mutations and deletions in mtDNA and decreased levels of total mtDNA (Trifunovic *et al.* 2003). In addition, defects in Twinkle (mitochondrial DNA helicase) and POLG enhance the age-dependent accumulation of mutations in the control region of mtDNA in humans (Wanrooij *et al.* 2004). This control region contains binding sites for various proteins involved in the maintenance of mtDNA, and an association between the control region variant 150T and longevity has been reported (Zhang *et al.* 2003). Further epidemiological evidence has been provided here that mtDNA control region variants may play a role in longevity. Mutations in the control region may thus affect the replication and transcription of mtDNA and lead to differences in the rate of mutation accumulation. This may in turn lead to differences in the production of ROS, which can further damage mtDNA, telomeres and other macromolecules. An mtDNA genome that harbours deleterious alleles may enter a vicious cycle that does not favour longevity. On the other hand, an mtDNA genome that harbours advantageous alleles, or allele combinations, may be in the inside lane in the lifelong race for longevity.

Further research is needed to assess the differences in the production of ATP and ROS between mtDNA lineages and whether these differences correlate with the length of the lifespan of a cell line or of the human subjects who belong to these lineages. In addition, further studies are needed to assess whether the efficiency and fidelity of mtDNA replication varies in cell lines of different mtDNA lineages and whether this correlates with the maximum number of cell divisions, and the length of the lifespan of individuals of these lineages. The role of nuclear genes that encode proteins involved in the metabolism of mtDNA and the role of various environmental factors in determining these

functions will also need further assessment in order to obtain a better understanding of the role of mtDNA in longevity and other complex traits.

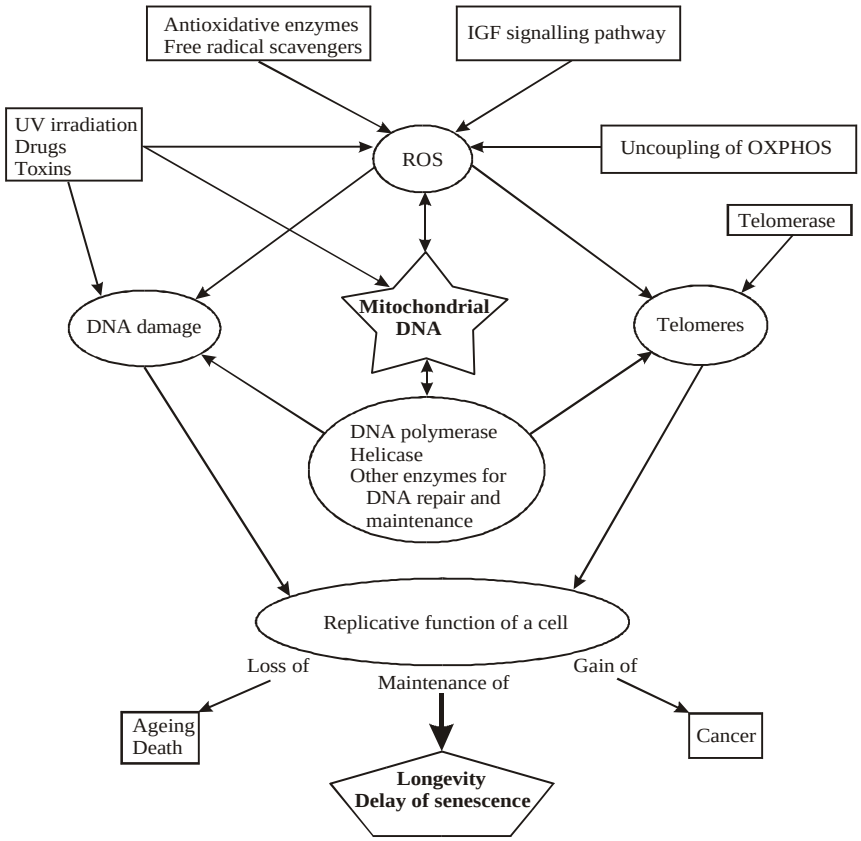


Fig. 6. The role of mtDNA in the network of factors and functions proposed as playing a role in ageing and longevity. IGF = insulin like growth factor.

7 Conclusions

1. Significant differences in mtDNA haplogroup frequencies were found between Finnish very old subjects and controls, supporting previous reports of associations between mtDNA polymorphisms and longevity. MtDNA haplogroup frequencies among endurance and sprint athletes were also assessed, and interestingly, haplogroup K and subhaplogroup J2, which were found at a higher frequency among the very old Finnish subjects, were not found in Finnish endurance athletes
2. Endurance athletes should be a highly selected group in terms of the efficiency of ATP production. The main function of the mitochondria is to produce ATP by OXPHOS. Uncoupling of OXPHOS generates heat, but concomitantly also reduces the production of ATP. Uncoupling also lowers the production of ROS, which may play a role in ageing. An “uncoupling genotype” leading to less efficient OXPHOS and lower ATP production would therefore produce less ROS as well, and probably promote longevity. The present data are consistent with this view, suggesting that subhaplogroup J2 and haplogroup K are “uncoupling genomes” encoding respiratory chain components that produce less ROS and therefore promote longevity. Due to uncoupling, these genomes may not be favourable in situations where highly efficient ATP production is required, such as endurance athletic performance.
3. Previous reports on associations between mtDNA and longevity have focused on single polymorphisms and not taken into account nucleotide variation elsewhere in the mtDNA. Novel approaches for association analyses of large sets of genomic mtDNA sequences were developed here in which it was possible to systematically assess evidence for the association of a trait not only with a single allele but also with allele combinations thereby revealing epistatic interactions between alleles of mtDNA. In addition, a permutation-based approach used here can be employed to compare overall nucleotide diversity in mtDNA sequences of cases (here very old subjects) with that expected. These novel methods allow association studies to be focused on single alleles and allele combinations as well as evaluating the significance of overall mtDNA variation for a given trait.
4. Association analysis revealed a combination of three common mtDNA polymorphisms that was significantly more frequent among the very old in both Finland and Japan. This provides the first epidemiological support for the assumption that the nature of a variant is influenced by epistatic interactions between different

loci in mtDNA. The most significant findings in the association analyses concerned alleles located in the control region of the mtDNA, among the tRNA and rRNA alleles and among the repeat sequences. In particular, longer repeat sequences were completely absent in the very old subjects. These data suggest that the control region, tRNA and rRNA genes and repeat sequences of mtDNA may have an unprecedented role in longevity.

5. Analysis of the overall sequence diversity of non-synonymous and synonymous sites of protein-coding genes in mtDNA revealed that there were no significant differences at non-synonymous sites but that the variation at synonymous sites differed between the very old subjects and the controls. The average number of pairwise differences and the number of singleton alleles were higher than expected in the sequences for the very old, who also had an excess of private synonymous alleles that clustered in the region of certain mtDNA genes. These data may be a sign of selection and suggest that even the synonymous sites of mtDNA may have functional roles, e.g. in the regulation of mtDNA transcription and replication.
6. In addition to neutral polymorphisms, mtDNA may harbour slightly deleterious or advantageous polymorphisms that modify complex traits such as longevity and elite athletic performance. The nature of an allele or allele combination may vary between traits, however. A genotype that promotes longevity, such as haplogroup K, may not be advantageous for elite endurance performance, or it may even be deleterious and increase the risk of a disease phenotype, as in the case of diabetes.
7. Further evidence is provided here that mtDNA may play a role in longevity, and also in elite athletic performance. Further epidemiological studies are needed to assess mtDNA variation systematically in these traits in different populations. In addition, functional studies are needed to assess differences in the production of ATP and ROS, in the level of uncoupling and in the efficiency and fidelity of mtDNA replication between various mtDNA lineages.

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