Mitochondrial hearing loss mutations in Northern Finnish preterm and term-born babies

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Abstract

Introduction: Approximately 3400 babies are born prematurely each year in Finland of which 1-2 % will develop a permanent hearing impairment. Premature babies are prone to severe infections and sepsis, therefore antibiotics are often needed during the first few months of their lives. Mitochondrial DNA mutations in the gene coding for mitochondrial ribosomal RNA (*MTRNR1*), can cause maternally inherited non-syndromic hearing loss. Sometimes the use of a special group of antibiotics, aminoglycosides, can induce the hearing loss in an individual carrying the mutation in *MTRNR1*. Aminoglycosides are commonly used for treating severe infections in premature infants. Aminoglycosides target the bacterial ribosome and block the protein translation process, so that eventually the bacterial cell dies. It has been speculated that *MTRNR1* mutations make the human mitochondrial ribosome more bacterial-like; thus enhancing the affinity of aminoglycosides to clamp on to the human ribosome as well. The sound-sensing cells in the inner ear have a high energy demand and have an abundance of mitochondria. The ribosomal RNA mutation and aminoglycosides together cause apoptosis and permanent damage in these sensitive cells. Materials and methods: In this study, 813 newborns (preterm and term-born babies) were screened for m.1555A>G, m.1095T>C and m.1495C>T mutations in the *MTRNR1* gene using PCR, restriction fragment length polymorphism, Sanger sequencing and radioactive S-labeled heteroplasmy quantification techniques. Results and conclusions: The prevalence of m.1555A>G was determined to be 0.12 % in the population of Northern Finland. This concurs with the findings from the rest of Europe. As a result, family carrying a heteroplasmic m.1555A>G mutation was identified for the first time from this geographical region. Previously a few large families with m.1555A>G had been identified from Northern Finland. This should be taken into account if non-syndromic hearing loss occurs in a premature baby's family. In such a case, genetic testing for the m.1555A>G mutation is recommended before administering aminoglycosides. M.1494T>C and m.1095T>C mutations were absent from the study cohort. This result suggests that either the mutations are very rare and the sample size was too small to detect them, or that the mutations do not occur in our population. The results can be directly utilized for safety considerations of aminoglycoside use on preterm babies in Finland. The need for genetic testing of aminoglycoside sensitivity mutations in preterm babies is assessed.

CONTENTS

1. INTRODUCTION 5

1.1. Inheritance of genetic information 6

1.1.1. Autosomal dominant inheritance 7

1.1.2. Autosomal recessive inheritance 7

1.1.3. X- and Y-chromosomal inheritance 8

1.1.4. *De novo* mutations 11

1.1.5. Multifactorial inheritance 11

1.2. The mitochondrion – energy for the cell 11

1.2.1. Mitochondrial DNA (mtDNA) 14

1.2.2. Mitochondrial DNA-nuclear DNA crosstalk 16

1.2.3. Mitochondrial DNA of Northern Finland 20

1.2.4. Pathogenic mtDNA mutations 21

1.2.5. Hearing loss mutations in mtDNA 23

1.2.6. Aminoglycoside sensitivity 24

1.3. Neonatal screening of genetic diseases 33

1.4. Genetic burden of small populations 35

1.4.1. The Finnish disease heritage 36

1.5. Preterm birth 37

1.5.1. Common health issues of preterm babies 41

1.5.2. Hearing loss among premature babies 43

1.5.3. Survival and long-term outcome of premature birth 44

2. OBJECTIVES OF THE STUDY 46

3. METHODS 46

3.1. Study population 46

3.2. DNA extraction 47
3.3. DNA amplification by polymerase chain reaction (PCR) 47
3.4. Sanger sequencing 48
3.5. Restriction fragment length analysis (RFLP) 49
3.6. Heteroplasmy analysis 51
3.7. Ethical considerations 52
4. RESULTS 52
5. DISCUSSION 54
REFERENCES 57

1. INTRODUCTION

Genetic traits can influence susceptibility to drug induced side-effects (1). Aminoglycosides are strong antibiotics which, as a side-effect, can cause irreversible damage to the sensory hair cells of the inner ear leading to even deafness in the most severe cases (2). Certain mitochondrial DNA (mtDNA) mutations increase the risk of aminoglycoside-induced hearing loss and deafness. These mtDNA mutations and their frequency differ among populations (3-6). The frequency of aminoglycoside-sensitivity hearing loss mutations in the healthy population has been previously determined in many Asian countries, such as China and Japan. Reported population estimates are available for e.g. United Kingdom, Italy, Greece, Spain and New Zealand, but not for Finland (7-11).

As a consequence of prematurity and intensive care, 1-2 % of children born prematurely today will have permanent hearing impairments (12, 13). Aminoglycosides are commonly used in the care of premature babies due to their efficiency in treating large scale infections and sepsis. Babies born before term are especially susceptible to the adverse effects of these antibiotics due to their underdeveloped hearing and sensory system (9). If a mitochondrial sensitivity mutation is also present, the risk of permanent hearing loss and deafness increases dramatically. The same effect can also be seen in mutation carriers in connection with the aminoglycoside usage later in life (4).

Pathogenic mitochondrial DNA mutations have been reported to be more prevalent in the healthy population than previously assumed. It has been estimated that 1 in 200 carries a harmful mitochondrial DNA mutation. These frequencies vary for each mutation and population. Some mtDNA mutations can be population-specific, meaning that a certain mutation can be more harmful in one population compared to another. This is due to the interaction of the mutation in the population-specific genetic background (nuclear genome) (14). Therefore, it is imperative to study mutation frequencies in each population separately. Frequencies of the aminoglycoside sensitive mutations are important to determine because the deafness/hearing loss they cause is avoidable. And if these mutations prove to be very common, a number of preterm-born children with permanent hearing loss could be prevented. Also the recommendations of aminoglycoside use in preterm infant care should then be revised. The consequences are life-long for the individual carrying the mutation.

1.1. Inheritance of genetic information

Genetic information is stored as double stranded deoxynucleic acid (DNA) and packed into chromosomes (Figure 1). Humans have 22 autosomal chromosome pairs inherited from both mother and father. In addition, there are two sex-determining chromosomes; the X and Y chromosomes. DNA holds the blueprint of the organism, organized in genes and intergenic non-coding regions. Genes contain a high level of variation, both benign and sometimes harmful. DNA is folded onto chromosomes during cell division and stored in a relaxed state in the nucleus after cell division is complete. Two copies of each gene are present in nuclear DNA. Mutations in nuclear DNA can be heterozygous (two different bases) or homozygous (two same bases). Mitochondrial DNA is a circular DNA molecule approximately 16 000 bp long. It is present in multiple copies in mitochondria, an energy producing organelle. The mitochondrion forms an ever changing organic network inside the cell. The mitochondrial DNA molecules exist as folded coils. Mitochondrial DNA can be heteroplasmic if a tissue/cell contains a mix of mutated and normal mtDNA. Heteroplasmy is measured in percentages and is an important feature in mitochondrial diseases (15).

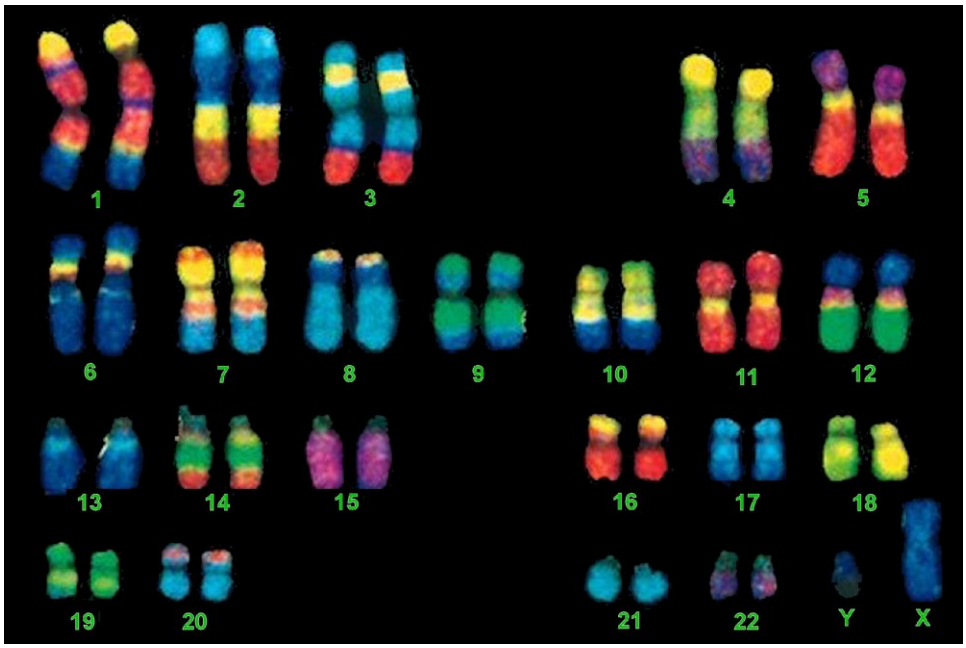

Figure 1 Human chromosomes (www.mun.ca)

1.1.1. Autosomal dominant inheritance

As chromosomes come in pairs, all autosomal genes have two copies: alleles. If a disease is inherited in the Mendelian autosomal dominant way (Figure 2), it means that the disease causing gene with a mutation is dominant over the healthy allele. This causes a disease in the individual even if he only carries a single mutated gene copy (heterozygosity): this gene copy is enough to impair the normal gene function. Hereditary diseases with autosomal dominant inheritance affect individuals in each generation and males and females in the same proportion, and the disease can be inherited either from the father or the mother. If a person with an autosomal dominant disease has children, each child has a 50 % risk of inheriting the disease causing allele and a 50 % chance of being healthy. Examples of some autosomal dominant diseases are Huntington's disease, Marfan syndrome, myotonic dystrophy and osteogenesis imperfecta (type VII excluded) (15-20).

1.1.2. Autosomal recessive inheritance

Recessive disease alleles will only impair gene function if they are present in both the paternal and the maternal gene copies. One healthy copy is enough to sustain normal gene function. This type of inheritance causes fewer cases of disease in families, and these can be generations apart (Figure 2). In recessive diseases, the parents are healthy carriers. They usually both carry one disease allele and one healthy allele. They are both healthy themselves. In the case where the child of these carriers inherits the mutated allele from both parents (therefore becoming homozygous), the disease will manifest itself. Carriers have a 25 % risk of having a child with the disease and a 75 % chance of having a healthy child. 50 % of the children will be healthy carriers and 25 % will be healthy with two normal gene copies. Consanguinity (parents are distant relatives) and population isolation increases the risk of recessive diseases. Common recessive inheritable diseases include sickle cell anemia and cystic fibrosis. A number of recessive conditions can be lethal when present in homozygous form while heterozygous carriers remain healthy. These can manifest themselves as repetitive stillbirths or miscarriages. People are quite commonly carriers of a multitude of rare recessive disease causing alleles, but since the alleles are so rare, the chance of the other parent carrying

the same rare allele is slim (15, 21-23). Sometimes a disease condition can be caused by a combination of two different heterozygous mutations, which together disrupt the function of the gene. This is called compound heterozygocity (15).

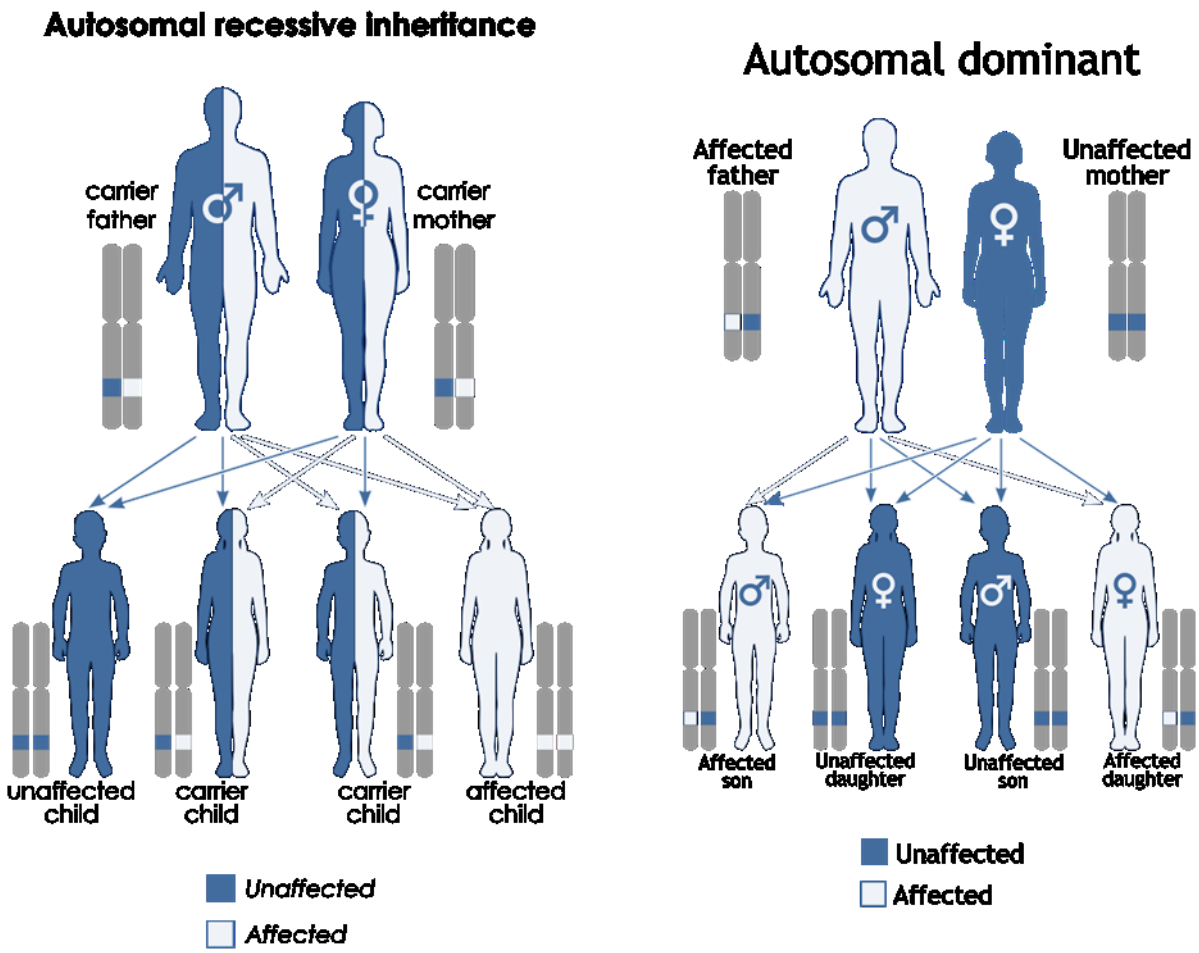

Figure 2 Recessive and dominant autosomal inheritance (en.wikipedia.org)

1.1.3. X- and Y-chromosomal inheritance

Sex chromosomes are inherited differently from autosomes, due to the fact that females inherit one X-chromosome from their mother and the other from their father. A male on the other hand inherits one X-chromosome from his mother and a Y-chromosome from his father. If a disease allele is located in one of the sex chromosomes (Y or X) the inheritance of the disease can depend on the sex of the individual and whether or not it is a dominant or

recessive allele. If the disease causing allele is in the X-chromosome, females can be affected (if dominant) or be carriers of the disease (if recessive), having two X-chromosomes (Figure 3). Males will automatically inherit the disease if they get the X-chromosome carrying the disease gene (recessive or dominant), because males only have one copy of the X-chromosome. Male children are therefore automatically homozygous for the maternal X-chromosome. As a result, sons will always be either healthy or affected, whereas daughters will be either healthy or carriers. Having only a mutated allele in the only X-chromosome of the male means no normal gene product will be produced, whereas in females the healthy X-chromosome will compensate the production of the protein in question. All daughters of an affected father and a normal mother will always be affected. Males can be more severely affected, or if the disease allele is lethal as a homozygote, the male embryos will not develop at all. In such a case, the pedigree of the family is missing male offspring and has only affected females. The women of such families can suffer multiple miscarriages (of sons) (24, 25).

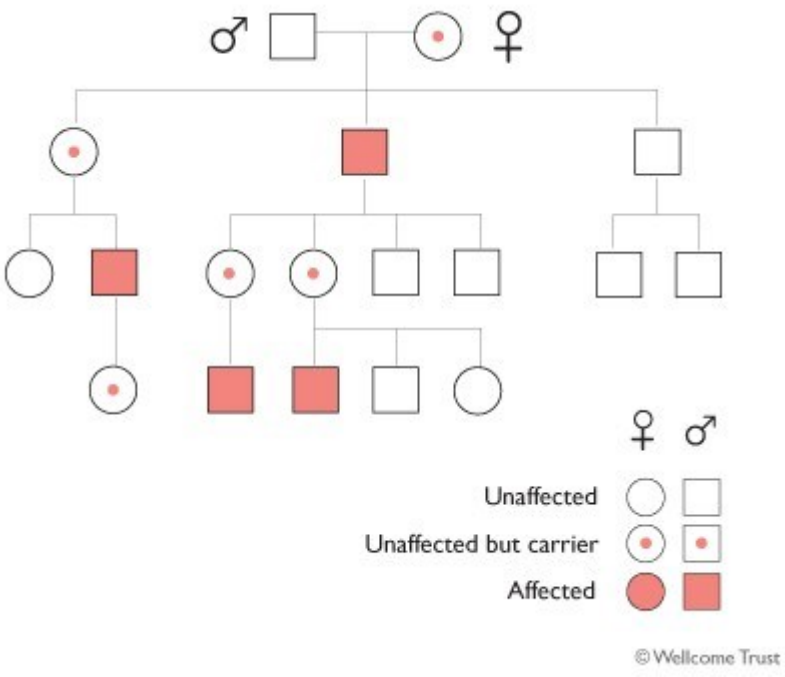

Figure 3 X-linked inheritance. A family pedigree for an X-linked recessive disease such as red-green color blindness, where affected males can reproduce (http://genome.wellcome.ac.uk)

In females, only one X-chromosome is active in each cell at a time (Figure 4). One copy is switched off at an early embryonic state. This is called X-inactivation. Sometimes due to the ratio of inactivated X-chromosomes, female carriers of a recessive X-linked disease can have

symptoms of the disease (26). Examples of hereditary X-linked diseases are hemophilia (27), Duchenne muscular dystrophy (28) and red-green color blindness (29).

Y-chromosomally inherited diseases are not as common as X-chromosomally inherited ones. Some examples of Y-chromosomally diseases are 47XYY-syndrome, where an individual has two Y-chromosomes. This causes tall height and potential learning difficulties (30). Another condition is Y-chromosomal infertility, which is caused by deletions in the azoospermia factor (AZF) site on the Y-chromosome (31, 32).

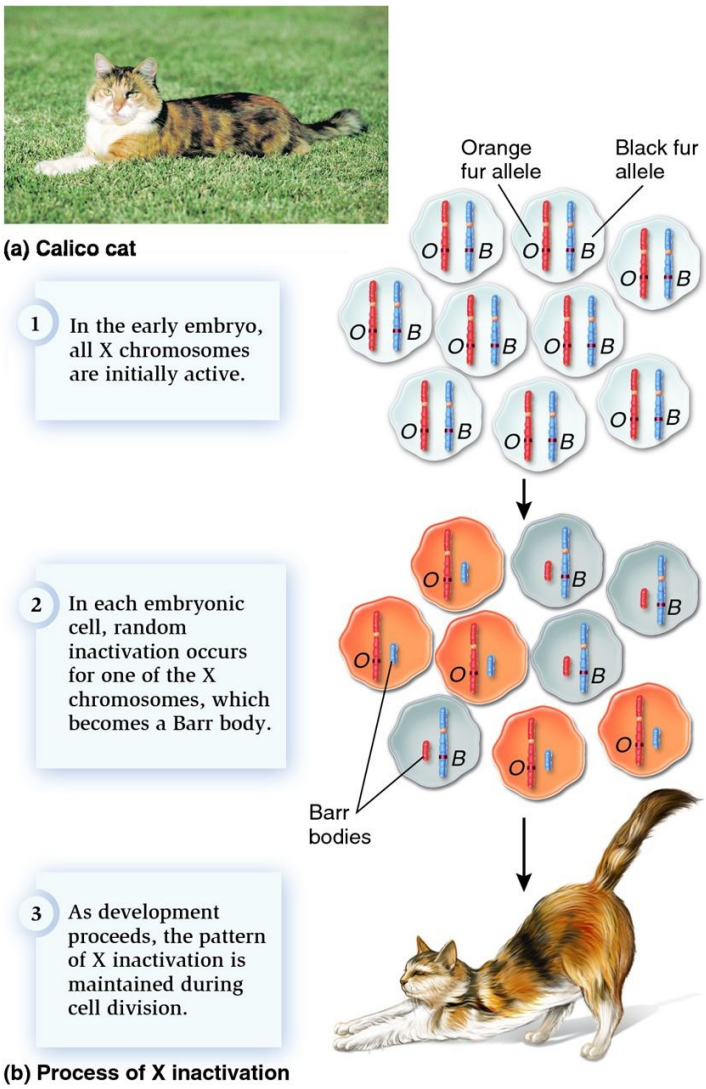

Figure 4 Example of X inactivation, the color pattern of calico-colored cats.

1.1.4. *De novo* mutations

If a mutation is referred to as '*de novo*', it means that the mutation has been newly formed during embryogenesis (or in the egg/sperm cell before conception) of the individual. *De novo* mutations are therefore not inherited from a mother or a father but become present in the child for the first time by random mutagenesis. The individual carrying the *de novo* mutation will then continue to pass it on to their children (and subsequent generations), but the mutation will be absent in grandparents (33, 34).

1.1.5. Multifactorial inheritance

Some of the most common diseases affecting millions of people are inherited in a multifactorial way (non-Mendelian), for example type 2 diabetes or lung cancer. There are several genes which increase the risk of getting the disease together with environmental and life-style factors. Sometimes these risk alleles are aggregated in families, and multiple cases of the disease can be observed in the family. Individual's risk can be measured by calculating relative risk ratios:

$$\lambda_r = \frac{\text{Prevalence of the disease in the relatives of the person}}{\text{Prevalence of the disease in the general population}}$$

Some examples of complex diseases with multifactorial inheritance are schizophrenia, autism, Crohn's disease and multiple sclerosis. Twin studies can be used to study the differences between genetic and environmental influences in a condition (15).

1.2. The mitochondrion – energy for the cell

The mitochondrion is the battery of the cell, producing the bulk of cellular energy. The mitochondria form networks inside the cell, constantly moving in fission and fusion and providing energy where it is needed. Tissues with high energy demand are packed with mitochondria, such as nervous tissue and muscle. The density of the mitochondrial network is

directly associated with the energy needs of the cell and tissue. The denser the mitochondrial network, the more energy the cell type needs (35).

The mitochondrion is a double-membrane cell organelle (Figure 5). It has an outer membrane and a folded inner membrane. These membrane folds form cristae formations inside the mitochondria. Mitochondrial DNA and ribosomes are located inside the matrix and inter-membrane space (36, 37).

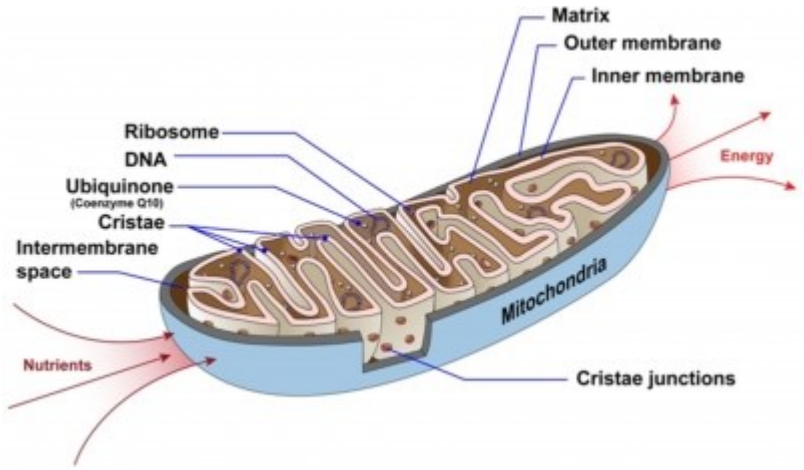

Figure 5 Structure of the mitochondrion (www.ispub.com)

Enzyme complexes, which produce cellular energy molecules, adenosine triphosphates (ATP) through oxidative phosphorylation, are located on the inner membrane. The mitochondrion has five enzyme complexes (I to V), which are formed from both mitochondrial and nuclear proteins (36).

The mitochondrion also acts as calcium-ion storage, capable of sudden release/intake of Ca-ions. This function is especially important in nerve cell function and in disease conditions such as epilepsy. Other functions of the mitochondrion include heat production, hormone and heme-precursor synthesis (Table 1) (38). The mitochondrion also has a role in apoptosis, programmed cell death. As a side product of oxidative phosphorylation, reactive oxygen species (ROS) are being formed. These superoxides damage tissues and DNA. MtDNA mutations often cause an abnormal increase in the ROS production, causing additional damage to the tissues and DNA. Therefore, the mitochondrion has a role in aging; ROS production naturally increases with age, further deteriorating cells

and DNA in the natural aging process. The energy production machinery slows down and stops working as efficiently (39).

The evolution of the mitochondrion is of bacterial origin. It evolved through endosymbiosis of an alpha-proteobacterium circa 2 billion years ago. As the level of oxygen rose in the atmosphere, eukaryotic primordial cells engulfed the alpha-proteobacterium to increase aerobic energy production in the oxygen rich environment (40). During evolution, the alpha-proteobacterium genome integrated into the eukaryotic cell; its unimportant genes were transferred to nuclear DNA as pseudogenes. Pseudogenes are non-functional copies or 'relatives' of genes that are no longer expressed. These pseudogene sequences lie in the intergenic intron spaces between functional genes. During evolution, the oxidative phosphorylation reaction of the mitochondria was honed to perfection adding nuclear proteins to the enzyme complexes. Also, many of the upkeep functions of the mitochondrion and the maintenance of mitochondrial DNA was moved to be handled by the nuclear genome (41).

The mitochondrion has its own ribosomes, which are encoded in the mitochondrial DNA. Ribosomes are the encryption machinery that produces the final protein product from the amino acid code encoded in a DNA sequence. Two mitochondrial ribosomal genes, 12S ribosomal RNA (*MTRNR1*) and 16S ribosomal RNA (*MTRNR2*) encode two components of the mitochondrial ribosome. Due to the bacterial origin of the mitochondrion, the mitochondrial ribosome resembles a bacterial ribosome, although new features have been added during evolution (42).

Table 1 The mitochondrion has many functions

Functions of the mitochondrion	
Aerobic energy production	**Aging – through ROS production**
Heat production	**Hormone production – steroids**
Calcium storage and homeostasis	**Heme production**
Apoptosis signaling	**Cell signaling**
Membrane potential regulation	**Cell cycle/growth**
Cellular differentiation	

1.2.1. Mitochondrial DNA (mtDNA)

The mitochondrion has its own, independent genome (mtDNA). It is circular, double-stranded and equals ~16 000 bp in length. MtDNA is maternally inherited. The mother will pass on her mtDNA much unchanged throughout the generations. The paternal mtDNA is lost during fertilization, as the male mitochondria are located in the tail of a sperm cell, which is left outside the egg. The female mitochondria are inherited randomly (mitochondrial bottleneck), so that a random number of mitochondrial DNA molecules ends up in the precursor egg cells (37).

The mtDNA encodes 37 genes. It is very compact, no introns (non-coding areas) are found in between the genes. It has a non-coding control region (D-loop), which is very variable and contains a collection of polymorphisms as well as replicatory maintenance sequences. MtDNA encodes 12 structural genes of the energy producing enzyme complexes, 22 mitochondrial transfer RNA (tRNA) genes and two ribosomal genes (36) (Figure 6).

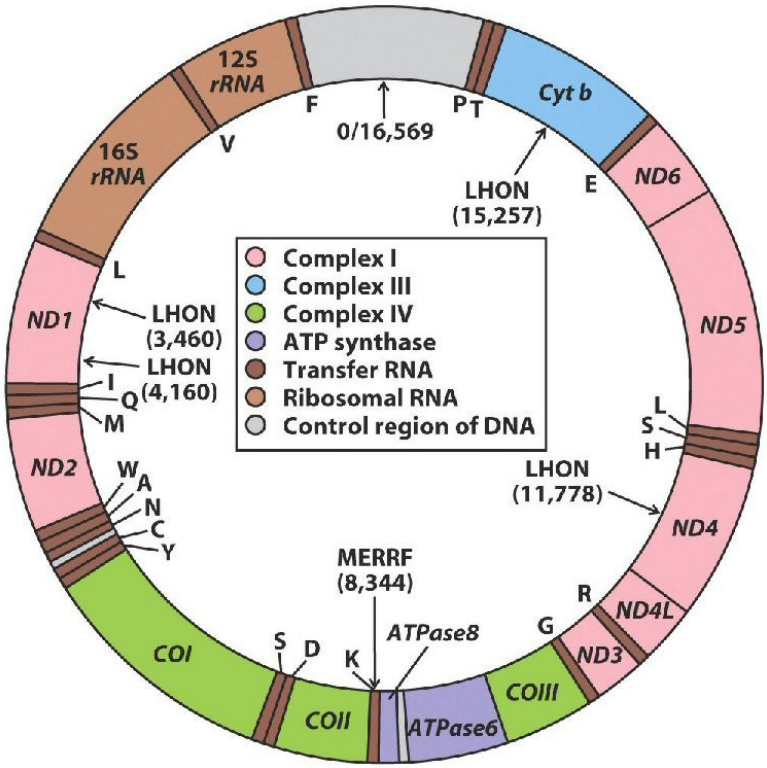

Figure 6 Organization of the mitochondrial DNA

Multiple copies of mtDNA exist in each mitochondrion and cell. A peculiar phenomenon called heteroplasmy occurs when a mutation exists in some mtDNA molecules but not in all (both pathogenic and benign mutations can be heteroplasmic). This means that a cell has a mixed collection of both mutated and normal mtDNA. This is an important phenomenon in mitochondrial diseases; heteroplasmy/mutation load often correlates with disease severity (Figure 7). Heteroplasmic mutations are inherited randomly, which makes genetic counseling difficult in mitochondrial diseases (43, 44).

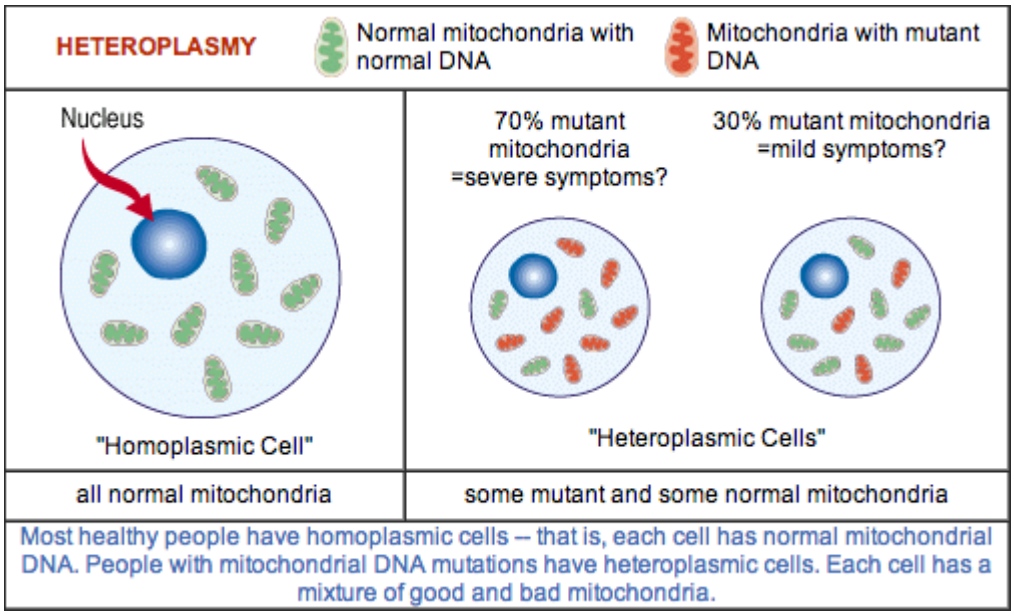

Figure 7 Heteroplasmy in mtDNA (www.quora.com)

Mitochondrial DNA evolves fast, so fast that polymorphisms and mutations occur 10 times more often than in nuclear DNA. This is due to the increased flexibility of the mitochondrial genetic code where more variation is tolerated in the third amino acid of the mitochondrial genetic code. Also, the mitochondrial DNA repair system is not as efficient as that of nDNA. Moreover, mtDNA has a fast turnover rate. The mutation rate of mitochondrial DNA is considered to be stable, so that it can be used as an 'evolutionary clock'. Evolution of animal species and genetic bottlenecks and other evolutionary occurrences can be timed with the

amount of mtDNA mutations that have occurred between, for example, two subspecies of animals. Maternal inheritance of mtDNA has made it possible to trace mankind's ancestry back to the 'Mitochondrial Eve', our most recent common ancestor (MRCA) (45). The origin of modern humans has been located to East-Africa. Through mitochondrial haplogroups (combinations of polymorphisms), the origins of humans and migrations of populations can be studied (46-48).

1.2.2. Mitochondrial DNA-nuclear DNA crosstalk

The energy production of the cell takes place in the mitochondrial inner membrane, where the respiratory chain pumps protons through four protein complexes to ultimately produce the energy harboring ATP molecules (Figure 8). A considerable part of the mitochondrial respiratory chain proteins are of nuclear origin. The eukaryotic respiratory chain consists of four complexes and ATP synthase, which have 12 mitochondrial proteins and the remainder of the proteins are encoded by nuclear genes (Figure 9). Mutations in these nuclear-mitochondrial structural genes (Table 6) can also be the cause of mitochondrial disease, but the inheritance pattern is autosomal dominant or autosomal recessive instead of maternal (49).

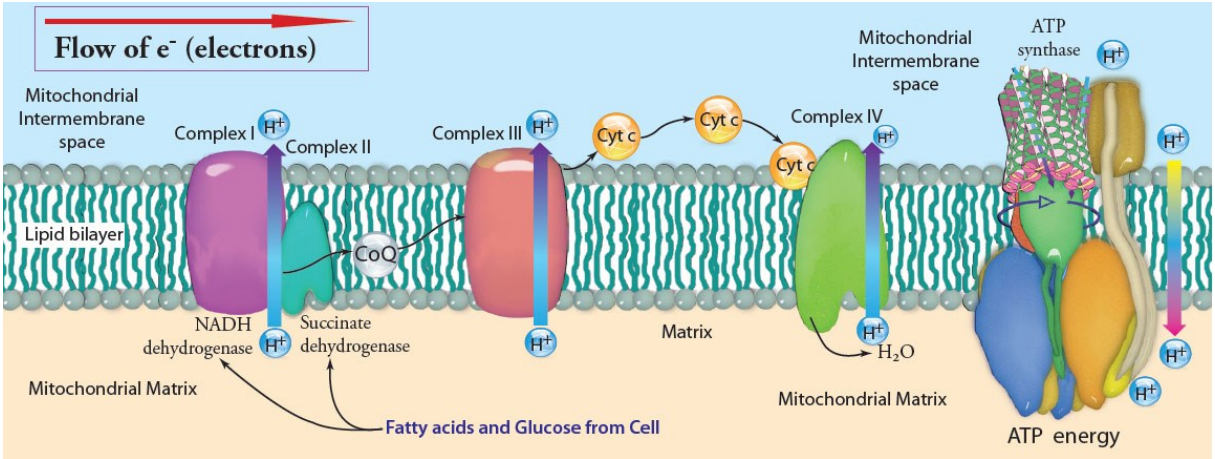

Figure 8 Mitochondrial respiratory chain for oxidative phosphorylation (Access revision/biology)
https://sites.google.com/site/accessrevision/biology/cell-form-and-function/cellular-respiration

Nuclear encoded proteins are used for replication and maintenance of mtDNA molecules (Figure 9 - 10, Tables 2 - 3). Other important mitochondrial functions handled by nDNA are respiratory chain complex assembly, mtDNA import, mitochondrial protein synthesis, iron homeostasis, coenzyme Q10 biogenesis, chaperone function, mitochondrial integrity and mitochondrial metabolism (49).

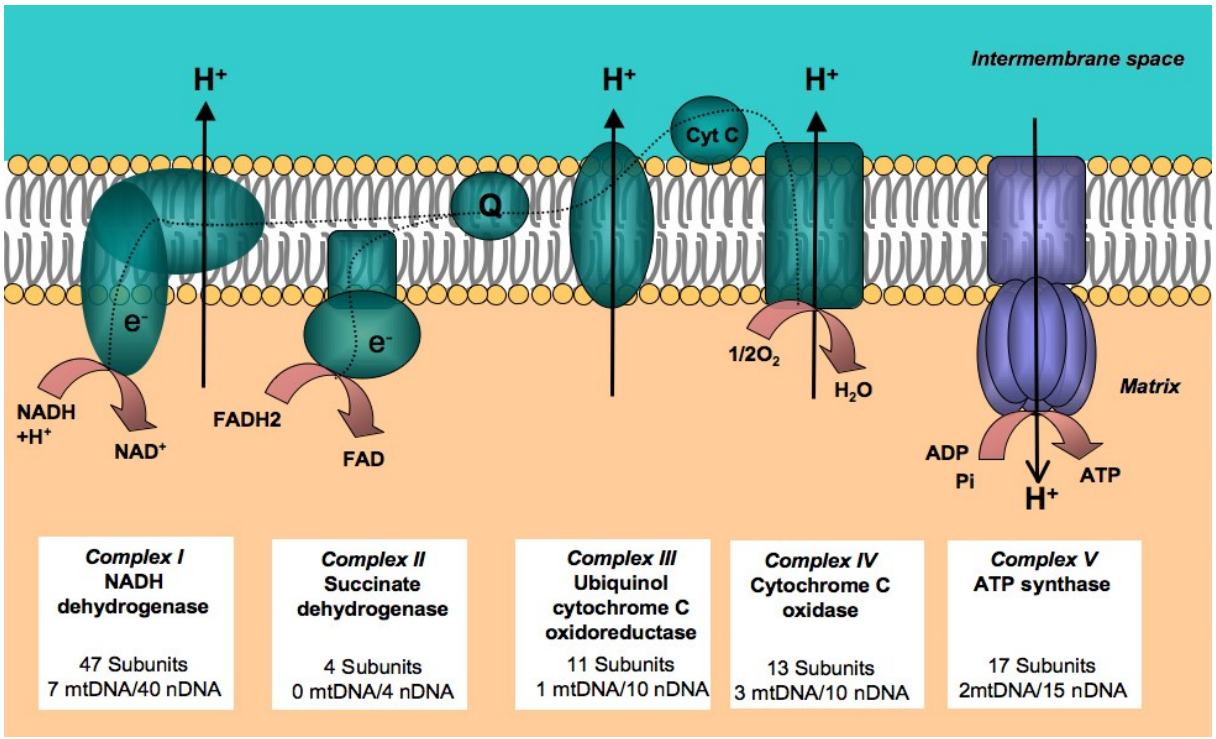

Figure 9 Mitochondrial and nuclear genome encoded protein subunits of oxidative phosphorylation (http://flipper.diff.org/).

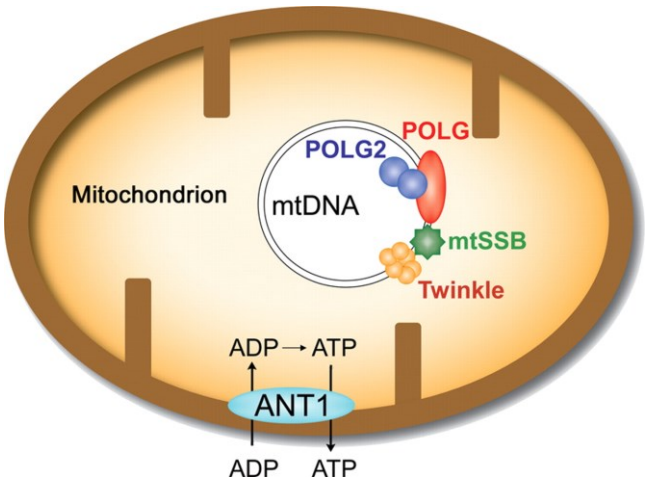

Figure 10 Nuclear mtDNA maintenance genes. Nuclear maintenance genes in color font. (www.neurology.org)

Table 2 Structural nuclear genes in mitochondrial disease (49). *AR = autosomal recessive inheritance, AD = autosomal dominant inheritance, X = X-chromosomal inheritance.*

Gene	Chromosome position	Clinical phenotype
NDUFS1	2q33-q34	Leigh syndrome (AR)
NDUFS2	1q23	Encephalopathy, cardiomyopathy (AR)
NDUFS3	11p11.11	Leigh syndrome (AR)
NDUFS4	5q11.1	Leigh syndrome (AR)
NDUFS6	5pter-p15.33	Fatal infantile lactic acidosis (AR)
NDUFS7	19p13.3	Leigh syndrome (AR)
NDUFS8	11q13	Leigh syndrome (AR)
NDUFB3	2q31.3	Fatal infantile lactic acidosis (AR)
NDUFV1	11q13	Leigh syndrome (AR)
NDUFV2	18p11	Cardiomyopathy, hypotonia, encephalopathy (AR)
NDUFA1	Xq24	Leigh syndrome, progressive neurodegenerative disorder (X)
NDUFA2	5q31.2	Leigh syndrome (AR)
NDUFA10	2q37.3	Leigh syndrome (AR)
NDUFA11	19p13.3	Fatal infantile lactic acidosis, encephalocardiomyopathy (AR)
SDH-A	5p15	Leigh syndrome (AR)
SDH-B	1p36.1-p35	Phaeochromocytoma and paraganglioma (AD)
SDH-C	1q21	Autosomal dominant paraganglioma type 3 (AD)
SDH-D	11q23	Autosomal dominant paraganglioma type 1, phaeochromocytoma (AD)
UQCRB	8q22	Hypoglycemia, lactic acidosis (AR)
UQCRQ	5q31.1	Severe neurological phenotype (AR)
COX6B1	19q13.1	Encephalomyopathy (AR)
ATP5E	20q13.3	Lactic acidosis, mental impairment, peripheral neuropathy (AR)

Table 3 Nuclear mitochondrial maintenance and stability genes (49). *AR = autosomal recessive, AD = autosomal dominant, PEO = autosomal dominant progressive external ophtalmoplegia, SANDO syndrome = sensory ataxic neuropathy, dysarthria & ophtalmoparesis, SCAE = Spinocerebellar ataxia with epilepsy, MDDS = Mal de Debarquement syndrome; imbalance sensation after movement, MNGIE = Mitochondrial neurogastrointestinal encephalopathy.*

Gene	Function	Clinical phenotype
POLG	Polymerase gamma, mtDNA replication	Alper's syndrome, AD-PEO, AR-PEO, male infertility, SANDO syndrome, SCAE (AR, AD)
POLG2	Catalytic subunit of polymerase gamma, mtDNA replication	AD-PEO (AD)
ANT1	Adenine nucleotide translocator isoform 1, transport of ADP and ATP	AD-PEO, multiple mtDNA deletions (AD)
MPV17	Maintenance of mtDNA copy number	Hepatocerebral MDDS (AR)
C10ORF2	Twinkle helicase, mtDNA replication	AD-PEO, SANDO syndrome (AD)
TYMP	Thymidine phosphorylase, catalyzes the reversible phosphorylation of thymine	MNGIE, mtDNA depletion
DGUOK	Deoxyguanosine kinase, mitochondrial dNTP-pool maintenance	Hepatocerebral mtDNA depletion syndrome
RRM2B	Ribonucleotide reductase M2 B, mitochondrial dNTP-pool maintenance	Encephalomyopathic renal tubulopathy MNGIE, AD-PEO (AR)
SUCLA2	Succinate-CoA ligase; β-subunit, ADP-forming	Encephalomyopathy with methylmalonic aciduria (AR)
SUCLG1	Succinate-CoA ligase; α-subunit	Encephalomyopathy with methylmalonic aciduria (AR)
TK2	Thymidine kinase, mitochondrial dNTP-pool maintenance	Myopathic mtDNA depletion (AR)

One of the most common mitochondrial diseases caused by a nuclear gene mutation is Leigh syndrome. It can be caused by multiple mutations in multiple mitochondrial structural genes (30 different genes). It is usually manifests itself in early childhood (during the first year) and can be autosomal recessive or dominant. It causes progressive loss of movement abilities, mental impairment and is often fatal within a few years. An adult-onset type also occurs, where the symptoms are milder, start in adulthood or late childhood and worsen slowly. Leigh

syndrome presents in babies as failure to thrive, excessive vomiting, diarrhea and dysphagia (difficulties in swallowing). Growth is slow and muscle tone is weak, eye problems such as nystagmus and ophtalmoplegia are often observed. Balance problems (ataxia) and peripheral neuropathy are common, as well as general movement difficulties. Sometimes the heart is affected and cardiomyopathy is observed. Lactic acid is usually elevated in blood due to muscle tissue involvement and decompensation of the poorly functioning oxidative phosphorylation. Breathing can weaken progressively, eventually causing death due to respiratory failure (50).

1.2.3. Mitochondrial DNA of Northern Finland

Finland was settled by the Fenno-Ugric people originating from the Volga-Ural region (51). The southern and western parts of Finland were settled first, with villages concentrated in the fertile river valley regions. Northern Finland was inhabited later and more slowly (52). The population of Finland was scarce at first, but during the 1800's there was rapid growth in population size. This fast increase in population from a restricted number of common ancestors caused a genetic bottleneck effect driving up the frequency of rare alleles (53, 54).

The mitochondrial DNA of Finland consists of haplotypes which are common in Europe and additionally eastern Siberian and Saami sub-haplotypes, e.g. U5b1b (Table 4). The population haplotype composition differs quite drastically between southern and northern Finland. For example haplotype V is more common in the northern parts, as it is one of the most common Saami haplotypes. The Southern part of Finland has haplotype frequencies similar to other parts of Europe, as most European settlers settled in the south of Finland. Haplotypes H, J and I especially increase toward the south while U, V, T and M increase toward the north (55).

Table 4 European and Saami mtDNA haplotypes

	Haplotypes
Saami	U5b1b, V, D5, H
European	H, V, U, K, J, T, W, I, X

Haplotype Z is observed in both Finns and in the Saami people. It is considered to be of Volga-Uralic origin (56). D5 haplotype is also seen in the Saami. It is an East Asian haplogroup, which is seen in low frequencies representing the Asian influence in the gene pool of the Saami people (47). The most common mtDNA haplotype of Europe, haplotype H can also be seen in the north and in the Saami people. It is considered to exist due to more recent admixture (55).

1.2.4. Pathogenic mtDNA mutations

Mutations in mitochondrial genes can cause maternally inherited mitochondrial diseases. Mitochondrial diseases are caused by a lack of cellular energy due to non-functioning mitochondria and respiratory chains. Mitochondrial diseases cause symptoms in multiple organs, especially in the ones which need a lot of energy (e.g. brain and muscle). Mitochondrial diseases cause symptoms such as epilepsy, encephalopathy, stroke-like episodes, diabetes, hearing impairment, pigmentary retinopathy in the eyes, bilateral ptosis of the eyelids, cardiomyopathy, muscle weakness and exercise intolerance. Liver malfunction is common in syndromes manifesting in childhood (Figure 11) (14, 57).

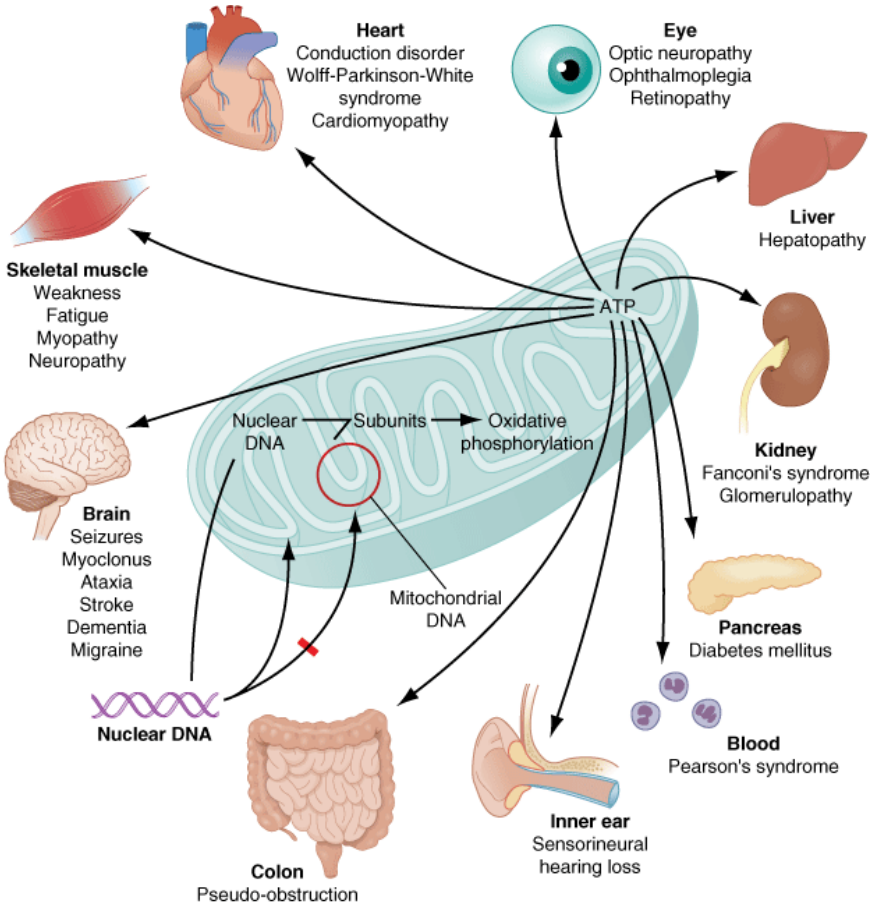

Figure 11 Symptoms of mitochondrial disease according to organ (www.accessmedicine.com)

Some of the most common mitochondrial diseases caused by mtDNA mutation are MELAS syndrome (mitochondrial encephalopathy lactic acidosis and stroke-like episodes) caused by the m.3243A>G mutation, NARP syndrome (neuropathy, ataxia and retinitis pigmentosum) caused by m.8993T>C/G mutation and LHON syndrome (Leber's hereditary optic neuropathy) caused by for example m.11778G>A (48).

There is no cure for mitochondrial diseases, they are often progressive and lethal. The age of onset varies from a few days old to adulthood. Also, the disease severity varies tremendously. Heteroplasmy is one of the main reasons for this phenotypic variability. The higher the mutation heteroplasmy, the more severe the disease. Siblings typically have a variety of heteroplasmy of the mutation, manifesting in either no symptoms, mild, or very severe symptoms (Figure 12) (58-60). All children of the affected mother inherit the mutation

without exception. The only thing which varies is the level of heteroplasmy. Pathogenic mtDNA mutations can be both homoplasmic and heteroplasmic. Nuclear genes are responsible for a part of mitochondrial diseases. In these cases, the inheritance pattern is not maternal, but autosomal dominant or autosomal recessive (61). In addition to pathogenic point mutations in mtDNA, larger rearrangements and deletions/insertions can also be the cause of mitochondrial disease (36, 62).

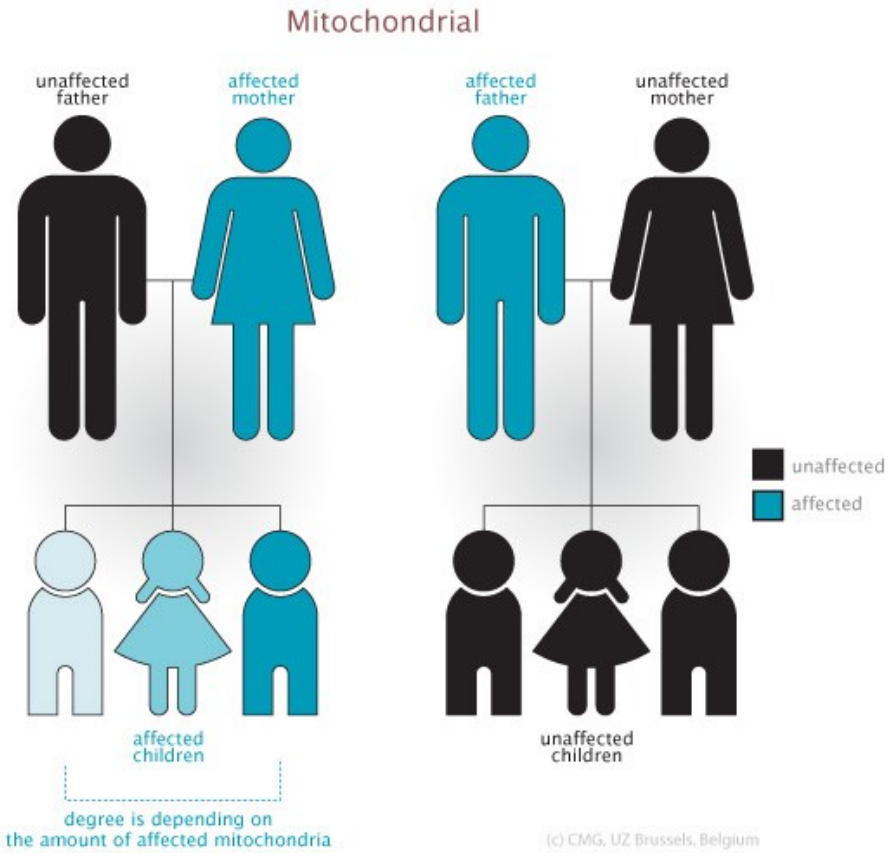

Figure 12 Inheritance of mitochondrial diseases (www.brusselsgenetics.be)

1.2.5. Hearing loss mutations in mtDNA

Hearing loss is a common symptom of mitochondrial diseases, but there are several mutations which cause only deafness/hearing loss (48, 63-65). These mtDNA mutations account for a small number of non-syndromic hearing loss cases. Hearing loss caused by mitochondrial

mutations is often bilateral, sensorineural and progressive. Like in other mitochondrial diseases, the severity or the degree of hearing loss is variable from mild to total deafness. Other symptoms of neurodegeneration are rarely associated with mitochondrial DNA hearing loss mutations, although they are not unheard of (66).

The most common and confirmed mtDNA hearing loss mutations include m.1555A>G, m.1494C>T, m.7445A>G and m.7511T>C. The ribosomal *MTRNR1* gene and the tRNA gene for Serine^{UCN} (*MTTS1)* are considered to be hot spots for hearing loss mutations in the mtDNA. Several other hearing loss mutations have been reported, but these are not confirmed and their status as pathogenic is still uncertain and under debate. In total, over 40 mtDNA variants have been associated with non-syndromic hearing loss (48). It has also been suggested, that increased overall variation of mtDNA may increase the risk for hearing impairment (67). The frequency of pathogenic mtDNA hearing loss mutations seems to be higher in Asian countries, such as China and Japan (63, 64, 68).

1.2.6. Aminoglycoside sensitivity

Aminoglycosides are a class of antibiotics effective against a large group of bacteria. These include gram negative bacteria, anaerobic bacilli and mycobacteria. Examples of aminoglycosides include gentamycin, streptomycin, tobramycin, neomycin and kanamycin. Streptomycin was in its time a huge discovery, finally providing the cure for tuberculosis. Aminoglycosides inhibit bacterial protein synthesis by binding to the bacterial ribosome (Figure 13). The binding of the antibiotic impairs translational proofreading and as a consequence wrong amino acids are attached to the manufactured protein, making it inactive and this way inhibiting bacterial growth (69).

As with all drugs and antibiotics, aminoglycosides can cause side effects. The adverse effects associated with aminoglycosides range from mild gastro-intestinal irritation to inner ear and acute kidney damage. Aminoglycoside-induced kidney damage is usually reversible but can also lead to chronic kidney disease. Hearing loss is irreversible and it has been reported to occur in 2 to 25 % of patients receiving aminoglycoside treatment (2, 70).

Aminoglycosides can also bind to mitochondrial ribosomes, especially, if a certain mtDNA mutation is present in the mitochondrial 12S ribosome subunit gene

MTRNR1, such as m.1555A>G. It has been suggested, that these mutations make the mitochondrial ribosome more bacterial-like, enabling the aminoglycoside to bind to it with higher affinity compared to wild-type human mitochondrial ribosome. Sound sensing hair cells of the inner ear have an abundance of mitochondria and are very susceptible to adverse effects of aminoglycosides, if a mutation in the ribosomal gene *MTRNR1* is present. Aminoglycosides damage the hair cells in the organ of Corti by triggering an apoptotic effect and increasing ROS (reactive oxygen species) production. Additionally, sensory nerve neurons in the spiral ganglion are afflicted. The apoptotic effect in the hair cells is due to activation of cell cycle regulatory genes, disrupting the post-mitotic state of the hair cell and activating a pro-apoptotic pathway. The increase of ROS also activates an oxidative stress response in the cell (71). These fragile sensory cells are unable to recover once damaged, much like in high volume noise damage (Figure 14). This results in permanent hearing loss or deafness (72).

A single dose of aminoglycosides can cause the above mentioned ototoxic effect at any age (6, 73, 74) but preterm babies are especially vulnerable. Infections are common during the first months of a premature baby's life. Aminoglycosides have a low level of antibiotic resistance and are very effective, so they are often used if a baby suffers from sepsis/infection. High noice level in the NICU with a lot of beeping and bleeping machines is also thought to add to the ototoxic effects of aminoglycosides (75).

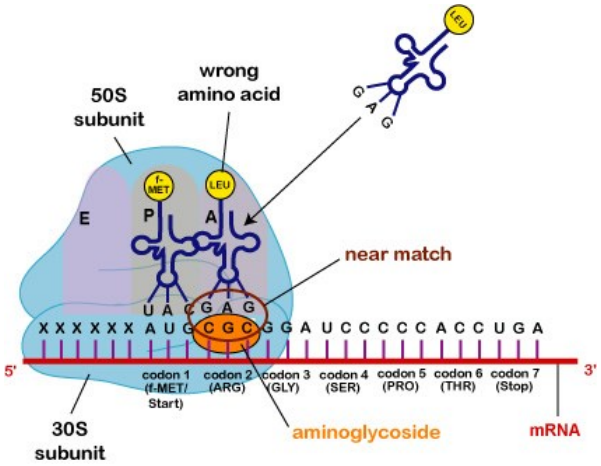

Figure 13 Aminoglycoside affects the bacterial ribosome disrupting translation (faculty.ccbcmd.edu)

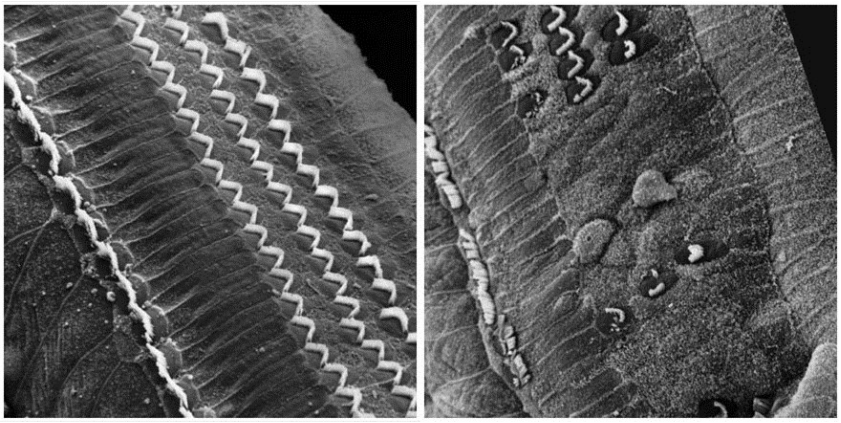

Figure 14 Cochlear hair cell damage (www.auditoryneuroscience.com)

m.1555A>G in 12S ribosomal RNA (*MTRNR1*)

Although sensorineural hearing loss is a common symptom of syndromic mitochondrial disease, mtDNA mutations which specifically cause sensorineural hearing loss have been identified. Overall, 5 % of non-syndromic, post-lingual hearing loss patients carry a pathogenic mitochondrial mutation (76). In these families, the hearing disability follows a matrilineal inheritance pattern. The most common mitochondrial disease mutation to cause hearing loss is the MELAS mutation m.3243A>G in the mitochondrial tRNA $^{Leu\ 1}$ (*MTTL1)* gene. It's prevalence in Finnish children has been estimated to be 18.4:100 000 (77) and 1:500 in Europe (70).

Several mitochondrial sensorineural hearing loss mutations have been found in *MTRNR1* and mitochondrial tRNA^{Ser} (*MTTS1*). The most common mtDNA hearing loss mutation is the m.1555A>G mutation in *MTRNR1*, which encodes a mitochondrial ribosomal subunit 12S. This mutation was first described in an American patient who had an ototoxic effect to aminoglycosides (74). During the following years, several reports from different population backgrounds confirmed the m.1555A>G to be indeed pathogenic causing sensorineural hearing loss and aminoglycoside sensitivity (Table 5) (78-80). The pathogenic and ototoxic effects were also confirmed by functional studies (81-83).

The m.1555A>G mutation causes a variable hearing loss phenotype (Figure 15). The hearing loss is usually bilateral and progressive, ranging from mild to severe to complete deafness. Like in many mitochondrial diseases, the age of onset varies greatly, from early childhood to adulthood. Its prevalence has been estimated mostly in non-syndromic hearing loss patients. Among Finnish hearing loss patients it is estimated to explain the etiology of the hearing loss in about 2.6 % of cases (66, 84). The prevalence of m.1555A>G is much higher in Asia; up to 3 % of sensorineural hearing loss patients and 10 % of cochlear implant patients in Japan carry the m.1555A>G mutation (64, 85). Among European nations, it is has been especially frequently reported in Spain (7).

Table 5 Prevalence of aminoglycoside sensitivity mtDNA mutations in sensorineural hearing loss patients or populations around the world. Hearing loss patients (H), population (P).

Population	m.1555A>G	m.1494C>T	m.1095T>C	Reference
Finland	4.4/100 000 (H)	n.a.	n.a.	(86)
Spain	15-20 % (H)	n.a.	n.a.	(11)
Greece	0.4 % (H)	n.a.	n.a.	(7)
Italy	5.4 % (H)	n.a.	n.a.	(10)
United Kingdom	1/300 (P)	n.a.	n.a.	(87)
China	4-6 % (P)	0.2 % (P)	0.6 % (P)	(8, 88)
Japan	3 – 5 % (H)	n.a.	n.a.	(89)
Australia	0.2 % (H)	n.a.	n.a.	(90)

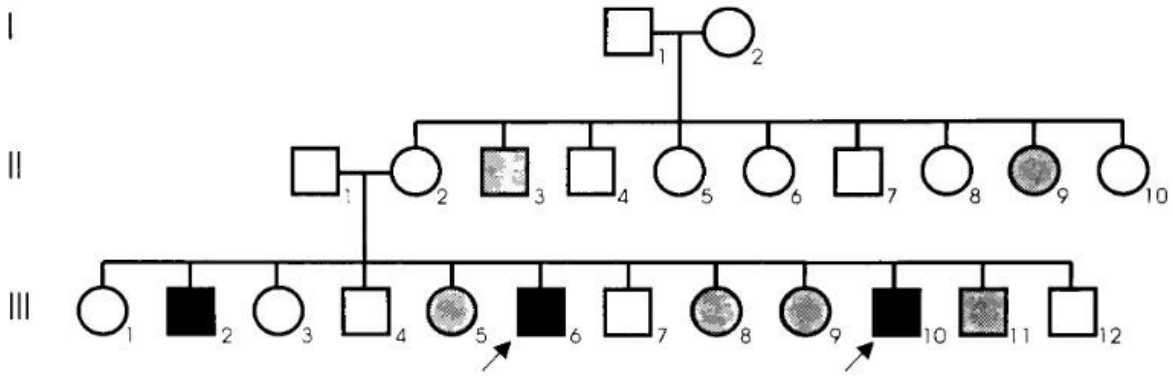

Figure 15 Example of a Finnish pedigree with m.1555A>G mutation. Black denotes profound hearing impairment, grey denotes hearing impairment in high frequencies, white denotes normal hearing. Arrows indicate probands in the study (84).

Nuclear modifying factors for m.1555A>G phenotype

Clear evidence exists of a nuclear modifying factor for the variance in the m.1555A>G phenotype. This has been observed in patients and in functional studies using lymphoblastic cell lines (5, 91). Most often the mutation is 100 % homoplasmic, but still some mutation carriers have normal hearing while others have very severe hearing loss or deafness. The variable level of hearing loss does not concur only with the heteroplasmy level of m.1555A>G. The age of onset also varies from childhood to adult-onset and so does the penetrance of the mutation (~ 20 %) (92).

Genes for mitochondrial transcription factor B1 (*TFB1M*), mitochondrial tRNA optimization 1 (*MTO1*), GTP binding protein 3 (*GTPBP3*), tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (*TRMU*) are some of the known suggested nuclear modifier genes (Table 6). A mutation in these genes can lead to deafness when a mtDNA mutation is present (Figure 16) (93). *GJB2* mutations in the background of the m.1555A>G phenotype have also been suggested (94), but research results remain inconclusive (95). Haplotypes of m.1555A>G carriers have been thoroughly investigated (92), but no haplotype or single mtDNA polymorphism could be found to explain the phenotypic variation of m.1555A>G

hearing loss. Compensatory mechanisms have been suggested to be compromised when a nuclear modifier gene mutation is present (Figure 17) (96).

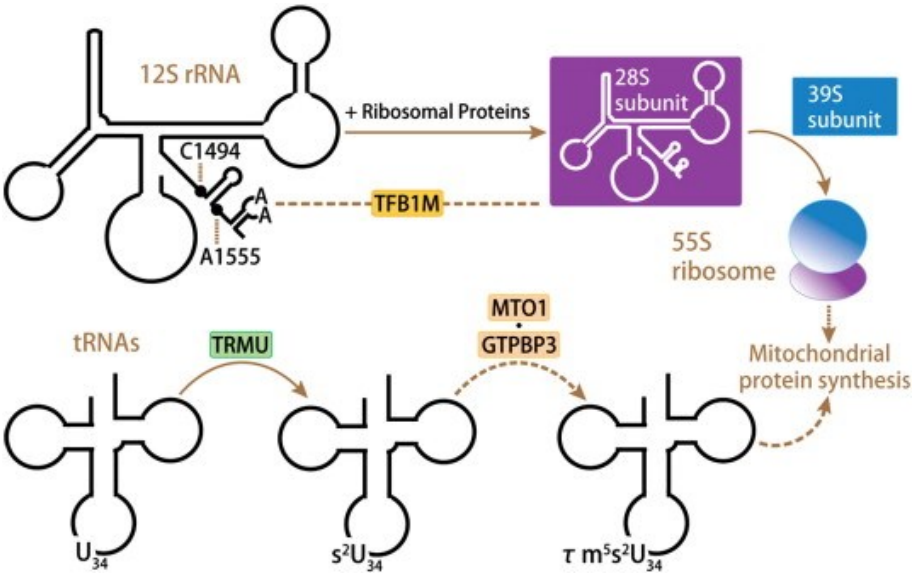

Figure 16 Four modifier genes and their functions in the mitochondrial protein synthesis (93).

Table 6 Nuclear modifier genes for hearing loss mtDNA mutations (93)

Gene	Location	Function	Common mutations
TMRU	22q13.31	tRNA thiolation	A10S
TFB1M	6p25.3	rRNA methylation	-
MTO1	6q13	tRNA taurinomethylation	A428T, R620Kfs*8
GTPBP3	19q13.11	tRNA taurinomethylation	-

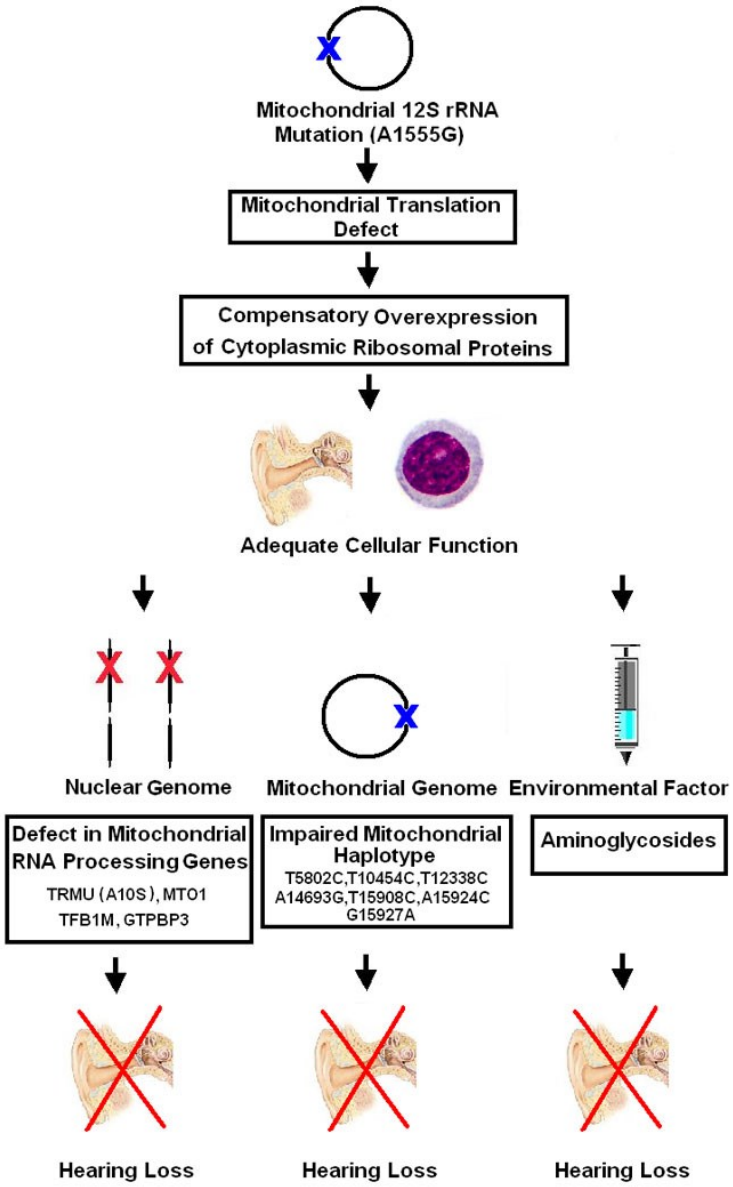

Figure 17 Mechanisms of compensation and pathogenesis of hearing loss associated with mitochondrial A1555G mutation (96)

m.1495C>T in *MTRNR1*

The m.1494C>T mutation is another confirmed cause of hearing loss in the *MTRNR1* gene. It was first discovered in a Chinese family, in which hearing loss was maternally inherited through generations in a large family suggesting a mitochondrial DNA mutation. (97). The mutation causes both aminoglycoside induced and non-syndromic hearing loss. The onset of

hearing loss ranged from 10 years to 55 years of age. The age in which the person was subjected to aminoglycosides correlated to the severity of the hearing loss.

Much like m.1555A>G, the m.1494C>T mutation is located in the same *MTRNR1* ribosomal mitochondrial gene. It forms a new A-U base in the same conserved position where the m.1555A>G mutation forms a new C>G base (Figure 18). The detrimental effect of aminoglycosides in patients with m.1494C>T was later confirmed with functional studies (98), where a significant increase in doubling time of aminoglycosides in patient lymphocytes was observed along with a significant decrease in total cell oxygen consumption of mutant cells. Yu et al. in 2014 reported an increase in mitophagy and a decrease of up to 35 % of ATP production in m.1494C>T cells treated with gentamycin (4).

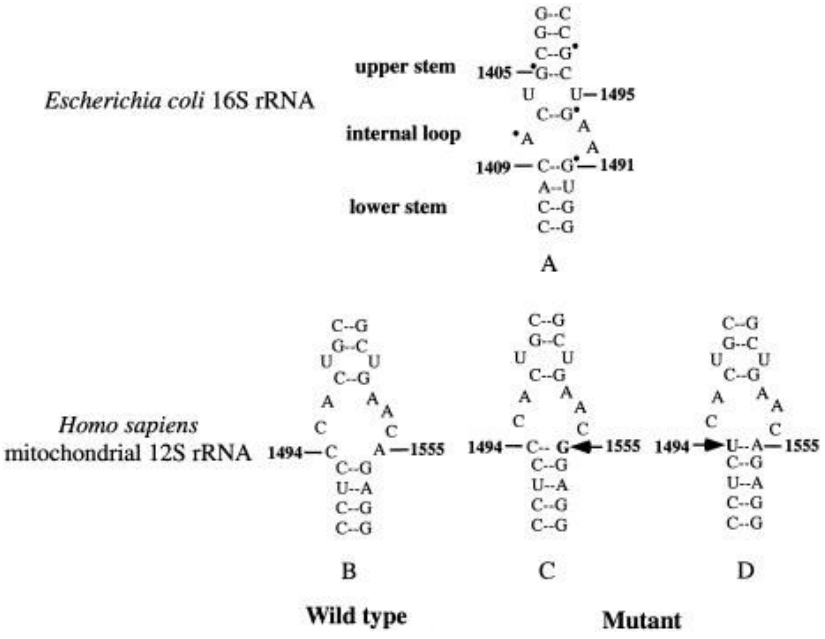

Figure 18 Position of the m.1494C>T and m.1555A>G mutations in mitochondrial 12S rRNA. A) E. coli 16S rRNA position sensitive to aminoglycosides marked with a dot B) Wild type human 12 S rRNA C) M.1555A>G 12S rRNA D) m.1494C>T 12S rRNA (97).

The m.1494C>T mutation is not as common as the m.1555A>G mutation (99). It has been found mainly in Chinese patients (8, 97, 100, 101). It is thought to be absent from some populations, as possibly indicated by the lack of m.1494C>T among Brazilian hearing loss patients (102).

m.1095T>C and other *MTRNR1* hearing loss mutations

The m.1095T>C mutation has been reported to occur in 1.25 % of Chinese non-syndromic hearing loss patients and in 2.24 % of Mongolian hearing loss patients (103, 104). In Caucasians, m.1095T>C has been reported in two Italian families with deafness, maternally inherited hearing impairment, Parkinsonism and neuropathy (105, 106). Dai et al. reported co-segregation of m.1095T>C and m.1555A>G in a Chinese hearing loss patient (107), who also suggested that m.1095T>C mutation impairs mtDNA translations and leads to a reduction of cytochrome C oxidase activity. Another study concluded that m.1095T>C increases aminoglycoside sensitivity similarly to m.1555A>G (108).

The *MTRNR1* gene certainly seems to be a hot spot for hearing loss mutations. Additional, less prevalent hearing loss mutations have been reported in this gene (Table 7). Most of them have been discovered a handful of times in hearing loss patients and their pathogenicity remains to be confirmed by functional studies and/or additional cases. MtDNA position m.956_965 holds a C-tract consisting of nine cytosines and a single thymine in the middle. The length variation of the C-tract and the occurrence or deletion of the T in the middle has been suggested to be associated with hearing loss, but remains to be functionally proven. These kinds of long repeat sequences normally hold a lot of variation in mtDNA (109). Only m.1555A>G and m.1494C>T mutations remain to have been robustly confirmed to be associated with both non-syndromic and aminoglycoside induced hearing loss and deafness.

Table 7 MTRNR1 variants reported to be associated with hearing loss (110). *Only the first reported reference for each mutation is included in the table.*

Variant	Note	Reference	Variant	Note	Reference
669T>C	Reported	Rydzanicz et al. 2009	**792C>T**	Reported	Lu et al. 2010
735A>G	Reported	Mkaouar-Rebai et al. 2010	**801A>G**	Reported	Lu et al. 2010
745A>G	Reported	Lu et al. 2010	**827A>G**	Reported	Bardien et al. 2009
			839A>G	Reported	Lu et al. 2010

Variant	Note	Reference
856A>G	Reported	Sawano et al. 1996
960delC	m.956_965	Elstner et al. 2008
960insCC	m.956_965	Elstner et al. 2008
961T>C	m.956_965	Li et al. 2005
961delT + insC	m.956_965	Shoffner et al. 1993
961T>G	m.956_965	Li et al. 2004
961insC	m.956_965	Zhao et al. 2004
988G>A	Reported	Rydzanicz et al. 2010
990T>C	Reported	Konings et al. 2008
1005T>C	Unclear	Li et al. 2005
1027A>G	Reported	Lu et al. 2010
1095T>C	Reported	Tessa et al. 2001

Variant	Note	Reference
1116A>G	Reported	Li et al. 2005
1180T>G	Reported	Li et al. 2004
1192C>A	Reported	Lu et al. 2010
1192C>T	Reported	Lu et al. 2010
1226C>G	Reported	Li et al. 2004
1291T>C	Reported	Ballana et al. 2006
1310C>T	Reported	Lu et al. 2010
1331A>G	Reported	Lu et al. 2010
1374A>G	Reported	Lu et al. 2010
1452T>C	Reported	Lu et al. 2010
1494C>T	Confirmed	Zhao et al. 2004
1517A>C	Reported	Mkaouar-Rebai et al. 2008
1537C>T	Reported	Leveque et al. 2007
1555A>G	Confirmed	Fischel-Ghodsian et al. 1993

1.3. Neonatal screening of genetic diseases

For historical practical reasons, Finland has not screened newborns extensively for very many diseases in the past. It is only now, during the 21st century that more diseases will be screened at the population level. In the past, newborns were routinely screened for hearing loss and hypothyroidism. In 2014 the Ministry of Social Affairs and Health Finland issued a letter for all the health care regions to start screening newborns for five metabolic diseases: congenital adrenal hyperplasia (CAH), medium-chain acyl-CoA dehydrogenase deficiency (MCAD),

long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD), glutaric acidemia type 1 (GA1) and phenylketonuria (PKU), in which early detection of the disease is crucial, as a special diet can make a difference between an individual with normal cognition and a severely mentally retarded individual. Currently more than 20 metabolic diseases are included in the Finnish newborn metabolic screening protocol provided by Turku University Hospital. This expanded newborn screening was initiated at Oulu University Hospital on 1st October 2015. It has been estimated that the costs of newborn screening would be much less than the costs of treating sick individuals, if the conditions were not diagnosed as early as possible. Many of these diseases are treatable when identified early, but can be devastating if they are diagnosed late.

The number of diseases screened for varies among countries. In other Nordic countries newborns are routinely screened for 25-27 diseases. In the USA, conditions screened for vary from state to state from around 30 to up to 57 conditions. Different genetic diseases are common in different parts of the world. Therefore a universal newborn screening system would not be effective, but each population should have a screening panel for the most common genetic diseases in that specific population. Also, the conditions screened for need to be easily detectable in a laboratory setting and have an available treatment. There is a big difference between Finland and other countries concerning neonatal screening programs for cystic fibrosis. It is quite rare in Finland, compared for example with America. Over 1000 mutations have been associated with cystic fibrosis. Cystic fibrosis is estimated to occur 1:3000 in Caucasians but only 1:25000 in Finns.

Screening for genetic diseases requires only a simple laboratory test, but there are other ethical considerations when screening is done across an entire population. The accessibility of the test should be equal for everyone, no matter whether they live in a big city or in a remote rural area. Parents should be given enough information beforehand, so that they understand what is being tested, and why and what it means to get a positive/negative result for their child. Testing has to be voluntary and parents should be able to decide without prejudice whether or not they want their child to be tested (111)

1.4. Genetic burden of small populations

A small population with limited gene flow will eventually gather rare, often pathogenic gene variants. Factors that can limit gene flow and genetic variation of breeding individuals are isolation (e.g. geographical, cultural and linguistic), small population size, low migration rate, inbreeding and genetic bottleneck events. Studies of human genetic isolates have helped map rare disease genes of monogenic disorders and benefitted the understanding of genetic diseases in general (112). Genetic diversity at the molecular level is smaller in humans compared to other primates (113). Y-chromosomal and mitochondrial DNA have been proven to be powerful tools to assess the level of genetic isolation of populations (114).

The fitness of a population is directly associated with its gene pool. High genetic load in a population means fewer reproductive individuals and individuals in optimal state of fitness. The fitness of an individual is manifested through his/her phenotype (115). As a population suffers from inbreeding and isolation, rare pathogenic alleles become more common. This causes an increase in hereditary diseases. In a large population with rapid gene flow, these rare allele carriers rarely happen to produce offspring. In a small isolated population, the probability of two rare allele carriers reproducing becomes higher. First cousin-marriage will greatly increase the risk of genetic diseases being passed on to children. Consanguineous parents carry more homozygous chromosomal regions due to their joined heritage. Consanguineous marriage is more common in certain cultures, such as in Saudi-Arabia. The adverse effects are often unknown to the general public and a strong cultural tradition overrides the awareness of risk for genetic disease (116).

Population genetic bottleneck is a rapid reduction in the size of a population, followed by a rapid expansion. After the bottleneck event, rare alleles are enriched by chance into the next generation. Some alleles will also become scarce or extinct (Figure 19).

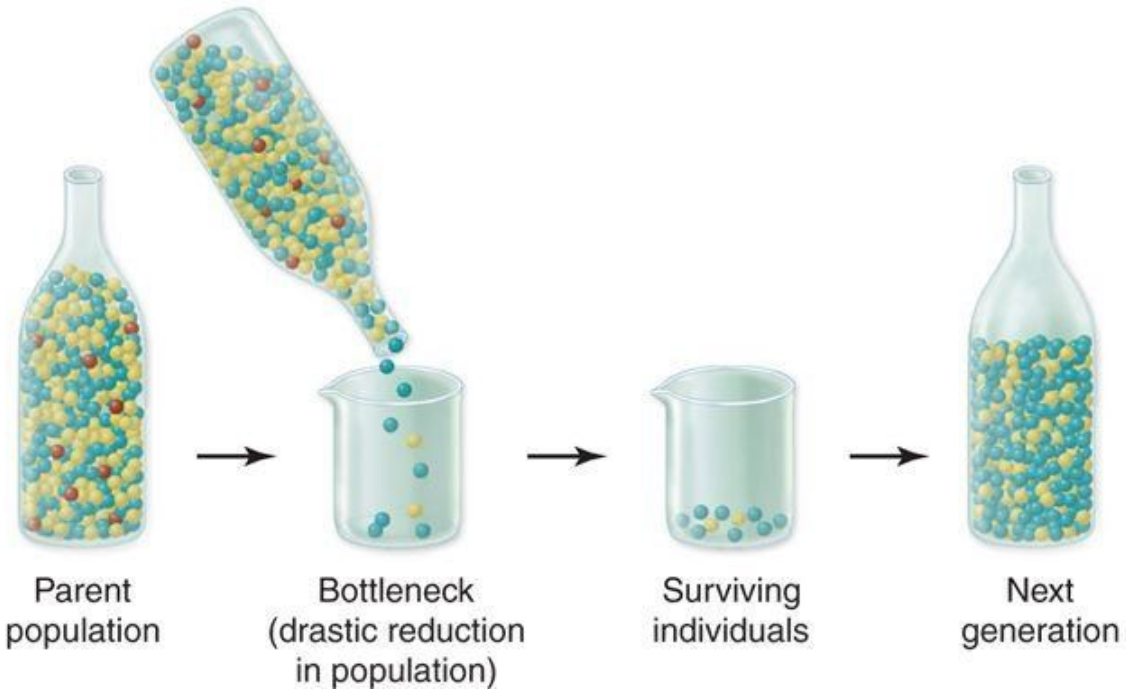

Figure 19 Genetic bottleneck event (www.planttreaty.org)

1.4.1. The Finnish disease heritage

Finland is among the most isolated human genetic populations in the world. Not only are we geographically in a remote area, but we are linguistically isolated as well. This has led to a relatively high frequency of rare, monogenic genetic disorders. Also, disorders specific to the Finns (the Finnish disease heritage) are observed in our population (117). The Finns are descendants of two major migration events that took place 2000 and 4000 years ago. It has been suggested, that the great famine years of 1696 – 1698 reduced the population by a third. Since then the Finnish population has grown rapidly to its over 5 million citizens observed today. Not only is the whole of Finland isolated, but regional isolates also occur, such as the Kainuu-region. The Finnish disease heritage consists of around 30 recessively inherited diseases, which have been enriched in our population. These diseases such as the cartilage-

hair hypoplasia occur in regional isolates (Figure 20). Some inheritable diseases which are more common in other parts of the world are almost nonexistent, such as cystic fibrosis (118, 119).

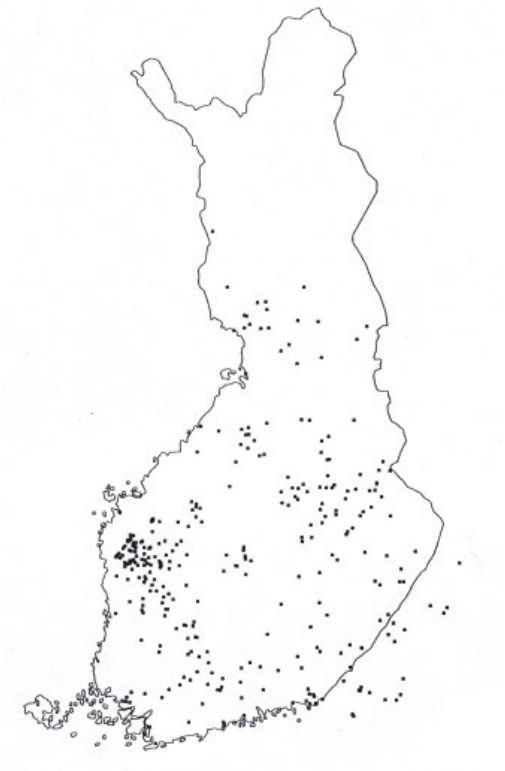

Figure 20 Incidence of cartilage-hair hypoplasia in Finland according to birth place (Reijo Norio, Suomi-neidon geenit, Otava, Helsinki 2000)

1.5. Preterm birth

The length of a normal pregnancy is 38 weeks. The worldwide incidence of preterm birth is 9.6 % of all births, 85 % of them are concentrated in Africa and Asia (120). Preterm birth is the number one cause of perinatal mortality (perinatal period: from 22+0 gestational weeks to 7 days after birth), accounting for over 75 % of perinatal deaths in developed countries (121). The mean length of a pregnancy in 2012 in Finland was 39+4 gestational weeks. If a baby is born before week 37, he/she is considered to be born prematurely. Preterm births can be further classified into near term (34-36 weeks), moderately preterm (32-33 weeks), very preterm (28-31 weeks) and extremely preterm (under 28 weeks) (Figure 21). Premature birth

poses a serious risk for later neurodevelopmental impairment and other, often life-long health complications and special needs. In Finland during the year 2012, 3387 babies out of a total of 59 856 babies were born prematurely before gestational week 37, which accounts for 5.6 % of all babies (Table 8). 4.2 % had a very low birth weight (under 2500 g) and 0.8 % had an extremely low birth weight (under 1501 g). The mean weight for a baby born at term in Finland in 2012 was 3498 g. For singleton pregnancies the frequency of preterm births was 4.5 %, for twin pregnancies 45.8 % and for triplet and multiple babies the number rose up to 100 % (www.thl.fi 2011-2012 statistics). Of low birthweight preemies 12.5 % were babies of mothers who had had infertility treatments. Neonatal mortality rate (death < 7 days of age) in Finland is 0.9 ‰, which is one of the lowest in the world (Table 9, Figure 22) (www.thl.fi).

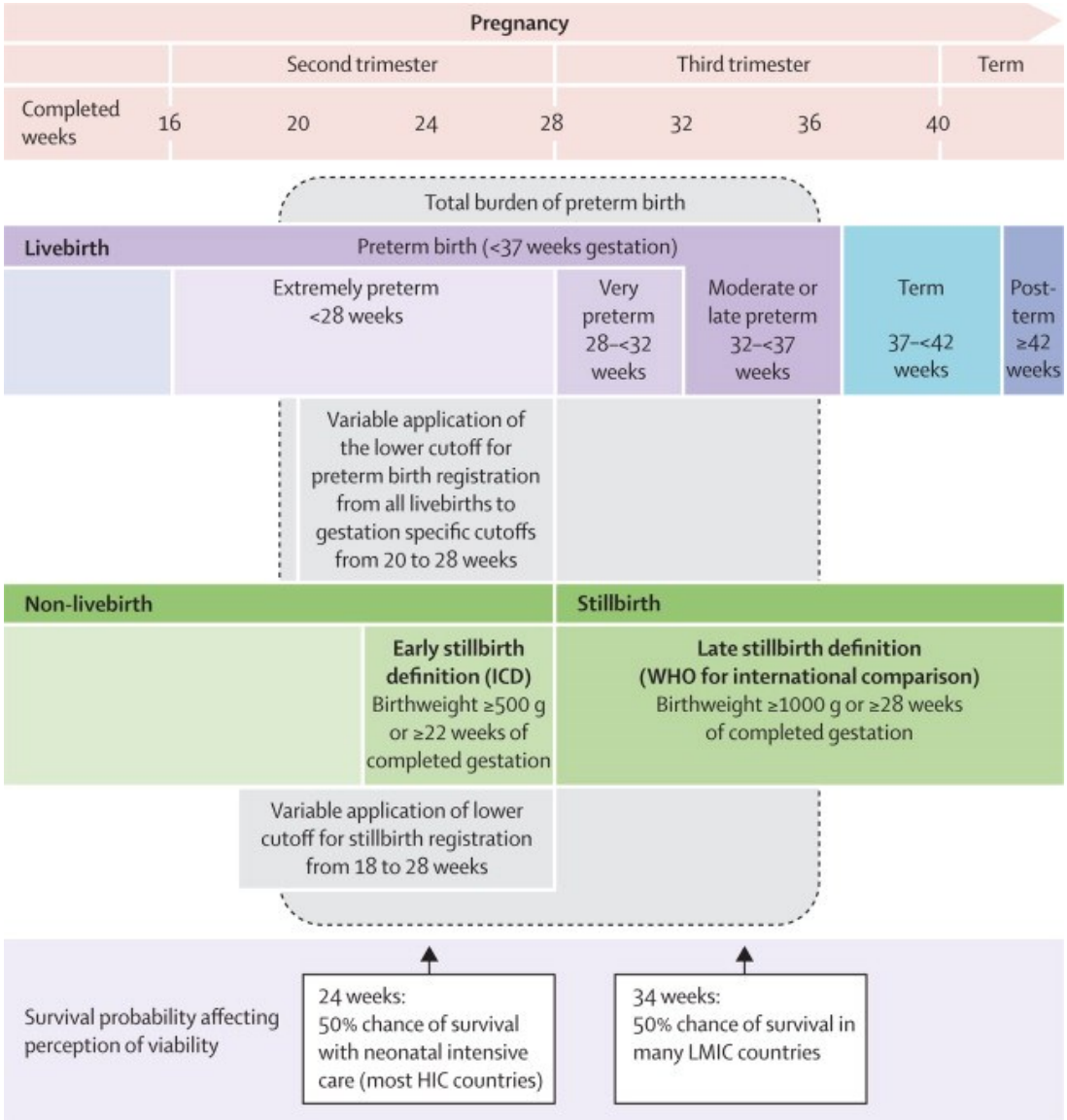

Figure 21 Classification of preterm birth & survival probability (122)

Table 8 Preterm babies born in Finland in 2012 (www.thl.fi)

Pregnancy length (weeks gestation)	**Babies born in 2012 (*N*)**
< 27	187
28 – 31	307
32 – 35	1559
36	1334
Total	3387

Table 9 Live births, stillbirths and death at age 0 - 6 days among preterm babies in Finland during 2011-2012 (www.thl.fi)

Weeks of gestation	**Live births (*N*)**	**Stillbirths (*N*)**	**Death at age 0-6 days (*N*)**
< 22 – 25	156	72	35
26 – 28	275	34	14
29 – 31	498	25	14
32 – 34	1636	42	17
35 – 36	4005	28	15

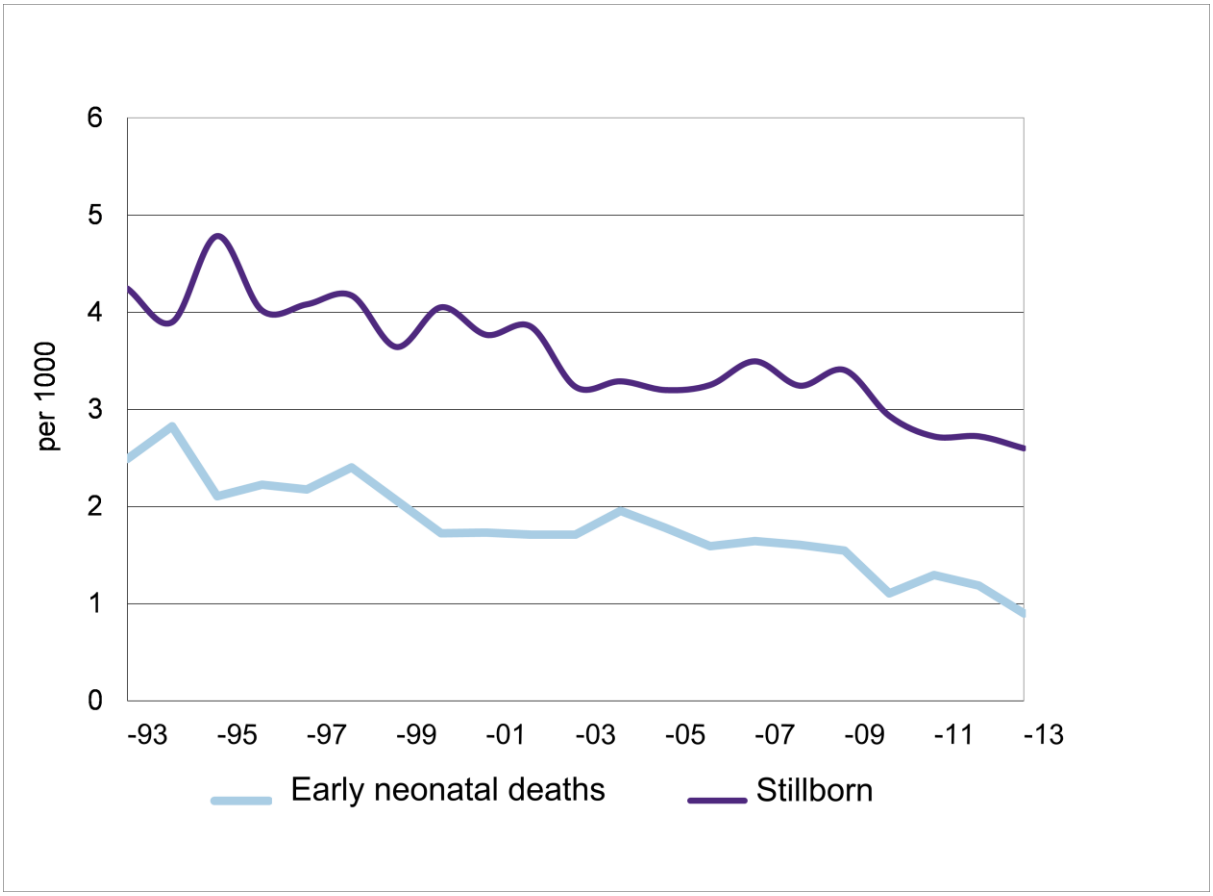

Figure 22 Number of stillbirths and early neonatal deaths in Finland during the year 2013. Purple denotes stillbirths, light blue denotes early neonatal deaths (www.thl.fi)

The causes for premature birth can include early planned delivery for maternal or fetal health reasons (30 %), spontaneous preterm labor with intact membranes (40 %) and premature rupture of membranes leading to premature labor (30 %) (123). Some of the risk factors for premature birth include pregnancy complications, such as pre-eclampsia, gestational diabetes, intrauterine infections, fetal exposure to smoking/alcohol/drugs, congenital abnormalities of the fetus, invasive prenatal testing (e.g. amniocentesis), placental problems such as placenta previa/hemorrhage/abruption, stress, short interval between pregnancies, low maternal body-mass-index (BMI), previous preterm birth, twin/triplet etc. pregnancy, assisted reproduction techniques (e.g. IVF), excess or low amniotic fluid, very young or old maternal age and maternal chronic diseases and conditions (thyroid disease, asthma, hypertension, gynecological procedures and uterus/cervix anomalies) (Figure 23).

Then again, often there are no risk factors or warning signs for a preterm birth. For unknown reasons, black women are more likely to go into preterm labor compared to women of other ethnicities (121, 122, 124-126).

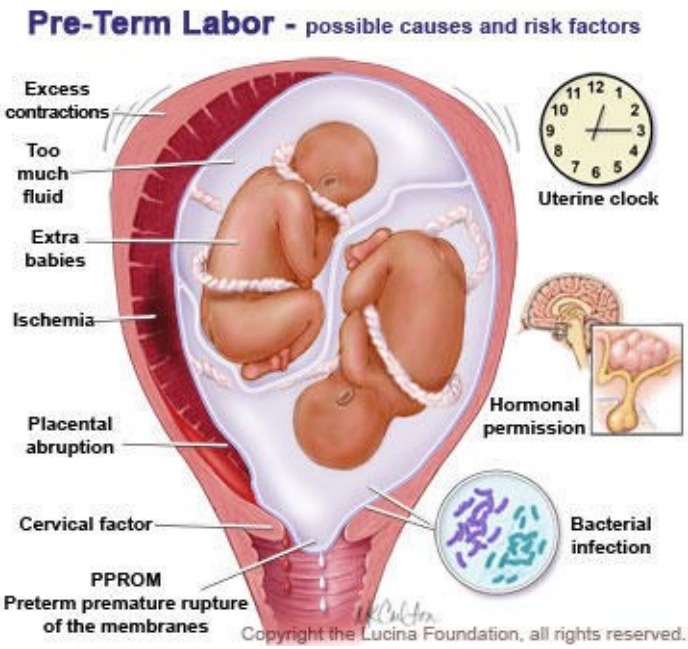

Figure 23 Causes and risk factors of preterm labor (lucinafoundation.org)

1.5.1. Common health issues of preterm babies

A preterm baby born at an early gestational age is very small, with a disproportionally large head, and often weighs considerably less than a baby born at term (Table 10). Body fat deposits are almost non-existent, because they are formed during the last weeks of a normal pregnancy and this is why the baby appears to be very thin. The lack of body fat also causes problems in the baby's body temperature regulation and there is a tendency towards low body temperature. The baby's body can be covered with fine hair, called lanugo. This hair normally disappears before the end of a full-term pregnancy.

A preterm baby's lungs can be underdeveloped, which causes troubles with breathing and maintaining a good oxygen level in the body. Respiratory distress syndrome (RDS) is one of the major complications of premature birth, along with dangerous sepsis and other infections. Lungs of premature babies lack surfactant, a substance which helps the lungs to stay open. Lung surfactants and steroids can be administered before birth if a premature birth is suspected to occur, and this has made a huge difference in preemie survival rates. Apnea is also common, where the baby will have long pauses in his breathing, leading to bradycardia (slowing of the heart beat). RDS may cause bronchopulmonary dysplasia (BPD) which can predispose to asthma later in life (127-129).

The heart of a premature baby is often affected and it is quite common for premature babies to undergo heart surgery after birth. A hole in between two blood vessels of the heart (patent ductus arteriosus, PDA) often needs surgery to be closed (130). Low blood pressure is also common. Heart rate variability can be reduced, which reflects the underdevelopment of autonomous cardiovascular control of the heart (131). Sudden death syndrome (SIDS) is more likely to occur in preterm babies than in babies born at term (132). Anemia and infant jaundice are frequent in preterm babies: up to 80 % of preterm babies develop jaundice, as well as hypoglycemia (133, 134). Due to organ immaturity, GI tract problems such as necrotizing enterocolitis (NEC) can develop when the child starts feeding.

Table 10 Weight, length and head circumference according to gestational age in premature babies in the USA, boys (mayoclinic.org)

Gestational age (weeks)	Weight (kg)	Length (cm)	Head circumference (cm)
40	3.6 kg	51 cm	35 cm
35	2.5 kg	46 cm	32 cm
32	1.8 kg	42 cm	29.5 cm
28	1.1 kg	36.5 cm	26 cm
24	0.65 kg	31 cm	22 cm

1.5.2. Hearing loss among premature babies

Hearing loss is one of the most common disabilities of premature birth. The most frequent type is sensorineural hearing loss, in which the inner ear nerves are damaged. The hearing loss/deafness is permanent, but it can range from mild hearing loss to complete deafness. It has been estimated that up to 7 % of premature babies suffer from hearing loss or deafness. A Dutch study concluded that the earlier a baby was born as measured in weeks of gestation, the higher the risk of non-syndromic hearing loss. Also, a low birth weight in itself was found to increase the risk of non-syndromic hearing loss (135). Another study reported that the gestational age would be a more relevant factor contributing to hearing loss compared to birth weight alone, as the auditory system is not fully developed before term (12). Severe jaundice or hyperbilirubinemia will also increase the risk of sensorineural hearing loss. About 80 % of preterm babies develop hyperbilirubinemia during the first weeks of life (136).

The development of hearing loss is not exclusively linked to low birth weight or gestational age, but prematurity adds additional risk factors for hearing loss. These risk factors are a) The use of aminoglycoside antibiotics to treat infections b) Noise made by the beeping/bleeping NICU machines and life support c) Lack of oxygen which causes damage to the cochlea. Sometimes mechanical ventilation can also cause hearing loss. The hearing loss can be progressive or delayed-onset, in which case the child will develop hearing loss by three years of age (www.sense.org.uk).

Signs of hearing loss in babies consist of not being startled by loud noises, not turning towards a sound, not imitating sounds after 6 months of age, not babbling by 1 year old, not using single words by 1.5 years and not using two-word sentences by 2 years of age. The hearing of babies is tested before the age of 1 month or preferably sooner with an automated auditory brain stem response system (AABR) and/or an otoacoustic emissions test (OAE). If the baby fails to pass these tests, more extensive testing and further treatment is done by an audiologist (137). In Finland, this hearing test is done in the hospital within the first days of life.

Treatment of sensorineural hearing loss includes hearing aids and cochlear implants. Speech therapy and learning sign language can help the child to communicate more

and help to boost their self-esteem. Cochlear implants can give a child a sense of hearing, but it cannot restore normal hearing (138).

1.5.3. Survival and long-term outcome of premature birth

The outcome of a premature birth depends on the gestational age of the baby and the circumstances leading to the premature birth. Neurological disabilities (e.g. cerebral palsy), mental deficits, sensorineural impairments (deafness, blindness etc.) and cognitive dysfunction are some of the main consequences of premature birth. Some signs and symptoms are more subtle and can be detected only as the child grows older, such as developmental delay and other cognitive impairments along with behavioral/social problems. It has been estimated that around 68 % of preterm born babies will have disabilities, 15 % of them severe (139).

Survival rates have continued to rise due to the steroid and lung surfactant replacement treatments available. Also, the technology of neonatal intensive care units has improved greatly. Today, around 50-67 % of extremely preterm babies will survive (born during 20-26 weeks of gestation) (140, 141). Most common morbidities for extremely preterm babies are brain injury, bronchopulmonary dysplasia, retinopathy of prematurity (ROP), severe infections and poor growth (139).

Low birth weight has been associated with later manifesting visuo-perceptual problems (142). Long-term effects of corticosteroid treatment of very preterm babies and early blood fusions have been linked to chronic lung disease (143, 144). Preterm infants have higher growth hormone levels at the time of birth compared to full term babies. This has been linked to neurodevelopmental anomalies such as working memory and attention problems at a later age (145). Time estimation and attentional task difficulties have also been detected at a higher rate in school aged preterm born children (146). Very preterm babies frequently suffer from anemia due to low iron storage. Excess iron treatment can lead to neurodevelopmental problems such as impaired memory and behavioral problems. The timing of the iron treatment is important for preventing side-effects and future problems (Hong-Xing et al. 2015).

Visual impairment is regularly diagnosed in preterm babies. This condition is called the retinopathy of prematurity (ROP), which is the second leading cause of childhood blindness. Preterm babies have underdeveloped blood vessels in the retina, which begin to grow abnormally after the untimely birth. This forms scar tissue inside the eye, which damages or even detaches the retina. ROP develops in 65 % of babies born before term. Retinopathy of prematurity can be treated with laser and/or freezing treatment of the retina. Early treatment can help to maintain good eyesight in the future. Severe ROP can cause myopia (nearsightedness) later in childhood. Sometimes visual problems arise due to hypoxia in the brain and damage to brain areas with vision functions. This can also cause problems in three dimensional vision, recognizing faces etc. (147). The full scale of long-term disabilities and consequences of premature birth are still continuing to unravel (Figure 24).

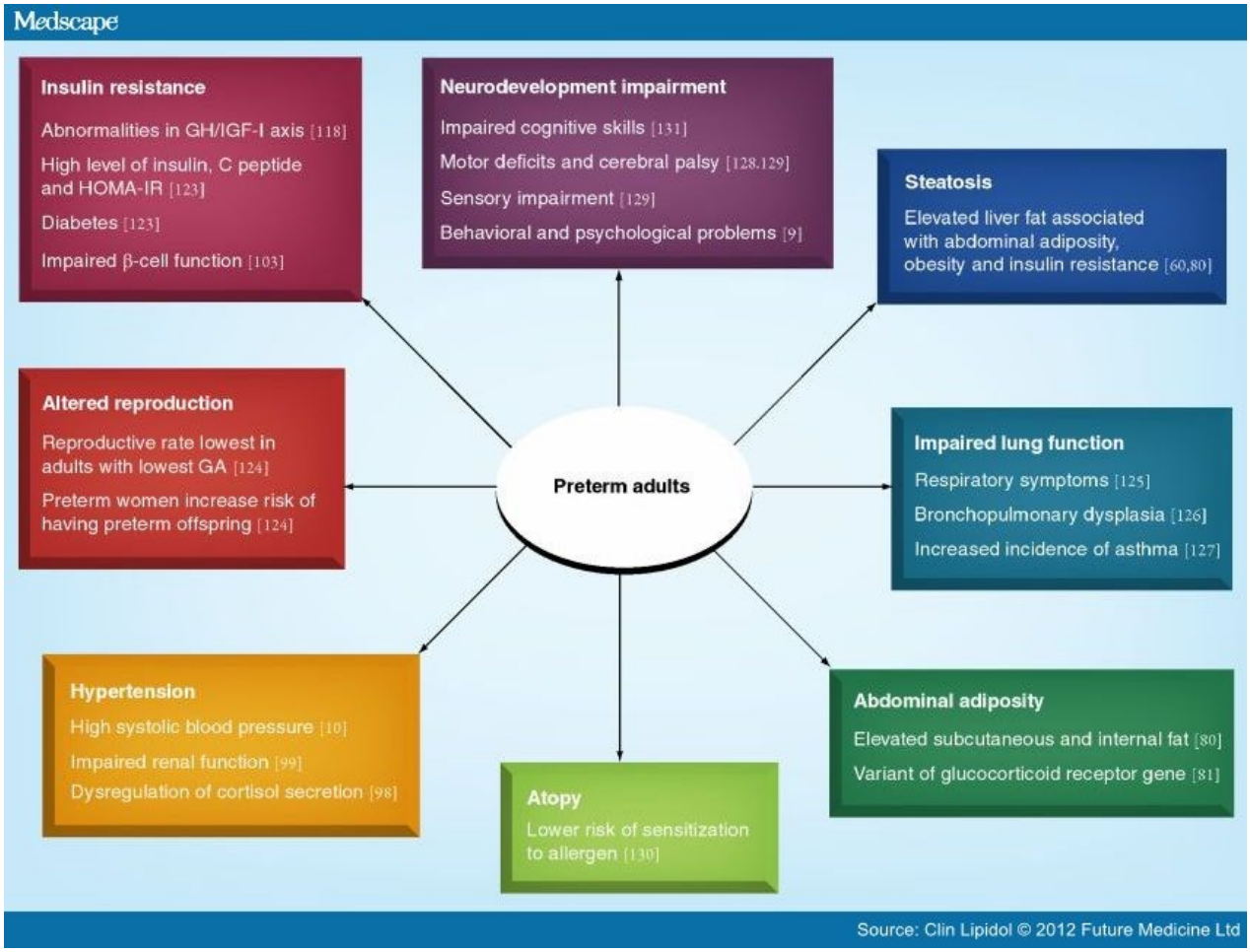

Figure 24 Common long-term effects of prematurity (148)

2. OBJECTIVES OF THE STUDY

The aim of this study is to determine the frequency of aminoglycoside sensitivity mutations m.1555A>G, m.1494C>T and m.1095T>C in preterm and term-born babies born in Northern Finland.

3. METHODS

3.1. Study population

The subjects of this study consist of a total of 866 newborns (Table 11). These babies were born in the Oulu University hospital during the years 1973 to 2012. Buccal, blood or umbilical cord samples were collected for DNA extraction. At first a total of 866 children's DNA samples were selected for the study. 677 of these were prematurely born babies. Of these preterm babies, 93 samples came from 40 families with more than one child in the study. To estimate the population frequency, we only studied one sibling per family, excluding 53 preterm baby samples from the study. This left us with 624 preterm babies and 189 term-born babies; a total of 813 children.

Table 11 Study samples of preterm and term-born babies

Samples	*N*	Gestational age at birth
Preterm babies	624	< 36 wk
Term-born babies	189	≥ 37 wk
Total	813	

3.2. DNA extraction

Genomic DNA was extracted from whole blood with the UltraClean DNA Blood Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) or UltraClean DNA BloodSpin Isolation Kit (MOBIO) and from umbilical cord tissue with the Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany). DNA was extracted from buccal cells with Chelex 100 (Bio-Rad, Hercules, CA, USA). Buccal cell DNA was whole-genome amplified with the Illustra GenomePhi V2 DNA Amplification Kit (GE Healthcare Sciences, Cardiff, USA) and the resulting samples were purified with Illustra Microspin G-50 columns (GE Healthcare Sciences).

3.3. DNA amplification by polymerase chain reaction (PCR)

The mtDNA fragment surrounding the hearing loss mutations was amplified using standard polymerase chain reaction (PCR) (Tables 12 - 13). Phire Hot Start II DNA polymerase (ThermoFisher Scientific, Waltham, MA, U.S.A.) was used for amplification of the PCR-product. PCR fragments were amplified through 30 cycles in a fast cycling PCR machine. First, the starting denaturation at 98 °C for 30 s, followed by 30 cycles of 5 s 98 °C denaturation, annealing at 65 °C or 67.3 °C, extension at 72 °C for 10-15 s. After 30 cycles, a longer 1 min extension was done at 72 °C followed by cooling down the sample to + 4 °C.

Table 12 PCR-conditions for mtDNA MTRNR1 mutations.

Mutation	Forward-primer position	Reverse-primer position	Annealing Temp (°C)
m.1555A>G **m.1495C>T**	m.1357	m.1696	65 °C
m.1095T>C	m.861	m.1246	67.3 °C

Table 13 Reaction mix for PCR-amplification.

Reaction mix	Volume (µl)
H_20	26.5
5 x Phire buffer	8
F-primer	1
R-primer	1
dNTP 10 mM	0.8
DMSO	1.2
Phire HotStart II	0.8
DNA-sample	1
Total	40

3.4. Sanger sequencing

Sequencing was done using the standard Sanger sequencing protocol (Table 14) (149). The same primers that were used for amplification of the PCR-product were also used for sequencing. First, the PCR-product was purified using *ExoI* and Rapid alkaline phosphatase enzymes. Sequences were analyzed using Sequencher 5.1. (GeneCodes Corporation, Ann Arbor, MI, U.S.A.). A common mtDNA control sequence, the Cambridge Reference Sequence (GenBank: NC_012920), was used to analyze mtDNA sequences.

Table 14 Sequencing reaction mix.

Reaction mix	Volume (µl)
H_20	3.5
PCR-product	1
Rapid alkaline phosphatase	0.5
ExoI	0.3
Primer (5µM)	0.7

3.5. Restriction fragment length analysis (RFLP)

Restriction fragment length analysis (RFLP) is a method where the template DNA is multiplied by PCR, followed by a digestion using a restriction enzyme. These enzymes bind to a specific DNA sequence (for example CGT) and then cut the DNA. This can be used to detect the presence of a mutation; whether or not the enzyme cuts the DNA. The resulting fragments are then be electrophoresed through 2 % agarose gel to detect the size difference and to spot the samples containing the mutation. RFLP is easy to use for large scale population screening for mutations (Figure 25). RFLP protocols used for each mutation in this study are shown below (Table 15, Figures 26 – 28).

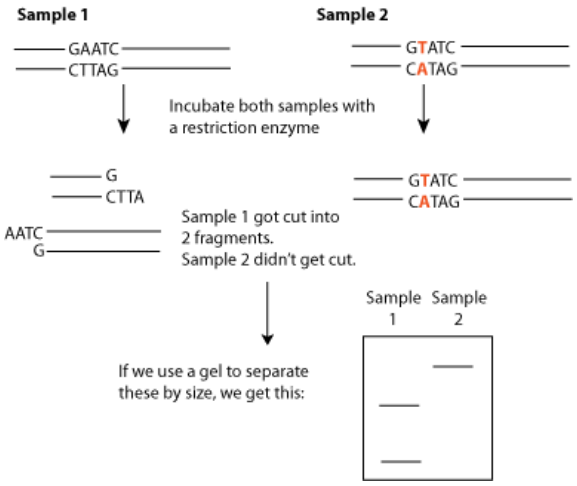

Figure 25 Restriction fragment length polymorphisms analysis (RFLP) (www.scienceblogs.com)

Table 15 RFLP protocols used in this study. WT = wild type, M = mutation.

Mutation	Enzyme	DNA fragment size (bp)
m.1555A>G	*Alw26I*	WT: 189 + 130 bp M: 319 bp
m.1494C>T	*HphI*	WT: 194 + 125 bp M: 319 bp
m.1095T>C	*BspCNI*	WT: 116 + 270 bp M: 130 + 136 + 140 bp

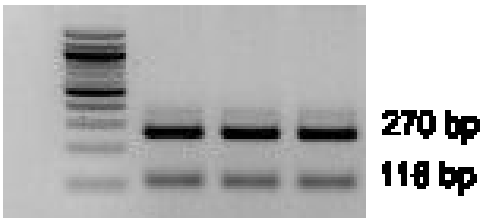

Figure 26 Example RFLP analysis of m.1095T>C mutation. Lane 1: size standard, lanes 2-4: mutation negative wild type samples (270 bp + 116 bp bands).

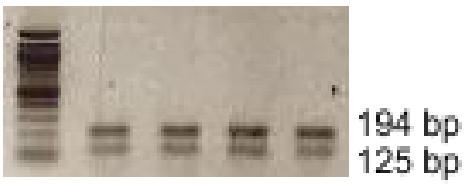

Figure 27 Example RFLP analysis of m.1494C>T. Lane 1: size standard, lanes 2-5: mutation negative wild type samples (194 bp + 125 bp bands)

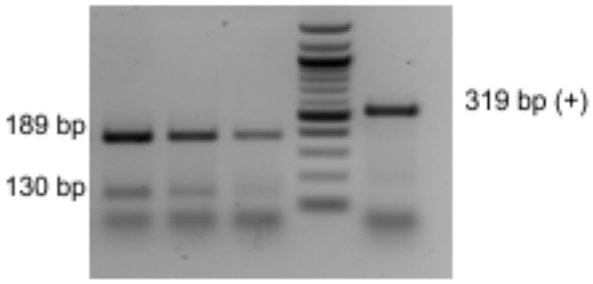

Figure 28 Example of RFLP analysis of m.1555A>G. Lane 1-3: mutation negative wild type samples (189 bp + 130 bp), lane 4: size standard, lane 5: m.1555A>G mutation positive (+) control (uncut 319 bp band)

3.6. Heteroplasmy analysis

RFLP analysis results show both wild type DNA fragments and mutated DNA fragments if a mutation is heteroplasmic. In the case of a heteroplasmic mutation, the RFLP analysis visualizes it, but the heteroplasmy percentages cannot be quantified. Radioactively labeled ^{35}S-dATP (Perkin-Elmer, Wellesley, MA, U.S.A.) is added to the PCR reaction, the digestion is carried out with the same enzyme and the fragments are electrophoresed through a PAGE-gel (polyacrylamide gel electrophoresis). Then the radioactive gel is exposed onto a film and developed. Finally, the intensities of the wild type and mutated DNA fragments are quantified from the film using Quantity One program (BioRad, Hercules, CA, U.S.A.). The heteroplasmy levels are then calculated and presented in percentage of mutated mtDNA, e.g. m.1555A>G in 80 % heteroplasmy.

3.7. Ethical considerations

The ethical committee of Oulu University Hospital (PPSHP) approved the study protocol. Each child's guardian signed an informed, written consent for the child's participation in the study. All samples were numbered and no names were used, except by the lead investigators. Participants were only to be informed of the findings of this study if it was imperative to their individual health and well-being.

4. RESULTS

Out of the 813 DNA samples investigated, a single individual was found to harbor the m.1555A>G mutation (Table 16). This individual was a baby born in normal gestational time. The heteroplasmy analysis revealed that the m.1555A>G mutation load in his blood was 81 %. The family was informed about the findings and we were also able to obtain a DNA sample from the patient's mother. The DNA sample was digested using a radioactively labelled RFLP protocol. Heteroplasmy level of m.1555A>G was quantified to 68% in her blood sample (Figure 29 - 30). Neither the mother nor the child reported any hearing loss (no hearing tests had been performed) nor to their knowledge did it occur in their family.

Based on our results, the population frequency for m.1555A>G hearing loss mutation was calculated to be 1.23:1000 individuals in this region (0.5 % of term-born babies in this study). No samples were found to harbor m.1494C>T or m.1095T>C hearing loss mtDNA mutation out of the studied 813 DNA samples. None of the screened hearing loss mutations were found in preterm babies.

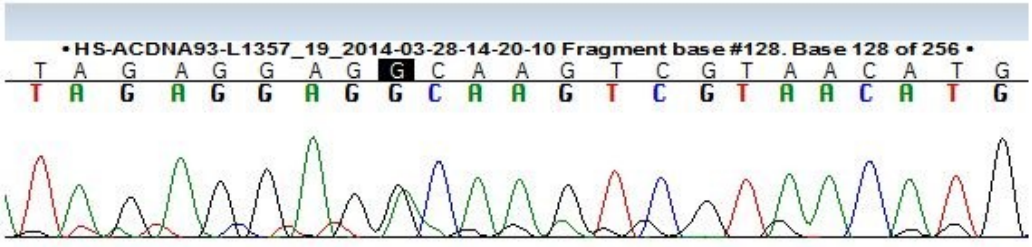

Figure 29 MtDNA sequence of m.1555A>G positive sample. Heteroplasmy is visible in the two overlapping peaks for both G and A.

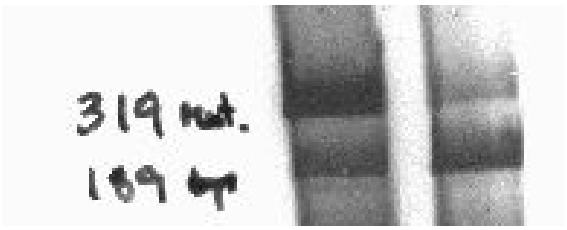

Figure 30 Radioactive RFLP analysis for measuring heteroplasmy. Lane 1: m.1555A>G child (80% heteroplasmy), lane 2: m.1555A>G mother (68 % heteroplasmy). Band 1 from top: mutation band 319 bp, band 2: wild type band 189 bp.

Table 16 Results of mtDNA hearing loss mutation screening in 813 preterm and term-born babies

Mutation	Preterm infants *(N)*	Term infants *(N)*	Whole study population (%)	Heteroplasmy (%)
m.1555A>G	0	1	0.12	81
m.1494C>T	0	0	0	n.a
m.1095T>C	0	0	0	n.a

5. DISCUSSION

Hearing loss is one of the consequences of premature birth. Brain hypoxia, infections and intensive care treatment can permanently damage the underdeveloped inner ear of premature babies. Aminoglycosides are commonly used in the treatment of sepsis and infections in premature babies. A known side effect, hearing loss, can occur in a small percentage of babies due to aminoglycoside use (2, 9). Babies with a predisposing mtDNA mutation for aminoglycoside sensitivity are at a greater risk for developing a permanent hearing impairment or deafness, even after a single dose of aminoglycoside antibiotics (3, 4, 6, 102, 150). The general frequency of pathogenic mtDNA mutations in the healthy European population has turned out to be much more substantial than expected. It has been estimated that 1:200 carries a deleterious mtDNA mutation (87).These aminoglycoside sensitivity mutations occur in varying frequencies in different populations. M.1555A>G, m.1494C>T and m.1095T>C are all most commonly reported in Asian countries, such as in China and Japan (64, 65, 97, 100, 101, 104). But, these mutations do exist among Europeans and have been noted to be surprisingly common in some European populations, such as m.1095T>C in Italy and m.1555A>G in Spain (10, 78, 105, 106). The frequencies of m.1095T>C and m.1494C>T mutations has never been screened on population level in Finland.

M.1555A>G mutation has been previously screened among Finnish non-syndromic hearing loss patients (84, 86). It is a well-known fact that Finns are the genetic outliers of Europe; a unique genetic heritage has evolved through the centuries with a trend towards genetic isolation. Finns are separated from the main Indo-Europeans linguistically, geographically and genetically (51, 52, 55, 119, 151). As with any disease causing mutations, it is imperative that the frequency of the mutation is screened in different populations, as the genetic composition of each population varies.

In our study, we found a single baby with m.1555A>G mutation among 813 DNA samples studied. This 0.12 % frequency concurs with the previously estimated European mutation frequencies of 0.19 % in British children and 0.21 % in European descended Australians (152-154) and can be even considered to be slightly lower. The absence of m.1095T>C and m.1494C>T in Finns either means that they are too rare to occur in our study population of 813 individuals or that they do not exist in our population altogether.

The single discovered m.1555A>G mutation was discovered at an 81 % heteroplasmy level in the blood. The child's mother harbored the mutation at 68 % heteroplasmy in her blood. It is common that a lower heteroplasmy mother passes on the mutation in a higher percentage to her children. On the other hand, a high heteroplasmy mother often has low heteroplasmy children even though heteroplasmy is considered to be inherited randomly. The reasons for this remain unclear. In the literature, the m.1555A>G is almost always reported to be homoplasmic (11, 155, 156). This family reported in our study is the first heteroplasmic m.1555A>G family in Northern Finland. The m.1555A>G mutation and the hearing loss it causes behaves differently compared to other mitochondrial diseases, where the percentage of heteroplasmy is directly associated with the severity of the symptoms and tissues with high heteroplasmy have more symptoms compared to low heteroplasmy level tissues e.g. muscle symptoms in mitochondrial diseases. In m.1555A>G patients, varying levels of hearing loss are reported from normal hearing to complete deafness. These phenotypic differences do not follow heteroplasmy levels, as most mutation carriers are homoplasmic. Also, as heteroplasmy is tissue specific, the heteroplasmy levels are unknown in the hair cells of the inner ear. Also the age of onset varies from early childhood to adulthood, the median being 14 years (66). Even in heteroplasmic m.1555A>G families, the severity of the hearing defect does not follow the mutation load. In addition, aminoglycoside exposure alone does not explain the phenotypic variation either. Hearing loss can develop without aminoglycoside use but is highly aggravated if these antibiotics are used (one dose can lead to total permanent deafness) (6). Due to this phenotypic variation, the existence of a modifying nuclear factor has been suggested. The main candidate gene has been *TRMU* but other mitochondrial protein synthesis related genes have also been theorized. The data is quite inconclusive and the real reason(s) for the phenotypic variation remains to be found (94, 95, 150).

Screening newborn babies for rare hereditary diseases is becoming increasingly commonplace as DNA screening methods become more affordable, automated, sensitive and robust. The most regularly screened diseases are usually metabolic diseases which are treatable with early diagnosis. A child can live a normal or better life if a condition is treated as early as possible (normal development vs. severe intellectual disability). The price of population level screening for treatable congenital diseases far surpasses the health care costs of treating patients with devastating diseases for the remainder of their lives (157).

As for the mitochondrial non-syndromic hearing loss mutations in this study, the mutation frequencies for m.1555A>G, m.1095T>C and m.1494C>T are rare and general screening for pre-term babies is not necessary. In specific circumstances though, genetic screening of m.1555A>G is advisable, for example, if a premature baby is born to a family with a history of non-syndromic hearing loss on the mother's side. A substantial difficulty is that there is often little time to wait the results of genetic testing, as premature babies often need urgent treatment and care. Not to mention the fact that premature birth is frequently an unplanned event. Tests for specific mtDNA mutations are not routinely performed in hospital laboratories and results can take a long time. Nonetheless, a few large families of m.1555A>G do exist in Northern Ostrobothnia and this should be taken into account (84, 86). In the case of positive family history of maternal hearing loss, the mother of the premature baby could be tested for the mutation and the baby would not have to go through a blood test, as the m.1555A>G mutation would be present in both the mother and the baby. This would be especially advisable if it were known that the birth would most likely happen before term.

It would be imperative for the members of m.1555A>G families to be fully aware of the risks for aminoglycoside induced hearing loss. Genetic testing and counseling should be offered to the matrilineal family members to fully understand the inheritance of the mutation (from the mother to all her offspring), understand the need to avoid aminoglycosides, get a marking in their medical records, also with regard to their babies when pregnant. More detailed audiological investigations for both children and adults should be organized if indicated.

The results of this study can be directly utilized in the clinical care of preterm born babies in terms of aminoglycoside use safety and the need for genetic screening of aminoglycoside sensitivity mutations. The mutations are not more common in Finland and Northern Ostrobothnia compared to rest of Europe. It should be kept in mind though, that a few large families with m.1555A>G exist in our region and their babies will be born in our regional hospitals.

It is not only important to know the frequency of these aminoglycoside predisposing mutations in our population, but also to assess the most vulnerable population of patients, preterm babies, who are often treated with aminoglycosides. If these mutations turn out to be more common in our population, aminoglycoside treatment of preterm babies should

be reconsidered and/or genetic screening for these mutations would be advisable. Also, it is extremely important to identify the families carrying these mutations, so that they are aware of the increased hearing loss risk associated with aminoglycosides. This knowledge that aminoglycoside-group antibiotics should be avoided could save several individuals and whole families from life-long hearing defects and deafness.

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