# CHAPTER 1

## 1.1 INTRODUCTION

In the last two decades there has been an increasing interest in human aging and its genetic basis. In the first instance, this might be explained by the increased number of elderly subjects in the developed countries, due to the ameliorated life condition (Kannisto, 1994). But to live longer implies higher healthcare costs necessary to treat the aging-related diseases that at present amount to approximately 20% of total healthcare costs. The mitigation of these costs, that may increase in the next years, seems necessary and the only way to realize this intent is to guarantee a healthy aging.

Aging is a complex trait characterized by the interaction between environmental, genetic and epigenetic factors (Capri M et al, 2006*).* Certainly, over the last centuries environmental conditions have had an important role in the radical change in life expectancy; on the other hand, genetic factors have also a large influence on the rate and the quality of aging (Perls et al, 2000, 2002; Herndon et al, 2002; Kirkwood and Finch, 2002; Kirkwood, 2005; Lescai et al, 2009a, b). In particular twin studies reported that the percentage of the variation in human life span which can be attributed to genetic differences among individuals ranges from 22% to 33% (McGue et al, 1993; Ljungquist et al, 1998; Bishop and Guarente, 2007;).

Longevity, defined as survival beyond the specie-specific average age of death (De Benedicis and Franceschi, 2006), is a phenotypic expression of successful aging. Although longevity is not sufficient for describing a successful aging process because, for example, it provides little information about functioning and quality of life, it is the most considered and the most simple phenotype to study. In this frame, association studies on long-lived subjects or centenarian, together with functional studies, contributed to identify the specific genes that influence variation in human

lifespan, and highlight genotypes that can predispose individuals to an increased or decreased risk of reaching extreme old age in a healthy status.

In the last years, the technological progress occurring have made possible the identification of genetic determinants of aging and longevity. Twin studies, large scale linkage studies on long-lived families, case-control association studies on candidate gene, longitudinal studies and studies in model organisms agree in detection of a common 'core' of genes belonging to different metabolic pathways and defence systems and shared by all the species studied (Kenyon, 2005; Christensen et al, 2006; Chung et al, 2010). All these studies indicate that aging is the result of the decline, that cause a less efficient conservation, mobilization and use of nutrients, and a worse ability to respond to external and internal stress.

### 1.1.1 Aim of the work

The intent of the present thesis is to highlight some genetic variants that influence this phenotype. In particular, in my PhD researches I have studied the variability of both mitochondrial and nuclear DNA in relation to aging and longevity. In the following chapters I'm describing the state of art of the well known interaction between DNA variability (mitochondrial and nuclear) and aging and longevity. Then, I report the results of the studies conducted during my PhD appointment, which are described in three manuscripts. The first and the second manuscripts have as their topic mitochondrial DNA variability. In particular, in the first manuscript we propose an analysis of the whole sequences of mtDNA to evaluate if mtDNA point mutations or group of mutations occurring in different haplogroups are involved in longevity; it is titled "*The analysis of mitochondrial DNA variability in a large sample of ultranonagenarians across Europe allows a reappraisal of the mtDNA correlation with longevity: a GEHA Project Study*" and has been submitted to the "Hamerican Journal of Human Genetics. The second manuscript is titled *"The interaction between*

*Mitochondrial DNA and APOE variability in a large sample of ultranonagenarians across Europe: a GEHA Project Study*" and it is submitted to "Age". In this work we analyze the interaction between the mtDNA variability and variability of nuclear genes such as mitochondrial haplogroups and APOE gene variability. The third manuscript titled "*Two-stage case-control association study of candidate genes and human longevity*", which is submitted to "Age" is focused on the nuclear DNA variability. Exactly, in this work we have conducted a multilocus analysis on hundreds of Single Nucleotide Polymorphisms (SNPs) located in candidate genes to individuate variables associated to human longevity.

## 1.2 MTDNA VARIABILITY AND AGING

### 1.2.1 Mitochondrial theory of aging

Over the years more than 300 aging theories have been postulated, but an universally accepted definition of the process of aging doesn't exist (Vina et al, 2007). A common opinion is to consider aging as an inevitable result of accumulation in an organism of occasional injuries. Among the several theories proposed, the oldest and most accredited is the Harman "Free radical theory" (Harman D, 1956). According to this theory aging is promoted by ROS (Reactive Oxygen Species) that, over the time, lead to cellular damage, including injure to DNA, proteins, and cellular membranes. Although several enzymes (NADPH oxidase family, enzymes of the lipid metabolism in peroxisomes, and several other cytosolic enzymes including cyclooxygenase, ecc) contribute to the ROS production, the 90% of ROS present in the organisms is due to the activity of the mitochondrial respiratory chain (Balaban et al, 2005). In this frame, in 1972 Harman (Harman, 1972) proposed a new version of the "Free radical theory" where mitochondria assume a central role in the aging process. Afterwards, in 1980 Miquel and co-workers proposed the "mitochondrial theory of aging" based on the exclusive role of mitochondria in the aging process (Miquel et al, 1980). However, mitochondria are not only the principal producers of ROS, but they are also the first targets. It is supposed that the mitochondrial accumulation of damage, and in particular the mtDNA impairment, is one of the most important factors in determining age-related cellular decline (Kirkwood, 2005) (Fig.1).

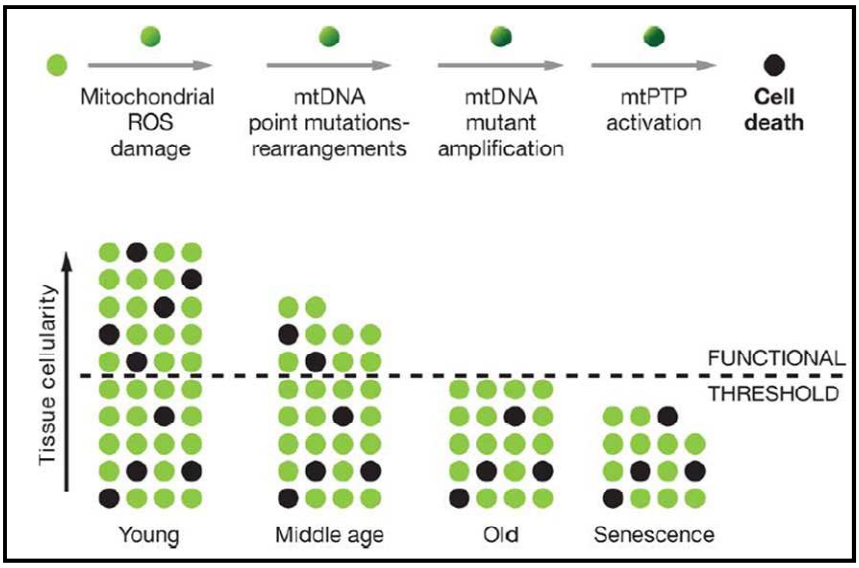


**Figura 1. Mitochondrial role in the energetic life and death of a cell**

The diagram represents the loss of cells in a tissue over the life. The minimum number of cells for the tissue to function normally is indicated by the dashed line (modified from Wallace, 2005).

During the last decades, a number of experimental evidences supported the hypothesis of oxidative damage accumulation as a cause of aging. It was found that levels of oxidated lipids, proteins and DNA increased with age (Van Remmen and Richardson, 2001). This is in line with several other literature data indicating that mitochondria of aged animals produce more ROS than mitochondria of younger one (Sohal et al, 1990), and that the introduction in young rat cells of mitochondria isolated from fibroblasts of aged rats cause premature aging (Corbisier and Remacle, 1990). Another evidence derives from an experimental work in which a reduction in mitochondrial ROS production was observed in animals with a controlled dietetic condition called caloric restriction, characterized by a reduced food intake that cause an increase in lifespan (this will be better explained in the next sections) (Sohal et al, 1994).

However, apart from the central role in ROS production, mitochondria carry out many crucial activities for cell survival and, as a consequence, for aging and longevity (Passarino et al, 2010). Thus, the next session will describe the structural and functional mitochondrial characteristics, with a particular attention to the peculiar features of mtDNA.

### 1.2.2 Mitochondrial structure and function

*Structure*

Structurally, the mitochondrion is a membrane-enclosed organelle ranging from 1–10 micrometers in size (Henze and Martin, 2003). As shown in Fig.2, mitochondria contain both an inner and an outer membrane, leading to the formation of two aqueous compartments, the matrix and the inter-membrane space, where several specialized functions take place. Each compartment, having a specific function, cooperate to carry out all mitochondrial functions.

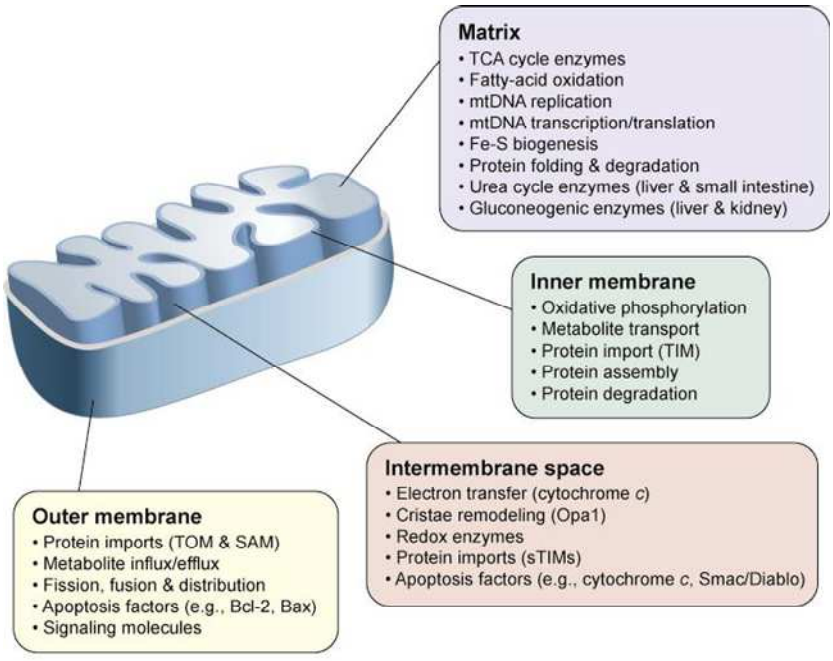


**Figura 2. Mitochondrial structure and functions.**

The compartment-specific processes and proteins are described in the colored boxes. Abbreviations: Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma protein 2; Opa1, Optic atrophy 1; SAM, Sorting and Assembly Machinery; sTIMs, small TIM proteins; TIM, Translocase of the mitochondrial Inner Membrane; TOM, Translocase of the mitochondrial Outer Membrane (modified from Ryan and Hoogenraad , 2007).

The outer membrane allows exchange of metabolites between the cytoplasm and intermembrane space through passive exchange and protein imports (TOM-Transporter Outer Membrane and SAM-Sorting and Assembly Machinery). Furthermore it takesa a central role in apoptosis and in signal transduction (Ryan and Hoogenraad, 2007).

The intermembrane space contains some proteins, principally implicated in electron transfer and apoptosis. Its most prominent member is cytochrome c, which in normal cells is involved both in respiration and in apoptotic induction. In addition, other potential pro-apoptotic factors are present as well as a variety of small proteins that contain cofactors or are disulfide bound (Koehler et al, 2006; Webb et al, 2006).

The inner membrane, delimiting the mitochondrial matrix, is protein rich and it has specific transporters inside, demonstrating a highly controlled permeability. Moreover, proteic complex are located across the inner membrane where ETC (Electron Transport Chain) take place (Arco et al, 2005).

The mitochondrial matrix contains various enzymes involved in metabolic processes: oxidation of fatty acids and tricarboxylic acid cycle, Fe-S biogenesis, and heme synthesis. The matrix also harbors a number of copies of mtDNA and the protein machinery involved in its maintenance and replication as well as components involved in transcription/translation.

Mitochondria are not static organelles: they come under structural modification and turn-over. These complex events, known as mitochondrial dynamics, allow the recruitment of mitochondria to cellular sites where they are needed. To maintain a healthy population of mitochondria, there is in mammalian cells a continuous turn-over: the damaged organelles are eliminated and new mitochondria are generated through processes called mitophagy and biogenesis. The regulation of these complex mechanisms require more than 1000 genes (located in mitochondrial and nuclear genome), and imply the alteration of the transcription of more than 20% of cellular proteins (Lopez-

Lluch et al, 2008). The dynamicity of mitochondria emerge also because the number and the size of these organelles vary in the different tissues: some cells have only a single mitochondrion, whereas others can contain several thousand mitochondria; this is principally due to cellular metabolic demands and health status. Moreover, mitochondria are continuously in movement throughout the cells and undergo fusion and fission modifying the inner membrane surface to readily satisfy energy requirements (Ono et al, 2001; Bereiter-Hahn and Voth, 1994). Finally, it has been clearly documented that in mitochondria ultrastructural change occur in response to environmental stimulation (Bertoni-Freddari et al, 1993, 2007).

*Functions*

Mitochondria play an important role in cell metabolism: they are the organelles where Oxidative Phosphorylation (OXPHOS) takes place, and occupies a key position in apoptosis regulation, heat production, calcium homeostasis, and cellular differentiation. However the two functions principally involved in aging and longevity are the oxidative phosphorilation and the apoptosis.

The main mitochondrial function is the production of energy through Oxidative Phosphorilation (OXPHOS). This metabolic pathway, that through the oxidation of nutrients produces energy stored as adenosine triphosphate (ATP) molecules, counts 80 proteins that form five complexes: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone cytochrome c oxidoreductase (complex III), cytochrome oxidase (complex IV), and, finally, F1Fo-ATP synthase (complex V).

The OXPHOS consists of two steps: 1) a series of redox reactions along four complexes (I–IV) that through the transfer of $H^+$ ions (protons) across the electron transfer chain (ETC) creates an electrochemical gradient ($\Delta\Psi$) and 2) the ATP formation by the F1Fo-ATP synthase (complex V), that uses this electrochemical gradient to generate chemical energy (ATP).

In detail, electrons are donated to complex I from NADH+ or to complex II via succinate, and passed to coenzymeQ (CoQ) to give ubisemiquinone (CoQH) and then ubiquinol (CoQH2). Electrons are then donated to complex III, which transfers the electrons to cytochrome c, and, eventually, to complex IV. Finally, they are moved to 1/2 O2 to give H2O. The energy released along the pathway is used to pump protons across the mitochondrial inner membrane, creating the transmembrane electrochemical gradient. Complex V uses the energy stored in the $\Delta\Psi$ to condense ADP and Pi to make ATP. ATP is then carried to cytosol and exchanged with ADP.

Another important mitochondrial function is the regulation of apoptosis. This is a fundamental cellular mechanism in order to remodel organisms during development or get rid of damaged or unnecessary cells. The apoptotic cell death can be triggered by external signals detected by cell surface membrane proteins, or alternatively by mitochondrial signals. The initiation of the mitochondrial path for apoptosis is mainly regulated by the opening of a non-specific channel that is located on the inner membrane of mitochondria: the mitochondrial permeability transition pore (mtPTP) (Green et al, 1998; Chipuk et al, 2006). The opening of this pore leads to a dramatic collapse of the transmembrane $\Delta\Psi$. This event causes the swelling of the inner membrane, and then the disruption of the outer membrane, with a consequent release of the pro-apoptotic molecules enclosed in the inner membrane space, such as cytochrome c, apoptosis inducing factor (AIF), Smac/Diablo, and several pro-caspases (Hengartner, 2000; Caroppi et al, 2009). In the cytosol, cytochrome c, together with others factors (Apaf-1 and ATP) activates the caspases 9 and 3 that disrupt the cytosol. In succession, AIF moves into the nucleus and disrupts the DNA.

The opening of mtPTP can be stimulated by: a) the mitochondrial uptake of excessive Ca2+, b) an increase of the oxidative stress, c) a decrease of the mitochondrial membrane potential, d) a decrease of the intra-mitochondrial ATP concentration (Wallace DC, 2005). It is intuitively evident that an efficient apoptosis machinery would eliminate unwanted cells.

### 1.2.3 The influence of mitochondrial structure and function on aging and longevity

The importance of mitochondrial functions are underlined by the dynamicity of these organelles that change in size and shape to satisfy cellular needs in the different physiological states. It is obvious that a so crucial mechanism of regulation for cell activities have also an essential role in aging and longevity. One example of this is given by the work of Kissova and colleagues (Kissova et al, 2004), that suggest an efficient mitophagy is essential to attain longevity. In fact, in yeast the deletion of the mitochondrial protein Uth1p was found to lead to a selective defect in mitophagy and to a shortened lifespan in yeast during nutrient deprivation. Moreover, in recent years, studies show that mitochondria turn over may be considerably influenced by the physiological status of tissues and organs. For example physical activity promote mitochondrial biogenesis; on the contrary, physiological aging is characterized by a reduction of mitochondrial biogenesis (Holloszy, 2004), and degradation (Terman et al, 2007), causing a reduction of mitochondrial turnover that, as consequence, lead to the accumulation of oxidized components lipids, proteins and DNA. Also the modification of ultrastructural features is related to aging. In fact, it was observed that in old rats less mitochondria are present than in young rats, but to compensate for the numeric loss of organelles, there is an increase in mitochondrial size (which is presumed to increase the potential area for respiration). Comparing the data from young and old rats, the outcome of these balanced changes is a constancy in the overall volume fraction of cytoplasm (volume density) occupied by mitochondria (Bertoni-Freddari et al. 1993, 2007).

Furthermore, several studies have shown how the state of the mitochondrial network morphology influences a variety of other cellular functions: mitochondrial proliferation (Möpert et al, 2009), propagation of signals such as those of energy deficiency (Chan 2006), apoptotic events (Suen et al, 2008), and cell differentiation (Park et al, 2001). All these cellular functions are involved in both cell survival and death; thus, it appears clear how impairments in mitochondrial dynamics affect

pathological conditions such as neurodegeneration (Chen and Chan 2009), type 2 diabetes (Molina et al, 2009), as well as normal aging (Lòpez-Lluch et al, 2008).

As previously mentioned, mitochondria play important activities essential for cell survival. So, also the regulation of mitochondrial functions results particularly correlated to the phenotypes under discussion.

In the energy production process, if the principal result of OXPHOS is the production of ATP molecules, mitochondrial respiration generates ROS as by-product. In physiological conditions a small percentage of electrons may prematurely reduce oxygen, forming toxic product such as superoxide, and peroxyl radical. If these molecules are present in a small percentage, they are neutralized by the scavenger molecules present in cells, but if they are present in a higher percentage they can cause oxidative stress. Because the diffusion capability of most ROS is limited by their lipid solubility the main harmful effects are exerted on mitochondrial molecules, contributing to the decline in mitochondrial function and, as a consequence, to aging process.

ROS may damage lipids, proteins and nucleic acids.

ROS may act on molecules directly by peroxidation or indirectly through the production of highly reactive aldehydes. In the direct action, ROS acts “stealing” electrons: this causes the production of radical molecules. In turn, these molecules, that are not very stable, react with other molecules causing a "chain reaction mechanism". In the indirect mechanism, of action the production of highly reactive aldehydes play a central role. One example of reactive aldehydes is the 4-hydroxy-2,3-transnonenale (4HNE) aldehyde, one of the main products of lipid peroxidation. It causes a variety of harmful effects on the molecules with which it comes into contact.

The action of ROS on phospholipids of mitochondrial inner and outer membranes cause, principally, the decreases of their fluidity. Since the function of these membrane plays a critical role especially in the regulation of some mitochondrial proteins such as cytochrome oxidase and adenine

nucleotide translocase (ANT) (Paradies et al, 1998), it is clear that membrane's oxidation may have very harmful effect on mitochondrial functions.

The effect of ROS on proteins lead to structural and functional changes (abnormal aggregation, degradation, loss of function, etc.). Damage to mitochondrial proteins, especially those present in the inner mitochondrial membrane, are the direct result of oxidative stress or the consequence of lipid peroxidation. Several enzymes of the electron transport chain, such as ATPase and ANT, are particularly susceptible to oxidative stress inactivation, and the principal consequence observed is a decrease in OXPHOS efficiency (Le Bras et al, 2005).

Because of the proximity with ROS production, and the lack of protective structure, such as histone proteins, the mtDNA is the primary target of ROS. The oxidative damage to mtDNA may be detected through the presence of some indicators; an example is the presence of 8-hydroxy-2-deoxyguanosine which is the most abundant among the products of nucleotides oxidation (Chomyn and Attardi, 2003). The accumulation of mtDNA damage may be dangerous for the cells, modifying the efficiency of mitochondrial function directly controlled by this genome, especially OXPHOS.

The importance of apoptosis in aging is documented by several studies that show as the reduction in number of myocytes (Kajstura et al, 1996), skeletal muscle (Dirks and Leeuwenburgh, 2002) and T-cells (Phelouzat et al, 1997) is the result of apoptotic cell death (Higami and Shimokawa, 2000). It is clear that the modulation of apoptosis results to be a decisive process in determining a balance between healthy versus senescent cells and tissues. In fact, a low level of apoptosis is probably deleterious for tissues and organs, because it leads to the accumulation of damages, but it might be useful for attaining longevity, even though in this cause an accumulation of damaged cells and consequent frailty is expected in oldest-olds (Rose et al, 2002). In line with this assumption is the observation that lymphocytes from very old people and centenarians are quite resistant to apoptosis

when compared with similar cells from young individuals (Salvioli et al, 2001). Moreover, the apoptotic process is doubly correlated with aging, because it is influenced also by ROS production (Kroemer and Reed, 2000): ROS damaging mitochondrial structure increases mitochondrial Ca2+ and consequently activates apoptosis (Lemasters et al, 2009).

### 1.2.4 Mitochondrial genome and aging

#### 1.2.4.1 Genome structure

During the last decades, along with the importance of mitochondrial function, also the relevance of the mitochondrial genome is progressively emerging. Mitochondria are the only cellular organelles, in addition to nucleus and chloroplasts of plant cells, to possess their own genome. It is located in the matrix and might be present in one or more copies per mitochondrion.

Mitochondrial DNA is a circular double-strand genome 16,569 bp long (Anderson et al, 1981), composed of two strands, a heavy strand (H), rich in G and a lightweight strand (L) rich in C. It has a compact structure, where introns are not present, the intergenic sequences are few in number and short, and overlapping genes are present. It has been tightly conserved for more than half a billion years, coding in every multicellular animal so far investigated for 37 genes. 2 genes code for ribosomal RNA (rRNA), 22 for transport RNA (tRNA), and the remaining 13 genes code for all structural subunits of oxidative phosphorylation enzyme complexes. They include 7 out 46 of the complex I polypeptides (NADH dehydrogenase), ND1-6 and NDL 4, cytochrome b, which is one of the 11 proteins of complex III, 3 (COI-III) out 13 proteins of complex IV (cytochrome oxidase) and 2 (ATP 6 and 8) out the 16 proteins of complex V (ATP synthase) (Fig. 3).

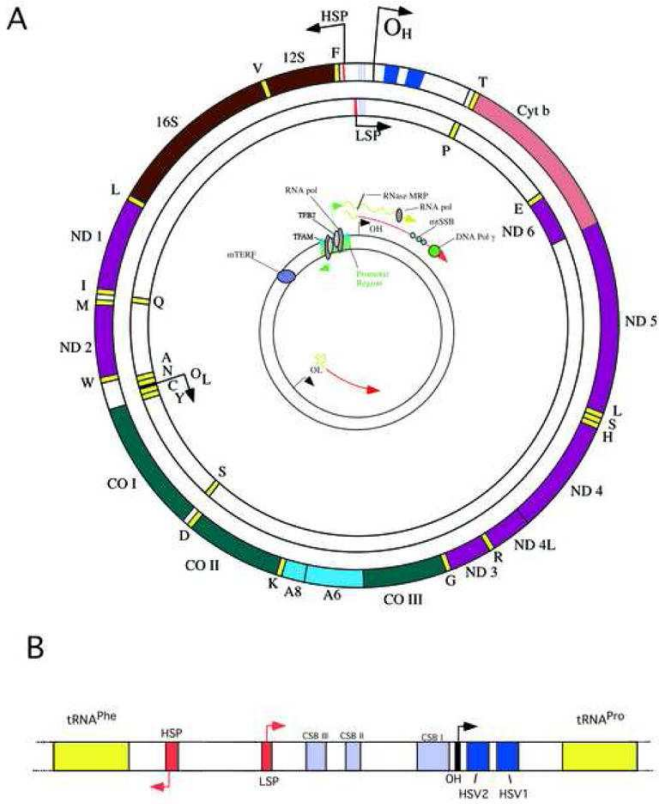


**Figure 3. Structure and expression of the human mitochondrial DNA.**The panel A represent the mitochondrial genome showing 13 protein coding genes as well as 2 rRNA- and 22 tRNA-coding genes. Genes coding for subunits of different complex are shown by different colors. The origins of replication for the H- and L- (OH and OL) strands are also shown. It also shows the binding sites for the mitochondrial RNA polymerase, the mitochondrial transcription factor TFAM, the RNA processing enzyme RNAse MRP and the transcription termination factor mTERF. In panel B the structure of the regulatory D-loop region is shown two hypervariable regions (HSV1 and HSV2) commonly used for evolutionary studies. Modified by Diaz and Moraesa, 2008.

The "displacement-loop" (D-loop), also defined as the mitochondrial regulatory region, is a non-coding sequence which regulates mtDNA's replication and transcription. It contains the promoters of the H and L strands (PH and PL), four binding sites for mitochondrial transcription factor A (mtTFA), three blocks of conserved sequences (CSB I, II, III), the origin of H strand replication (OH) and the termination associated sequences (TAS).

The mtDNA transcription and replication take place inside the organelle (Kasamatsu et al, 1973; Montoya et al, 1983; Clayton, 1987) although many proteins involved in these processes are encoded from nuclear DNA (nDNA). Among others the subunits of mitochondrial DNA polymerase γ (POLG), mitochondrial RNA polymerase components, mitochondrial transcription

factor (mtTFA), mitochondrial ribosomal proteins, elongation factors and metabolic enzymes are coded by nDNA.

#### 1.2.4.2 Mitochondrial genetics

Mitochondrial DNA shows a series of peculiar characteristics that differ from nuclear genome.

One of these characteristics is the relatively high mutation-fixation rate with respect to nuclear genome (Brown et al, 1979; Torroni et al, 2006; Wallace, 2007). Among the reasons that explain the presence of these peculiar feature are included: mtDNA limited repair ability, loss of histones and physical association with the mitochondrial inner membrane where damaging reactive oxygen species are generated (Pinz and Bogenhagen, 1998).

A second characteristic of mtDNA is its mode of inheritance. It is transmitted only maternally through the oocyte cytoplasm (asexual reproduction). In fact, the few mitochondria from the sperm cell that could enter the oocyte during fertilization are actively eliminated by an ubiquitin-dependent mechanism (Sutovsky et al, 2000). Through this way of transmission mtDNA escapes recombination and it is transmitted unaltered from mothers to children.

Finally, the cells are polyploid with respect to mtDNA: most mammalian cells contain hundreds of mitochondria and, in turn, each mitochondrion contains several (2–10) copies of mtDNA (Wiesner et al, 1992). If all mtDNA copies are identical, we have a condition known as homoplasmy, but if mtDNA copies are not identical, we have a condition known as heteroplasmy. At cell division mitochondria and their genomes are randomly distributed to daughter cells and hence, starting from a given heteroplasmic situation, different levels of heteroplasmy up to homoplasmy can arise in different cell lineages.

The uniparental mode of inheritance and the elevated mutation rate, have led to the presence in human populations of mtDNA lineages evolved independently from each other by sequential accumulation of mutations. Consequently, mutations which occurred ten thousands of years ago are nowadays present in high frequency, and are population- and continent-specific. This individual haplotypes may be grouped through phylogenetic analysis in groups, called haplogroups, sharing a specific set of mutation (Torroni and Wallace, 1994; Wallace, 1994; Passarino et al, 1998; Richards et al, 2000, 1996; Torroni et al, 2006, 1996; Kivisild et al, 2006; Underhill and Kivisild 2007).

The classification of mtDNA haplogroups was based on information obtained from RFLP analysis of the coding region and from the nucleotide sequence of the control region (Torroni et al, 1996). Today, thanks to the deep knowledge of mtDNA sequence, it is ever more frequent the identification of haplogroups through the analysis of complete sequence or of the d-loop sequence.

The first mtDNA haplogroups, discovered in Native Americans, were named A, B, C, and D (Torroni et al, 1993). Subsequently, detected haplogroups were designated using other letters of the alphabet, and subcluster with a running number (Ballinger et al, 1992; Torroni et al, 1996). By now, all letters of the alphabet, except O (although once proposed), have been used. The main identified haplogroups are divided between the three major ethnic groups: Africans ($L_1$, $L_2$, $L_3$), Asians (C, D, G, E, A, B, F,) and Europeans (H, U, T, I, J, K, V, W, X) (Torroni et al. 1994, 1996) (Fig 4), that cover almost the totality of human population.

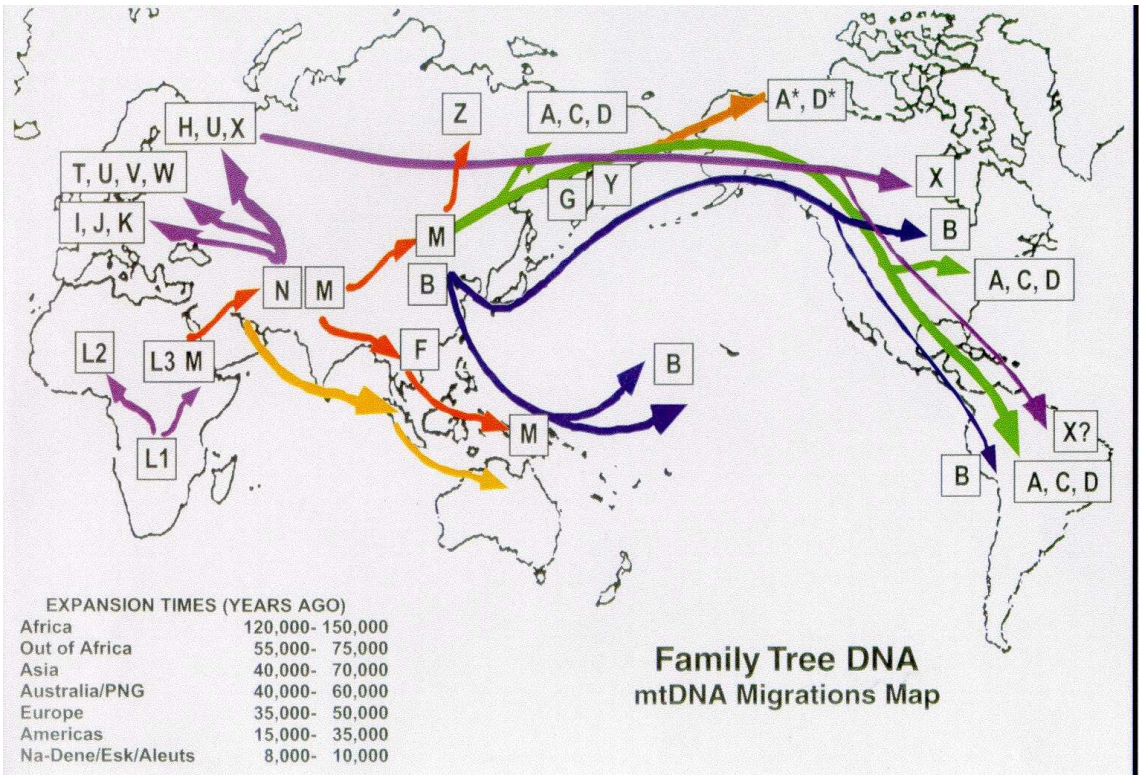


**Figure 4 Mitochondrial haplogroups distribution around the world.**
In this image are indicated the principal mitochondrial haplogroups localization. The arrows indicate the direction of homo sapiens migrations. Modified from Bryan Syke's book: The Seven Daughters of Eve.

For example, the nine Europe haplogroups cover more than 95% of mtDNA of all subjects (Wallace 2007; Torroni et al. 2006, 1996). The philogenetics of haplogroups is complex and multiple subclade exist. For example Haplogroup U comprises phylogenetically different subhaplogroups such as U1, U2, U3, U4, and U5, the oldest subclade, U6, U7, U8, U9, and K (Achilli et al, 2005). Also the prevalent European haplogroup H, that cover 30%-50% of the population, comprises numerous sub-haplogroups (H1- H21) that have very different spatial frequency patterns in European regions (Achilli et al, 2004). Among the sub-haplogroups, H1 and H2 have been identified in a sample of Finns (Finnilä et al, 2001). Based on mtDNA complete sequences, two further sub-haplogroups were described by Herrnstadt and coworkers (Herrnstadt et al, 2002): H3, the next most common sub-haplogroup after H1, and the rare H4. Moreover, Quintàns and colleagues (Quintàns et al, 2004) further identified H5, H6, and H7. Additional sub-haplogroups (H16-21) have been recently proposed (Roostalu et al, 2006).

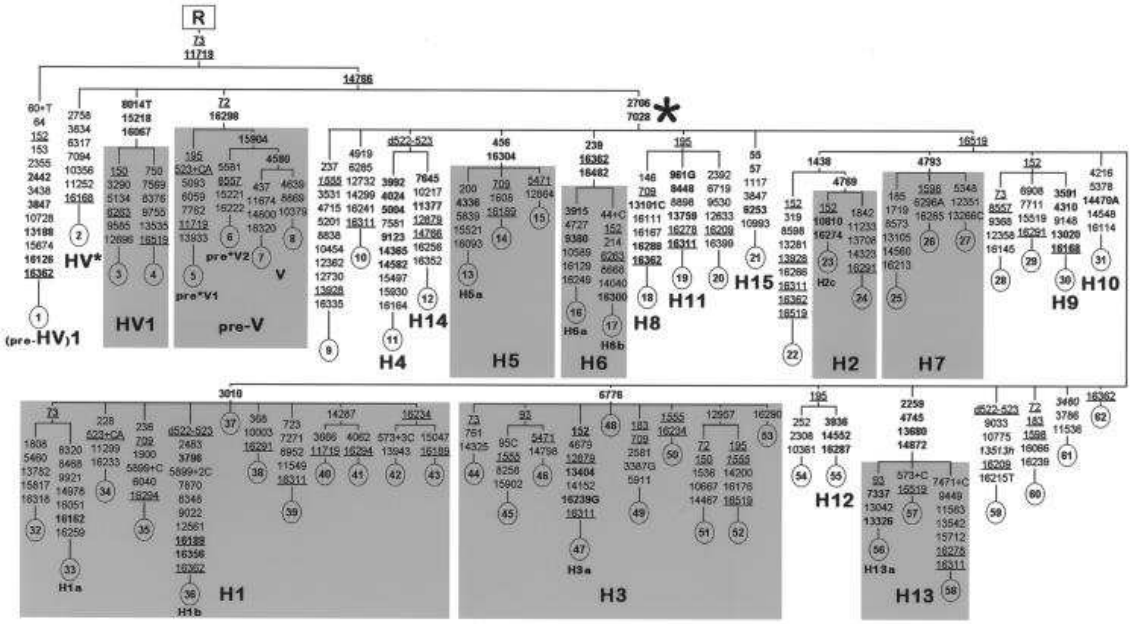


**Figura 5** It encompasses 62 entire myna sequences, all mutations relative to the root of R, and 15 sub-haplogrouos (H1-15) identified by Achilli et al, 2004.

Studies about mtDNA variability in the past decades were considered useful principally for the reconstruction of human population history. That is possible because each lineage shares the most ancient mutations, and distinguish itself from others for the presence of more recent ones, probably risen during the last glaciations, when the groups of humans were isolated from each other. The analysis of the phylogenetic relationships among haplogroups allowed some interesting inferences about the origin of human beings: *Homo sapiens* appeared in Africa about 120.000-150.000 years ago, and only 55.000-75.000 years ago he migrated first in middle east, and then in the other country regions (Figure 4) (Cavalli Sforza et al, 1994; Torroni et al, 1996; Quintana-Murci et al. 1999; Macaulay et al 1999; Richards et al, 2000; Underhill et al, 2001; Kivisild et al. 2006; Olivieri et al. 2006).

In the last two decades the interest of medical scientists on mtDNA is greatly increased (for a review see Wallace and Fan 2009; Dimauro 2011) as result of the discovery of various mitochondrial mutations leading to degenerative disorders that mainly affect the nervous and muscular systems (Wallace et al. 1988 a, b; Zeviani et al. 1988; Schon et al. 1994). Over the years

other mitochondrial diseases have been identified, such as mitochondrial encephalomyopathy, lactic acidosis (a condition however that can also be due to pharmacological treatment of infectious diseases), myoclonic epilepsy, raggedred fibers disease (MERRF), stroke-like symptoms (MELAS), Leber's hereditary optic neuropathy (LHON) (Vilkki et al, 1989; Lestienne and Bataillé 1994; Chinnery et al, 2000; for a review see Wallace 2005; Dimauro 2010).

Successively, studies have shown that also human haplogroups are qualitatively different from each other, due to their defining mutations. For instance, two European haplogroups, H and T which displayed a significant difference in the activity of complexes I and IV of OXPHOS (Ruiz Pesini et al, 2000), leading to a worse motility of sperms for haplogroup T carriers. From this evidence of different haplogroup energetic efficiency, recently confirmed by in vitro studies (Gómez-Durán et, 2010), has grown the belief that haplogroups can play an important role in predisposing to disorders. Some examples of mtDNA haplogroups associated with particular diseases are: Wolfram syndrome, also known as DIDMOAD syndrome (diabetes insipidus, diabetes mellitus, optic atrophy and deafness), as well as to LHON (Hofmann et al. 1997; Torroni et al. 1997; Barrett et al. 2000; Hudson et al. 2007).

Apart from the correlation with human disease, the polymorphic variations of mtDNA are involved in determining the inter-individual susceptibility to a number of complex traits, either pathological (such as ophtalmolog- ical disorders, cardiovascular diseases, cancer, dementias) or physiological such as aging (Wallace 2001; Rose et al, 2002; Santoro et al, 2006; Zeviani and Carelli 2007).

#### 1.2.4.3 mtDNA variability, aging and age-related diseases

With aging mitochondria progressively lose their functionality. In fact, in aged tissues, a decrease in the number of mitochondrial DNA (mtDNA) copies is observed (Barazzoni et al, 2000) and an

accumulation of mtDNA large deletions and point mutations (Cortopassi et al, 1992, Michikawa et al, 1999). These damages mainly originate from the impairment of respiratory function causing a reduction in ATP production and an increase in ROS production. Today it is well established that the enhanced production of ROS, accompanied by a decreased activity of free radical-scavenging enzymes, is the principal cause of the age-associated decline (Di Mauro et al, 2002). As previously mentioned, ROS attack organelle constituents by oxidizing them, and mtDNA is one of the principal targets. ROS have a mutagenic effect on DNA and the accumulation of mutations lead to the production of less efficient OXPHOS subunits (Drouet et al, 1999; Fannin et al, 1999; Terzioglu and Larsson 2007), exacerbating the production of ROS that, in turn, aggravates the decay of the organelle. This "vicious cycle" is at the basis of the aging process. Many studies on animal models have demonstrated how ROS are responsible for mitochondrial decay of aged tissues: in vitro studies have shown that an increased ROS production, induced pharmacologically, amplify mtDNA impairment in fibroblasts (Esposito et al, 1999). Moreover, it is also observed that the administration of antioxidant compounds (acetyl cysteine, GSH, vitamin C) is able to attenuate the age-related mtDNA damage (Melov et al, 2000; Figueiredo et al, 2008, 2009). Other studies have shown as the accumulation of mtDNA somatic mutations results to be correlated with aging phenotypes: high levels of both point mutations and large-scale deletions of mtDNA induce in animal models many features of premature aging. This is the case of mice expressing a proofreading-deficient version of the catalytic subunit of mtDNA polymerase (PolgA), and of the so-called ''mutator mice'' (Trifunovic et al, 2004; Kujoth et al, 2005; Edgar et al, 2009). Some studies have also highlighted the correlation between the presence of mtDNA mutations and an increase in ROS production: for example mice with an increased lifespan show a decreased damage to mtDNA and increased mitochondrial resistance to ROS damage (Schriner et al, 2005). But this connection is still debated because in other studies high mutation rate in mtDNA was not associated with increased oxidative damage to macromolecules (Trifunovic et al, 2005).

Human studies have been focused on mutations that define mitochondrial haplogroups, mutations well characterized and, for this reason, more simple to study.

In one of the first association studies, the Asian haplogroup D, which is characterized by mutations present in protein subunits belonging to OXPHOS complex I, is overrepresented in Japanese centenarians (Tanaka et al, 1998, 2000). In line with this finding, it was observed that in northern Italians haplogroup J, is by far more frequent among centenarians than among younger controls (De Benedictis et al, 1999), suggesting a haplogroup-specific effect on rate and quality of aging. Further support for this results has been confirmed by additional studies in northern Irish (Ross et al, 2001), and in Finns (Niemi et al, 2003),but not in southern Italians (Dato et al, 2004). Previously, a study conducted by Torroni e colleagues (Torroni et al, 1997), revealed that the mutations causing LHON were much more likely to cause the disease if they occurred on molecules belonging to haplogroup J, presumptively because of the genetic background of the J haplogroup. In fact, it is characterized by missense mutations falling in ND1 and ND5 subunits of the OXPHOS complex I (4216C, 13708A), by the 5633T-7476T-15812A haplotype that has been related to a predisposition to the Alzheimer's disease, and by the 3010A mutation falling in a very conserved region of the rRNA gene 16S, and previously associated to many complex diseases (Rose et al, 2002).

From this data it seems that the same group of mutations can induce longevity or diseases. It seems that Haplogroup J, because of the presence of missense mutations in complex I genes, have a low efficiency of OXPHOS, putting the cell in a vulnerable situation. From a hand a low production of ATP, may be detrimental for the cell, especially in presence of a certain environmental or genetic condition, like the presence of a further mutation. It is the example of LHON mutations occurring in molecules belonging to haplogroup J. On the other hand a low OXPHOS efficiency, often associated with a higher production of ROS, may be beneficial for the cell if associated with other environmental or genetic condition. For example, when the increased ROS production induce a tissue-specific over expression of nuclear genes coding for detoxifying enzymes (Esposito et al,

1999), the cell may benefit from a lower presence of ROS, that may result in a more healthy aging (Rose et al, 2001).

The primary role occupied by mitochondria in energy production and calorie uptake is also at the basis of several age-related diseases, in which was observed mt DNA impairment (mtDNA somatic alterations and rearrangements) (Wallace 2001, 2005). But also in these cases the low efficiency of OXPHOS and the consequent production of ROS may be modulated by other environmental conditions like physical activity. For example, the availability of calories and the absence of muscular exercise, largely diffused in human western societies, provoke a reduction of ATP production, and an increase of heat and ROS production (Wallace 2005; Hepple 2009). The accumulation of the problems associated with this ‘‘well being’’ combination, together with mtDNA mutations, either inherited or acquired with aging, has important effects on the onset of many age-related diseases as Type II Diabetes (Wilson et al, 2004), cardiac and coronary diseases (Das et al, 1989; Corral-Debrinski et al, 1992), neurodegenerative diseases (Alzheimer Disease, Parkinson Disease, etc.) (Zeviani and Carelli 2007), and cancer (Wallace 2005).

All these intricate events shed light on the complexity of the aging process, in which the effect of mtDNA inter-individual variability on mitochondrial-related phenomena may be attained either directly, or by interaction with the nuclear genome, and it may be modulated by environmental circumstances (Rose et al, 2002).

#### 1.2.4.4 Mitochondria-nucleus cross talk and aging

mtDNA inherited variability doesn’t operate on human phenotypes independently of nuclear variability. One of the first evidence is the observation that when OXPHOS is temporarily reduced and levels of ROS are increased, a higher transcription of nuclear genes coding for detoxifying

enzymes occurs in order to get rid of ROS (Esposito et al, 1999). Today it is well known that mtDNA works in strict connection with the nuclear genome, and all the processes involving the mitochondrion, are regulated both by proteins coded in nuclear and mitochondrial genomes. Many studies, dealing with the so called nucleus–mitochondria cross talk, show that a correct communication between mtDNA and nDNA is an essential process in cell biology (Garesse et al, 2001).

One method to better discriminate how the mitochondrial and cellular functionality are due to mitochondrial or nuclear variation is the cybrid technology, engineered cells where the mitochondria are completely deplete of their DNA and new mitochondria are introduced into the cells by using platelets obtained from blood donors (King and Attardi, 1989). The presence of cells with the same nuclear genome and different mtDNA, is a good chance to understand how the polymorphic variation of mtDNA affects mitochondria and cells health.

In the last years the idea that the combined effect of nuclear and mitochondrial DNA are often stronger than mtDNA main effects became stronger, leading the scientist to talk of nuclear–mitochondrial epistatic effects (Tranah, 2011). Some evidences exist about the importance of nuclear-mitochondrial cross talk on complex traits like aging and age-related diseases in animal models (Rand et al, 2006) like in humans (Rose et al, 2002), and their epistastic effect may explain why some mtDNA mutations have very different phenotypic effects in different individuals.

One of first studies about the interaction between the two genomes, was conducted by De Benedictis and collegues (2000) about a possible interaction between the mtDNA inherited variants and a polymorphic site of tyrosine hydroxylase (THO) gene, implicated in stress-response. The polymorphism under study is a microsatellite, that has a role in the regulation of transcription the THO, as demonstrated by in vitro studies (Meloni et al, 1998). This study indicated that U haplogroup was over-represented in centenarians carrying the THO genotype unfavorable to longevity (homozygosity for long alleles of the HUMTH01-STR) (De Benedictis et al, 1998).

An in vitro experiment that confirm the importance of the nuclear mitochondrial cross talk in stress-response was carried out by Bellizzi and collegues (2006) on expression levels of cytokines and cytokine receptors in cybrids cells. In fact the transcription patterns of some are specifically modulated by the variability of the mtDNA under stress conditions (interleukin-6) and also at basal conditions (interleukin-1 β and tumor necrosis factor receptor 2).

Another support to the influence of nucleus–mitochondria cross talk on aging and age-related diseases are given by the results obtained by studying a large group of patients affected by sporadic Alzheimer's disease. Carrieri and colleagues (Carrieri et al, 2001), in a case-control study, have demonstrated, in the Italian population, some mtDNA haplogroups, K and U, seem to neutralize the deleterious effect of the ε4 allele, a variant of APOE, a nuclear stress-responder gene. Unfortunately, other studies conducted in different populations (Tuscany: Mancuso et al, 2007; Eastern European population: Maruszak et al, 2009), haven't confirmed the idea that the mitochondrial mtDNA variability and the APOE gene alleles interact in the modulation of some complex traits such as the Alzheimer Disease (AD), suggesting that this interaction may be population specific.

Other evidences are given by studies on different complex phenotype like: maternally inherited deafness, LHON. For the development of the maternally inherited deafness associated with the A1555G mutation in the mitochondrial 12S ribosomal RNA (rRNA) gene additional environmental or genetic changes are required, as identified in aminoglycosides or nuclear modifier genes (Bykhovskaya et al., 2000; 2004a; 2004b; Bindu et al, 2008). The LHON is caused by missense mutations in mtDNA, but a recent study has shown that also a nuclear LHON susceptibility locus on chromosome Xq25–27.2 exist (Shankar et al, 2008).

Lastly, another case of interaction between nuclear and mitochondrial genomes is shown by somatic mutations, non-randomly distributed along the mitochondrial genome and with different rate of accumulation in different tissues. These mutations seem to vary according to the inherited

mitochondrial genome (Gadaleta et al, 1999; Wang et al, 2001; Tanaka et al, 2000), but are also influenced by nuclear genome (Rose et al, 2010, 2007; Attardi, 2002).

In conclusion, it is possible to contend that a complex interplay among mtDNA inherited variation, nDNA inherited variation, and stochastic accumulation of DNA damages probably affects rate and quality of aging.

## 1.3 VARIABILITY OF NUCLEAR DNA AND AGING

Aging is driven by diverse molecular pathways and biochemical events; in fact, studies have shown that during the aging process the intensity of the main cell signaling pathways change dramatically, especially in presence of age-related diseases (Carlson and al, 2008). In this context, it is clear that aging is not controlled by few individual genes, but rather by many genes belonging to key signaling pathways, that many studies have demonstrated to be well-conserved in different species from yeast to humans (Kim, 2007).

Thus, the study of the major cell signaling pathways and their specific mechanisms of transduction have occupied a central role for the detection of genetic factors involved in aging and longevity. In humans, studies aimed to genetic dissection of complex traits (and of longevity in particular) are very difficult to conduct. Linkage analysis is the traditional means of genetic mapping in humans. But in the case of longevity studies it is often difficult to use because of the scarce availability of multi-generational DNA from long-lived individuals. The best model for the study of human longevity are centenarians, who have avoided or survived the most important pathologies that affect old people, causing morbidity and mortality. However, despite the increasing number of old people, centenarians are still few (Franceschi and Bonafè, 2003). The most commonly used studies for the individuation of genetic factor of longevity are linkage analysis, case controls studies and longitudinal studies (Cristensen et al, 2006; Wheeler and Kim, 2011).

The candidate-gene association studies have the advantage to detect also the variants with small effects by comparing the genotypes of centenarians at specific loci with those of younger cohorts. Some limits of this kind of studies are that a biological knowledge of the phenomenon under study is required, that it may suffer for population stratification, and that it is often difficult to define an appropriate control group.

In longitudinal studies a cohort of individuals is followed over time, avoiding in this way the problems about the selection of controls. However, although this methodological approach provides a powerful opportunity to study the determinants of survival in advanced age, for longevity studies exist some logistic difficulties, first of all the necessity to recruit thousands of people to conduct a study on 200 centenarians.

All these studies have given more consistent results, but the only two genes associated with human longevity that have been replicated in multiple populations are FOXO3A and APOE (Corder et al, 1993; Kervinen et al, 1994; Schachter et al, 1994; Willcox et al, 2008; Anselmi et al, 2009; Flachsbart et al, 2009; Li et al, 2009; Pawlikowska et al, 2009), suggesting a population specific effect.

More useful results have given the studies on animal models, where it is possible the identification of molecular mechanisms that regulate a healthy lifespan through in vivo experiment. In fact, the possibility to induce mutation in various genes belonging to integrated molecular pathways has given the chance to characterize genes that dramatically increase or reduce life span. In particular, mutations in genes affecting endocrine signaling, stress response, metabolism, or telomeres, have been reported to increase lifespans of several model organisms (Kenyon, 2005; Fontana et al, 2010).

According to the data obtained from humans and animal models, several biological genes and related pathways have been identified as being involved in affecting lifespan, although the underlying mechanisms involved in the aging process are not completely understood. The intent of the following paragraph is to summarize the main pathways and biological mechanism associated with aging and longevity.

### 1.3.1 INS/IGF1 pathways

Experimental evidence is accumulating that aging is hormonally regulated by the evolutionarily conserved insulin/IGF-1 signalling (IIS) pathway (Kenyon, 2005; Bartke, 2008).

This IIS pathways is the first discovered to affect aging and longevity and it is also the most prominent and thus far best studied (Kenyon, 2005; Cohen and Dillin, 2008). It involves a cascade of phosphorylation events that include phosphatidylinositol 3-kinase (PI3K)/AKT/pyruvate dehydrogenase kinase (PDK) which regulates the nuclear translocation and activity of FOXO (a forkhead transcription factor) protein (Fig. 6) (Christensen et al, 2006).

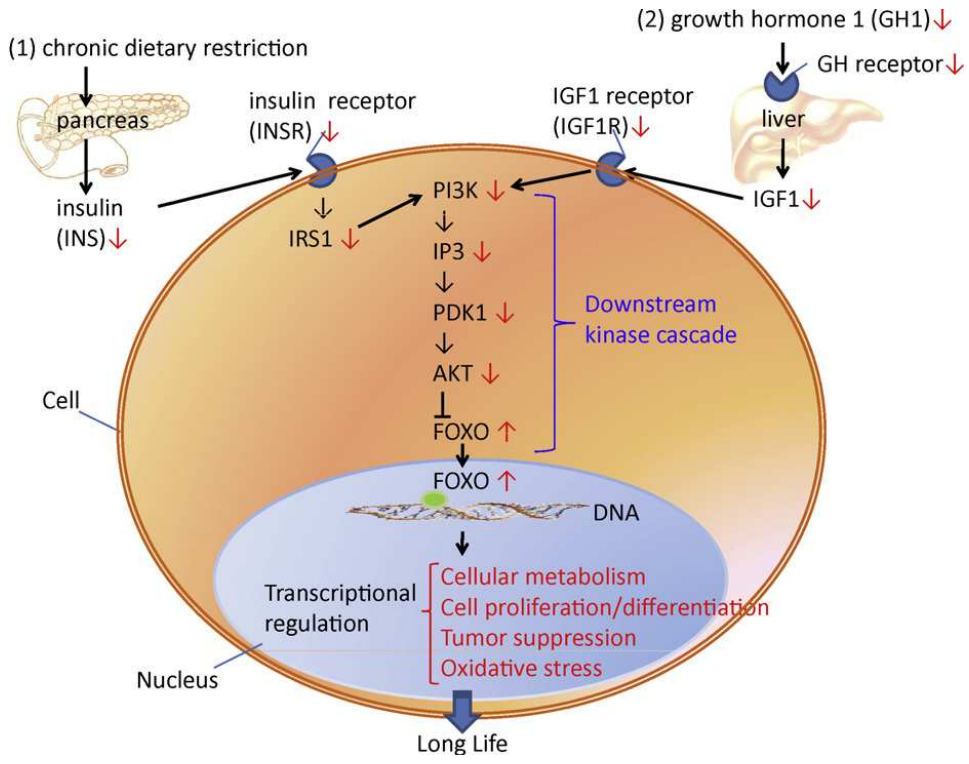


**Figura 6.** The Insulin/IGF1 pathway involves a cascade of phosphorylation events that ultimately regulate the translocation and activity of FOXO proteins, leading to a change in lifespan; the activation of this pathway may be induced by exogenous stimulation (e.g., diet) or growth hormone, that induce the secretion of insulin into the plasma, and the production of IGF-1. Modified from Chung et al 2010.

In particular mutations that cause a decrease in IIS downstream cascade activity were found to extend lifespan (Cohen and Dillin, 2008). Other transcription factors that are inhibitors of IIS signaling, able to extend life-span are HSF-1 (the heat-shock transcription factor) and SKN-1 (a Nrf-like xenobiotic- response factor) (Lin et al, 1997; Brunet et al, 2004; Tullet et al, 2008).

Mutations in IIS components affect lifespan in all model organisms studied (Kuningas et al, 2008).

In *C. elegans*, one of the first genes identified was Daf-2 which encodes for the insulin receptor-like gene involved in insulin signaling; mutations in this gene cause a significant increase in lifespan (Kimura et al, 1997). Following this finding, also mutations in AGE-1 gene, homologous to the mammalian phosphatidylinositol-3-OH kinase catalytic subunits, which are located downstream of the IR and IGF-1R, cause an increase in lifespan (Morris et al, 1996). Moreover, expression studies have shown how the inhibition of IIS cause changes in gene expression of several transcription factors such as DAF-16 (a FOXO transcription factor), the heat-shock transcription factor HSF-1, and SKN-1 (a Nrf-like xenobiotic-response factor) (Tullet et al, 2008) that, as previously mentioned, controls IIS pathways.

For *D. melanogaster* the increase in lifespan was observed in presence of mutations in insulin-like receptor (InR) or in its substrate (chico), and in flies with ablated insulin-producing cells (Giannakou & Partridge, 2007). It has been also shown that the inhibition of insulin/IGF-1 signalling or the increase of FOXO (the orthologue of DAF-16 in *Drosophila*) activity have as a consequence a lifespan increase.

Mammals are more complex: they present separate receptors for insulin (IR) and IGF-1 (IGF-1R) (Navarro et al., 1999); however, all the experimental evidence to date collected in mouse models shows that reduced IIS can extend lifespan also in mammals. In the mouse model, the complete disruption of the IR gene causes many pathological phenotype (insulin resistance, diabetes, etc.), but doesn't influence lifespan extension (Okamoto and Accili, 2003). Also tissue-specific IR knockout mouse models doesn't show beneficial effect for lifespan except that for fat-specific IR knockout mice (FIRKO) which in addiction shows reduced fat mass (Okamoto and Accili, 2003).

The IGF-1 branch acts through the growth-hormone-releasing hormone, growth hormone (GH) and IGF-1; mice mutated for the IGF-1 receptor suggest a direct role for reduced IGF-1 signalling in mammalian longevity: in fact, Igf1r +/– females exhibit a long-lived phenotype (Holzenberger et al, 2003). Moreover, a deficiency in GH and the disruption of GH receptor, causes reduced fertility but extends lifespan (Brown-Borg et al, 1996; Bartke et al, 2001). In mice, another gene able to modulate aging and life span is the *Klotho* gene, which encodes a hormone known to inhibit IIS; in fact, mice deficient for this hormone show an acceleration of aging and age-ralated diseases (Kuro-o et al, 1997), on the contrary over-expression of *Klotho* results in IIS inhibition and increased lifespan (Kurosu et al, 2005).

In humans, the data collected by case/control association studies, reveal a modulation of human lifespan, but not in a magnitude that would come close to what is seen by analogous defects in some of the model organisms.

The influence of IIS on longevity is highlighted by the evidence that longlived subjects, such as centenarians, have decreased plasma IGF-1 levels and preserved insulin action (Paolisso et al, 1997). This result was confirmed in a number of genetic association studies; for example polymorphisms in the IGF-1R locus, able to lower plasma IGF-1 levels, are significantly more represented among Italian centenarians (Bonafe et al, 2003). Moreover, in Dutch population a polymorphism in the GH1 gene is associated with longevity, and a combined effect of variation at the GH1, IGF-1 and IRS1 loci is also suggested as associated with reduced IIS signalling on human longevity (van Heemst et al, 2005). Genetic association studies have revealed other second messengers of IIS associated with human longevity: INSR, AKT, FOXO1A and FOXO3A (Chung et al, 2010).

### 1.3.2 Caloric restriction, and longevity

Caloric restriction (CR) is usually defined as a moderate (normally 20–40%) reduction in caloric intake compared with an ad libitum diet, without malnutrition (Piper and Bartke, 2008). The initial

evidence of the influence of this dietary regime on longevity was observed in rats (McCay et al, 1989). Subsequently similar evidences that CR increases maximum lifespan up to 50% has been reported for yeast, rotifers, spiders, worms, flies, fish, mice and rats (Koubova and Guarente, 2003). In humans, very preliminary evidence based on surrogate measures show how CR exerts similar adaptive responses as in laboratory animals, reducing the risk of developing age-associated pathological complications (Holloszy and Fontana, 2007). Then is no doubt that CR modulates longevity and that, from an evolutionary point of view, it represents an adaptation to food scarcity exempt from doubt (Harrison et al, 1989; Holliday, 1989); but the mechanisms that underliethis phenomenon is not completely understood. The class of proteins primarly correlated to CR are sirtuins.

The Sirtuins represent an evolutionarily conserved family of silent information regulator 2 (Sir2) gene, and are NAD+-dependent protein deacetylases, that if overexpressed extend lifespan in yeast, worms and flies (Kenyon, 2005); moreover genetic studies have suggested that at least one member of the SIRT family is involved in human lifespan regulation (Rose et al, 2003; Bellizzi et al, 2005). These proteins could contribute to longevity influencing the activity of various transcription factors and co-regulators (Bordone and Guarente, 2005). For this reason Sirtuins were heralded as the "master controllers of a regulatory system for aging" because not only do they modify hormonal networks (e.g. IIS pathways), inflammation (e.g. inhibition of NFKB) and other genes associated with longevity (e.g. p53, FOXO), but they may also provide a link between diet, longevity and epigenetic regulation (Sinclair and Guarente, 2006; Martin et al, 2007).

Many experimental evidence on animal models report the implication of this class of proteins in CR.

In yeast Sir2 gene seems to be essential to mediate lifespan extension in CR: in fact, where the gene coding for Sir2 was deleted the reduction in glucose was unable to increase lifespan (Lin et al, 2000). Also in *D. melanogaster* CR efficiently extends lifespan and increases Sir2 mRNA (Clancy et al, 2001; Rogina et al, 2002). In *C. elegans* is Sir-2.1, one of the four Sir genes, seems to regulate

lifespan under a restricted feeding regime (Wood et al, 2004). In mammals, there are seven Sir2 homologues (SIRT1-7), of which SIRT1 is the most closely related to Sir2 (Frye, 2000). SIRT1, that has been associated with glucose and fat metabolism, stress resistance and cell survival (Haigis & Guarente, 2006), is related to CR. In fact SIRT1 protein levels are increased in response to CR in many key metabolic tissues (Chen et al, 2008), and mice lacking SIRT1 are metabolically inefficient and, importantly, the longevity response to CR is blunted (Boily et al, 2008).

Although many studies demonstrated that Sir2/SIRT1 is necessary for CR, not all the studies point in that direction. The scenery seems more complex: some experiments suggest that the beneficial effect of CR might be a consequence of the balance of more signaling networks rather than being defined by single elements. For example, CR might not only be sensed by SIRT1 as a change in the NAD+/NADH ratio but also by AMPK as a change in the AMP/ATP ratio. In turn, AMPK can regulate mitochondrial respiration, which can positively regulate SIRT1. Both AMPK and SIRT1 can impact the activity of FOXO transcription factors, which also have been extensively linked to the regulation of metabolism and longevity. Additionally, CR promotes the downregulation of insulin-derived signals, a candidate longevity pathway, which also interacts with FOXO transcription factors (Cantò and Auwerx, 2009) (Figure 7).

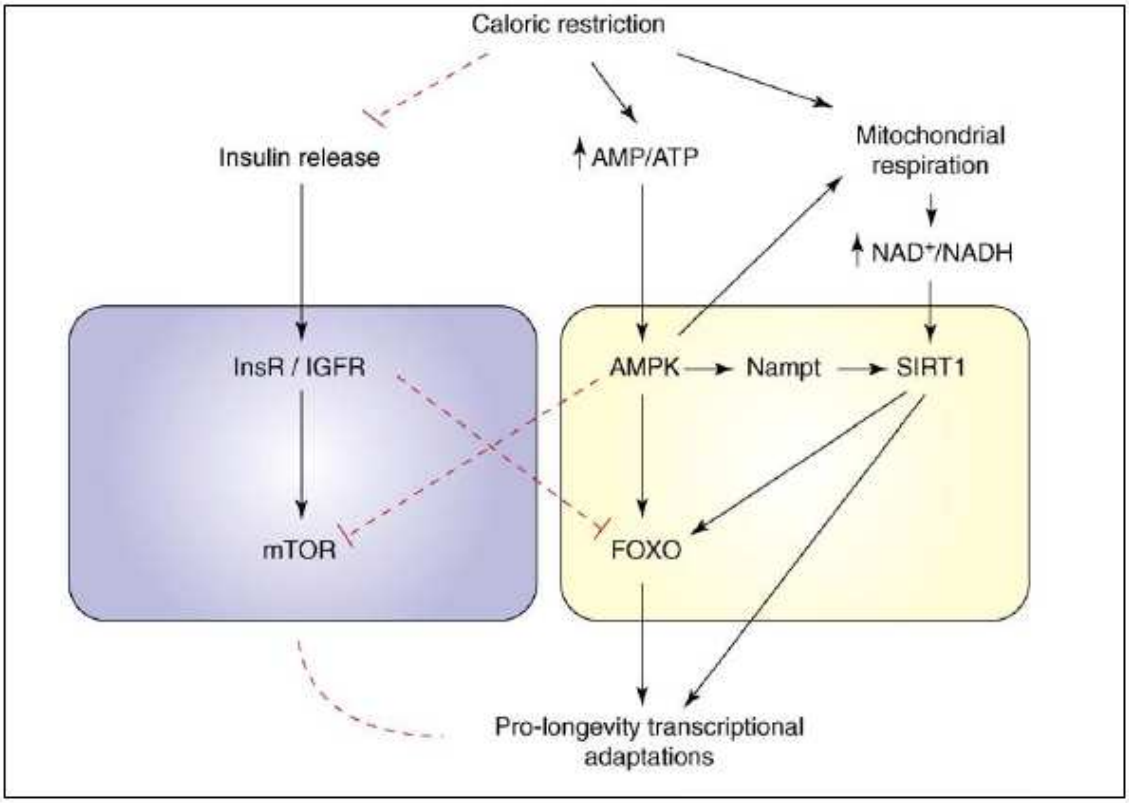


**Figure 7** Integrative view of mammalian signaling pathways involved in regulating the effects of caloric restriction (CR). CR, caloric restriction; SIRT1, silent information regulator T1; AMPK, AMP-activated protein kinase; FOXO, forkhead box O1. Modified by Cantò and Auwerx, 2009.

### 1.3.3 Cellular and systemic response to damage

Aging is due to a progressive accumulation of damage in macromolecules, and as a consequence in tissues, that cause the disruption of physiological function. Efficient defensive and reparatory systems, that are essential for survival, were developed during evolution. The cellular response to damage may be observed at different levels: macromolecular, cellular and systemic.

At molecular level antioxidant defenses such as catalase and superoxide dismutase (SOD) enzymes help to prevent cellular damage caused by oxidative stress. However, their association with longevity results controversial (Hekimi, 2006; Kuningas et al, 2008). In fact in *D. melanogaster* genes encoding catalase and superoxide dismutase (SOD) showed to influence lifespan (Orr and Sohal, 1994), but additional experiment showed that the results may be straightly influenced by genetic background of all lines (Orr and Sohal, 2003). Also in mammals, where one catalase and

three SOD are present, there are contrasting results. The disruption of the SOD2 gene is lethal (Li et al, 1995; Melov et al, 1998), and the overexpression of SOD2 leads to increased lifespan (Hu et al., 2007), but mice heterozygous for SOD2, that have high levels of DNA oxidation in multiple organs, didn't show decline in lifespan and acceleration in aging (Van Remmen et al, 2003). However, all these data illustrate the existence of a balance between the production of free radicals and of antioxidative enzymes. In humans, genetic variations in antioxidant genes were shown to be associated with normal cognitive aging, cancer risk, etc., but not with mortality (Kachiwala et al, 2005; Taufer et al, 2005; Liu et al, 2004; De Benedictis et al, 1998). Likewise, no associations between genetic variants in the catalase gene and mortality have been found (Christiansen et al, 2004).

When DNA, proteins and membranes are damaged, mechanisms of repair evolutionarily conserved (Eisen and Hanawalt, 1999) occur. However, there are many studies which have demonstrated the detrimental effects of impaired repair systems on lifespan, but only few have demonstrated beneficial effects of increased repair capacity. In *D. melanogaster* the correlation between repair systems and longevity was detected: the absence of the excision repair gene mei-41 reduces lifespan, and the presence of extra copies of the gene significantly increases lifespan (Symphorien and Woodruff, 2003). Likewise, over-expression of protein carboxyl methyltransferase (PCMT), which is another protein repair enzyme, is correlated with enhanced longevity in a temperature dependent manner (Chavous et al, 2001). In mammals studies on homologues of these genes, and on other DNA repair enzyme have shown the correlation with various pathological phenotype, but hasn't shown an unambiguous association with aging and longevity (Pascucci et al, 2011).

At cellular level one example of defense mechanism is the tumor suppressor gene p53 that eliminates damaged cells through apoptosis or cell-cycle arrest. In aged tissue p53 is less expressed (Feng et al, 2007), causing the accumulation of mutations in individual cells during the life that may promote cancer formation (Tyner et al, 2002).

At systemic levels, the mechanism of defense from external pathogenic agents are immune response and inflammation. And, in effect, association studies have shown how the genes that take part in these two processes (TNF-α, IL-6, IL-1 cluster, and IL-10), may contribute to human longevity (Franceschi et al, 2005).

Moreover, every day organisms are exposed to many hydrophobic compound present in food and in environment, that are often toxic. To cope with these hydrophobic toxic substances two different strategies are possible: one is to conjugate them enzymatically with cofactors (glutathione, glucuronate, or sulfat) making this substance more hydrophilic and detoxified; the other strategy is to recognize hydrophobic compounds as they pass through the plasma membrane and to excrete them directly out of the cells (Ueda, 2011). Defects on the enzyme that carried out these important functions are related to various diseases (Vasieva, 2011; Polimanti et al, 2011; Ueda, 2011). In particular, some of these genes (of GST family) turned out to be associated also with aging markers (telomeres shortening, respiratory function, hand grip strength reduction, and cognitive decline) (Starr et al, 2008).

### 1.3.4 Lipoprotein genes and longevity

The best-known example of a potential longevity gene has an important role in regulating lipoproteins: apolipoprotein E (APOE) gene that is also known to be related to susceptibility to age-related diseases, as well as being a recognized risk factor for coronary heart disease and Alzheimer's disease (Kolovou et al, 2004, 2002). Since coronary heart disease and Alzheimer's disease are common in the elderly, increasing genetic association studies revealed that APOE is an important genetic variable in regulation of human longevity (Christensen et al, 2006; Wilson et al, 1996; Rubinsztein and Easton, 1999; Smith, 2002). It has various isoforms, which are encoded by different alleles (ε2, ε3 and ε4) and interact differently with specific lipoprotein receptors that alter circulating levels of cholesterols. As previously mentioned, ε4 allele results negatively associated with longevity (Schachter et al, 1994). In contrast to other candidate genes, cross-sectional studies

of APOE allele frequency differences between age groups have been remarkably consistent. Although APOE ε4 frequency varies considerably between populations of younger adults (about 25% among Finns, 17–20% among Danes and about 10% among French, Italians and Japanese), in all these populations the frequency among centenarians is about half these values (Christensen et al, 2006). At the contrary, APOE ε2 allele shows an increased frequency at older ages in different populations (Louhija et al, 1994; Blanché et al, 2001; Frisoni et al, 2001; Seripa et al, 2006).

Between the other APO genes, polymorphic variant have also been reported associated with longevity in different populations (Pepe et al, 1998; De Benedictis et al, 1997; Anisimov et al, 2001; Atzmon et al, 2006). An example is APOC3, a major component of very LDLs and a minor component of HDL, that results associated with survival longevity in Ashkenazi Jewish. In particular, APOC3 -641CC genotypes were more frequent among long-living individuals. In addition this genotype results to be correlated also with a lower prevalence of hypertension and greater insulin sensitivity (Atzmon et al, 2006).

PON1 gene codify for a paraoxonase, an enzyme that hydrolyzes lipoperoxides and various toxic metabolites (Draganov et al, 2005). PON1 is synthesized in the liver and secreted into the blood, where it is associated exclusively with high density lipoproteins (HDLs). For its role and its localization it is considered to be a protective factor against oxidative modification of LDL, assuming a potential role in the prevention of the atherosclerotic process (Draganov et al, 2005). Many studies have been conducted an association between PON1 variants and human longevity, and because of the association individuated in some populations was not replicated in all studies, it is possible that the effects observed are population-specific (Rea et al, 2004; Lescai et al, 2009b; Caliebe et al, 2010).

### 1.3.5 **Cell cycle regulation and longevity**

In the history of aging research, there has been the idea that the life-long sequence of age associated events might be controlled by a sort of "clock." This idea of "the aging clock" was first proposed by Arthur Everitt in Australia in 1973 (Everitt et al, 1973). However, only recent studies have demonstrated that circadian clock proteins may be involved in DNA repair and in regulating accumulation of cellular ROS, thus making them plausible actors in the aging processes (Antoch et al, 2010). In particular, the connection between aging and circadian rhythm was provided by the phenotypes of Bmal1-deficient mice and Per1/2-deficient mice, that show the pathological features typical of aged mice (Kondratov et al, 2006; Lee, 2005). Moreover, it has been demonstrated that SIRT1, the master controllers of a regulatory system for aging, regulates the amplitude and the duration of circadian gene expression through the interaction and the deacetylation of key circadian clock regulators, such as BMAL1 (or ARNTL-aryl hydrocarbon receptor nuclear translocator-like) and PER2 (period homolog 2) (Asher et al, 2008; Nakahata et al, 2008).

The importance of the molecular clock machinery is also underlined by the fact that it is one of the most powerful modifiers of metabolism (Green et al, 2008; Ramsey et al, 2007). An example is provided by the homozygous Clock mutant mice that exhibit the metabolic syndrome (Turek et al 2005).

All these findings suggest that a connection exist between physiological rhythmicity, metabolism and aging, and that SIRT1 might function at a central interface connecting these fundamental biological events. A key molecule in this events may be NAD (Imai, 2010) that from an hand have been shown to directly modulate activity of circadian clock proteins (Eckel-Mahan and Sassone-Corsi, 2009), and on the other hand is essential for SIRT activity.

### 1.3.6 Telomere length maintenance

Many studies have shown how the process of aging is mitigated by the maintenance of telomeres (chromosome ends, composed by TAGGG tandem repeats), and this results in a life span extension. Early evidence about the biological importance of telomeres shortening was given by the observation of telomere shortening in primary human cells culture: when telomere erosion reaches a critical point, cells cease to proliferate and undergo senescence or apoptosis (Harley et al, 1990; Allsopp et al, 1992; Wright and Shay, 1992; Counter, 1996).

Only recently, studies on peripheral white blood cells have demonstrated the correlation with life-span extension: in fact, although telomere length is inversely correlated with increasing age (Njajou et al, 2007; Kimura et al, 2008; Njajou et al, 2009), healthy centenarians possessed significantly longer telomeres than their unhealthy counterparts (Terry et al, 2008). Studies on twins have given a big support to this finding. In fact, analyses of Swedish twins demonstrated that telomere length at advanced age is a biomarker that predicts survival beyond the impact of early familial environment and genetic factors (Bakaysa et al, 2007). By contrast, in Danish population, the co-twin with the shorter telomeres died first, suggesting that telomere length is not only a biomarker of aging, but also a determinant of lifespan (Kimura et al, 2007).

Thus, in the elderly it is possible to use telomere length as a biomarker of disease risk (like as cognitive decline) and progression as well as mortality (Devore et al, 2011).

The mechanism of telomeres length regulation is complex, and many proteins are involved in telomere maintenance, including the telomerase complex in proliferating cells and other capping proteins involved in protection of chromosome termini (Cohen et al, 2007; Palm and de Lange 2008).

In the last years experiments have provided solid evidence supporting a role for telomerase activation in longevity: for example in mice an over-expression of telomerase reverse transcriptase (TERT) in various tissues resulted in an extension of maximum lifespan of up to 10% and a low incidence of age-related diseases, although this caused a higher incidence of both induced and spontaneous tumors, causing increased mortality in the first year of life (González-Suárez et al, 2001). These studies indicate that activation of telomerase provides anti-aging activity in mammals. In humans evidence demonstrate that telomere length has heritability estimates ranging from 44 to 80% (Slagboom et al, 1994; Andrew et al, 2006). Hovewer, only a few studies have been conducted about the correlation of telomerase genes and longevity. An example is the work of Atzmon and collegues that demonstated how a common telomerase reverse transcriptase haplotype is associated with longer telomeres in Ashkenazi centenarians and their offspring, confirming that human telomerase is involved in lifespan determination for longevity (Atzmon et al, 2010).

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## CHAPTER 2

### 2.1 The analysis of mitochondrial DNA variability in a large sample of ultranonagenarians across Europe allows a reappraisal of the mtDNA correlation with longevity: a GEHA Project Study

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**Astract**

The involvement of mitochondrial function in the aging process has been widely studied from different standpoints. In this frame, many studies have suggested that mitochondrial DNA variability affects the quality of aging and longevity. In particular, some specific groups of haplotypes, (such as haplogroup J in Europe and D in Asia) sharing mutations in the subunits of the OXPHOS complex I, turned out to be more frequent in centenarians than in younger controls. However, J haplogroup was also found to predispose to Leber Disease. In addition, new surveys in a number of populations did not confirm the association between J haplogroup and longevity. In order to reappraise the correlation between mtDNA variability and longevity, we took advantage of the large GEHA recruitment, which has carried out an unprecedented collection 2200 ultranonagenarians and an equal number of younger controls across Europe. The samples, have been categorized by high resolution haplogroup classification, while about 1300 mtDNA molecules from 650 nonagenarians and an equal number of controls have been completely sequenced. The large sample size and the extensive molecular analyses allowed to better clarify the correlation between mtDNA mutations and longevity. In addition they allowed to highlight the importance of the complete mtDNA sequences as it allows to evaluate the effects rare and repeated mutations which are not fully appreciated when haplogroup analysis is carried out.

## Introduction

The research on mitochondrial biology has demonstrated the central role played by the mitochondrion in a number of complex traits, including ageing and numerous degenerative diseases. This is basically due to the crucial role of mitochondrion in the energetic balance of the cell. In fact, mitochondria are the organelles where Oxidative Phosphorylation (OXPHOS) takes places, and this implies that mitochondria have a central position between energy uptake (that is food uptake and metabolism) and energy production. As a consequence of this, mitochondria are also involved in a number of crucial cellular processes such as heat production, apoptosis regulation, cellular differentiation, but especially in the production and the regulation of one of the most important byproduct of the cellular metabolism: the Reactive Oxigen Species (ROS) (Passarino et al. 2010; Wallace 2010). Many studies have addressed the role played by the variability of mitochondrial DNA in modulating the individual variability in these processes and, consequently on the susceptibility to the different phenotypes influenced by mitochondrial activity (Montiel-Sosa et al. 2006; Niemi et al. 2003; Santoro et al. 2006; Wallace 2005; van der Walt et al. 2003; Rose et al. 2002; Ross et al. 2001; Ruiz-Pesini et al. 2000; De Benedictis et al. 1999; Ivanova and Lepage 1998; Brown et al. 1997). These studies have mainly been managed by taking advantage of the haplogroup classification of mtDNA molecules, which has been carried out by population geneticists, due to the non recombinant nature of mtDNA. This has allowed to find out that haplogroup J, which is diffused over Europe and Near East, might be beneficial to longevity, as it has been found more frequently in centenarians than in relevant younger controls in northern Italians (De Benedictis et al. 1999) in Irish (Ross et al. 2001) and in Finns (Niemi et al. 2003). On the other hand, the same haplogroup J has been found to be strongly associated with LHON (Leber's Hereditary Optic Neuropathy), as it increases the penetrance of some specific mutations which are significantly less harmful when present in different haplogroups (Man et al. 2004; Brown et al. 1997; Torroni et al. 1997). It was then proposed that the mutations defining the J haplogroup

(falling in the protein subunits of OXPHOS complex I) lower the OXPHOS activity, putting the cell in a vulnerable situation where a further mutation (such as the LHON mutations) would be more likely to be harmful. But, if the nuclear response stimulated by the higher ROS production is adequate, the low OXPHOS efficiency may become advantageous and lead to a lower presence of ROS, and this would result in a more healthy ageing (Rose et al. 2001). In this case, then, the final phenotype (Leber Neuropathy, normal lifespan or longevity) depends on the interaction among mtDNA, nuclear genome and environment. Similarly, the Asian haplogroup D (which is characterized by mutations affecting Complex I as haplogroup J), has been found overrepresented in Japanese centenarians (Tanaka et al. 2000; Tanaka et al. 1998). However, different studies have failed to find any association between mtDNA and longevity (Pinos et al, 2011; Dato et al, 2004), suggesting that the possible effect of mtDNA on longevity might be population specific, but also that such effect might be very low and then a very numerous sample is necessary in order to have the necessary statistical power to observe such effect.

In this study we present the results of a very large study, where more than 2200 subjects older than 90 years, recruited in the frame of the GEHA project and coming from most of the European populations, have been compared to a similar number of younger controls matched for sex and geographic origins. In this study we also present data on complete sequencing of a subgroup of GEHA samples (650 ultra nonagenarians and a comparable number of controls, coming from Denmark, Finland, southern Italy and Greece), tested to find out if recurrent mutations may influence longevity without being detected by haplogroup analysis or if different mutations accumulate in some specific genes.

## Materials and Methods

### *Sampling*

Samples were collected in the frame of the GEHA research project (2004-2010) in 11

European Countries. Each Institution providing blood samples received the approval from its own ethical committees and all the recruited subjects provided written informed consent for the use of their phenotypic and genetic data in studies on human ageing (Skytthe et al. 2011).

DNA was recovered from fresh blood by automated and standardized protocol in order to guarantee quality and concentration uniformity among samples. The GEHA Partner in charge of the DNA extraction (KTL - Helsinki, Finland) provided 4 µg of genomic DNA for mtDNA variability analysis.

A total of 4,239 samples were available for mitochondrial haplogroups classification and for exploring the possible association between mtDNA inherited sequence variation and longevity.

Samples were distributed among ultra nonagenarians (90+) and younger controls as in Table 1. The control group was comparable for sex and ethnicity to the 90+ group. The number of available samples for each recruitment centre is reported in Table 1S (Supplementary materials).

*mtDNA Sequence Variation Screening*

From the 4,239 total samples, 1,292 (637 nonagenarians and 655 controls) were selected for complete sequencing in order to analyze the differences between Northern and Southern European populations in the whole DNA molecule. In the remaining 2,947 samples (1,449 nonagenarians and 1,498 controls) the mtDNA subhaplogroup was determined in order to verify possible haplogroups association with longevity and to compare haplogroups distribution among different European countries.

The complete sequencing was performed using two different protocols previously compared for results reliability by testing the same samples. A total of 125 sequences (109 calabrians and 16 greeks) were produced using the MitoALL Resequencing kit (Applera, Foster City, CA) introducing four alternative primer pairs in order to improve the amplification rate (Table 2S). Each amplification step was followed by the purification with EXOSAPit (U.S. Biochemical,Cleveland,Ohio). The sequencing reaction was conducted using the BigDye kit version

3.1 (Applera) and M13 universal primers (forward and reverse), followed by purification of the sequences by using ethanol precipitation. Electropherograms were analyzed with SeqScape version 2.5 software (Applera) which allows to assembly all the 46 fragments belonging to the sample and to align and compare the obtained consensus sequence with the revised Cambridge Reference Sequence (rCRS, NCBI: NC_012920.1 gi:251831 106) (Andrews et al. 1999). Sequences were manually verified for phantom mutations by reads of both strands, by two independent operators.

The remaining samples were sequenced in Beijing Genomics Institute (BGI) as previously described (Wang et al. 2008).

The definition of mtDNA subhaplogroups in the remaining 2947 samples was conducted by resequencing the D-loop region from nucleotide position (np) 16024 to np 576 followed by RFLP analysis in specific coding region traits as previously described (Torroni et al. 1996).

For the haplogroups and subhaplogroups assignment we have followed the Phylotree nomenclature (van Oven et al. 2009; www.phylotree.org). Considering the number of samples to classify we have developed a custom tree search algorithm that, coupled with a highly-efficient SNP discovery pipeline, is able to find the haplogroup that better matches the sample mutational motifs, starting from the raw sequence in FastA format (manuscript in preparation).

*Statistical analysis*

R statistical software was used for all statistical analyses.

MtDNA subhaplogroups and relevant frequencies were stratified by gender and were compared between 90+ subjects and their ethnically-matched controls using the $\chi 2$ with Pearson correction or Fisher-exact test. The comparison of frequencies in 90+ cases and younger controls of each mtDNA subhaplogroup has been computed by applying Pearson's Chi-squared test with Yates' continuity correction. Tests for statistical significance were two-sided with = 0.05; we performed also a logistic regression to generate odds ratio (OR) with their associated 95% confidence intervals (CI), to assess odds of carrying each mtDNA haplogroups in cases compared with controls.

A non-metric MDS was performed separately on both 90+ subjects and controls. This analysis was implemented in the Sammon function in MASS library of R program.

## Results

### *Haplogroup analysis.*

The different mtDNA haplogroups found in cases and controls, with frequencies, standard errors and relative references for the European population, are summarized in Table 2. Table 3a and 3b report the summary of observed haplogroups for males and females, which are also graphically reported in Fig.1. Statistically significant differences were observed in males for haplogroups J2 (p=0.0214) and H1 (p=0.0427). More in details, the higher frequency of haplogroup J2 in male controls is attributable to an increase in subcluster J2a (p=0.017), even though the same trend was observed for J2b.

Fig. 2 reports a MDS for each group of cases and controls. The analysis shows a geographic pattern with northern European countries quite separated from southern Europeans. Interestingly, we observe that northern European cases are more scattered with respect to the relevant controls.

### *Sequence analysis*

We first compared all complete sequences with the reference sequence (rCRS). Then we counted for each position the number of subjects mutated with respect to the reference. Fig 1S (a,b and c) reports a summary of all the mutations (subdivided by population), and it highlights the differences between cases and controls. Tables 3S (A, B and C) reports in details the mutations with a significant frequency difference between cases and controls in each population.

We then focused on non synonymous mutations by evaluating the presence of such mutations in genes belonging to the different mitochondrial complex. In supplementary Table 4S (A, B and C)

we report the possible effect of each observed mutation, as estimated by PolyPhen (http://genetics.bwh.harvard.edu/pph/).

When we considered the subunits of Complex I (ND 1-6 and ND4L), all the three populations show a significant difference between cases and controls. But if in Danish and southern European populations there is a higher frequency of non synonymous mutations in controls than in cases (respectively p=0,01 in Danmark and p< 0,0001 in south Europe), in Finnish we observe the opposite situation (p=0,005) (Fig. 3).

In complex 3 (Cyt b) Danish and Southern European population show a significant higher frequency of mutations in controls than in cases (p=0,004 in Danmark and p=0,042 in South Europe).

In complex 4 (COI-III) all the populations show a higher frequency of mutation in controls than in cases (p<0,0001 in Denmark and p<0,0001 in Finland). The difference is not significant only in Southern European population, probably due to the low sample size.

In complex 5 (ATPase 6 and 8) a different frequency of mutations is present in the southern European population (p=0,002) while in the other two population the mutation frequency does not differ between cases and controls.

We have then analyzed the difference of mutation frequency between cases and controls gene by gene. Within the subunits of complex I, Danish and South European populations show a higher occurrence of mutations in controls than in cases in the ND5 gene (p= 0,003 in Denmark p= 0,007 in South Europe). The Finnish population show instead a significant higher frequency in controls than in cases in ND6 gene (p=0,048) (Fig.4). As to complex IV, the genes belonging to this complex show in all the populations a higher frequency of mutations in controls than in cases with a significative difference for COI in Danish (p=0,00006) and in Finnish (p=0,014) (Fig. 5). A

different mutation frequency is present in southern European population for ATP6 gene (p= 0,002) (Fig.6)

The analysis of the whole tRNA sequences shows no significant differences (data not shown). The further analysis of each tRNA was carried out by pooling the data of all the three populations due to the small length of each tRNA. The most significant differences were found for tRNA Leu (p=0,035), tRNA Trp ( p<0,001), tRNA Arg (p=0,031), tRNA Thr (p<0,001) and tRNA Pro (p=0,002) (Fig. 7).

The analysis of rRNAs did not show any significant differences between cases and controls.

Table 4, shows the frequency in cases and controls, of mutations which have been previously reported to be associated to degenerative diseases. It is interesting to see that in most cases these mutations are not absent in cases and in some cases are more frequent in cases than in controls.

Finally it is worth mentioning that synonymous mutations did not highlight significant results, taking into account the number of comparisons.

**Discussion.**

Previous analysis on the correlation between mitochondrial DNA variability and longevity has given interesting but contradictory results. In fact, mutations linked to haplogroup J, mainly falling in the subunits of the OXPHOS complex I seemed to be associated to longevity in different populations (De Benedictis et al. 1999; Ross et al. 2001; Niemi et al. 2003), suggesting the hypothesis that mutations in these proteins may lower OXPHOS activity and consequently ROS production contrasting the oxidative related aging process. On the other hand, the same haplogroup J turned out to be not associated with longevity in other population (Pinos et al. 2011; Dato et al. 2004) and, what is more, it has been consistently found in different studies to be associated to a

degenarative disease such as the LHON disease (Man et al, 2004; Brown et al. 1997; Torroni et al. 1997). These further findings, suggested that lower OXPHOS, in some cases, either environmental or genetic due to the occurrence of additional mutations, may be detrimental.

The present study, by taking advantage of the large GEHA sampling of European ultranonagenarians with sex and age matched controls, has tried to shed a light in this contradictory scenario. The unprecedented number of samples collected and analyzed makes these result very relevant in the longevity and mtDNA world; furthermore the specific attention dedicated to the recruitment process in order to avoid sampling bias is proved by Table 2.

The analysis of haplogroups has shown that haplogroups H1 and J2 are associated with longevity. Interestingly, the association of haplogroup J2 shows an opposite trend with respect to previous studies (Dominguez-Garrido et al. 2009; Niemi et al. 2003). In fact, J2 turned out to be less frequent in cases than in controls.

This result is in line with the result obtained by sequence analysis. In fact, Complex I, the most affected region from mutations characterizing haplogroup J, turned out to have more mutations in cases than in controls in Finns (in line with the hypothesis that mutations in these subunits may be beneficial for longevity), but it showed more mutations in controls than in cases in Danish and in Southern Europeans (in line with the opposite hypothesis that mutations in the subunits of complex I may be detrimental for longevity). The results, both statistically significant, confirm the idea that the mutations which may modulate the efficiency of complex I do affect ageing, but they might be beneficial or deleterious possibly due to external factors or to additional genetic factors.

Studies on LHON mutations, have shown that the penetrance of these mutations is greatly increased if mutations on complex III occur together with those occurring in the subunits of complex I and defining J haplogroups (Carelli et al. 2006). In fact, we noticed that Danes and Southern Europeans which show higher frequency of complex I mutations in controls with respect to sibs also show a higher frequency of complex III mutations in controls with respect to sibs. By contrast in Finns,

where we observed higher frequency of complex I mutations in sibs with respect to controls, no differences could be detected between sibs and controls as to mutations in complex III subunits. We then investigated if the different results obtained in our different samples could be due to different distribution of the simultaneous presence of mutations in subunits of complex I and III. As most of the samples showed at least one mutation in both complex I and III, for each sample we counted the subjects where we observed two or more mutations in the subunits of both complex I and complex III. Table 6 reports the results obtained. Indeed we observed that, when the controls are considered, both Danes and southern Europeans show higher frequency of the simultaneous presence of two mutations in complex I and III than Finns (p=0,004 and p=0,0008 respectively). This is not observed in long lived subjects, due to the demographic selection of this feature.

We believe this result may be of great interest as it might explain why J haplogroup is associated to longevity in some area but not in others. In the populations where J sub-haplogroups with additional mutations in complex III are more frequent we may expect to find no association between J and longevity.

The analysis of complete sequences also shows a significant result that had never been highlighted by haplogroup analysis. In fact, in the mtDNA sequences encoding for the subunits of complex 4, we observe a higher frequency of mutations in controls than in cases. This has been consistently observed in all the populations we examined. The analysis of the most updated literature suggested that only very rare haplotypes are characterized by mutations falling in Complex IV sequences (H15, H1f, N22, U8b). This may explain why previous analyses were unable to spot a correlation between the variability of Complex IV sequence and longevity. This result, indicate that variability of CO genes, especially COI, are detrimental for longevity, possibly because they alter OXPHOS by making it less efficient and raising OXPHOS production. In addition this result suggest that complete sequencing may highlight some effects of mtDNA variability that cannot be captured by DNA haplogroup classification.

The complexity of the correlation between mtDNA variability and longevity is also highlighted by the analysis of the variability falling in tRNA sequences, and also by the mutations that previous studies have consistently shown to be associated with degenerative diseases. For instance the 4336 mutation, falling in the $tRNA^{gln}$, has been found to be more frequent in AD patients than in controls by a number of studies between 1993 and 2010 (Santoro et al. 2010; Brown et al. 1996; Hutchin et al. 1995; Shoffner et al. 1993). We found this mutation to be twice as frequent in controls than in nonagenarians. Other supposed deleterious mutations have been found to be quite frequent in nonagenarians (Table 4). Similarly, mutations in tRNA, which have been found to be largely correlated with degenerative diseases have been observed to frequently occur in nonagenarians and for a number of tRNAs even more frequently than in controls. In particular, it is of note the result obtained for the $tRNA^{thr}$, which has significantly more mutations in cases than in controls. In fact, many mutations on this tRNA have been reported to be associated with a number of diseases (Table 5) (Scaglia and Wong 2008; Howell 1999).

These results support the idea that certain mtDNA inherited mutations could induce longevity or disease according to the individual-specific genetic background as well as to stochastic events (Rose et. al 2001). In this frame, it might be important to mention the studies on the import of tRNA into mitochondria. Although for mammals this has been proved only for $tRNA^{gln}$ (Rubio et al. 2008), it is possible that also other tRNA undergo a similar phenomenon (Schneider 2011 and references therein), and then mutations on tRNA could be beneficial or detrimental according to the efficiency of the import process. The coincidence that the 4336 mutation, which has been largely studied for predisposing to AD and which we found twice as frequent in long lived than in controls, falls in the one tRNA which has been proved to be imported into mammalian mitochondria is very intriguing and suggests further studies.

On the whole our study has allowed a reappraisal of the correlation between mtDNA variability and longevity. It has shown that complete sequencing and analysis of complete variability can highlight correlations between mtDNA variability and a phenotype (longevity) which escape haplogroup analysis. In fact, complete sequence analysis allows to appreciate the effects of rare or repeated mutations which are not accounted for in haplogroup analysis. In addition we have better highlighted the beneficial effects on longevity of mutations in the subunits of OXPHOS complex I and the detrimental effects of mutations in subunits of complex III (also in presence of mutations in complex I) and IV. In addition we have highlighted the positive effects of mutations in tRNA genes, which had previously been found to be associated to a number of disorders. This suggests that tRNA mutations can be either positive or negative according to other factors, certainly including nuclear genome response.

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**Table 1**: Sample distribution and general characteristics of the study participants.

| | **Males (N = 1,327)** | | **Females (N = 2,912)** | |
|---|---|---|---|---|
| | **90+ (%)** | **Controls (%)** | **90+ (%)** | **Controls (%)** |
| **N** | 628 (47) | 699 (53) | 1,458 (50) | 1,454 (50) |
| **Mean Age** | 94,1 ± 2,5 | 61,7 ± 6,2 | 94,5 ± 2,6 | 61,7 ± 6,2 |
| **Age range** | 89-103 | 43-79 | 88-107 | 49-83 |

**Table 2.** mtDNA Haplogroups distribution in GEHA samples. Haplogroups and subhaplogroups with frequencies higher than 1.5% are reported. OTHER includes N1a, N1b, N1c,N9a, A4, D5, C1d, M1, L1b1.

| | Controls (n=2153) | | | 90+ (n=2086) | | | Reference | |
|---|---|---|---|---|---|---|---|---|
| **sub-haplogroups** | **N** | **%** | **SE** | ***N*** | **%** | **SE** | **%** | **Literature** |
| **HV*** | 1042 | 48.40 | .0108 | 1052 | 50.43 | .0109 | 50.4-54.1 | *Richards et al. 2000* |
| **HV0*** | 68 | 3.16 | .0038 | 83 | 3.98 | .0043 | | |
| **H*** | 924 | 42.92 | .0107 | 927 | 44.44 | .0109 | 44.5-48.2 | *Richards et al. 2000* |
| **H1** | 325 | 15.10 | .0077 | 292 | 14.00 | .0076 | 19.0 | *Brandstatter et al. 2006* |
| **H2** | 23 | 1.07 | .0022 | 42 | 2.01 | .0031 | 1.5 | *Brandstatter et al. 2006* |
| **H3** | 85 | 3.95 | .0042 | 71 | 3.40 | .0040 | 2.2 | *Brandstatter et al. 2006* |
| **H5** | 63 | 2.93 | .0036 | 68 | 3.26 | .0039 | 4.8 | *Brandstatter et al. 2006* |
| **H6** | 47 | 2.18 | .0031 | 52 | 2.49 | .0034 | 2.9 | *Brandstatter et al. 2006* |
| **I** | 44 | 2.04 | .0030 | 46 | 2.21 | .0032 | 1.6-2.7 | *Richards et al. 2000* |
| **J** | 210 | 9.75 | .0064 | 183 | 8.50 | .0060 | 8.3-10.4 | *Richards et al. 2000* |
| **J1** | 153 | 7.11 | .0055 | 144 | 6.90 | .0056 | | |
| **J2** | 57 | 2.65 | .0035 | 39 | 1.87 | .0030 | | |
| **K** | 161 | 7.48 | .0057 | 137 | 3.98 | .0054 | 4.9-6.6 | *Richards et al. 2000* |
| **K1** | 140 | 6.50 | .0053 | 117 | 5.61 | .0050 | | |
| **T** | 211 | 9.80 | .0064 | 223 | 10.69 | .0068 | 7.2-9.2 | *Richards et al. 2000* |
| **T1** | 53 | 2.46 | .0033 | 43 | 2.06 | .0000 | | |
| **T2** | 152 | 7.06 | .0055 | 174 | 8.34 | .0061 | | |
| **U** | 313 | 14.54 | .0076 | 272 | 13.04 | .0074 | 20.1-23.2 | *Richards et al. 2000* |
| **U2** | 39 | 1.81 | .0029 | 38 | 1.82 | .0029 | | |
| **U4** | 48 | 2.23 | .0032 | 34 | 1.63 | .0028 | | |
| **U5a** | 109 | 5.06 | .0047 | 90 | 4.31 | .0044 | | |
| **U5b** | 56 | 2.60 | .0034 | 51 | 2.44 | .0034 | | |
| **W** | 39 | 1.81 | .0029 | 51 | 2.44 | .0034 | 1.5-2.5 | *Richards et al. 2000* |
| **X** | 41 | 1.90 | .0029 | 42 | 2.01 | .0031 | 1.2-2.0 | *Richards et al. 2000* |
| **OTHER** | 92 | 5.53 | .0049 | 80 | 5.08 | .0048 | | |

**Table 3A.** Haplogroups distribution in males. SE= Standard Error. Sub-haplogroups with frequencies lower than 1.5% were grouped. HV0* includes HV0a, OTHER includes K, K2, T, R0, R0a, R1, R2, HV1, HV2, N1a, N1b, N1c,N9a, A4, D5, C1d, M1, L1b1, with frequencies lower than 1.5%.

**Males (N=1327)**

| | Controls (N= 699) | | | 90+ (N=628) | | |
|---|---|---|---|---|---|---|
| | **N** | **%** | **SE** | **N** | **%** | **SE** |
| **HV*** | 347 | 49.6 | .0189 | 318 | 50.6 | .0200 |
| **HV0*** | 24 | 3.4 | .0069 | 24 | 3.8 | .0077 |
| **H*** | 306 | 43.8 | .0188 | 280 | 44.6 | .0198 |
| **H1** | 108 | 15.5 | .0137 | 75 | 11.9 | .0129 |
| **H2** | 11 | 1.6 | .0047 | 10 | 1.6 | .0050 |
| **H3** | 16 | 2.3 | .0057 | 18 | 2.9 | .0067 |
| **H5** | 20 | 2.9 | .0063 | 24 | 3.8 | .0077 |
| **H6** | 16 | 2.3 | .0057 | 19 | 3.0 | .0068 |
| **I** | 13 | 1.9 | .0051 | 7 | 1.1 | .0042 |
| **J1** | 44 | 6.3 | .0092 | 40 | 6.4 | .0097 |
| **J2** | 22 | 3.1 | .0066 | 6 | 1.0 | .0039 |
| **K1** | 46 | 6.6 | .0094 | 47 | 7.5 | .0105 |
| **T1** | 11 | 1.6 | .0047 | 11 | 1.8 | .0052 |
| **T2** | 54 | 7.7 | .0101 | 46 | 7.3 | .0104 |
| **U** | 20 | 2.9 | .0063 | 21 | 3.3 | .0072 |
| **U2** | 13 | 1.9 | .0051 | 12 | 1.9 | .0055 |
| **U4** | 13 | 1.9 | .0051 | 13 | 2.1 | .0057 |
| **U5a** | 30 | 4.3 | .0077 | 24 | 3.8 | .0077 |
| **U5b** | 17 | 2.4 | .0058 | 18 | 2.9 | .0067 |
| **W** | 16 | 2.3 | .0057 | 13 | 2.1 | .0057 |
| **X** | 19 | 2.7 | .0062 | 18 | 2.9 | .0067 |
| **OTHER** | 34 | 4.9 | .0081 | 34 | 5.4 | .0090 |

**Table 3B.** Haplogroups distribution in females. SE=Standard Error; Sub-haplogroups with frequencies lower than 1.5% were grouped. HV0* includes HV0a, OTHER includes K, K2, T, R0, R0a, R1, R2, HV1, HV2, N1a, N1b, N1c,N9a, A4, D5, C1d, M1, L1b1, with frequencies lower than 1.5%.

| Females (N = 2912) | | | | | | |
|---|---|---|---|---|---|---|
| | Controls (N= 1454) | | | Cases (N= 1458) | | |
| | N | % | SE | N | % | SE |
| **HV*** | 695 | 47.8 | .0131 | 734 | 50.3 | .0131 |
| **HV0*** | 44 | 3.0 | .0045 | 59 | 4.0 | .0052 |
| **H*** | 618 | 42.5 | .0130 | 647 | 44.4 | .0130 |
| **H1** | 217 | 14.9 | .0093 | 217 | 14.9 | .0093 |
| **H2** | 12 | 0.8 | .0024 | 32 | 2.2 | .0038 |
| **H3** | 69 | 4.7 | .0056 | 53 | 3.6 | .0049 |
| **H5** | 43 | 3.0 | .0044 | 44 | 3.0 | .0045 |
| **H6** | 31 | 2.1 | .0038 | 33 | 2.3 | .0039 |
| **I** | 31 | 2.1 | .0038 | 39 | 2.7 | .0042 |
| **J1** | 109 | 7.5 | .0069 | 104 | 7.1 | .0067 |
| **J2** | 35 | 2.4 | .0040 | 33 | 2.3 | .0039 |
| **K1** | 94 | 6.5 | .0064 | 70 | 4.8 | .0056 |
| **T1** | 42 | 2.9 | .0044 | 32 | 2.2 | .0038 |
| **T2** | 98 | 6.7 | .0066 | 128 | 8.8 | .0074 |
| **U** | 41 | 2.8 | .0043 | 38 | 2.6 | .0042 |
| **U2** | 26 | 1.8 | .0035 | 26 | 1.8 | .0035 |
| **U4** | 35 | 2.4 | .0040 | 21 | 1.4 | .0031 |
| **U5a** | 79 | 5.4 | .0059 | 66 | 4.5 | .0054 |
| **U5b** | 39 | 2.7 | .0042 | 33 | 2.3 | .0039 |
| **W** | 23 | 1.6 | .0033 | 38 | 2.6 | .0042 |
| **X** | 22 | 1.5 | .0032 | 24 | 1.6 | .0033 |
| **OTHER** | 85 | 5.8 | .0062 | 72 | 4.9 | .0057 |

**Table 4.** mtDNA rare mutations associated to diseases and their distriburion in cases and controls.

| | | Controls | | Cases | | | |
|---|---|---|---|---|---|---|---|
| **position** | mutation type | N. | % | N. | % | region | disease |
| **827** | 'A/G' | 1 | 1,39 | 0 | 0,00 | 12 S | Deafness MTRNR1 DEAF |
| **961** | T/C | 1 | 0,19 | 5 | 0,99 | 12S | Deafness |
| **1005** | 'T/C' | 1 | 1,39 | 0 | 0,00 | 12 S | Deafness MTRNR1 DEAF |
| **1438** | A/G | 499 | 95,95 | 469 | 92,66 | 12S | Diabetes Mellitus |
| **1555** | A/G | 0 | 0,00 | 3 | 0,60 | 12S | Deafness |
| **3196** | G/A | 1 | 0,19 | 0 | 0,00 | 16S | Alzheimer & Parkinson Disease |
| **3308** | 'T/C' | 1 | 1,39 | 0 | 0,00 | ND1 | Encephalomyopathy, MELAS |
| **3460** | G/T | 0 | 0,00 | 1 | 0,20 | ND1 | LHON |
| **3796** | A/G | 2 | 0,39 | 5 | 0,79 | ND1 | Dystonia |
| **4295** | A/G | 9 | 1,73 | 4 | 0,79 | TI | Maternaly Inherited Hypertrophic Cardiomyopathy |
| **4336** | T/C | 5 | 0,96 | 10 | 1,79 | TQ | Alzheimer & Parkinson Disease |
| **5460** | G/A | 32 | 5,01 | 30 | 5,36 | ND2 | Alzheimer & Parkinson Disease |
| **5628** | 'T/C' | 1 | 1,39 | 0 | 0,00 | tRNA A | Mitochondrial Myopathy, CPEO MTTA CPEO |
| **5843** | A/G | 1 | 0,19 | 0 | 0,00 | TY | Mitochondrial Myopathy Cytopathy |
| **6489** | C/A | 4 | 0,77 | 1 | 0,20 | CO1 | Epilepsy |
| **7444** | G/A | 4 | 0,77 | 4 | 0,79 | CO1 | Deafness-Sensory Neural Hearing Loss |
| **8108** | A/G | 0 | 0,00 | 1 | 0,20 | CO1 | Deafness-Sensory Neural Hearing Loss |
| **8302** | 'A/G' | 0 | 0,00 | 1 | 1,39 | tRNA k | MTTK Encephalopathy |
| **8993** | T/C | 1 | 0,19 | 0 | 0,00 | ATP6 | Leigh Syndrome |
| **10044** | A/G | 7 | 1,35 | 3 | 0,60 | TG | Encephalomyopathy Gastrointestinal Reflux and Sudden Infant Death Syndrome |
| **11084** | A/G | 1 | 0,19 | 0 | 0,00 | ND4 | Encephalomyopathy, MELAS |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **12026** | A/G | 0 | 0,00 | 1 | 0,20 | ND4 | Diabetes Mellitus |
| **12192** | G/A | 0 | 0,00 | 2 | 0,40 | TH | Maternally Inherited Cardiomyopathy |
| **12308** | A/G | 140 | 23,31 | 105 | 18,45 | TL2 | Mitochondrial Myopathy, CPEO |
| **15497** | G/A | 2 | 0,39 | 1 | 0,20 | CYTB | MM, Execise Intolerance |
| **15498** | G/A | 0 | 0,00 | 2 | 0,40 | CYTB | Hypertrophic Cardiomyopathy |
| **15924** | A/G | 13 | 2,50 | 22 | 4,37 | TT | Mitochondrial Myopathy Lethal Infantile Mitochondrial Myopathy |
| **15940** | T/- | 1 | 0,19 | 1 | 0,20 | TT | Mitochondrial Myopathy |

**Table 5.** mtDNA mutations in tRNAthr genes associated to disease.

| Location | Disease | Mutation |
|---|---|---|
| **tRNA Thr** | DEAF helper mut. | T15908C |
| **tRNA Thr** | Encephalomyopathy | G15915A |
| **tRNA Thr** | Lethal Infantile Mitochondrial Myopathy | A15923G |
| **tRNA Thr** | Lethal Infantile Mitochondrial Myopathy | A15924G |
| **tRNA Thr** | Multiple Sclerosis/DEAF1555 increased penetrance | G15927A |
| **tRNA Thr** | Multiple Sclerosis | G15928A |
| **tRNA Thr** | Dopaminergic nerve cell deat (PD) | G15950A |
| **tRNA Thr** | LHON modulator | A15951G |

**Table 6.** Samples with almost 2 mutation in complex I and III.

| Population (n. total sample) | n. sample | % |
|---|---|---|
| Danmark controls (394) | 150 | 38,07 |
| Finland controls (125) | 30 | 24,00 |
| South Europe controls (72) | 34 | 47,22 |
| Danmark cases (377) | 125 | 33,16 |
| Finland cases (127) | 42 | 33,07 |
| South Europe cases (72) | 22 | 30,56 |

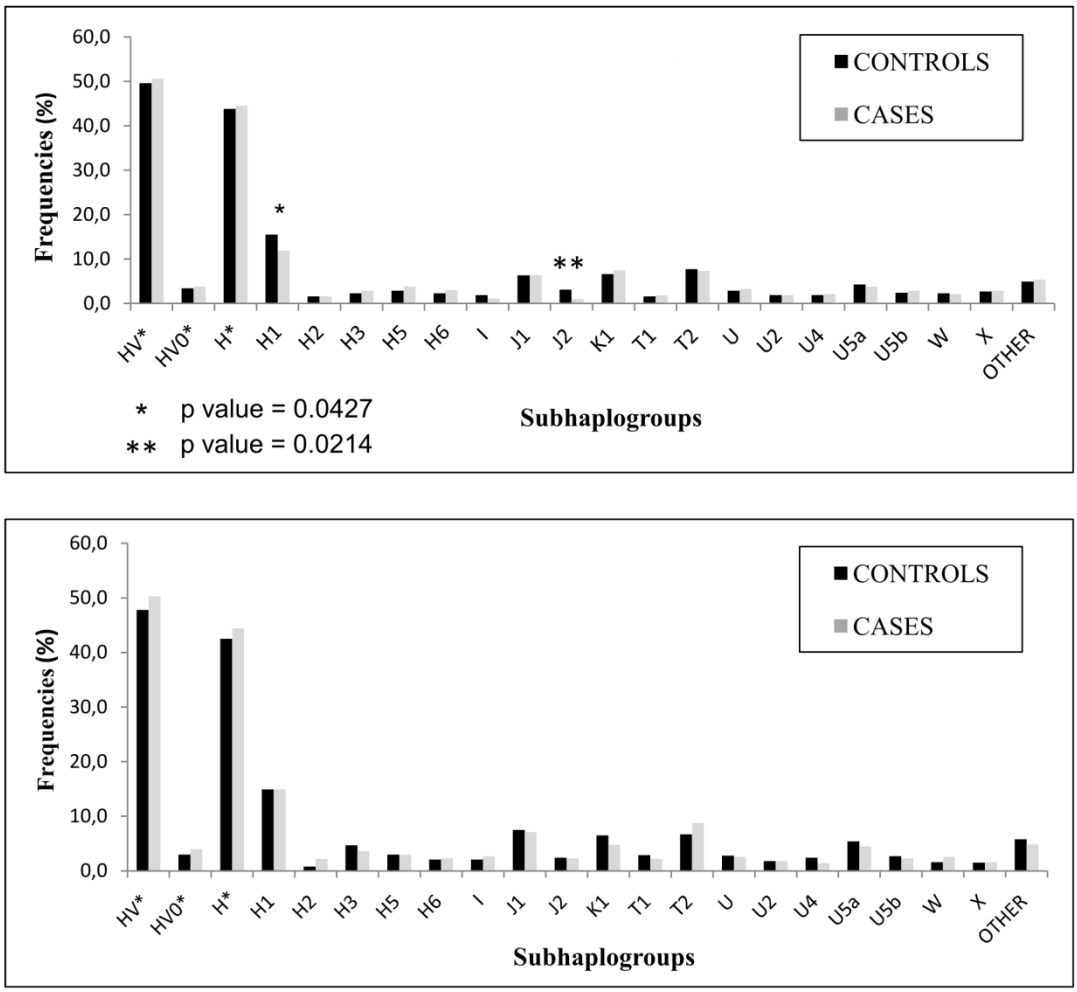


**Figure 1** Haplogroups distribution in male (upper table) and female (inferior table).

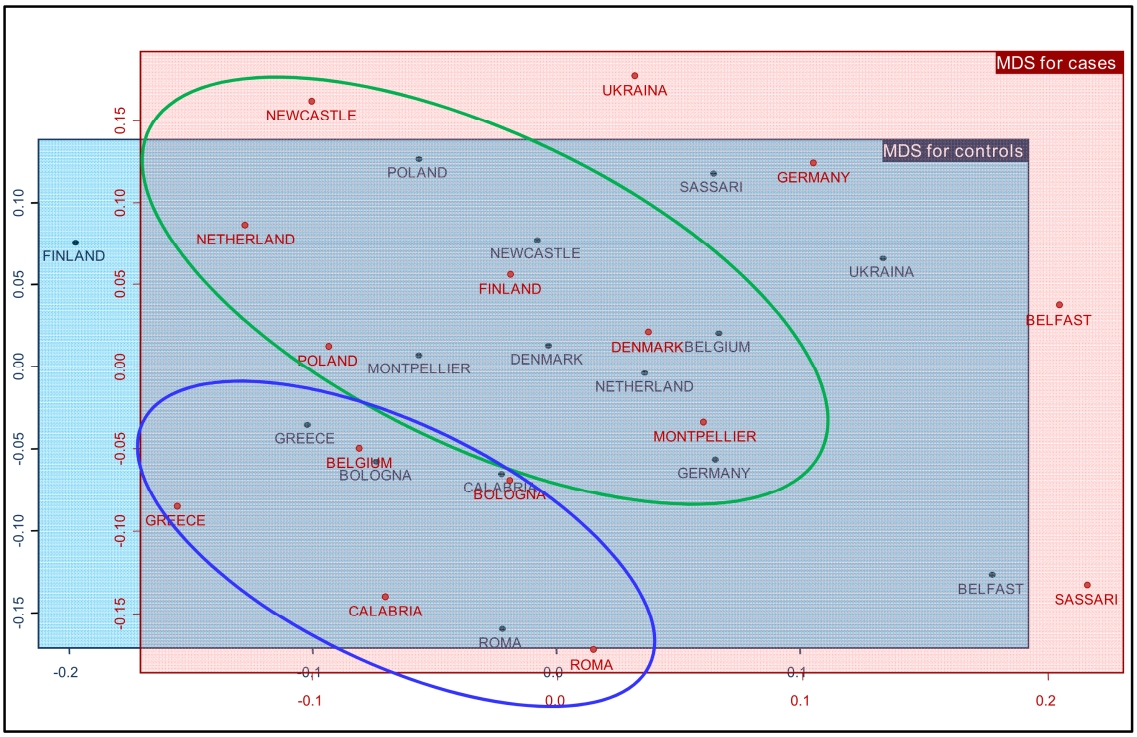


**Figure 2** Multidimensional Scaling (MDS) for cases and controls.

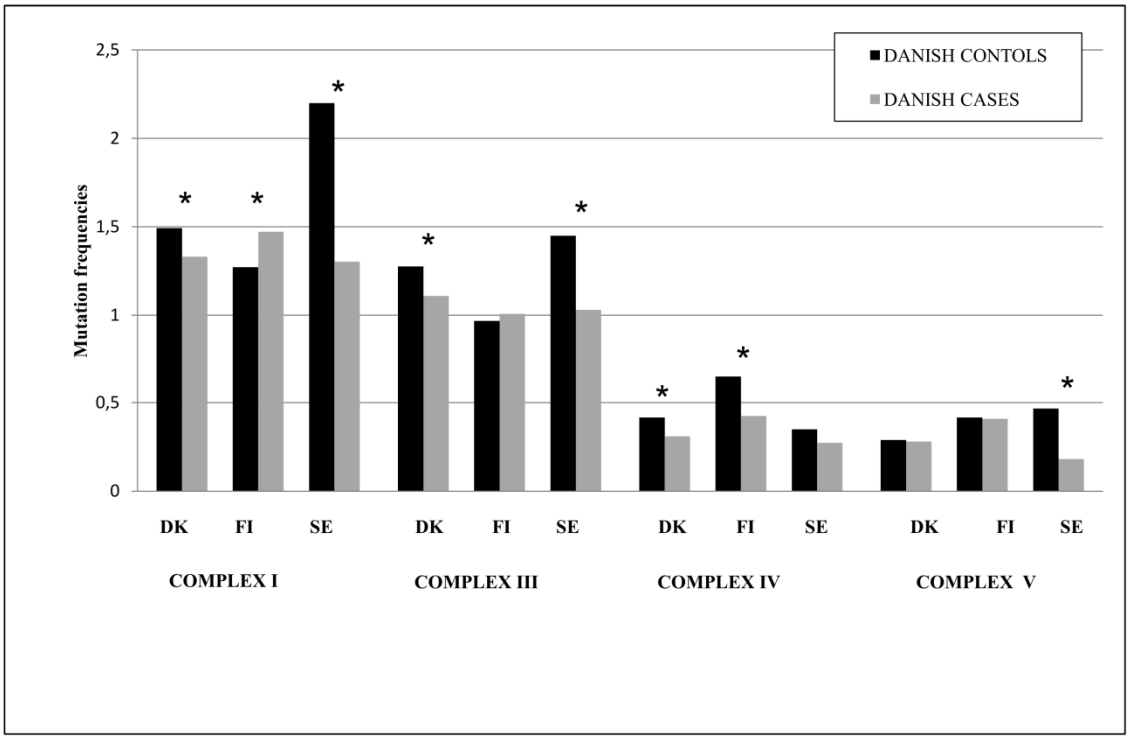


**Figure 3** Non synonimous mutation frequencies in cases and controls in complexes I-V. * indicate statistic significative differences between case and controls ($p<0,05$).

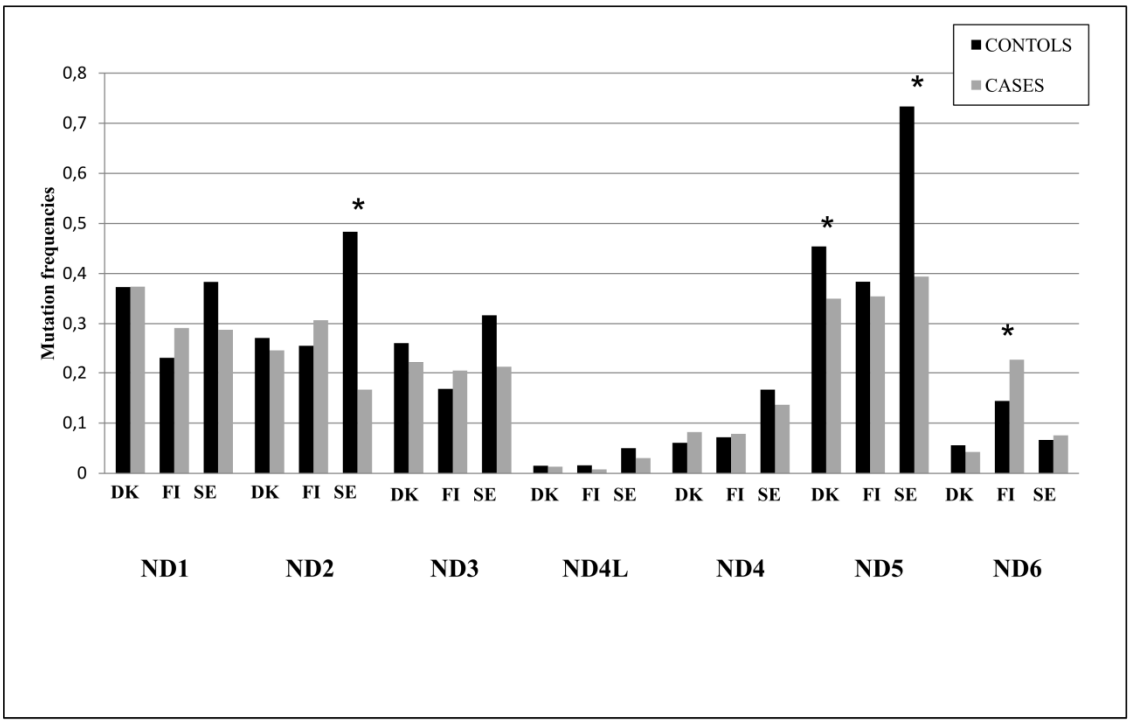


**Figure 4** Non synonimous mutation frequencies in complex I genes.
* indicate statistic significative differences between case and controls ($p<0,05$).

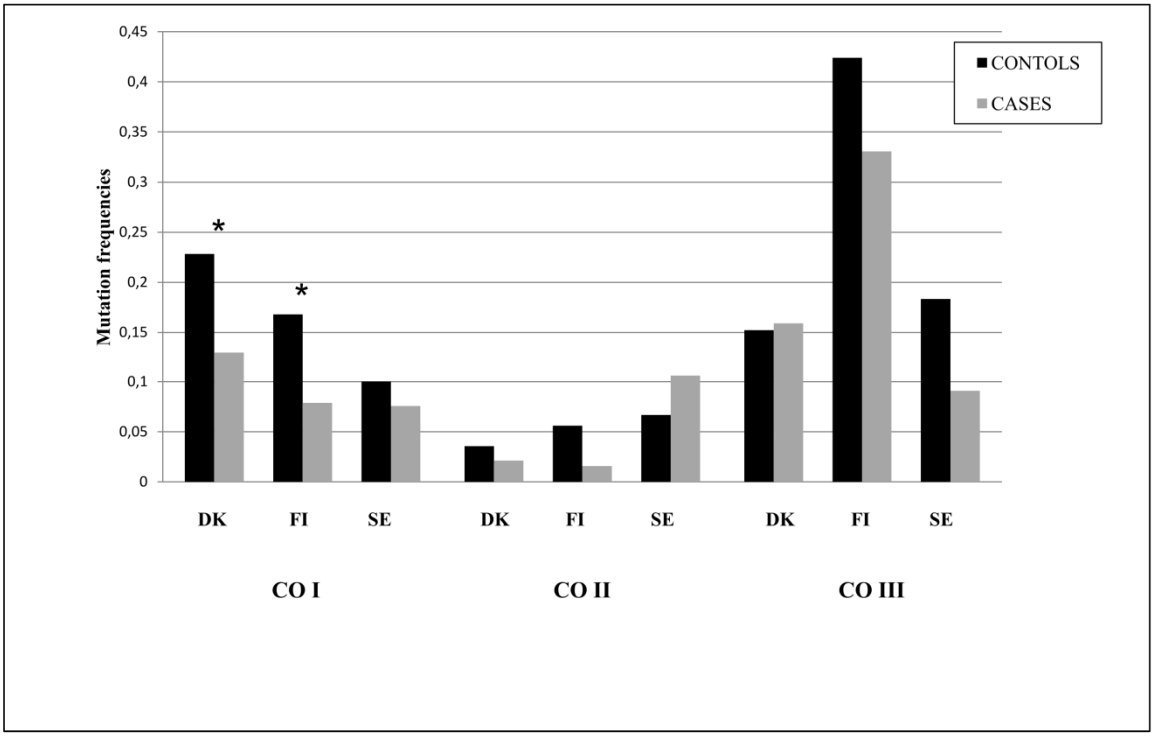


**Figure 5** Non synonimous mutation frequencies in complex IV genes.
* indicate statistic significative differences between case and controls ($p<0,05$).

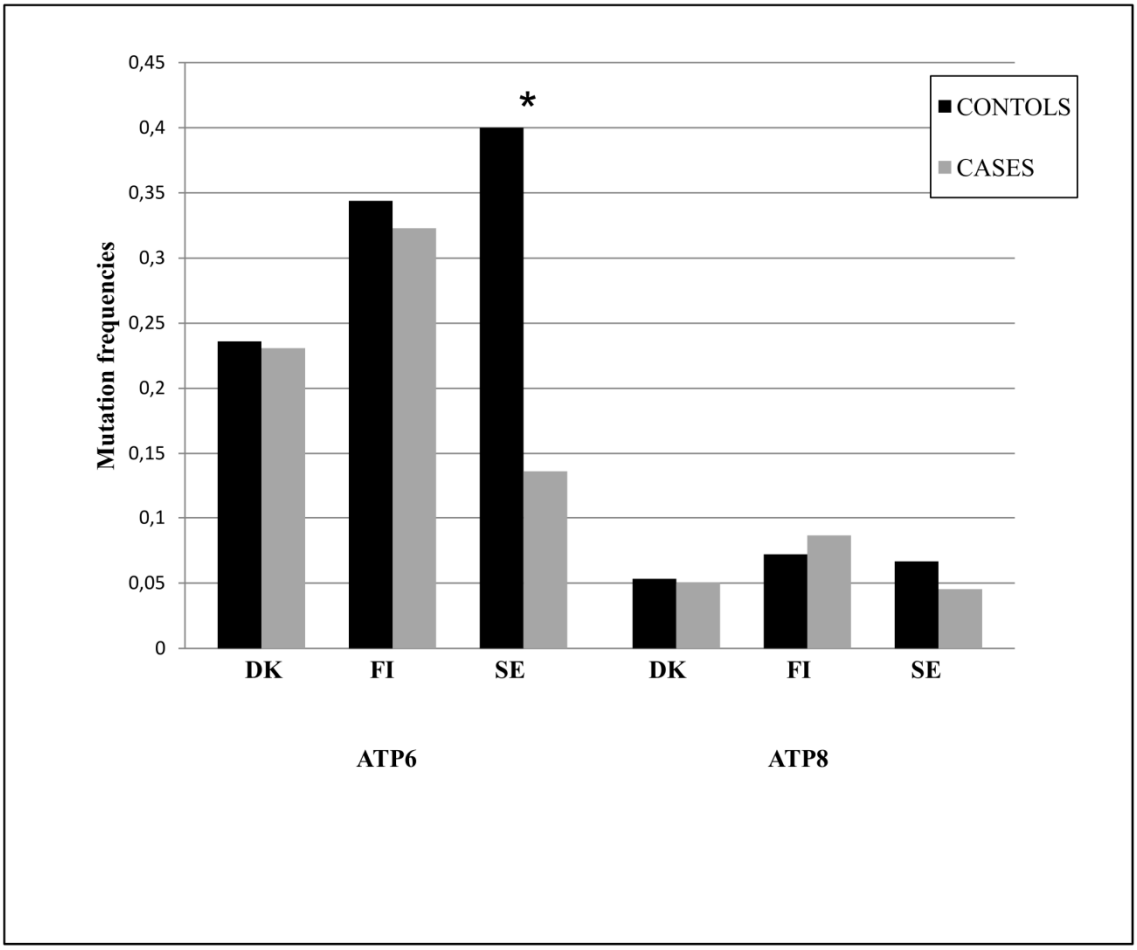


**Figure 8** Non synonimous mutation frequencies in complex V genes.
* indicate statistic significative differences between case and controls ($p<0,05$).

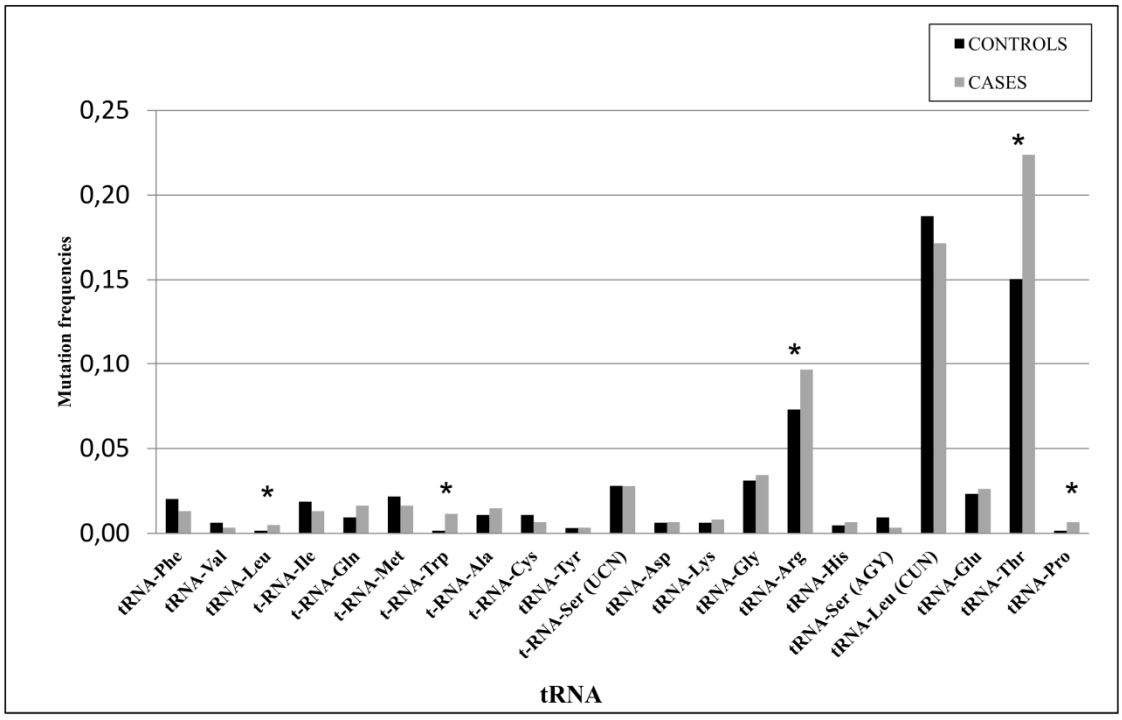


**Figure 79** tRNA mutation's frequencies (all samples).
* indicate statistic significative differences between case and controls ($p<0,05$).

**Table 1S**: Distribution of cases and controls in each country.

| Recruitment center | Country | No. 90+ | No. younger controls |
|---|---|---|---|
| Belfast | Northern Ireland | 64 | 64 |
| Louvain | Belgium | 80 | 88 |
| Bologna | Italy | 213 | 214 |
| Kiel | Germany | 94 | 96 |
| Tampere | Finland | 153 | 145 |
| Montpellier | France | 274 | 275 |
| Newcastle | UK | 99 | 100 |
| Odense | Denmark | 428 | 441 |
| Leiden | Netherland | 162 | 167 |
| Warsaw | Poland | 129 | 132 |
| Roma | Italy | 75 | 75 |
| Sassari | Italy | 47 | 52 |
| Kiev | Ukraina | 49 | 49 |
| Calabria | Italy | 125 | 152 |
| Athens | Greece | 94 | 103 |
| **Total** | | **2,086** | **2,153** |

**Table 2S**: Modified primer pairs for the complete resequencing (M13 tag is not reported).

| | **Forward** | **Reverse** |
|---|---|---|
| **Primer 41b** | 5'- CTG CTT CTT CTC ACA TGA-3' | 5'- TAG GTA GGA GTA GCG TG-3' |
| **Primer 43b** | 5'- ATC AAT TGG CTT CCT AGG-3' | 5'- GGC ATC CAT ATA GTC ACT-3' |
| **Primer 44b** | 5'- CCT TAT CTG CTT CCT AGT-3' | 5'-GGG TGC TAT AGG GTA AAT -3' |
| **Primer 45b** | 5'- ATT TTG ACT ACC ACA ACT CA-3' | 5'-ATA TGT GTT GGA GAT TGA GA -3' |

**Table 3S a.** List of the mtDNA mutations with the most significant differences between cases and controls in Danes.

| Position | Region | Controls (N= 394) | Cases (N= 377) | OR | P- value |
|---|---|---|---|---|---|
| **73** | Control-Region | 232 | 182 | 0,652 | 0,003 |
| **150** | Control-Region | 36 | 18 | 0,499 | 0,020 |
| **195** | Control-Region | 79 | 52 | 0,638 | 0,021 |
| **263** | Control-Region | 5 | 16 | 3,448 | 0,017 |
| **389** | Control-Region | 5 | 15 | 3,223 | 0,025 |
| **524** | Control-Region | 12 | 3 | 0,255 | 0,036 |
| **930** | rRNA | 10 | 24 | 2,611 | 0,012 |
| **2706** | rRNA | 224 | 187 | 0,747 | 0,044 |
| **3480** | ND1 | 35 | 19 | 0,544 | 0,039 |
| **4580** | ND2 | 10 | 22 | 2,380 | 0,026 |
| **4769** | ND2 | 14 | 26 | 2,010 | 0,040 |
| **7768** | CO2 | 12 | 3 | 0,255 | 0,036 |
| **8860** | ATP6 | 5 | 15 | 3,223 | 0,025 |
| **10550** | ND4L | 34 | 18 | 0,531 | 0,035 |
| **11299** | ND4 | 33 | 18 | 0,549 | 0,047 |
| **11467** | ND4 | 87 | 55 | 0,603 | 0,008 |
| **11719** | ND4 | 211 | 167 | 0,690 | 0,010 |
| **12308** | tRNA | 86 | 59 | 0,664 | 0,029 |
| **12372** | ND5 | 87 | 59 | 0,655 | 0,023 |
| **14167** | ND6 | 35 | 19 | 0,544 | 0,039 |
| **14766** | CYB | 210 | 160 | 0,646 | 0,003 |
| **14798** | CYB | 75 | 47 | 0,606 | 0,013 |
| **15326** | CYB | 6 | 16 | 2,866 | 0,030 |
| **15904** | tRNA | 10 | 23 | 2,495 | 0,018 |
| **16224** | Control-Region | 34 | 18 | 0,531 | 0,035 |
| **16304** | Control-Region | 20 | 36 | 1,974 | 0,019 |

**Table 3S b.** List of the mtDNA mutations with the most significant differences between cases and controls in Finns.

| Position | Region | Controls (N. 125) | Cases (N. 127) | OR | P-value |
|---|---|---|---|---|---|
| **72** | Control-Region | 15 | 3 | 0,178 | 0,007 |
| **185** | Control-Region | 1 | 8 | 8,305 | 0,048 |
| **462** | Control-Region | 1 | 7 | 7,207 | 0,067 |
| **524** | Control-Region | 2 | 10 | 5,247 | 0,035 |
| **1888** | rRNA | 2 | 8 | 4,128 | 0,077 |
| **4580** | ND2 | 14 | 4 | 0,258 | 0,020 |
| **5936** | CO1 | 7 | 1 | 0,134 | 0,062 |
| **6776** | CO1 | 11 | 3 | 0,251 | 0,037 |
| **8697** | ATP6 | 2 | 8 | 4,128 | 0,077 |
| **8705** | ATP6 | 10 | 2 | 0,184 | 0,031 |
| **11251** | ND4 | 7 | 16 | 2,429 | 0,060 |
| **12705** | ND5 | 8 | 16 | 2,107 | 0,100 |
| **12811** | ND5 | 9 | 3 | 0,312 | 0,086 |
| **13708** | ND5 | 5 | 12 | 2,503 | 0,094 |
| **14182** | ND6 | 9 | 18 | 2,128 | 0,079 |
| **14793** | CYB | 14 | 4 | 0,258 | 0,020 |
| **15452** | CYB | 7 | 17 | 2,604 | 0,041 |
| **15884** | CYB | 6 | 1 | 0,158 | 0,090 |
| **15904** | tRNA | 15 | 4 | 0,239 | 0,013 |
| **16189** | Control-Region | 19 | 34 | 2,039 | 0,026 |
| **16192** | Control-Region | 15 | 6 | 0,364 | 0,043 |
| **16193** | Control-Region | 3 | 9 | 3,099 | 0,096 |
| **16256** | Control-Region | 14 | 4 | 0,258 | 0,020 |
| **16298** | Control-Region | 16 | 4 | 0,222 | 0,009 |
| **16526** | Control-Region | 7 | 1 | 0,134 | 0,062 |

**Table 3S c.** List of the mtDNA mutations with the most significant differences between cases and controls in Southern European Population.

| Position | **Region** | **Controls (N. 72)** | **Cases (N. 72)** | **OR** | **P-value** |
|---|---|---|---|---|---|
| 73 | Control-Region | 43 | 34 | 0,604 | 0,134 |
| 1811 | rRNA | 12 | 5 | 0,374 | 0,079 |
| 2706 | rRNA | 49 | 37 | 0,496 | 0,043 |
| 7028 | CO1 | 48 | 36 | 0,500 | 0,044 |
| 11719 | ND4 | 43 | 31 | 0,510 | 0,047 |
| 14766 | CYB | 44 | 32 | 0,509 | 0,046 |
| 15607 | CYB | 8 | 3 | 0,348 | 0,131 |
| 16189 | Control-Region | 11 | 19 | 1,987 | 0,104 |

**Table 4S a.** PolyPhen prediction of functional effect of the observed mtDNA mutations in Danish.

| Position | Mutation | Controls | Cases | Amino acid substitution | Substitution effect | Prediction data |
|---|---|---|---|---|---|---|
| **ND1** | | | | | | |
| 3335 | 'T/C' | 1 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 3337 | 'G/A' | 0 | 2 | V-M | benign | |
| 3338 | 'T/C' | 1 | 1 | V-A | benign | |
| 3350 | 'T/C' | 0 | 4 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 3355 | 'A/G' | 3 | 1 | M-V | benign | |
| 3388 | 'C/A' | 0 | 1 | L-M | possibly damaging | PSIC score difference: 1.515 |
| 3394 | 'T/C' | 11 | 4 | Y-H | benign | |
| 3398 | 'T/C' | 0 | 2 | M-T | benign | |
| 3434 | 'A/G' | 1 | 0 | Y-C | benign | |
| 3460 | 'G/T' | 0 | 1 | A-S | benign | |
| 3487 | 'C/A' | 0 | 1 | L-M | benign | |
| 3496 | 'G/A' | 1 | 1 | A-T | benign | |
| 3505 | 'A/G' | 4 | 6 | T-A | benign | |
| 3511 | 'A/G' | 0 | 1 | T-A | benign | |
| 3565 | 'A/G' | 0 | 1 | T-A | benign | |
| 3613 | 'C/A' | 1 | 0 | L-M | benign | |
| 3616 | 'T/G' | 1 | 0 | F-V | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 3617 | 'T/G' | 1 | 0 | F-C | benign | |
| 3626 | 'C/T' | 0 | 1 | A-V | benign | |
| 3644 | 'T/C' | 0 | 2 | V-A | benign | |
| 3745 | 'G/A' | 0 | 3 | A-T | benign | |
| 3746 | 'C/T' | 1 | 0 | A-L | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 3760 | 'T/G' | 1 | 0 | S-A | benign | |
| 3796 | 'A/G' | 0 | 3 | T-A | benign | |
| 3803 | 'T/C' | 0 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3890 | 'G/T' | 1 | 0 | R-L | possibly damaging | PSIC score difference: 1.847 |
| 3892 | 'A/T' | 1 | 0 | T-S | benign | |
| 3991 | 'A/G' | 0 | 1 | T-A | benign | |
| 3992 | 'C/T' | 12 | 9 | T-M | benign | |
| 4024 | 'A/G' | 7 | 4 | T-A | benign | |
| 4025 | 'C/T' | 0 | 1 | T-M | benign | |
| 4029 | 'C/A' | 0 | 1 | I-M | benign | |
| 4216 | 'T/C' | 97 | 87 | Y-H | benign | |
| 4232 | 'T/C' | 2 | 0 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 4246 | 'A/C' | 0 | 1 | I-L | benign | |
| **ND2** | | | | | | |
| 4491 | 'G/A' | 4 | 1 | V-I | benign | |
| 4543 | 'A/G' | 1 | 0 | H-R | Improper substitution in the transmembrane region | PHAT matrix element difference: -4 |
| 4544 | 'C/G' | 1 | 0 | H-Q | benign | |
| 4561 | 'T/C' | 3 | 4 | V-A | benign | |
| 4629 | 'G/A' | 0 | 1 | E-K | probably damaging | PSIC score difference: 2.243 |
| 4639 | 'T/C' | 1 | 2 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 4640 | 'C/A' | 1 | 1 | I-M | benign | |
| 4732 | 'A/G' | 5 | 2 | N-S | benign | |
| 4763 | 'C/A' | 1 | 1 | I-M | benign | |
| 4767 | 'A/G' | 1 | 0 | M-V | benign | |
| 4824 | 'A/G' | 5 | 1 | T-A | benign | |
| 4917 | 'A/G' | 37 | 46 | N-D | benign | |
| 4920 | 'G/A' | 1 | 0 | V-M | benign | |
| 4924 | 'G/C' | 0 | 1 | S-N | benign | |
| 4924 | 'G/A' | 5 | 1 | S-T | benign | |
| 4960 | 'C/T' | 1 | 0 | A-V | benign | |
| 5046 | 'G/A' | 4 | 6 | V-I | benign | |
| 5095 | 'T/C' | 1 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 5262 | 'G/A' | 1 | 0 | A-T | benign | |
| 5263 | 'C/T' | 2 | 1 | A-V | benign | |
| 5277 | 'T/C' | 4 | 1 | F-L | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5290 | 'A/G' | 1 | 0 | N-S | benign | |
| 5302 | 'T/C' | 1 | 0 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 5307 | 'A/G' | 1 | 0 | T-A | benign | |
| 5310 | 'A/G' | 0 | 1 | I-V | benign | |
| 5319 | 'A/G' | 1 | 1 | T-S | benign | |
| 5322 | 'A/C' | 1 | 1 | I-L | benign | |
| 5367 | 'A/G' | 1 | 0 | T-A | benign | |
| 5427 | 'A/G' | 1 | 0 | T-A | benign | |
| 5437 | 'C/T' | 0 | 1 | T-I | benign | |
| 5440 | 'C/T' | 1 | 0 | P-L | benign | |
| 5460 | 'G/A' | 20 | 18 | A-T | benign | |
| **CO1** | | | | | | |
| 5913 | 'G/A' | 7 | 4 | D-M | benign | |
| 5935 | 'A/T' | 1 | 0 | N-I | probably damaging | PSIC score difference: 3.231 |
| 5936 | 'C/A' | 20 | 10 | N-K | probably damaging | PSIC score difference: 2.556 |
| 6019 | 'C/G' | 1 | 0 | A-G | benign | |
| 6024 | 'C/A' | 1 | 0 | L-M | benign | |
| 6025 | 'T/A' | 1 | 0 | L-Q | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 6027 | 'G/A' | 1 | 0 | G-S | benign | |
| 6040 | 'A/G' | 0 | 2 | N-S | benign | |
| 6253 | 'T/C' | 1 | 5 | M-T | benign | |
| 6261 | 'G/A' | 2 | 3 | A-T | benign | |
| 6345 | 'T/C' | 0 | 1 | F-L | benign | |
| 6366 | 'G/A' | 1 | 0 | V-I | benign | |
| 6436 | 'A/C' | 2 | 0 | Q-P | benign | |
| 6439 | 'A/C' | 1 | 0 | Y-S | Disruption of ligand binding site | PSIC score difference: 1.099 ligand name: THR distance: 2.846 Å |
| 6445 | 'C/A' | 3 | 0 | T-K | Hydrophobicity change at buried site | PSIC score difference: 1.759 normed accessibility: 0.00 hydrophobicity change: 3.57 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6445 | 'C/T' | 2 | 1 | T-M | Hydrophobicity change at buried site | PSIC score difference: 0.938 normed accessibility: 0.00 hydrophobicity change: 1.02 |
| 6447 | 'C/T' | 6 | 0 | P-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 6448 | 'C/A' | 7 | 1 | P-H | Improper substitution in the transmembrane region | PHAT matrix element difference: -6 |
| 6465 | 'G/A' | 0 | 5 | V-I | benign | |
| 6489 | 'C/A' | 4 | 1 | L-I | benign | |
| 6546 | 'C/T' | 1 | 1 | L-F | benign | |
| 6762 | 'G/C' | 1 | 0 | V-L | benign | |
| 6909 | 'G/C' | 1 | 0 | A-P | benign | |
| 6915 | 'G/A' | 2 | 0 | V-M | benign | |
| 6996 | 'A/G' | 1 | 0 | I-V | benign | |
| 7021 | 'T/G' | 0 | 1 | V-G | probably damaging | PSIC score difference: 2.839 |
| 7023 | 'G/A' | 0 | 1 | V-M | benign | |
| 7024 | 'T/G' | 1 | 0 | V-G | possibly damaging | PHAT matrix element difference: -2 |
| 7041 | 'G/A' | 0 | 1 | V-I | benign | |
| 7059 | 'G/C' | 0 | 1 | V-L | benign | |
| 7060 | 'T/C' | 3 | 0 | V-A | benign | |
| 7063 | 'T/A' | 2 | 2 | F-Y | benign | |
| 7063 | 'T/G' | 0 | 1 | F-C | benign | |
| 7101 | 'T/C' | 1 | 0 | F-L | benign | |
| 7119 | 'G/A' | 1 | 1 | D-N | benign | |
| 7149 | 'A/G' | 0 | 1 | I-V | benign | |
| 7245 | 'A/G' | 1 | 0 | T-A | Hydrophobicity change at buried site | PSIC score difference: 0.719 normed accessibility: 0.00 hydrophobicity change: 1.08 |
| 7269 | 'G/A' | 1 | 1 | V-M | benign | |
| 7270 | 'T/C' | 1 | 1 | V-A | benign | |
| 7299 | 'A/G' | 1 | 1 | M-V | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 7372 | 'T/C' | 1 | 0 | M-T | benign | |
| 7419 | 'G/T' | 1 | 0 | E-Ter | | |
| 7419 | 'G/C' | 3 | 0 | E-Q | benign | |
| 7420 | 'A/T' | 1 | 0 | E-V | benign | |
| 7444 | 'G/A' | 3 | 3 | Ter-K | | |
| **CO2** | | | | | | |
| 7598 | 'G/A' | 2 | 0 | A-T | benign | |
| 7604 | 'G/A' | 3 | 0 | V-M | benign | |
| 7694 | 'C/A' | 1 | 0 | L-M | benign | |
| 7724 | 'A/G' | 0 | 1 | T-A | benign | |
| 7754 | 'G/A' | 1 | 0 | D-N | benign | |
| 7763 | 'G/T' | 0 | 1 | E-Ter | | |
| 7775 | 'G/A' | 1 | 0 | V-I | benign | |
| 7805 | 'G/A' | 2 | 1 | V-I | benign | |
| 7853 | 'G/A' | 0 | 2 | V-I | benign | |
| 7941 | 'A/G' | 2 | 0 | N-S | benign | |
| 7979 | 'G/A' | 1 | 0 | D-N | benign | |
| 8022 | 'T/C' | 1 | 0 | I-T | benign | |
| 8108 | 'A/G' | 0 | 1 | I-V | benign | |
| 8256 | 'T/C' | 0 | 2 | V-A | benign | |
| **ATP8** | | | | | | |
| 8388 | 'T/C' | 1 | 1 | V-A | benign | |
| 8393 | 'C/T' | 2 | 1 | P-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 8417 | 'C/G' | 0 | 1 | L-V | benign | |
| 8432 | 'A/C' | 0 | 1 | I-L | benign | |
| 8448 | 'T/C' | 6 | 4 | M-T | benign | |
| 8462 | 'T/C' | 0 | 1 | Y-H | benign | |
| 8472 | 'C/T' | 1 | 0 | P-L | probably damaging | PSIC score difference: 2.434 |
| 8483 | 'A/G' | 0 | 1 | K-E | possibly damaging | PSIC score difference: 1.735 |
| 8492 | 'A/G' | 1 | 1 | K-E | benign | |
| 8504 | 'T/C' | 1 | 0 | Y-H | benign | |
| 8519 | 'G/A' | 1 | 1 | E-K | benign | |
| 8535 | 'A/T' | 0 | 1 | K-M | probably damaging | PSIC score difference: 2.191 |
| 8558 | 'C/T' | 3 | 3 | P-S | probably damaging | PSIC score difference: 2.506 |
| 8567 | 'T/C' | 2 | 2 | S-P | possibly damaging | PSIC score difference: 1.924 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8572 | 'G/C' | 3 | 1 | Ter-Y | | |
| **ATP6** | | | | | | |
| 8535 | A/T | 0 | 1 | E-D | possibly damaging | PSIC score difference: 1.594 |
| 8545 | G/A | 0 | 1 | A-T | benign | |
| 8557 | G/A | 8 | 3 | A-T | benign | |
| 8558 | C/T | 3 | 3 | A-V | benign | |
| 8567 | T/C | 2 | 2 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 8572 | G/A | 1 | 2 | G-S | benign | |
| 8572 | G/C | 3 | 1 | G-A | benign | |
| 8573 | 'G/A' | 2 | 2 | G-D | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 8584 | 'G/A' | 1 | 0 | A-T | benign | |
| 8602 | 'T/C' | 0 | 4 | F-L | benign | |
| 8604 | 'T/A' | 0 | 1 | F-L | benign | |
| 8616 | 'G/T' | 0 | 4 | L-F | benign | |
| 8618 | 'T/C' | 0 | 2 | I-T | benign | |
| 8648 | 'G/C' | 0 | 1 | R-P | benign | |
| 8684 | 'C/T' | 0 | 1 | T-I | benign | |
| 8696 | 'T/C' | 0 | 1 | M-T | possibly damaging | PSIC score difference: 1.594 |
| 8701 | 'A/G' | 1 | 1 | T-A | benign | |
| 8705 | 'T/C' | 5 | 2 | M-T | benign | |
| 8752 | 'A/G' | 1 | 0 | I-V | benign | |
| 8762 | 'T/C' | 0 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 8765 | 'C/T' | 1 | 1 | A-V | benign | |
| 8794 | 'C/T' | 5 | 1 | H-Y | benign | |
| 8839 | 'G/A' | 2 | 0 | A-T | benign | |
| 8843 | 'T/C' | 0 | 2 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 8857 | 'G/A' | 1 | 0 | G-S | benign | |
| 8860 | 'A/G' | 5 | 15 | T-A | benign | |
| 8869 | 'A/G' | 1 | 2 | M-V | benign | |
| 8870 | 'T/C' | 1 | 0 | M-T | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8881 | 'T/G' | 0 | 1 | S-A | benign | |
| 8887 | 'A/G' | 1 | 1 | I-V | benign | |
| 8902 | 'G/A' | 1 | 0 | A-T | benign | |
| 8944 | 'A/G' | 0 | 1 | M-V | benign | |
| 8950 | 'G/A' | 0 | 1 | V-I | benign | |
| 8993 | T/C | 1 | 0 | L-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 9007 | 'A/G' | 0 | 1 | T-A | possibly damaging | PSIC score difference: 1.501 |
| 9038 | 'T/C' | 1 | 1 | M-T | benign | |
| 9052 | 'A/G' | 1 | 0 | S-G | benign | |
| 9053 | 'G/A' | 3 | 0 | S-N | benign | |
| 9055 | 'G/A' | 34 | 19 | A-T | benign | |
| 9070 | 'T/G' | 1 | 0 | S-A | benign | |
| 9073 | 'A/G' | 1 | 0 | T-A | benign | |
| 9098 | 'T/C' | 1 | 2 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 9098 | 'T/G' | 0 | 2 | I-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 9116 | 'T/C' | 0 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 9145 | 'G/A' | 1 | 3 | A-T | benign | |
| 9151 | 'A/G' | 1 | 0 | I-V | benign | |
| 9181 | 'A/G' | 2 | 0 | S-G | benign | |
| 9196 | 'G/A' | 1 | 0 | D-M | probably damaging | PSIC score difference: 2.148 |
| **CO3** | | | | | | |
| 9210 | 'A/G' | 0 | 2 | T-A | benign | |
| 9300 | 'G/A' | 0 | 1 | A-T | benign | |
| 9325 | 'T/C' | 0 | 1 | M-T | benign | |
| 9438 | 'G/A' | 0 | 1 | G-S | benign | |
| 9469 | 'C/T' | 0 | 2 | T-I | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 9477 | 'G/A' | 35 | 22 | V-I | benign | |
| 9500 | 'C/G' | 0 | 1 | F-L | benign | |
| 9591 | 'G/A' | 1 | 0 | V-I | benign | |
| 9592 | 'T/C' | 1 | 0 | V-A | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9612 | 'G/A' | 1 | 0 | V-M | benign | |
| 9664 | 'A/G' | 0 | 3 | E-G | benign | |
| 9667 | 'A/G' | 4 | 4 | N-S | benign | |
| 9670 | 'A/G' | 2 | 0 | N-S | benign | |
| 9682 | 'T/C' | 1 | 0 | M-T | benign | |
| 9741 | 'T/C' | 1 | 0 | S-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 9756 | 'T/G' | 0 | 1 | S-A | benign | |
| 9783 | 'T/A' | 1 | 1 | Y-N | probably damaging | PSIC score difference: 3.213 |
| 9784 | 'A/C' | 1 | 1 | Y-S | probably damaging | PSIC score difference: 3.174 |
| 9790 | 'C/T' | 4 | 4 | S-L | probably damaging | PSIC score difference: 2.277 |
| 9804 | 'G/A' | 2 | 2 | A-T | benign | |
| 9843 | 'A/G' | 1 | 0 | T-A | benign | |
| 9857 | 'C/G' | 1 | 0 | I-M | benign | |
| 9866 | 'C/G' | 0 | 2 | I-M | benign | |
| 9887 | 'T/C' | 0 | 2 | F-S | probably damaging | PSIC score difference: 2.951 |
| 9912 | 'G/C' | 0 | 1 | E-Q | Disruption of ligand binding site | PSIC score difference: 1.856 ligand name: ZN distance: 2.102 Å |
| 9913 | 'A/C' | 0 | 1 | E-A | Disruption of ligand binding site | PSIC score difference: 2.402 ligand name: ZN distance: 2.102 Å |
| 9915 | 'G/T' | 0 | 2 | A-T | possibly damaging | PSIC score difference: 1.960 |
| 9921 | 'G/A' | 1 | 2 | A-T | benign | |
| 9948 | 'G/A' | 1 | 1 | V-I | benign | |
| 9966 | 'G/A' | 2 | 2 | V-I | benign | |
| **ND3** | | | | | | |
| 10084 | 'T/C' | 1 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 10111 | 'T/A' | 0 | 2 | M-K | Improper | PHAT matrix |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | substitution in the transmembrane region | element difference: -6 |
| 10116 | 'A/G' | 0 | 1 | I-V | benign | |
| 10188 | 'A/G' | 0 | 1 | M-V | benign | |
| 10192 | 'C/T' | 6 | 11 | S-F | possibly damaging | PSIC score difference: 1.534 |
| 10243 | 'T/C' | 1 | 0 | F-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 10336 | 'T/C' | 1 | 1 | L-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 10370 | 'T/G' | 1 | 0 | Y-Ter | | |
| 10398 | 'A/G' | 92 | 67 | T-A | benign | |
| 10399 | 'C/G' | 1 | 0 | T-S | benign | |
| **ND4L** | | | | | | |
| 10506 | 'A/G' | 2 | 1 | T-A | benign | |
| 10554 | 'T/G' | 0 | 1 | S-A | benign | |
| 10555 | 'C/G' | 0 | 1 | S-C | benign | |
| 10609 | 'T/C' | 1 | 0 | M-T | benign | |
| 10750 | 'A/G' | 3 | 2 | N-S | benign | |
| **ND4** | | | | | | |
| 10799 | 'C/G' | 0 | 1 | L-V | benign | |
| 10863 | 'G/A' | 1 | 0 | S-N | benign | |
| 10877 | 'C/G' | 0 | 1 | L-V | benign | |
| 10877 | 'C/A' | 2 | 3 | L-M | benign | |
| 10878 | 'T/C' | 2 | 0 | L-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 10878 | 'T/G' | 0 | 1 | L-R | Improper substitution in the transmembrane region | PHAT matrix element difference: -6 |
| 10907 | 'T/C' | 1 | 0 | F-L | benign | |
| 10919 | 'C/T' | 0 | 1 | P-S | benign | |
| 10920 | 'C/T' | 0 | 2 | P-L | benign | |
| 10922 | 'A/C' | 0 | 1 | T-P | benign | |
| 10922 | 'A/G' | 0 | 1 | T-A | possibly damaging | PSIC score difference: 1.604 |
| 10923 | 'C/T' | 0 | 1 | T-I | benign | |
| 10997 | 'A/C' | 0 | 1 | S-R | Improper substitution in the transmembrane | PHAT matrix element difference: -6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | region | |
| 11016 | 'G/A' | 2 | 2 | S-N | benign | |
| 11025 | 'T/C' | 1 | 0 | L-P | benign | |
| 11031 | 'G/A' | 0 | 1 | R-Q | benign | |
| 11037 | 'A/C' | 0 | 1 | K-T | probably damaging | PSIC score difference: 2.047 |
| 11038 | 'A/C' | 0 | 1 | K-N | possibly damaging | PSIC score difference: 1.822 |
| 11039 | 'C/T' | 0 | 1 | L-F | benign | |
| 11040 | 'T/C' | 0 | 1 | L-P | possibly damaging | PSIC score difference: 1.646 |
| 11084 | 'A/G' | 1 | 0 | T-A | benign | |
| 11150 | 'G/A' | 0 | 1 | A-T | benign | |
| 11204 | 'T/C' | 0 | 1 | F-L | benign | |
| 11253 | 'T/C' | 5 | 3 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 11498 | 'A/C' | 1 | 0 | T-P | probably damaging | PSIC score difference: 2.007 |
| 11501 | 'C/T' | 1 | 0 | L-F | benign | |
| 11615 | 'A/C' | 1 | 0 | I-L | benign | |
| 11619 | 'C/A' | 1 | 0 | A-E | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 11696 | 'G/A' | 0 | 1 | V-I | benign | |
| 12020 | 'C/T' | 1 | 1 | H-Y | benign | |
| 12021 | 'A/C' | 1 | 0 | H-P | benign | |
| 12032 | 'A/G' | 0 | 1 | N-D | benign | |
| 12037 | 'A/T' | 1 | 0 | M-I | benign | |
| 12054 | 'G/C' | 1 | 0 | R-P | probably damaging | PSIC score difference: 2.992 |
| 12056 | 'G/A' | 1 | 0 | E-K | probably damaging | PSIC score difference: 2.194 |
| 12063 | 'C/T' | 0 | 1 | T-Y | benign | |
| **ND5** | | | | | | |
| 12337 | 'A/G' | 1 | 0 | M-V | benign | |
| 12341 | 'C/T' | 0 | 2 | T-I | benign | |
| 12346 | 'C/T' | 3 | 0 | H-Y | benign | |
| 12358 | 'A/G' | 2 | 3 | T-A | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 12397 | 'A/G' | 1 | 3 | T-A | benign | |
| 12406 | 'G/A' | 1 | 1 | V-I | benign | |
| 12454 | 'G/A' | 0 | 2 | V-I | benign | |
| 12491 | 'C/T' | 0 | 1 | T-M | benign | |
| 12509 | 'A/G' | 0 | 1 | D-G | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 12557 | 'C/T' | 1 | 4 | T-I | benign | |
| 12599 | 'T/C' | 1 | 0 | M-T | benign | |
| 12634 | 'A/G' | 3 | 0 | I-V | benign | |
| 12674 | 'A/G' | 0 | 1 | N-S | benign | |
| 12684 | 'G/C' | 1 | 0 | Q-H | benign | |
| 12730 | 'G/A' | 1 | 0 | V-I | benign | |
| 12775 | 'G/A' | 3 | 0 | V-M | benign | |
| 12776 | 'T/A' | 0 | 1 | V-E | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 12811 | 'T/C' | 1 | 0 | Y-H | benign | |
| 12903 | 'T/A' | 0 | 1 | F-L | benign | |
| 12907 | 'C/A' | 0 | 1 | L-M | benign | |
| 12927 | 'C/A' | 0 | 1 | D-E | benign | |
| 12937 | 'A/G' | 1 | 3 | M-V | benign | |
| 13063 | 'G/A' | 0 | 1 | V-I | benign | |
| 13094 | 'T/G' | 0 | 1 | V-G | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 13102 | 'G/A' | 1 | 0 | G-Ter | | |
| 13105 | 'A/C' | 6 | 1 | I-L | benign | |
| 13105 | 'A/G' | 3 | 5 | I-V | benign | |
| 13129 | 'C/A' | 1 | 0 | P-T | probably damaging | PSIC score difference: 2.626 |
| 13135 | 'G/A' | 3 | 0 | A-T | benign | |
| 13145 | 'G/A' | 4 | 6 | S-N | benign | |
| 13204 | 'G/A' | 1 | 0 | V-I | benign | |
| 13327 | 'A/G' | 0 | 1 | T-A | benign | |
| 13528 | 'A/G' | 1 | 1 | T-A | benign | |
| 13564 | 'T/C' | 0 | 1 | S-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 13565 | 'C/T' | 1 | 1 | S-F | Improper substitution in the transmembrane | PHAT matrix element difference: -2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | region | |
| 13581 | 'T/C' | 2 | 1 | A-R | Improper substitution in the transmembrane region | PHAT matrix element difference: -6 |
| 13630 | 'A/G' | 2 | 0 | T-A | benign | |
| 13637 | 'A/G' | 8 | 2 | Q-R | benign | |
| 13681 | 'A/G' | 0 | 1 | T-A | benign | |
| 13708 | 'G/A' | 68 | 47 | A-T | benign | |
| 13711 | 'G/A' | 1 | 0 | A-T | benign | |
| 13729 | 'G/A' | 0 | 1 | G-Ter | | |
| 13759 | 'G/A' | 8 | 6 | A-T | benign | |
| 13780 | 'A/G' | 6 | 9 | I-V | benign | |
| 13784 | 'C/T' | 1 | 0 | P-L | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 13879 | 'T/C' | 9 | 4 | S-P | benign | |
| 13928 | 'G/C' | 3 | 1 | S-T | benign | |
| 13933 | 'A/G' | 1 | 2 | T-A | benign | |
| 13934 | 'C/T' | 7 | 4 | T-M | benign | |
| 13948 | 'C/T' | 1 | 1 | P-S | possibly damaging | PSIC score difference: 1.571 |
| 13958 | 'G/C' | 1 | 0 | G-A | benign | |
| 13966 | 'A/G' | 10 | 7 | T-A | benign | |
| 13967 | 'C/T' | 6 | 2 | T-M | benign | |
| 14002 | 'A/G' | 3 | 1 | T-A | benign | |
| 14071 | 'A/G' | 1 | 0 | T-A | benign | |
| **ND6** | | | | | | |
| 14163 | 'C/T' | 1 | 0 | A-S | benign | |
| 14180 | 'T/C' | 1 | 1 | Y-C | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 14198 | 'G/A' | 0 | 1 | T-M | benign | |
| 14221 | 'T/C' | 1 | 0 | W-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -6 |
| 14249 | 'G/A' | 0 | 2 | A-V | benign | |
| 14258 | 'G/A' | 0 | 1 | P-L | benign | |
| 14318 | 'T/C' | 1 | 0 | N-S | benign | |
| 14319 | 'T/C' | 0 | 1 | N-D | benign | |
| 14325 | 'T/C' | 1 | 1 | N-D | benign | |
| 14405 | 'A/G' | 2 | 1 | V-A | benign | |
| 14502 | 'T/C' | 2 | 3 | I-V | benign | |
| 14552 | 'A/G' | 1 | 0 | V-A | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 14562 | 'C/T' | 0 | 1 | V-I | benign | |
| 14570 | 'C/T' | 1 | 0 | S-N | benign | |
| 14582 | 'A/G' | 8 | 4 | V-A | benign | |
| 14625 | 'C/A' | 1 | 0 | V-L | benign | |
| 14640 | 'A/T' | 1 | 0 | L-M | benign | |
| 14646 | 'C/G' | 1 | 0 | V-L | benign | |
| **Cytb** | | | | | | |
| 14748 | 'T/G' | 0 | 1 | M-Ter | | |
| 14750 | 'A/C' | 0 | 1 | T-P | possibly damaging | PSIC score difference: 1.946 |
| 14751 | 'C/A' | 0 | 1 | T-N | benign | |
| 14759 | 'C/A' | 2 | 3 | R-S | Disruption of ligand binding site | PSIC score difference: 2.448 ligand name: CDL distance: 2.681 Å |
| 14760 | 'G/A' | 0 | 1 | R-H | Disruption of ligand binding site | PSIC score difference: 2.311 ligand name: CDL distance: 2.681 Å |
| 14762 | 'A/G' | 3 | 4 | K-E | benign | |
| 14763 | 'A/G' | 1 | 2 | K-Ter | benign | |
| 14766 | 'C/T' | 210 | 160 | T-I | benign | |
| 14793 | 'A/G' | 24 | 20 | H-R | benign | |
| 14798 | 'T/C' | 75 | 47 | F-L | benign | |
| 14819 | 'T/C' | 0 | 1 | T-K | benign | |
| 14831 | 'G/A' | 0 | 1 | A-T | benign | |
| 14871 | 'T/C' | 0 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 14954 | 'A/G' | 1 | 0 | T-A | benign | |
| 14963 | 'G/C' | 1 | 0 | V-L | possibly damaging | PSIC score difference: 1.720 |
| 15014 | 'T/C' | 0 | 1 | F-L | benign | |
| 15047 | 'G/A' | 1 | 0 | G-S | benign | |
| 15110 | 'G/A' | 1 | 1 | A-T | benign | |
| 15159 | 'T/C' | 0 | 2 | M-T | benign | |
| 15164 | 'T/C' | 0 | 1 | F-L | benign | |
| 15218 | 'A/G' | 17 | 16 | T-A | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 15257 | 'G/A' | 17 | 10 | D-N | benign | |
| 15287 | 'T/C' | 2 | 0 | F-L | benign | |
| 15314 | 'G/A' | 0 | 1 | A-T | benign | |
| 15315 | 'C/T' | 0 | 1 | A-V | benign | |
| 15326 | 'A/G' | 6 | 16 | T-A | benign | |
| 15381 | 'C/T' | 0 | 1 | T-I | benign | |
| 15449 | 'T/C' | 1 | 0 | F-L | benign | |
| 15450 | 'T/C' | 1 | 0 | F-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 15452 | 'C/A' | 97 | 86 | L-I | benign | |
| 15479 | 'T/C' | 0 | 2 | F-L | benign | |
| 15497 | 'G/A' | 2 | 1 | G-S | benign | |
| 15498 | 'G/A' | 0 | 2 | G-D | possibly damaging | PSIC score difference: 1.695 |
| 15542 | 'C/T' | 1 | 0 | P-S | benign | |
| 15608 | 'C/G' | 1 | 0 | L-V | benign | |
| 15609 | 'T/G' | 2 | 0 | L-R | Improper substitution in the transmembrane region | PHAT matrix element difference: -6 |
| 15617 | 'G/A' | 0 | 1 | V-I | benign | |
| 15618 | 'T/C' | 1 | 0 | V-A | benign | |
| 15672 | 'T/C' | 1 | 0 | M-T | benign | |
| 15693 | 'T/C' | 9 | 6 | M-T | benign | |
| 15701 | 'C/T' | 0 | 1 | P-S | probably damaging | PSIC score difference: 2.731 |
| 15719 | 'T/C' | 0 | 1 | Y-H | benign | |
| 15758 | 'A/G' | 3 | 3 | I-V | benign | |
| 15773 | 'G/A' | 1 | 1 | V-M | benign | |
| 15804 | 'T/C' | 3 | 1 | V-A | benign | |
| 15812 | 'G/A' | 3 | 5 | V-M | benign | |
| 15813 | 'T/C' | 3 | 1 | V-A | benign | |
| 15884 | 'G/C' | 4 | 6 | A-P | benign | |
| 15884 | 'G/A' | 9 | 7 | A-P | benign | |

**Table 4S b.** PolyPhen prediction of functional effect of the observed mtDNA mutations in Finland.

| Position | Mutation | Controls | Cases | Amino acid substitution | Substitution effect | Prediction data |
|---|---|---|---|---|---|---|
| **ND1** | | | | | | |
| 3394 | 'T/C' | 1 | 2 | Y-H | benign | |
| 3398 | 'T/C' | 1 | 0 | M-T | possibly damaging | PSIC score: 2,231 |
| 3434 | 'A/G' | 0 | 2 | Y-C | benign | |
| 3505 | 'A/G' | 5 | 9 | T-A | benign | |
| 3547 | 'A/C' | 1 | 0 | I-V | benign | |
| 3644 | 'T/C' | 1 | 0 | V-A | benign | |
| 3736 | 'G/A' | 1 | 0 | V-I | benign | |
| 3796 | 'A/G' | 2 | 1 | T-A | benign | |
| 3992 | 'C/T' | 3 | 3 | T-M | benign | |
| 4024 | 'A/G' | 3 | 3 | T-A | benign | |
| 4093 | 'A/G' | 0 | 1 | T-A | benign | |
| 4216 | 'T/C' | 10 | 16 | Y-H | benign | |
| 4243 | 'A/C' | 1 | 0 | S-R | functional effect, functional site, transmembrane Improper substitution in the transmembrane region | PHAT matrix element difference: -6 |
| **ND2** | | | | | | |
| 4491 | 'G/A' | 0 | 1 | V-I | benign | |
| 4561 | 'T/C' | 1 | 1 | V-A | benign | |
| 4639 | 'T/C' | 6 | 2 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 4659 | 'G/A' | 0 | 1 | A-T | benign | |
| 4732 | 'A/G' | 1 | 2 | N-S | benign | |
| 4812 | 'G/C' | 1 | 0 | V-L | benign | |
| 4917 | 'A/G' | 2 | 7 | N-D | benign | |
| 5046 | 'G/A' | 5 | 8 | V-I | benign | |
| 5074 | 'T/C' | 0 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 5145 | 'A/C' | 1 | 0 | T-R | possibly damaging | PSIC score difference: 1.816 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5178 | 'C/A' | 0 | 1 | L-M | benign | |
| 5194 | 'C/T' | 1 | 1 | P-L | benign | |
| 5206 | 'C/G' | 0 | 1 | S-C | benign | |
| 5263 | 'C/T' | 6 | 2 | A-V | benign | |
| 5301 | 'A/G' | 0 | 1 | I-V | benign | |
| 5319 | 'A/G' | 1 | 0 | T-S | benign | |
| 5319 | 'A/T' | 0 | 1 | T-A | benign | |
| 5460 | 'G/A' | 6 | 9 | A-T | benign | |
| 5485 | 'T/G' | 1 | 0 | I-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| **CO1** | | | | | | |
| 5935 | 'A/T' | 3 | 0 | N-I | probably damaging | PSIC score difference: 3.231 |
| 5936 | 'C/A' | 7 | 1 | N-K | probably damaging | PSIC score difference: 2.556 |
| 6066 | 'A/C' | 1 | 0 | N-H | probably damaging | PSIC score difference: 2.331 |
| 6261 | 'G/A' | 0 | 1 | A-T | benign | |
| 6267 | 'G/A' | 1 | 0 | A-T | benign | |
| 6426 | 'G/A' | 1 | 0 | A-T | benign | |
| 6467 | 'C/T' | 0 | 1 | P-S | benign | |
| 6480 | 'G/A' | 1 | 1 | V-I | benign | |
| 6855 | 'G/A' | 1 | 0 | V-I | benign | |
| 7024 | 'T/G' | 1 | 0 | V-G | possibly damaging | PHAT matrix element difference: -2 |
| 7080 | 'T/C' | 1 | 0 | F-L | benign | |
| 7309 | 'T/C' | 3 | 4 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 7389 | 'T/C' | 0 | 1 | Y-H | benign | |
| 7444 | 'G/A' | 1 | 1 | Ter-K | | |
| **CO2** | | | | | | |
| 7685 | 'A/G' | 0 | 1 | I-V | benign | |
| 7761 | 'A/T' | 1 | 0 | Q-L | 2.2.3: functional effect, functional site, ligand binding Disruption of ligand binding site | PSIC score difference: 2.934 ligand name: PEK distance: 2.520 Å |

| | | | | | | |
|---|---|---|---|---|---|---|
| 7805 | 'G/A' | 0 | 1 | V-I | benign | |
| 7853 | 'G/A' | 2 | 0 | V-I | benign | |
| 8126 | C/A | 4 | 0 | Q-K | probably damaging | PSIC score difference: 2.440 |
| A**TP8** | | | | | | |
| 8393 | 'C/T' | 0 | 2 | P-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 8417 | 'C/G' | 0 | 1 | L-V | benign | |
| 8418 | 'T/G' | 0 | 1 | L-R | Improper substitution in the transmembrane region | PHAT matrix element difference: -6 |
| 8426 | 'T/C' | 1 | 0 | F-L | benign | |
| 8448 | 'T/C' | 1 | 2 | M-T | benign | |
| 8532 | 'C/A' | 1 | 0 | T-K | probably damaging | PSIC score difference: 2.171 |
| 8534 | 'A/G' | 1 | 0 | K-E | possibly damaging | PSIC score difference: 1.741 |
| 8535 | 'A/G' | 1 | 0 | K-Ter | X | |
| 8537 | A/G | 1 | 0 | I-V | benign | |
| 8559 | 'C/T' | 0 | 1 | P-L | probably damaging | PSIC score difference: 2.956 |
| 8564 | 'C/A' | 0 | 1 | Q-K | probably damaging | PSIC score difference: 2.029 |
| 8565 | 'A/C' | 0 | 1 | Q-P | probably damaging | PSIC score difference: 2.421 |
| 8572 | 'G/C' | 3 | 0 | Ter-Y | X | |
| **ATP6** | | | | | X | |
| 8532 | C/A | 1 | 0 | N-K | benign | |
| 8533 | G/C | 1 | 0 | E-Q | benign | |
| 8534 | A/G | 1 | 0 | E-G | possibly damaging | PSIC score difference: 1.893 |
| 8537 | A/G | 1 | 1 | N-S | benign | |
| 8563 | A/C | 0 | 1 | T-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -4 |
| 8564 | C/A | 0 | 1 | T-K | Improper substitution in | PHAT matrix element |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | the transmembrane region | difference: -6 |
| 8572 | G/C | 3 | 0 | G-R | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 8616 | 'G/C' | 1 | 0 | L-F | benign | |
| 8616 | 'G/T' | 1 | 1 | L-F | benign | |
| 8651 | 'T/C' | 0 | 1 | L-P | benign | |
| 8659 | 'A/G' | 0 | 2 | T-A | benign | |
| 8684 | 'C/T' | 1 | 0 | T-I | benign | |
| 8701 | 'A/G' | 0 | 1 | T-A | benign | |
| 8705 | 'T/C' | 10 | 2 | M-T | benign | |
| 8723 | 'G/A' | 0 | 5 | R-Q | benign | |
| 8764 | 'G/A' | 0 | 1 | A-T | benign | |
| 8765 | 'C/T' | 0 | 1 | A-V | benign | |
| 8812 | 'A/G' | 0 | 4 | T-A | benign | |
| 8833 | 'G/A' | 0 | 1 | A-T | benign | |
| 8842 | 'A/G' | 1 | 0 | I-V | benign | |
| 8843 | 'T/C' | 0 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 8860 | 'A/G' | 0 | 2 | T-A | benign | |
| 8869 | 'A/G' | 6 | 2 | M-V | benign | |
| 8887 | 'A/G' | 0 | 2 | I-V | benign | |
| 8902 | 'G/A' | 0 | 1 | A-T | benign | |
| 8923 | 'A/G' | 1 | 3 | T-A | probably damaging | PSIC score difference: 2.259 |
| 9053 | 'G/A' | 1 | 2 | S-N | benign | |
| 9055 | 'G/A' | 12 | 7 | A-T | benign | |
| 9058 | 'A/G' | 1 | 0 | T-A | benign | |
| 9088 | 'T/C' | 1 | 0 | S-P | benign | |
| **CO3** | | | | | | |
| 9214 | 'A/G' | 0 | 1 | H-R | Disruption of ligand binding site | PSIC score difference: 1.699 ligand name: CD distance: 2.180 Å |
| 9300 | 'G/A' | 0 | 1 | A-T | benign | |
| 9391 | 'C/T' | 2 | 1 | T-M | benign | |
| 9477 | 'G/A' | 24 | 23 | V-I | benign | |
| 9478 | 'T/C' | 0 | 1 | V-A | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9480 | 'T/C' | 1 | 0 | F-L | benign | |
| 9500 | 'C/G' | 0 | 2 | F-L | benign | |
| 9612 | 'G/A' | 2 | 2 | V-M | benign | |
| 9667 | 'A/G' | 5 | 4 | N-S | benign | |
| 9736 | 'A/C' | 0 | 1 | Q-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 9738 | 'G/A' | 4 | 0 | A-T | benign | |
| 9777 | 'G/A' | 0 | 1 | G-S | benign | |
| 9783 | 'T/A' | 2 | 0 | Y-N | probably damaging | PSIC score difference: 3.213 |
| 9784 | 'A/C' | 2 | 0 | Y-S | probably damaging | PSIC score difference: 3.174 |
| 9790 | 'C/T' | 3 | 0 | S-L | probably damaging | PSIC score difference: 2.277 |
| 9804 | 'G/A' | 0 | 1 | A-T | benign | |
| 9825 | 'C/G' | 0 | 1 | H-D | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 9856 | 'T/G' | 2 | 0 | I-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 9857 | 'C/G' | 2 | 0 | I-M | benign | |
| 9895 | 'A/C' | 1 | 0 | K-T | benign | |
| 9903 | 'T/C' | 3 | 2 | F-L | possibly damaging | PSIC score difference: 1.625 |
| 9921 | 'G/C' | 0 | 1 | A-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| **ND3** | | | | | | |
| 10068 | 'G/T' | 0 | 1 | A-S | benign | |
| 10084 | 'T/C' | 2 | 0 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 10086 | 'A/G' | 2 | 2 | N-D | benign | |
| 10111 | 'T/A' | 0 | 4 | M-K | Improper substitution in the | PHAT matrix element difference: -6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | transmembrane region | |
| 10237 | 'T/C' | 2 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 10307 | 'C/T' | 0 | 1 | N-K | benign | |
| 10398 | 'A/G' | 15 | 17 | T-A | benign | |
| **ND4L** | | | | | | |
| 10644 | 'G/A' | 1 | 0 | V-M | benign | |
| 10654 | 'C/T' | 1 | 1 | A-V | benign | |
| **ND4** | | | | | | |
| 10805 | 'T/G' | 0 | 1 | W-G | probably damaging | PSIC score difference: 3.844 |
| 10877 | 'C/G' | 1 | 0 | L-V | benign | |
| 10922 | 'A/T' | 1 | 0 | T-S | benign | |
| 11099 | 'G/A' | 0 | 1 | E-K | Improper substitution in the transmembrane region | PHAT matrix element difference: -4 |
| 11126 | 'G/A' | 0 | 1 | E-K | probably damaging | PSIC score difference: 2.194 |
| 11253 | 'T/C' | 0 | 3 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 11436 | 'C/A' | 0 | 1 | A-D | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 11589 | 'T/C' | 1 | 0 | L-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 11592 | 'G/C' | 1 | 0 | R-P | probably damaging | PSIC score difference: 2.908 |
| 12017 | 'A/C' | 1 | 0 | T-P | benign | |
| 12026 | 'A/G' | 0 | 1 | I-V | benign | |
| 12135 | 'C/A' | 0 | 1 | S-Y | possibly damaging | PSIC score difference: 1.985 |
| **ND5** | | | | | | |
| 12358 | 'A/G' | 0 | 1 | T-A | benign | |
| 12397 | 'A/G' | 0 | 1 | T-A | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 12406 | 'G/A' | 2 | 0 | V-I | benign | |
| 12557 | 'C/T' | 0 | 1 | T-I | benign | |
| 12580 | 'C/G' | 1 | 0 | L-V | benign | |
| 12613 | 'G/A' | 2 | 2 | A-T | benign | |
| 12613 | 'G/C' | 1 | 0 | A-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 12622 | 'G/A' | 1 | 0 | V-I | benign | |
| 12739 | 'A/G' | 1 | 0 | N-D | benign | |
| 12803 | 'G/C' | 0 | 1 | S-T | benign | |
| 12811 | 'T/C' | 9 | 3 | Y-H | benign | |
| 12814 | 'G/A' | 0 | 1 | A-T | benign | |
| 12893 | 'T/G' | 1 | 0 | L-W | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 12928 | 'C/G' | 1 | 0 | P-A | benign | |
| 13105 | 'A/G' | 1 | 0 | I-V | benign | |
| 13105 | 'A/C' | 3 | 1 | I-L | benign | |
| 13135 | 'G/A' | 0 | 1 | A-T | benign | |
| 13144 | 'A/C' | 0 | 1 | S-R | benign | |
| 13145 | 'G/A' | 3 | 1 | S-N | benign | |
| 13637 | 'A/G' | 1 | 2 | Q-R | benign | |
| 13651 | 'A/G' | 1 | 0 | T-A | benign | |
| 13681 | 'A/G' | 0 | 1 | T-A | benign | |
| 13708 | 'G/A' | 5 | 12 | A-T | benign | |
| 13759 | 'G/A' | 1 | 3 | A-T | benign | |
| 13762 | 'T/G' | 1 | 1 | S-A | benign | |
| 13780 | 'A/G' | 1 | 3 | I-V | benign | |
| 13928 | 'G/C' | 4 | 0 | S-T | benign | |
| 13933 | 'A/G' | 0 | 1 | T-A | benign | |
| 13934 | 'C/T' | 0 | 3 | T-M | benign | |
| 13953 | 'T/G' | 1 | 0 | Y-Ter | | |
| 13966 | 'A/G' | 2 | 3 | T-A | benign | |
| 13967 | 'C/T' | 1 | 1 | T-M | benign | |
| 14062 | 'A/G' | 4 | 0 | I-V | benign | |
| **ND6** | | | | | | |
| 14180 | 'T/C' | 1 | 1 | Y-C | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 14233 | 'A/G' | 2 | 6 | D-E | benign | |
| 14325 | 'T/C' | 1 | 0 | N-Y | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 14550 | 'T/C' | 2 | 0 | I-V | benign | |
| 14577 | 'T/G' | 0 | 1 | I-L | benign | |
| 14582 | 'A/G' | 3 | 3 | V-A | benign | |
| **Cytb** | | | | | | |
| 14759 | 'C/A' | 1 | 0 | R-S | Disruption of ligand binding site | PSIC score difference: 2.448 ligand name: CDL distance: 2.681 Å |
| 14760 | 'G/A' | 1 | 1 | R-H | Disruption of ligand binding site | PSIC score difference: 2.311 ligand name: CDL distance: 2.681 Å |
| 14762 | 'A/G' | 1 | 3 | K-E | benign | |
| 14763 | 'A/G' | 0 | 1 | K-Ter | | |
| 14766 | 'C/T' | 55 | 65 | T-I | benign | |
| 14775 | 'T/C' | 0 | 1 | L-P | benign | |
| 14793 | 'A/G' | 14 | 4 | H-R | benign | |
| 14798 | 'T/C' | 11 | 13 | F-L | benign | |
| 14817 | 'C/T' | 1 | 0 | P-L | probably damaging | PSIC score difference: 3.181 |
| 14828 | 'T/C' | 0 | 1 | S-P | probably damaging | PSIC score difference: 2.233 |
| 15077 | 'G/A' | 0 | 1 | E-K | benign | |
| 15213 | 'T/C' | 1 | 0 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 15218 | 'A/G' | 6 | 3 | T-A | benign | |
| 15257 | 'G/A' | 5 | 3 | D-N | benign | |
| 15314 | 'G/A' | 1 | 0 | A-T | benign | |
| 15326 | 'A/G' | 0 | 3 | T-A | benign | |
| 15452 | 'C/A' | 7 | 17 | L-I | benign | |
| 15459 | 'C/T' | 0 | 1 | S-F | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 15693 | 'T/C' | 0 | 1 | M-T | benign | |
| 15758 | 'A/G' | 0 | 2 | I-V | benign | |
| 15773 | 'G/A' | 1 | 0 | V-M | benign | |
| 15812 | 'G/A' | 2 | 0 | V-M | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 15831 | 'T/G' | 0 | 1 | I-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 15852 | 'T/C' | 3 | 0 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 15884 | 'G/C' | 5 | 9 | A-P | benign | |
| 15884 | 'G/A' | 6 | 1 | A-T | benign | |

**Table 4S c.** PolyPhen prediction of functional effect of the observed mtDNA mutations in South Europe

| Position | Mutation | Controls | Cases | Amino acid substitution | Substitution effect | Prediction data |
|---|---|---|---|---|---|---|
| **ND1** | | | | | | |
| 3308 | 'T/C' | 1 | 0 | M-T | probably damaging | PSIC score difference: 2.775 |
| 3335 | 'T/C' | 0 | 2 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 3388 | 'C/A' | 0 | 1 | L-M | possibly damaging | PSIC score difference: 1.515 |
| 3505 | 'A/G' | 2 | 0 | T-A | benign | |
| 3547 | 'A/G' | 1 | 0 | I-V | benign | |
| 3796 | 'A/G' | 0 | 1 | T-A | benign | |
| 3992 | 'C/T' | 1 | 1 | T-M | benign | |
| 4021 | 'A/T' | 0 | 1 | T-S | benign | |
| 4024 | 'A/G' | 0 | 1 | T-A | benign | |
| 4136 | 'A/G' | 0 | 1 | Y-C | benign | |
| 4216 | 'T/C' | 17 | 11 | Y-H | benign | |
| 4243 | 'A/G' | 1 | 0 | S-G | benign | |
| **ND2** | | | | | | |
| 4491 | 'G/A' | 0 | 1 | V-I | benign | |
| 4561 | 'T/C' | 2 | 0 | V-A | benign | |
| 4596 | 'G/A' | 1 | 0 | V-I | benign | |
| 4639 | 'T/C' | 2 | 0 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 4640 | 'C/A' | 1 | 0 | I-M | benign | |
| 4732 | 'A/G' | 0 | 1 | N-S | benign | |
| 4917 | 'A/G' | 8 | 4 | N-D | benign | |
| 4924 | 'G/A' | 1 | 0 | S-N | benign | |
| 4936 | 'C/T' | 1 | 0 | T-I | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 4960 | 'C/T' | 1 | 1 | A-V | benign | |
| 5046 | 'G/A' | 3 | 0 | V-I | benign | |
| 5263 | 'C/T' | 2 | 1 | A-V | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **CO1** | | | | | | |
| 6054 | 'G/T' | 0 | 1 | D-Y | probably damaging | PSIC score difference: 2.239 |
| 6340 | 'C/T' | 0 | 1 | T-I | benign | |
| 6366 | 'G/A' | 1 | 1 | V-I | benign | |
| 6423 | 'C/G' | 1 | 0 | P-A | benign | |
| 7146 | 'A/G' | 1 | 0 | T-A | benign | |
| 7270 | 'T/C' | 1 | 0 | V-A | benign | |
| 7299 | 'A/G' | 1 | 0 | M-V | benign | |
| 7389 | 'T/C' | 1 | 0 | Y-H | benign | |
| **CO2** | | | | | | |
| 7598 | 'G/A' | 1 | 0 | A-T | benign | |
| 7650 | 'C/T' | 0 | 1 | T-I | benign | |
| 7680 | 'T/C' | 0 | 1 | F-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 7941 | 'A/G' | 1 | 0 | N-S | benign | |
| 7958 | 'C/T' | 0 | 1 | P-S | benign | |
| 7979 | 'G/A' | 0 | 1 | D-N | benign | |
| 8058 | 'A/G' | 0 | 1 | D-G | Contact with functional site, indirect effect | PSIC score difference: 2.636 functional site: HIS 161B distance: 2.624 Å |
| 8261 | 'A/G' | 1 | 0 | T-A | possibly damaging | PSIC score difference: 1.559 |
| **ATP8** | | | | | | |
| 8393 | 'C/T' | 1 | 1 | P-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |
| 8426 | 'T/C' | 1 | 0 | F-L | benign | |
| 8472 | 'C/T' | 1 | 1 | P-L | probably damaging | PSIC score difference: 2.434 |
| 8496 | 'T/C' | 0 | 1 | M-T | benign | |
| 8519 | 'G/A' | 1 | 0 | E-K | benign | |
| **ATP6** | | | | | | |
| 8572 | 'G/A' | 1 | 0 | G-S | benign | |
| 8578 | 'C/T' | 1 | 0 | P-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8642 | 'A/G' | 1 | 0 | N-S | benign | |
| 8653 | 'A/G' | 1 | 0 | I-V | benign | |
| 8684 | 'C/T' | 1 | 0 | T-I | benign | |
| 8701 | 'A/G' | 4 | 2 | T-A | benign | |
| 8711 | 'A/G' | 0 | 1 | N-S | benign | |
| 8836 | 'A/G' | 1 | 1 | M-V | benign | |
| 8857 | 'G/A' | 1 | 0 | G-S | benign | |
| 8860 | 'A/G' | 0 | 0 | T-A | benign | |
| 8869 | 'A/G' | 2 | 0 | M-V | benign | |
| 8887 | 'A/G' | 1 | 0 | I-V | benign | |
| 8939 | 'T/C' | 0 | 1 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 8950 | 'G/A' | 1 | 0 | V-I | benign | |
| 9041 | 'A/G' | 1 | 0 | H-R | Improper substitution in the transmembrane region | PHAT matrix element difference: -4 |
| 9055 | 'G/A' | 4 | 4 | A-T | benign | |
| 9070 | 'T/G' | 2 | 0 | S-A | benign | |
| 9196 | 'G/A' | 2 | 0 | D-N | benign | |
| **CO3** | | | | | | |
| 9300 | 'G/A' | 2 | 1 | A-T | benign | |
| 9336 | 'A/G' | 1 | 0 | M-V | benign | |
| 9477 | 'G/A' | 3 | 2 | V-I | benign | |
| 9531 | 'A/G' | 1 | 0 | T-A | benign | |
| 9756 | 'T/G' | 4 | 0 | S-A | benign | |
| 9804 | 'G/A' | 0 | 1 | A-T | benign | |
| 9840 | 'T/A' | 0 | 1 | S-T | benign | |
| 9921 | 'G/A' | 0 | 1 | A-T | benign | |
| **ND3** | | | | | | |
| 10086 | 'A/G' | 0 | 1 | N-D | benign | |
| 10112 | 'A/T' | 0 | 1 | M-I | benign | |
| 10336 | 'T/C' | 1 | 0 | L-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 10398 | 'A/G' | 18 | 13 | T-A | benign | |
| **ND4L** | | | | | | |
| 10506 | 'A/G' | 1 | 1 | T-A | benign | |
| 10609 | 'T/C' | 1 | 0 | M-T | benign | |
| 10680 | 'G/A' | 1 | 0 | A-T | benign | |
| 10687 | 'T/G' | 0 | 1 | V-G | Improper substitution in | PHAT matrix element |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | the transmembrane region | difference: -2 |
| **ND4** | | | | | | |
| 10784 | 'A/G' | 0 | 1 | I-V | benign | |
| 10907 | 'T/C' | 2 | 0 | F-L | benign | |
| 10922 | 'A/G' | 1 | 2 | T-A | benign | |
| 11016 | 'G/A' | 0 | 2 | S-N | benign | |
| 11069 | 'A/G' | 1 | 0 | I-V | benign | |
| 11204 | 'T/C' | 0 | 1 | F-L | benign | |
| 11253 | 'T/C' | 1 | 0 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 11447 | 'G/A' | 0 | 1 | V-M | benign | |
| 11582 | 'A/G' | 0 | 1 | I-V | benign | |
| 11752 | 'C/G' | 2 | 0 | N-K | probably damaging | PSIC score difference: 2.650 |
| 11936 | 'C/G' | 1 | 0 | L-V | benign | |
| 12017 | 'A/G' | 1 | 0 | T-A | benign | |
| 12084 | 'C/T' | 0 | 1 | S-F | benign | |
| 12135 | 'C/A' | 1 | 0 | S-Y | possibly damaging | PSIC score difference: 1.985 |
| **ND5** | | | | | | |
| 12376 | 'T/G' | 1 | 0 | S-A | benign | |
| 12403 | 'C/T' | 2 | 1 | L-F | benign | |
| 12490 | 'A/G' | 1 | 0 | T-C | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 12557 | 'C/T' | 0 | 1 | T-I | benign | |
| 12562 | 'C/G' | 4 | 1 | L-V | benign | |
| 12634 | 'A/G' | 2 | 1 | I-V | benign | |
| 12663 | 'C/A' | 1 | 0 | N-K | benign | |
| 12841 | 'A/G' | 1 | 0 | I-V | benign | |
| 12923 | 'G/T' | 1 | 0 | W-L | probably damaging | PSIC score difference: 2.733 |
| 12950 | 'A/C' | 2 | 1 | N-T | benign | |
| 13105 | 'A/G' | 1 | 0 | I-V | benign | |
| 13135 | 'G/A' | 3 | 0 | A-T | benign | |
| 13145 | 'G/A' | 2 | 0 | S-N | benign | |
| 13350 | 'A/C' | 0 | 1 | M-I | benign | |
| 13351 | 'C/A' | 1 | 0 | L-M | benign | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 13525 | 'G/A' | 1 | 0 | E-K | probably damaging | PSIC score difference: 2.143 |
| 13630 | 'A/G' | 0 | 1 | T-A | benign | |
| 13708 | 'G/A' | 9 | 7 | A-T | benign | |
| 13711 | 'G/A' | 0 | 1 | A-T | benign | |
| 13768 | 'T/C' | 1 | 0 | F-L | benign | |
| 13780 | 'A/G' | 1 | 1 | I-V | benign | |
| 13789 | 'T/C' | 1 | 0 | Y-H | benign | |
| 13834 | 'A/G' | 0 | 1 | T-A | benign | |
| 13880 | 'C/A' | 1 | 0 | S-Y | benign | |
| 13934 | 'C/T' | 2 | 1 | T-M | benign | |
| 13942 | 'A/G' | 1 | 0 | T-A | benign | |
| 13943 | 'C/T' | 0 | 1 | T-M | benign | |
| 13966 | 'A/G' | 3 | 6 | T-A | benign | |
| **ND6** | | | | | | |
| 14178 | 'T/C' | 1 | 0 | I-V | benign | |
| 14180 | 'T/G' | 0 | 1 | Y-S | Improper substitution in the transmembrane region | PHAT matrix element difference: -2 |
| 14180 | 'T/C' | 1 | 0 | Y-C | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 14258 | 'G/A' | 1 | 0 | P-L | benign | |
| 14394 | 'C/T' | 0 | 1 | V-M | benign | |
| 14552 | 'A/G' | 0 | 1 | V-A | benign | |
| 14582 | 'A/G' | 0 | 1 | V-A | benign | |
| 14634 | 'T/C' | 1 | 0 | M-V | benign | |
| 14655 | 'G/T' | 0 | 1 | L-M | benign | |
| **Cyt B** | | | | | | |
| 14750 | 'A/G' | 2 | 0 | T-A | benign | |
| 14766 | 'C/T' | 44 | 32 | T-I | benign | |
| 14769 | 'A/G' | 1 | 0 | N-S | benign | |
| 14793 | 'A/G' | 2 | 1 | H-R | benign | |
| 14798 | 'T/C' | 9 | 6 | F-L | benign | |
| 14831 | 'G/A' | 0 | 2 | A-T | benign | |
| 14856 | 'T/C' | 1 | 0 | L-P | Improper substitution in the transmembrane region | PHAT matrix element difference: -5 |
| 14870 | 'A/G' | 0 | 1 | I-V | benign | |
| 14927 | 'A/G' | 1 | 1 | T-A | benign | |
| 14979 | 'T/C' | 0 | 1 | I-T | Improper substitution in | PHAT matrix element |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | the transmembrane region | difference: -1 |
| 15218 | 'A/G' | 1 | 1 | T-A | benign | |
| 15257 | 'G/A' | 1 | 3 | D-N | benign | |
| 15326 | 'A/G' | 1 | 1 | T-A | benign | |
| 15452 | 'C/A' | 15 | 11 | L-I | benign | |
| 15456 | 'T/G' | 0 | 1 | L-R | Improper substitution in the transmembrane region | PHAT matrix element difference: -6 |
| 15467 | 'A/G' | 0 | 1 | T-A | benign | |
| 15506 | 'G/A' | 1 | 0 | D-N | possibly damaging | PSIC score difference: 1.968 |
| 15519 | 'T/C' | 0 | 1 | L-P | benign | |
| 15657 | 'T/C' | 1 | 0 | I-T | Improper substitution in the transmembrane region | PHAT matrix element difference: -1 |
| 15693 | 'T/C' | 3 | 1 | M-T | benign | |
| 15734 | 'G/A' | 1 | 1 | A-T | benign | |
| 15789 | 'C/T' | 1 | 0 | Q-H | benign | |
| 15812 | 'G/A' | 0 | 1 | V-M | benign | |
| 15884 | 'G/A' | 0 | 2 | A-P | benign | |
| 15884 | 'G/C' | 2 | 0 | A-T | benign | |

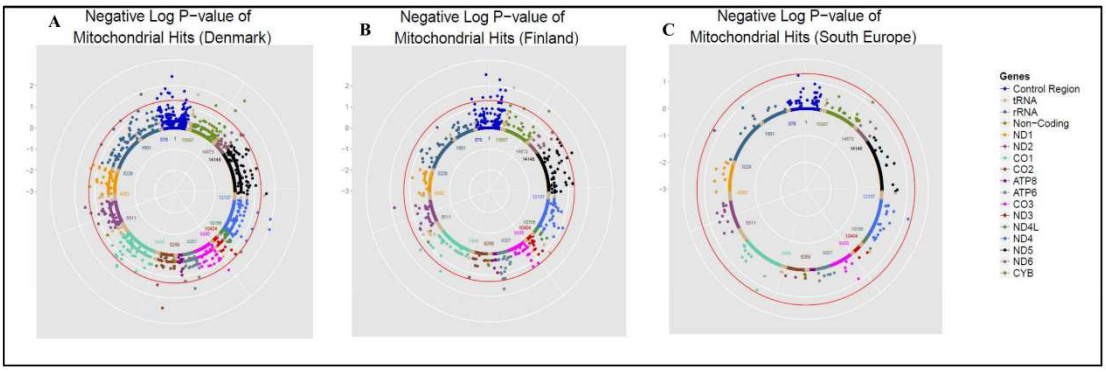


**Figure 1S** Differences in the frequency of each observed mtDNA mutations, represented as negative logaritm of the p-value, between cases and controls in Denmark (A), Finland (B); South Europe (C). The red circle represent the significative threshold (-logp 0.05).

## CHAPTER 3

### 3.1 The interaction between Mitochondrial DNA and APOE variability in a large sample of ultranonagenarians across Europe: a GEHA Project Study.

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*These Authors equally contributed to the study.

**Abstract**

The interaction between mitochondrial DNA variability and nuclear variability is one of the main issues in the research on mitochondrial biology. In this frame, the effect of the interaction between mtDNA variability and the variability of nuclear genes on longevity has been widely studied. We availed of a large recruitment project, the GEHA project, to analyze the interaction between mitochondrial DNA variability and APOE. The large sample size and the distribution of samples across the European countries, allowed us to highlight the interaction between mitochondrial DNA haplogroup U and APOE ε4 allele, but also the interaction between this "allele combination" and the environmental conditions.

## Introduction

The research on healthy aging and longevity, which is enormously increased in the last decade, has demonstrated the central role played by the variability of mitochondrial DNA (Passarino et al. 2010; Castri et al. 2009; Santoro et al. 2006; Rose et al 2002; Tanaka 1998) and by the variability of APOE (Drenos and Kirkwood 2010; Ang et al. 2008; Christensen et al. 2006; Smith 2002) in determining individual aging phenotype and individual chance to attain longevity. Many reports have also suggested that these complex phenotypes are influenced by the interaction between different genetic factors. In particular, the interaction between mitochondrial DNA variability and nuclear variability has been highlighted as crucial in determining the efficiency of the interaction between the two genomes and then the efficiency of a number of process (such as OXPHOS, metabolism, apoptosis and many others) which are essential for the cell life and in particular for preserving the cell from the age related decline (Tranah et al. 2011). In this frame, the interaction between APOE and mitochondrial DNA variability has been reported to influence the individual chance to be affected by Alzheimer Disease, one the major age related diseases correlated to the decline of the nervous system. In fact, mtDNA haplogroup U was reported to neutralize the effect of APOE allele ε4, which are widely known as risk factor for AD (Carrieri et al. 2001). The relative low frequency of these two variants (APOE and mtDNA haplogroup U) has made difficult to follow up on this observation and, for instance, to highlight the effect of this interaction on longevity as it would have required very large samples.

In this study we present the results of a very large study, where mitochondrial DNA and APOE variability have been studied in more than 1950 subjects older than 90 years, recruited in the frame of the GEHA project and coming from most of the European populations, and compared to a similar number of younger controls matched

for sex and geographic origins (1985 nonagenarians and 2036 controls). This has allowed to extensively reconsider the interaction between these two systems and the interaction between their variability, environment and genetic background of different populations. In fact, both mitochondrial DNA variability and APOE variability, which are differently distributed in different populations and in particular across European populations, have been reported to have been shaped by environmental conditions (Finch 2010; Kivisild et al. 2006; Singh et al 2006; Elson et al. 2004; Ruiz-Pesini et al. 2004; Mishmar et al. 2003; Moilanen and Majamaa 2003; Cann et al. 1984; Gerdes et al 2003; Lucotte et al 1997).

## Materials and Methods

### *Sampling*

Samples were collected in the frame of the GEHA research project (2004-2010) in 11 European Countries. Each Institution providing blood samples received the approval from its own ethical committees and all the recruited subjects provided written informed consent for the use of their phenotypic and genetic data in studies on human ageing (Skytthe et al. 2011).

DNA was recovered from fresh blood by automated and standardized protocol in order to guarantee quality and concentration uniformity among samples. The GEHA Partner in charge of the DNA extraction (KTL - Helsinki, Finland) provided 4 µg of genomic DNA for mtDNA variability analysis.

### *DNA analysis*

The definition of mtDNA subhaplogroups was conducted by resequencing the

D-loop region from nucleotide position (np) 16024 to np 576 followed by RFLP analysis in specific coding region traits as previously described (Raule et al Ms. Submitted, see chapter 2 of this thesis).

APO E variability was assessed by PCR amplification and RFLP digestion according to standardized protocol (Carrieri et al. 2001).

**Results**

Table 1. reports the distribution of APOE alleles in each population. Table 2 reports the distribution of mtDNA haplogroups in the different populations.

Fig. 1 shows the significance of the different distribution of APOE allele ε4 in nonagenarians and controls in each population. This allows to figure out the effect of this allele on the chance to attain longevity in different populations. No significant differences between nonagenarians and controls were observed for mtDNA haplogroups.

Fig.2 shows the influence of haplogroup U on the significance of the different distribution of APOE allele ε4 in nonagenarians and controls. In fact it compares, for each population, the OR of the differences between nonagenarians and controls observed in subjects with U and non U mtDNA molecules. Interestingly, in almost all cases the negative effect of APOE ε4 is diminished by the presence of haplogroup U. The only exceptions were in Denmark, Germany and Finland.

In Fig. 3 we summarized the information given in Fig.2 by pooling together the population where the presence of U decreased the negative effect of APO ε4 and the populations where this was not observed. We see that indeed the presence of

haplogroup U cancels the negative effect of APOE ε4 in most of the European populations we sampled. This is not observed in Danes, Germans and Finns.

**Discussion.**

In the last decades many evidences accumulated which show that a number of common polymorphisms affect longevity and age related phenotypes. Each polymorphisms has shown to exerts a little or even a very little effect on these phenotypes and this makes difficult to study such effect as it requires very big samples. The picture gets even more complicated by the fact that most of the polymorphisms exert population specific effects and by the interactions between polymorphisms occurring at different loci. It becomes then necessary that observed associations, are replicated in very large samples, possibly comparing different populations (De Benedictis and Franceschi 2006; Salvioli et al. 2006). In this constext, the GEHA sample, which has been recruited in the framework of a European project gathering labs from most of the European countries, represents an unprecedented resource. In fact it includes 15 samples from different European areas and gathers more than 2200 ultranonagenarian sibships and an equal number of geographic matched younger controls. In the present study we took advantage of such sample in order to test the described interaction between mtDNA variability and APOE variability, with haplogroup U which was reported to be protective with respect to the AD risk allele APOE ε4 (Carrieri et al. 2001).

Our results show that indeed in most of the European populations APOE ε4 is an important “risk” allele for survival to 90 years and over, but for the carriers of haplogroup U allele APO ε4 is a neutral allele. On the other hand this observation does not holds up if we consider German, Danish and Finnish populations. In fact, within

these populations, APOE ε4 is a risk allele for both U carriers and for the general population. It might be worth noticing in this frame, that the results does not change if we considers the different Haplogroup U subgroups instead of the whole haplogroup. The results are even more striking if we consider that Germans, Danes and Finns are the populations where both APOE and haplogroup U are more frequent than elsewhere. If the high frequency of haplogroup U is thought to be due to genetic drift (Soares et al. 2010; Achilli et al. 2005; Torroni et al. 2001), the high frequency of APO ε4 has been reported to be likely due to the advantage this allele may give in environments with poor day light (Huebbe et al. 2011). This might suggest that APOE ε4 is part of complex pathways meant to play a crucial role in the survival of Nordic populations and that this does not allow the supposed effect of haplogroup U molecules to interfere.

On the whole, although our results do not allow to understand the molecular basis of the interaction between mtDNA variability and APOE variability, they confirm that there is a positive interaction between APOE ε4 and haplogroup U, as previously suggested. In addition they show that this interaction is population specific and that it does not occur in some of the populations with the highest frequency of APOE ε4 and which have been reported to have an advantage from the high frequency of this allele due to the advantage it gives in environments with poor day light.

These results suggest that the demographic selection occurring after the age of 65 years is influenced by the combination of the different alleles at different loci and by their interaction with the environment. It might be intriguing to compare this to the classical "fitness landscapes metaphore" introduced by S Wright to explain the interaction between genes and environment to explain natural selection: "allelic combinations" hold different positions in the "fitness landscapes" according to the external environment and

other external forces as drift or migration (Wright 1932; Beerenwinkel et al. 2007). Obviously, demographic selection, unlike natural selection, does not affects the population gene pool but only the gene pool of the aging population.

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**Table 1** Distribution of APOE alleles in the different countries.

| COUNTRIES (N. of sample) | ε2 + ε2 | ε2 + ε3 | ε2 + ε4 | ε3 + ε3 | ε4 + ε3 | ε4 + ε4 |
|---|---|---|---|---|---|---|
| **BELFAST (127)** | 0 | 20 | 1 | 80 | 25 | 1 |
| **BELGIUM (152)** | 1 | 30 | 5 | 92 | 23 | 1 |
| **BOLOGNA (409)** | 2 | 52 | 2 | 307 | 45 | 1 |
| **CALABRIA (229)** | 1 | 32 | 3 | 172 | 20 | 1 |
| **DENMARK (844)** | 4 | 138 | 21 | 495 | 176 | 10 |
| **FINLAND (291)** | 2 | 29 | 6 | 181 | 66 | 7 |
| **GERMANY (181)** | 4 | 29 | 5 | 106 | 34 | 3 |
| **GREECE (179)** | 0 | 24 | 2 | 135 | 18 | 0 |
| **MONTPELLIER (527)** | 4 | 83 | 8 | 340 | 85 | 7 |
| **NETHERLAND (323)** | 2 | 53 | 7 | 206 | 50 | 5 |
| **NEWCASTLE (193)** | 0 | 32 | 6 | 116 | 38 | 1 |
| **POLAND (246)** | 1 | 32 | 0 | 179 | 33 | 1 |
| **ROMA (140)** | 0 | 19 | 2 | 102 | 17 | 0 |
| **SASSARI (89)** | 0 | 3 | 0 | 76 | 10 | 0 |
| **UKRAINA (91)** | 0 | 14 | 1 | 65 | 10 | 1 |

**Table 2** Distribution of mtDNA haplogroups in the different populations.

| COUNTRIES (N. of sample) | H | HV | I | J | K | T | U | W | X | OTHER |
|---|---|---|---|---|---|---|---|---|---|---|
| **BELFAST (127)** | 65 | 11 | 1 | 14 | 14 | 9 | 11 | 2 | 0 | 0 |
| **BELGIUM (152)** | 66 | 5 | 2 | 15 | 14 | 19 | 17 | 7 | 2 | 5 |
| **BOLOGNA (409)** | 179 | 30 | 5 | 29 | 29 | 43 | 58 | 7 | 17 | 12 |
| **CALABRIA (229)** | 95 | 12 | 8 | 22 | 22 | 18 | 26 | 6 | 9 | 11 |
| **DENMARK (844)** | 389 | 12 | 18 | 107 | 53 | 92 | 102 | 11 | 14 | 46 |
| **FINLAND (291)** | 133 | 4 | 3 | 19 | 18 | 11 | 56 | 14 | 5 | 28 |
| **GERMANY (181)** | 71 | 17 | 7 | 20 | 16 | 13 | 29 | 5 | 1 | 2 |
| **GREECE (179)** | 73 | 15 | 3 | 12 | 14 | 21 | 25 | 6 | 4 | 6 |
| **MONTPELLIER (527)** | 249 | 46 | 10 | 42 | 32 | 52 | 70 | 5 | 8 | 13 |
| **NETHERLAND (323)** | 134 | 20 | 9 | 24 | 33 | 43 | 44 | 7 | 7 | 2 |
| **NEWCASTLE (193)** | 75 | 14 | 8 | 24 | 12 | 28 | 23 | 4 | 3 | 2 |
| **POLAND (246)** | 101 | 24 | 6 | 22 | 7 | 28 | 45 | 5 | 3 | 5 |
| **ROMA (140)** | 60 | 15 | 3 | 9 | 9 | 22 | 13 | 0 | 4 | 5 |
| **SASSARI (89)** | 42 | 7 | 0 | 9 | 4 | 7 | 13 | 2 | 1 | 4 |
| **UKRAINA (91)** | 44 | 3 | 3 | 9 | 10 | 6 | 13 | 2 | 1 | 0 |

**Figure 10** Odds ratio and confidence interval (95%) for APOE allele ε4 distribution in nonagenarians and controls in the different population.

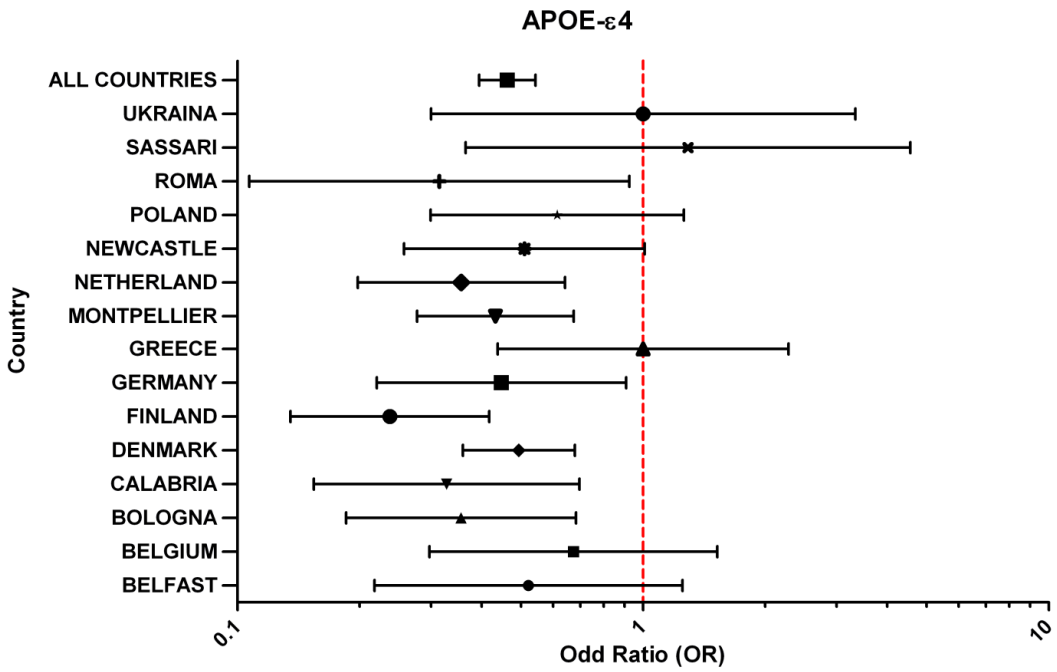


**Figure 2** Odds ratio value for APOE ε4 allele carriers in subjects with U and non U mtDNA molecules in each country.

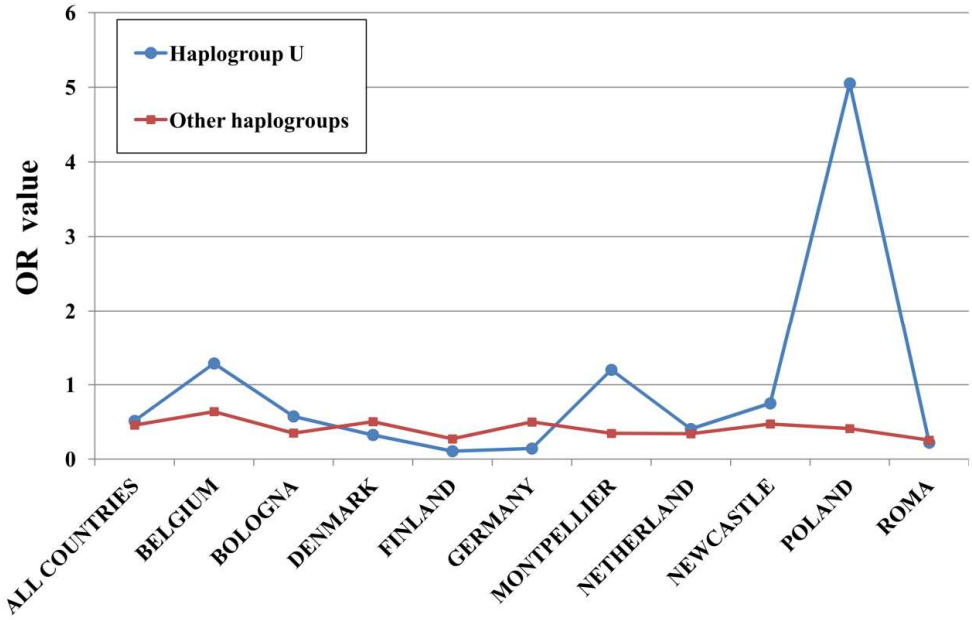
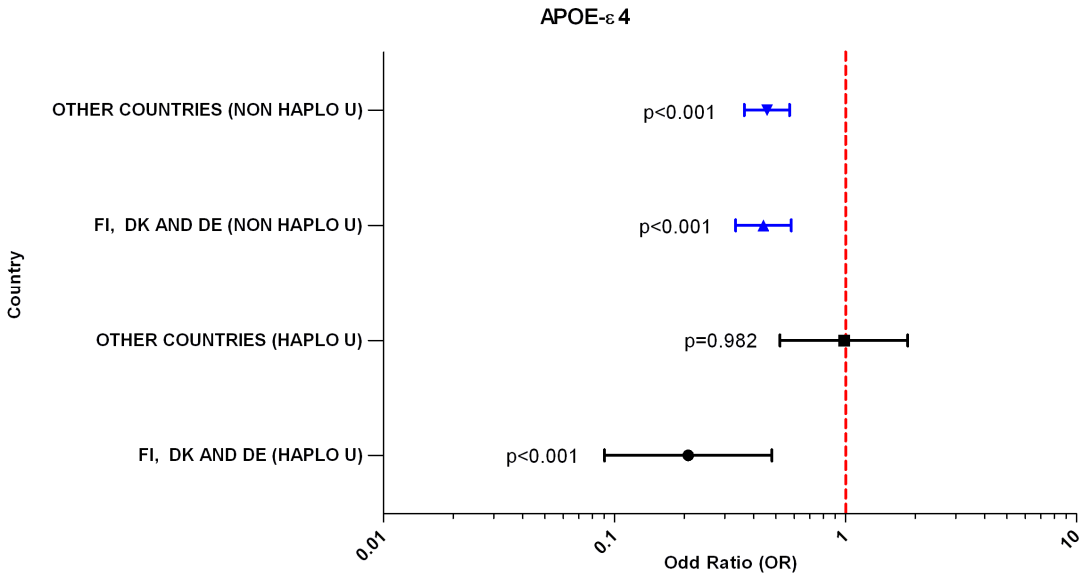


**Figure 3** Odds ratio and confidence interval (95%) for APOE allele ε4 carriers in subjects with U and non U mtDNA molecules in groups of populations pooled according to Figure 2.



## CHAPTER 4

### 4.1 Two-stage case-control association study of candidate genes and human longevity.

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**Abstract**

Human lifespan is determined in part by genetic factors. The pathways that regulate energy homeostasis, the mechanisms of damage repair, and the signaling response to internal environmental changes or external signals have been shown to be critical in modulating lifespan of model organisms and humans. We performed a two-stage case-control study in two samples from Calabria (Italy) to identify variants that contribute to survival at very old ages. Single-nucleotide polymorphisms (SNPs) were selected from genes encoding components of several metabolic pathways with known or supposed biological relevance to aging and longevity. In stage 1, 317 SNPs in 105 genes were analyzed in 78 cases (subjects older than 90 years) and 71 controls. In stage 2, 29 candidate SNPs identified in stage 1 (nominal p-value<0.15) were analyzed in an independent sample composed by 288 cases and 554 controls(subjects younger than 90 years). Two SNPs, rs282070 on intron 1 of *MAP3K7* gene, and rs2111699 on intron 1 of *GSTZ1* gene, were significantly associated with survival at advance age after adjustment for multiple testing in stage 2 ($P= 4.72x10^{-4}$ and 0.001 respectively). Interestingly, both genes are implicated in the cellular response to internal and external environmental changes, thus playing a crucial role in the inflammation processes that accompany aging. Our data, confirm that long-lived subjects are endowed with genetic variants that allow them to optimize these cellular responses, and to better deal with environmental and internal stresses.

**Key words:** longevity; two-stage analysis; single-nucleotide polymorphisms;

## Introduction

Over the past decades developed societies have experienced a gradual increase in life expectancy which sharply increased the percentage of elderly people in the population, and, at the same time, increased the number of subjects which are not autonomous and are affected by invalidating pathologies (Vaupel, 2010). Since this has important implications, especially from a social point of view, there is at present a considerable interest in understanding how individuals achieve a long and healthy life.

The determinants of human lifespan are multifactorial: environmental conditions (education, socio-economic status, and lifestyle choices such as diet, smoking habits, etc.), and the individual genetic make-up are the main factors responsible of the age-related changes in cell and tissue structure and function, that, inexorably, lead the organism to a condition of greater susceptibility and vulnerability to disease, and, ultimately, to death.

Family-based studies demonstrated the existence of a strong familial and genetic component of longevity. Indeed, parents, siblings and offspring of long-lived subjects have a significant survival advantage and a higher probability to have been or to became long-lived (Gudmundsson et al. 2000; Kerber et al. 2001; Cournil and Kirkwood 2001; Perls et al. 2000; Perls et al. 2002; Schoenmaker et al. 2006; Willcox et al. 2006). Moreover, they have a lower incidence of age related diseases and a higher degree of physical functioning and autonomy, when compared to appropriate selected controls (Terry et al. 2003; Terry et al. 2004; Atzmon et al. 2006). Studies comparing life span in twins have found that approximately 25% of the overall variation in human lifespan can be attributed to genetic factors (Herskind et al. 1996; Ljungquist et al. 1998; Skytthe et al. 2003), which become more relevant at advanced ages (Hjelmborg et al. 2006).

In the past several years, there has been a surge of activity aimed at unraveling the genetic processes that play key roles in aging and lifespan. Specific mutations in single genes (age-1,

daf2, sir2, methusela, p66) that extend or decrease lifespan in nematode worms (C. elegans), fruit flies (Drosophila), and mice, have revealed evolutionary, often interconnected, conserved pathways for aging which are related to nutritional sensing, stress response signalling, and DNA repair/maintenance (Kenyon 2010; Fontana et al. 2010). The insulin/insulin-like growth factor-I (IGF-I) signalling pathway, and downstream effectors such as the foxhead box O group (FoxO) family, the sirtuin family, the target of rapamycin (TOR) kinase, are perhaps the best studied pathway involved in aging and longevity.

Research in human longevity mainly focused on the orthologues of such candidate loci. Genetic association studies of centenarians or long-lived subjects, as well as functional studies, have been successful in identifying specific genes that influence variation in human lifespan, and in highlighting genotypes that can increase or decrease the individual probability to reach advanced age. Several reviews are now available that summarize and analyze gene variants associated with long life in humans (Christensen et al. 2006; Bonafè and Olivieri 2009; Chung et al. 2010; Slagboom et al. 2011). Particular striking examples include GH/IGF-1/Insulin signalling (*GHR, IGF1R, FOXO3A*), antioxidant (*SOD1, SOD2, PON1, FOXO3A*), inflammatory (*IL6, CETP, Klotho*) pathways, silencing genes (*SIRT1 and SIRT3*), elements of lipid metabolism (*APOE, APOB, ACE, APOC3*), to cite some of them.

Until recently, almost all gene-longevity association studies have focused on a single or few candidate genes. However, given the complexity of the longevity trait, single-locus methods may not be suitable, since it is likely that loci contribute to the trait by their epistatic interaction with other genes, while main effects of the individual loci may be small or absent. Thus, approaches have been developed that take into account that complex traits can be caused by an intricate pattern of genetic variants. Recent technological improvements in genotyping technology have made whole genome genotyping (> 100,000 SNPs) more accessible. Indeed, genome-wide association studies (GWAS) are at present widely used to find genetic variants contributing to variation in human lifespan (Puca et al. 2001; Lunetta et al. 2007; Newman et al. 2010; Walter et al. 2011; Deelen et al. 2011; Nebel et al. 2011; Malovini et al. 2011). Along

with GWAS, studies that consider candidate biochemical pathway consisting of many genes biologically related, and a minimal number of ‘‘tagging’’ SNPs that efficiently captures all the common genetic variation in the assayed genomic region, are also being routinely used (Nebel et al. 2009; Pawlikowska et al. 2009; Flachsbart et al. 2010). Taken together, these studies highlighted that the variation of several genes contribute to human longevity, although replication has often failed in independent studies in different populations. Exceptions are the APOE and FOXO3A genes, (Deelen et al. 2011; Nebel et al. 2011; Willcox et al. 2008; Anselmi et al. 2009; Flachsbart et al. 2009; Li et al. 2009). The lack of replication may be due to statistical limitations of large-scale association studies that require a large number of long-lived or centenarian subjects for adequate power. In addition, replication may be hampered by the fact that longevity genes can be population-specific (De Benedictis and Franceschi 2006).

In the last years, two-stage study designs have been proposed as an efficient means of addressing these challenges. In a typical two-stage design for association study, a first sample (stage 1) is used to test a large number of SNPs and only those showing some hint of association are subsequently re-tested in a second sample (stage 2), thus reducing the number of association tests. The second sample may be considered as a replication study (Zuo et al. 2006; Satagopan et al. 2003; Satagopan et al. 2004; Thomas et al. 2004; Skol et al. 2007; Zheng et al. 2008).

Here, using a two-stage approach, we performed a targeted investigation of selected genes involved in longevity pathways (DNA repair, apoptosis and cell cycles regulation, metabolism of xenobiotics, insulin /IGF-1, neuromodulation). Altogether, we tested 317 single nucleotide polymorphisms (SNPs) in 105 candidate genes for association with the longevity phenotype in two samples from South Italy for a total 366 long-lived (nonagenarians and centenarians) and 625 younger subjects.

## Materials and Methods

*Sample*

Our initial sample (stage 1) was recruited between 1994 and 1997 in Calabria (southern Italy) and included a sample of 149 unrelated subjects of which 78 were considered as cases (≥ 90 years; mean age 97.4 ±3.2 years, 36 males and 42 females ) and 71 as controls (< 90 years; mean age 70.0 ± 9.9 years, 39 males and 32 females). The replication study (stage 2) consisted of 288 cases (≥ 90 years; mean age 93.7 ± 4.0 years, 125 males and 163 females) and 554 controls (< 90 years; mean age 58.3 ± 20.0 years, 277 males and 277 females) recruited subsequently between 2005 and 2008, also in Calabria. Study participants, their parents, and grandparents were all born in Calabria, as ascertained from population registers.

Younger subjects were contacted through general physicians. Subjects older than 90 years were identified by screening of population registers in different municipalities distributed across the entire Calabria region. Subjects who were eligible for the study were contacted and asked for participating in the study. A written informed consent was obtained from all participants before enrolling in the study. The healthy status was ascertained by medical visit carried out by a geriatrician who also administered a structured questionnaire validated at European level. The questionnaire was aimed to the collection of socio-demographic information, evaluation of physical and cognitive status, medications, and information on common diseases occurred in the past. Subjects with dementia and/or neurologic disorders were not included. At the time of the visit, peripheral venous blood samples were also obtained. White blood cells (WBC) from blood buffy coats were used as source of DNA.

*Candidate gene and SNP selection*

Candidate genes were chosen from genetic pathways related to DNA repair, apoptosis and cell cycles regulation, metabolism of xenobiotics, insulin/IGF-1. The selection was based on their

biological relevance, and on published reports indicating their involvement in aging and longevity in either model organisms or humans. Using keywords as “aging AND longevity”, “human aging”, “human longevity”, “aging AND model organism”, a range of online databases were employed including pubmed (http://www.ncbi.nlm.nih.gov/pubmed/), GenAge (http://genomics.senescence.info/genes)/. Additionally, pathway analysis tools, such as “Biocarta” and “Kegg” were used for identification of pathway-specific genes.

The chromosomal region encompassing the candidate genes were identified through the databases http://www.ncbi.nlm.nih.gov/ (gene and mapview), http://www.ensembl.org/index.html. The gene region was defined as the region from up to 5Kb upstream of the translation start site to up to 5Kb downstream of the translation stop site, allowing no overlap with the neighboring genes. In the choice of candidate SNPs we availed of different online databases (es: http://www.ncbi.nlm.nih.gov/projects/SNP; http://www.ensembl.org/index.html; http://www.hapmap.org/cgi-perl/gbrowse/hapmap).

SNPs selection was based on the following criteria: minor allele frequency (MAF) > 10% in caucasian, coding SNPs and SNPs having potential functional impact (non-synonymous SNPs, SNPs located in potential regulatory region), SNPs previously identified in relevant association studies. Tagging SNPs were selected with the use of the Tagger program within Haploview (http://www.broad.mit.edu/mpg/haploview/; http://www.broad.mit.edu/mpg/tagger/; de Bakker et al., 2005), using pairwise tagging with a minimum r2 of 0.8.

In total 105 genes composing the above mentioned pathways, and 317 SNPs were identified. The complete list of genes, SNPs and other relevant information is provided in Supplementary Table S1

*Genotyping*

Genotyping of samples in stage 1 was carried out using a microarray technique based on the arrayed primer extension (APEX) (Kurg et al. 2000).

Genotyping of samples in stage 2 was carried out using an allele-specific PCR-based KASPar SNP genotyping system (KBiosciences, Hoddesdon, UK) (Cuppen 2007).

Genotyping quality control procedures consisted of inclusion of duplicates, and inclusion of both positive and negative (water blanks) controls in each assay reaction.

*Quality-control*

After genotype calling, in order to check errors at genotypic level, the dataset was subjected to a battery of quality-control (QC) tests. In particular, SNPs were excluded if they had (1) a significant deviation from Hardy-Weinberg equilibrium (HWE, $p<0.001$) in the control group, (2) a missing frequency (MiF) higher than 20%, and (3) a frequency of rare allele (MAF) <1%.

*Statistical analyses*

Single SNP analysis

Two different genetic association tests were adopted in the present study. In stage 1 the chi-square test was used to assess the association between the variability of the analysed polymorphisms and human longevity, while in the stage 2 the *MAX* test was used (Freidlin et al.2002; Zang et al.2010) As it regards the stage 1 association analysis, before to apply the chi-square test, each genotypic variable was first coded with respect dominant, recessive and heterozygote disadvantage/advantage model. For each of these models the corresponding chi-square value was obtained. The maximum of these three chi-square values ($\chi^2_{MAX}$) was used as a measure of association between the polymorphism and the analyzed phenotype:

$$\chi^2_{MAX} = \max(\chi^2_{DOM}, \chi^2_{REC}, \chi^2_{HET})$$

where $\chi^2_{DOM}$, $\chi^2_{REC\ AND}$ $\chi^2_{HET}$ represent the three chi-square statistics assuming a dominant, recessive and an heterozygote disadvantage/advantage model of inheritance of the analyzed polymorphism. The p-value of the proposed test statistics was obtained by the Monte-Carlo procedure described in Ziegler and König (2010).

As mentioned before, in stage 2 the *MAX* test, originally proposed by Freidlin et co-workers (2002) was adopted. The *MAX* test (or *MAX3* test) takes the maximum of three Cochran-Armitage Trend Tests ($Z_{REC}$, $Z_{ADD}$, $Z_{DOM}$) and it is given by:

$$MAX = MAX\{|Z_{Rec}|, |Z_{Add}|, |Z_{Dom}|\}$$

where $Z_{REC}$, $Z_{ADD}$, and $Z_{DOM}$ represent the three Cochran-Armitage Trend Test statistics assuming a recessive, additive or dominant mode of inheritance of the analyzed polymorphism. In order to estimate the p-value of *MAX*, a simple algorithm to calculate its asymptotic null distributions was recently suggested by Zang and co-workers (2010) was used.

In the present study the $\chi^2_{MAX}$ test (maximum of three different chi-square tests) was used in stage 1 to assess the associations between the selected markers (M=317) and the analysed phenotype. Then, the $\chi^2_{MAX}$ values were ranked and a proportion $\pi_{markers}$ of the top-ranked polymorphisms (p-value<0.15), corresponding approximately to the 10% of the initial set of SNPs (first decile), was selected for stage 2 analysis. By following the standard strategy to consider the stage 2 data as a replication study, we tested for association using the *MAX* test, adopting the Bonferroni-corrected significance level $\alpha/(\pi_{markers}*M)$.

*Interaction analysis*

In order to capture gene-gene interactions we availed of the Model-Based Multifactor Dimensionality Reduction (MB-MDR) method recently developed by Calle and co-workers (2010). It is an extension of the popular Multifactor Dimensionality Reduction (MDR) method in which risk categories are defined using a regression model that also allows adjustments for

main effects and covariates. By this approach, first, a logistic regression analysis is performed to define multilocus genotypes categorized as high (*H*), low (*L*) or no risk (0). Then, this multilocus genotypes of the same risk category are merged and two Wald statistics (WH and WL, one for each risk) with the relevant p-values (PH and PL) are obtained. The significance for the epistatic effect is be based on the minimum between PH and PL (MIN.P). Finally, the significance of a specified model is assessed through a permutation test on the maximum Wald statistic and implemented in the function mbmdr.PermTest of MB-MDR package.

In the present study we applied the MB-MDR algorithm to the stage 2 dataset. The permutation procedure (10,000 permutations) was applied to the interaction models with a MIN.P<0.05.

**Results**

*Dataset Stage 1*

Initially, 317 SNPs from 105 candidate genes (see Supplementary Table S1) were genotyped in 78 cases and 71 controls. After the QC phase, the final number of analysed polymorphisms in Stage 1 was 235. In particular, 49 SNPs were excluded from the analysis because of a significant deviation from HWE. 30 SNPs were excluded from the analysis because of a MiF value higher than 20%. Finally, 3 SNPs were excluded on the basis of MAF criterion (<1%). The cleaned dataset was then analyzed for association with longevity by using the $\chi^2_{MAX}$ test.

*Single-locus analysis*

The following Manhattan plot (Figure.1) displays the p-values computed on the basis of the $\chi^2_{MAX}$ test with respect to the analyzed polymorphisms.

Table 1 reports the list of the top-ranked SNPs (p-value<0.15) obtained according to the association results ($\chi^2_{MAX}$) and corresponding approximately to the top decile of the initial set of SNPs.

*Dataset Stage 2*

The 31 selected SNPs were genotyped in the additional 288 cases and 554 controls in the subsequent Stage 2. Two SNPs were excluded from the following association analysis because of a MiF value higher than 20%.

As mentioned in "Materials and Methods" section, the cleaned dataset was then analyzed for association with longevity by using the *MAX* test. In Table 2 the association results of the 29 selected SNPs ranked on the basis of their estimated p-values are reported.

From Table 2, it can be easily be seen that there is a major peak on the chromosome 6 with a p-value equal to $4.72 \times 10^{-4}$(rs282070). This SNP is located in *MAP3k7* gene. Another peak can be seen on chromosome 14 with a p-value equal to 0.001 (rs2111699). This SNP is located in *GSTZ1* gene. Also after the Bonferroni correction both the associations between the above mentioned variations and human longevity remained statistically significant (p-value < 0.05/29).

*Interaction analysis.*

Table 3 reports the significant second-order interactions results ($p<0.05$) in the Stage 2 dataset from the MB-MDR analysis.

In this table for each interaction model a permutation testing was performed for each of the top-ranked models (MIN.P <0.05) by permuting the outcome variable calling the *mbmdr.PermTest* function of the *mbmdr* package (10,000 permutations). By this approach we found that all these interactions resulted to be highly significant ($P \leq 0.05$). However, after a Bonferroni correction for multiple testing no significant differences remained.

**Discussion**

In the present study, we have investigated possible associations between longevity and 317 SNPs in 105 candidate genes involved in the regulation of aging related biological pathways. For testing associations we used a two-stage design, an approach that has proven useful for the identification of a number of genetic variants associated with pathological phenotypes such as

cancer, Crohn's Disease, migraine (Milne et al. 2006; Beckly et al. 2008; Corominas et al. 2009). To improve the efficiency of this study design, we used two robust tests which are powerful when the underlying genetic model is unknown, and which were successfully applied to genome-wide association studies (Joo et al. 2005; Tian et al. 2005; Zheng et al. 2007; Sladek et al. 2007; Li et al. 2008a; 2008b).

We found that two SNPs, rs282070 on intron 1 of *MAP3K7* gene, and rs2111699 on intron 1 of *GSTZ1* gene, were significantly associated with survival at advance age after Bonferroni correction.

MAP3K7 (also known as TAK1, Transforming growth factor-β activated kinase-1) is a serine/threonine kinase, member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, which plays a crucial role in interleukin 1 (IL-1), tumor necrosis factor (TNF), and Toll-like receptor (TLR) signaling pathways. Various stressors and inflammatory cytokines trigger the activation of MAP3K7 which in turn activates key intra-cellular kinases such as the p38 MAPK, c-jun N-terminal kinase (JNK) and I-kappa B kinase complex (IKK). These pathways ultimately activate transcription factors nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) which are critical regulators of genes of the immune-inflammatory responses (Ninomiya-Tsuji et al. 1999; Sato et al. 2005; Chen et al. 2006; Landström 2010). Studies have also demonstrated that the oxidative stress caused by reactive oxygen species (ROS) may induce or mediate the activation of the MAPK pathways, including the one mediated by MAP3K (Son et al. 2011 for a review). In particular, Omori and colleagues (2008) reported that MAP3K7 regulates the levels of ROS and prevent inflammation. Thus, MAP3K7 may be considered as a primary factor in the cascade of intracellular events initiated by environmental and/or physiological stressors that induce cells to survive or to die, and therefore may be important for regulating aging. However, although several components of MAPK signaling pathways play important roles in the regulation of cellular senescence (Maruyama et al. 2009; Debacq-Chainiaux et al. 2010), and have been also implicated in the pathogenesis of many human diseases (Kim and Choi 2010), no data has been documented so far about the

influence of MAP3K7 on human aging and longevity. The results of our study provide the first evidence of such an influence with the rs282070 positively affecting longevity in a dominant manner.

It is intriguing that the second gene, *GSTZ1*, we have identified as playing a role in longevity is also implicated in the cellular response to environmental insults. *GSTZ1* is in fact a member of glutathione S-transferase (GSTs) superfamily of multifunctional enzymes which are involved in the cellular detoxification, via conjugation with glutathione (GSH) of a wide range of endogenous and exogenous toxic compounds, and various reactive products generated under oxidative stress. Diverse cellular functions, ranging from apoptosis to cell survival, are also modulated by GSTs (Nebert et al. 2004; Hayes et al. 2005; Laborde 2010).

The GSTs comprise three families of genes designed as cytosolic, mitochondrial, and microsomal transferase. The cytosolic GSTZ class of GSTs is expressed in many eukaryotic species, including fungi, plants, insects, and mammals (Board et al. 1997). Human GSTZ1, which is identical to maleylacetoacetate isomerase, catalyses two distinct reactions: the biotransformation of a range of α-haloacids, including dichloroacetic acid (DCA), a drinking water contaminant, and the GSH-dependent isomerisation of maleylacetoacetate to fumarylacetoacetate, the penultimate step in the Phenylalanine–Tyrosine catabolic pathway (Board et al. 2005; Board and Anders 2011). Analysis of *Gstz1*-knockout mice provided more insight about the physiological role of Gstz1. Besides a range of pathological changes, *Gstz1*$^{-/-}$ mice display the induction of some of the cytosolic GST isoenzymes, and the constitutive expression of genes that are regulated by antioxidant-response elements (AREs) and that respond to oxidative stress via the Keap1/Nrf2 signaling pathway, which regulates the expression of numerous detoxifying and antioxidant genes (Lim et al. 2004; Lee and Johnson 2004;). All of these findings support the view that the lack of GSTZ1 may induce oxidative stress (Blackburn et al. 2006). The antioxidant role of GSTs is well known and derives essentially from their ability to regulate the concentration of GSH, the most powerful cellular antioxidant, in different cellular compartments including mitochondria, the principal source and

target of ROS. For instance, specific mitochondrial GST isoforms are able to protect mitochondria from oxidative insults either indirectly by maintaining appropriate levels of GSH or directly by reducing cellular peroxides and reactive aldehydes, such as 4-hydroxynonenal (HNE), generated from lipid peroxidation (Raza 2011). The recent discovery that GSTZ1 also localizes in mitochondria (Li et al. 2011), leads to assume that GSTZ1 may also contribute to the cellular redox status, and then may be relevant to aging and longevity. Interestingly Starr et colleagues (2008) showed that the *GSTZ1* R42G variant affects both telomere length, a biomarker of stress and aging, and physical aging and suggest that this may occur through oxidative stress. Moreover it has been shown that the *GSTZ1*- 1002 G>A variant influences cognitive ability in the elderly, probably because of its effect on dopamine production and dopamine by-product disposal (Starr et al. 2008). In our study, the rs2111699 resulted to be significant associated to the longevity phenotype. It seems to act in a recessive manner, with the minor allele favoring survival at very old age. To our knowledge this is the first study reporting an association between *GSTZ1* genetic variability and human survival.

Overall, based on our an previous data, it can be concluded that *MAP3K7* and *GSTZ1* are longevity genes whose variability actually affects the organism's chances of reaching advanced age.

As the rs282070 in *MAP3K7* and the rs2111699 in *GSTZ1* are intronic, their functional significance is unclear. It is possible that these SNPs are located in regions containing cis-regulatory elements essential for gene expression, and that the allelic variants differently affect the transcriptional rate, but linkage disequilibrium (LD) of these SNPs with other functional variants it also possible. This is an interesting issue that needs to be investigated in future research. Nevertheless, we believe that our results reinforce the view that the immune, inflammatory and stress responses, which are essential components of the body’s network of defense against external and internal damaging agents, play an essential role in life span and in age-related phenotypes.

In the 2000 Franceschi and colleagues used the term inflamm-aging to indicate that aging is accompanied by an age-dependent up-regulation of the inflammatory response due to the lifelong chronic antigenic load which impinges upon the immune system (immunosenescence), and is associated with increased morbidity and mortality rates in the elderly. In fact, chronic inflammation has been implicated in the pathogenesis of chronic age-related diseases including Alzheimer's disease, atherosclerosis, diabetes, and even cancer (Franceschi et al. 2000; Franceschi et al. 2007); on the contrary, studies of centenarians showed that variations in several pro- or anti-inflammatory molecules influence successful aging and longevity, and that longevity is partially due to the capability of maintaining an inflammatory response at relatively low level of intensity (Bonafè et al. 2001; Lio et al. 2002; Capri et al. 2006; Iannitti and Palmieri, 2011). There is a close link between inflammation and oxidative stress, in fact an excessive production of ROS can induce an inflammatory response. This fits in the frame of the free-radical theory of aging, developed in 1956, which asserts that free radical damages are responsible of many of the changes occurring over time. The recently proposed oxidation-inflammatory theory of aging (De la Fuente and Miquel 2009), brings together the concepts above mentioned, highlighting the key involvement of the immune/ inflammatory/oxidative stress axis in the rate and quality of aging. Our finding that the variability of two genes working along this axis promotes survival at very old age agrees with the above, and

confirms that long-lived subjects are equipped with gene variants that allow them to optimize the cellular response to environmental and internal stresses.

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**Figure 1**. Manhattan plot of the stage 1 association results using the $\chi^2_{MAX}$ test (maximum of three different chi-square tests). Red line represents the p-value threshold for the top ranked SNPs ($p<0.15$).

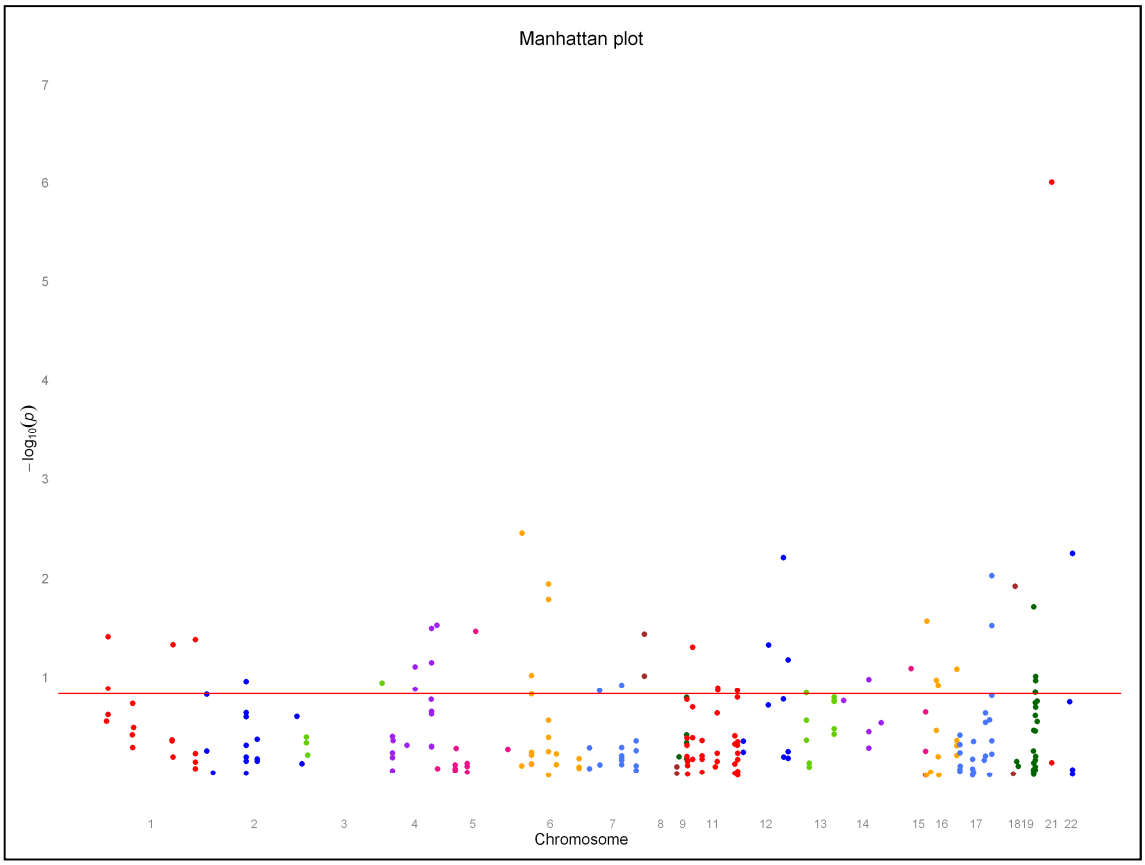

**Table 1.** Top-ranked SNPs (p<0.15) obtained according to $\chi^2_{MAX}$ test in the stage 1 sample.

| dbSNP | GENE | DOM | $P_{DOM}$ | REC | $P_{REC}$ | HET | $P_{HET}$ | MAX | P-value |
|---|---|---|---|---|---|---|---|---|---|
| rs12626475 | SOD1 | 16.997 | <0.001 | 2.236 | 0.151 | 5.587 | 0.022 | 16.997 | <0.001 |
| rs135539 | PPARa | 9.406 | 0.002 | 0.012 | 1.000 | 8.229 | 0.006 | 9.406 | 0.006 |
| rs6214 | IGF1 | 9.394 | 0.004 | 1.508 | 0.275 | 3.842 | 0.058 | 9.394 | 0.006 |
| rs1062935 | RAPTOR | 8.431 | 0.005 | 0.540 | 0.560 | 3.739 | 0.064 | 8.431 | 0.010 |
| rs7614 | MBD2 | 3.899 | 0.054 | 1.822 | 0.196 | 7.937 | 0.006 | 7.937 | 0.012 |
| rs282070 | MAP3K7 | 5.629 | 0.019 | 0.295 | 0.668 | 7.028 | 0.011 | 7.028 | 0.012 |
| rs1005273 | PDK1(PDPK1) | 1.494 | 0.271 | 3.262 | 0.088 | 6.838 | 0.015 | 6.838 | 0.028 |
| rs10470911 | EGF | 1.482 | 0.232 | 6.546 | 0.013 | 0.573 | 0.512 | 6.546 | 0.033 |
| rs2069762 | IL2 | 6.527 | 0.014 | 4.943 | 0.045 | 1.147 | 0.332 | 6.527 | 0.030 |
| rs16902632 | GAP(RASA1) | 6.488 | 0.017 | 0.174 | 0.710 | 3.740 | 0.069 | 6.488 | 0.035 |
| rs157703 | MAP3K7 | 4.370 | 0.040 | 1.179 | 0.352 | 6.254 | 0.015 | 6.254 | 0.016 |
| rs1799930 | NAT2 | 4.220 | 0.042 | 0.655 | 0.529 | 6.171 | 0.021 | 6.171 | 0.037 |
| rs7209040 | RAPTOR | 1.470 | 0.288 | 2.765 | 0.105 | 6.043 | 0.015 | 6.043 | 0.031 |
| rs6337 | NTRK1 | 5.877 | 0.022 | 0.934 | 0.400 | 3.497 | 0.072 | 5.877 | 0.047 |
| rs1800471 | TGFB1 | 5.708 | 0.020 | 5.708 | 0.019 | 0.000 | 0.000 | 5.708 | 0.020 |
| rs1800896 | IL10 | 3.413 | 0.092 | 0.857 | 0.378 | 5.587 | 0.018 | 5.587 | 0.042 |
| rs1625525 | MDM2 | 5.491 | 0.032 | 0.295 | 0.697 | 4.652 | 0.041 | 5.491 | 0.048 |
| rs3789327 | ARNTL | 1.076 | 0.358 | 5.398 | 0.023 | 1.016 | 0.393 | 5.398 | 0.050 |
| rs11066301 | SHP12 (PTPN11) | 3.243 | 0.108 | 4.905 | 0.036 | 0.016 | 1.000 | 4.905 | 0.067 |
| rs9991904 | EGF | 4.871 | 0.034 | 0.642 | 0.446 | 3.014 | 0.094 | 4.871 | 0.072 |
| rs6533485 | EGF | 4.814 | 0.044 | 0.890 | 0.444 | 1.272 | 0.319 | 4.814 | 0.073 |
| rs4073 | IL8 | 3.092 | 0.093 | 4.460 | 0.039 | 0.154 | 0.739 | 4.460 | 0.079 |
| rs1800566 | NQO1 | 0.340 | 0.621 | 4.452 | 0.055 | 0.011 | 1.000 | 4.452 | 0.084 |
| rs1549854 | MEK (MAP2K1) | 4.441 | 0.042 | 0.272 | 0.668 | 2.658 | 0.139 | 4.441 | 0.083 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| rs1799929 | NAT2 | 1.996 | 0.192 | 1.109 | 0.326 | 4.396 | 0.045 | 4.396 | 0.098 |
| rs1770345* | MTOR (FRAP1) | 4.330 | 0.046 | - | - | 4.330 | 0.042 | 4.330 | 0.040 |
| rs195990 | PRKCB1 | 0.596 | 0.477 | 3.343 | 0.079 | 4.276 | 0.050 | 4.276 | 0.108 |
| rs3212948 | ERCC1 | 0.071 | 0.861 | 4.111 | 0.050 | 3.176 | 0.099 | 4.111 | 0.099 |
| rs854556 | PON1 | 3.974 | 0.050 | 0.322 | 0.618 | 2.638 | 0.136 | 3.974 | 0.122 |
| rs2111699 | GSTZ1 | 3.964 | 0.056 | 1.664 | 0.328 | 1.836 | 0.220 | 3.964 | 0.107 |
| rs870995 | PI3K (PIK3 CA) | 1.618 | 0.230 | 1.030 | 0.332 | 3.874 | 0.062 | 3.874 | 0.116 |
| rs1800587 | IL1A | 3.856 | 0.071 | 0.583 | 0.590 | 2.366 | 0.174 | 3.856 | 0.112 |
| rs649392 | CCND1 | 0.218 | 0.724 | 3.817 | 0.081 | 1.436 | 0.248 | 3.817 | 0.130 |
| rs1042571 | POMC | 3.052 | 0.113 | 3.738 | 0.125 | 1.203 | 0.369 | 3.738 | 0.152 |
| rs603965 | CCND1 | 0.289 | 0.714 | 3.694 | 0.060 | 1.435 | 0.255 | 3.694 | 0.138 |
| rs2227306 | IL8 | 3.669 | 0.071 | 0.883 | 0.399 | 1.972 | 0.187 | 3.669 | 0.134 |
| rs2261434 | MTOR (FRAP1) | 1.308 | 0.314 | 1.755 | 0.241 | 3.554 | 0.068 | 3.554 | 0.132 |
| rs1799787 | ERCC2 | 2.170 | 0.177 | 0.368 | 0.642 | 3.542 | 0.068 | 3.542 | 0.145 |

*Since no subject homozygous for the rare allele was observed, the chi-square test for the recessive model was not reported.

**Table 2.** Association results of the 29 selected SNPs ranked on the basis of their estimated p-values using the *MAX* test in the stage 2 sample.

| dbSNP ID | Gene | CHR | BP | Best model | MAX3 | P-value | Q-value |
|---|---|---|---|---|---|---|---|
| **rs282070** | **MAP3K7** | **6** | **91296420** | **Dominant** | **3.733** | **4.72E-04** | **0.011** |
| **rs2111699** | **GSTZ1** | **14** | **77788597** | **Recessive** | **3.537** | **0.001** | **0.012** |
| rs11066301 | SHP12 (PTPN11) | 12 | 112871372 | Dominant | 2.906 | 0.009 | 0.069 |
| rs1042571 | POMC | 2 | 25383887 | Recessive | 2.101 | 0.075 | 0.459 |
| rs3212948 | ERCC1 | 19 | 45924362 | Recessive | 2.006 | 0.095 | 0.460 |
| rs1770345 | MTOR (FRAP1) | 1 | 11214580 | Dominant | 1.569 | 0.222 | 0.738 |
| rs1800587 | IL1A | 2 | 113542960 | Recessive | 1.363 | 0.321 | 0.738 |
| rs1062935 | RAPTOR | 17 | 78939857 | Additive | 1.306 | 0.349 | 0.738 |
| rs1549854 | MEK (MAP2K1) | 15 | 66696735 | Dominant | 1.276 | 0.365 | 0.738 |
| rs649392 | CCND1 | 11 | 69464793 | Dominant | 1.254 | 0.377 | 0.738 |
| rs12626475 | SOD1 | 21 | 33042929 | Recessive | 1.247 | 0.382 | 0.738 |
| rs1799787 | ERCC2 | 19 | 45856144 | Dominant | 1.225 | 0.393 | 0.738 |
| rs195990 | PRKCB1 | 16 | 23946385 | Recessive | 1.061 | 0.495 | 0.738 |
| rs135539 | PPARa | 22 | 46559267 | Recessive | 1.029 | 0.518 | 0.738 |
| rs3789327 | ARNTL | 11 | 13385316 | Additive | 1.017 | 0.521 | 0.738 |
| rs10470911 | EGF | 4 | 110865271 | Recessive | 1.010 | 0.524 | 0.738 |
| rs4073 | IL8 | 4 | 74606024 | Dominant | 0.962 | 0.558 | 0.738 |
| rs6214 | IGF1 | 12 | 102793569 | Additive | 0.925 | 0.584 | 0.738 |
| rs854556 | PON1 | 7 | 94944923 | Recessive | 0.885 | 0.609 | 0.738 |
| rs2069762 | IL2 | 4 | 123377980 | Recessive | 0.870 | 0.617 | 0.738 |
| rs1625525 | MDM2 | 12 | 69212831 | Dominant | 0.843 | 0.637 | 0.738 |
| rs870995 | PI3K (PIK3 CA) | 3 | 178913006 | Dominant | 0.771 | 0.686 | 0.741 |
| rs7209040 | RAPTOR | 17 | 78527294 | Dominant | 0.705 | 0.725 | 0.741 |
| rs1005273 | PDK1(PDPK1) | 16 | 2645965 | Additive | 0.696 | 0.731 | 0.741 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| rs1799930 | NAT2 | 8 | 18258103 | Recessive | 0.603 | 0.791 | 0.770 |
| rs7614 | MBD2 | 18 | 51681244 | Recessive | 0.457 | 0.872 | 0.816 |
| rs1800896 | IL10 | 1 | 206946897 | Recessive | 0.263 | 0.956 | 0.837 |
| rs16902632 | GAP(RASA1) | 5 | 86654813 | Additive | 0.129 | 0.989 | 0.837 |
| rs1800566 | NQO1 | 16 | 69745145 | Dominant | 0.050 | 0.998 | 0.837 |

**Table 3**. Interaction analysis results obtained using the Model-Based Multifactor Dimensionality Reduction approach proposed by Calle and co-workers (2010)

| SNP1 | SNP2 | NH | WH | PH | NL | WL | PL | MIN.P | P* |
|---|---|---|---|---|---|---|---|---|---|
| rs195990 | rs2111699 | 2 | 6.947 | 0.008 | 1 | 3.493 | 0.062 | 0.008 | 0.0056 |
| rs1770345 | rs3789327 | 1 | 4.032 | 0.045 | 2 | 6.870 | 0.009 | 0.009 | 0.0094 |
| rs16902632 | rs3789327 | 1 | 6.147 | 0.013 | 1 | 3.432 | 0.064 | 0.013 | 0.0118 |
| rs649392 | rs2069762 | 1 | 5.717 | 0.017 | 0 | - | - | 0.017 | 0.0128 |
| rs16902632 | rs6214 | 1 | 2.890 | 0.089 | 1 | 5.651 | 0.017 | 0.017 | 0.0122 |
| rs1800566 | rs2069762 | 1 | 5.188 | 0.023 | 0 | - | - | 0.023 | 0.0109 |
| rs12626475 | rs1799930 | 1 | 4.890 | 0.027 | 0 | - | - | 0.027 | 0.0141 |
| rs1549854 | rs1800896 | 1 | 4.661 | 0.031 | 0 | - | - | 0.031 | 0.0234 |
| rs1770345 | rs1625525 | 0 | - | - | 1 | 4.439 | 0.035 | 0.035 | 0.0281 |
| rs649392 | rs4073 | 1 | 4.434 | 0.035 | 0 | - | - | 0.035 | 0.0274 |
| rs1799930 | rs6214 | 1 | 4.381 | 0.036 | 0 | - | - | 0.036 | 0.0253 |
| rs1062935 | rs282070 | 1 | 4.372 | 0.037 | 0 | - | - | 0.037 | 0.0193 |
| rs11066301 | rs1800566 | 1 | 4.338 | 0.037 | 0 | - | - | 0.037 | 0.0242 |
| rs649392 | rs1770345 | 0 | - | - | 1 | 4.305 | 0.038 | 0.038 | 0.0345 |
| rs135539 | rs870995 | 1 | 3.461 | 0.063 | 1 | 4.236 | 0.040 | 0.040 | 0.0286 |
| rs1800566 | rs4073 | 1 | 4.065 | 0.044 | 0 | - | - | 0.044 | 0.0254 |
| rs282070 | rs1800896 | 1 | 4.042 | 0.044 | 0 | - | - | 0.044 | 0.0304 |
| rs135539 | rs7614 | 1 | 4.022 | 0.045 | 1 | 3.629 | 0.057 | 0.045 | 0.037 |
| rs1800896 | rs1799787 | 0 | - | - | 1 | 3.962 | 0.047 | 0.047 | 0.0382 |

NH, NL: the number of multi locus genotypes classified as high and low risk, respectively.

WH, WL: Wald statistic for the high and low risk categories, respectively.

PH, PL: unadjusted *P*-value for the high and low risk categories, respectively

MIN.P: The minimum between PH and PL.

P*: simulated p-values obtained calling the *mbmdr.PermTest* function of the *mbmdr* package (10000 permutations)

Table 1SM. Loci and polymorphisms analyzed in the stage 1.

| Gene Name | SNPs | Chr position | bp position | Type | Pos. in gene |
|---|---|---|---|---|---|
| MAP3K7 | rs157703 | 6q15 | 29047801 | Stress response | Intron 14 |
| MAP3K7 | **rs791063** | 6q15 | 29058479 | Stress response | Intron 13 |
| MAP3K7 | **rs13208824** | 6q15 | 29064902 | Stress response | Intron 13 |
| MAP3K7 | **rs1144159** | 6q15 | 29067873 | Stress response | Intron 12 |
| MAP3K7 | **rs2273567** | 6q15 | 29077101 | Stress response | Intron 12 |
| MAP3K7 | **rs282070** | 6q15 | 29116593 | Stress response | Intron 1 |
| MAP3K7IP1 | **rs7949** | 22q13.1 | 19218068 | Stress response | Intron 12 |
| CAT | rs1001179 | 11p13 | 33247472 | Stress response | Promoter |
| CAT | **rs11032700** | 11p13 | 33248944 | Stress response | Intron 2 |
| CAT | **rs533425** | 11p13 | 33256565 | Stress response | Intron 2 |
| CAT | **rs2300181** | 11p13 | 33263780 | Stress response | Intron 7 |
| CAT | **rs554576** | 11p13 | 33271135 | Stress response | Intron 10 |
| CAT | **rs10488736** | 11p13 | 33276493 | Stress response | Intron 11 |
| CD14 | rs2569190 | 5q31.1 | 1175843 | Stress response | Promoter |
| IL10 | **rs3024498** | 1q31-q32 | 459308 | Stress response | 3’ UTR |
| IL10 | **rs3024496** | 1q31-q32 | 459643 | Stress response | 3’ UTR |
| IL10 | **rs1518111** | 1q31-q32 | 462424 | Stress response | Intron 3 |
| IL10 | rs1800872 | 1q31-q32 | 464186 | Stress response | Promoter |
| IL10 | rs1800871 | 1q31-q32 | 464413 | Stress response | Promoter |
| IL10 | rs1800896 | 1q31-q32 | 464676 | Stress response | Promoter |
| IL1A | **rs2856838** | 2q14 | 2247895 | Stress response | Intron 4 |
| IL1A | **rs2071373** | 2q14 | 2248007 | Stress response | Intron 4 |
| IL1A | **rs1800587** | 2q14 | 2250883 | Stress response | 5’ UTR |
| IL1B | **rs1143643** | 2q14 | 2296225 | Stress response | Intron 7 |

| | | | | | |
|---|---|---|---|---|---|
| IL1B | **rs1143634** | 2q14 | 2298313 | Stress response | Exon 4 |
| IL1B | rs1143627 | 2q14 | 2302310 | Stress response | Promoter |
| IL1B | rs16944 | 2q14 | 2302790 | Stress response | Promoter |
| IL6 | **rs1800795** | 7p21 | 22255603 | Stress response | Promoter |
| IL6 | **rs1474347** | 7p21 | 22257082 | Stress response | Intron 3 |
| IL8 | rs4073 | 4q13-q21 | 3113034 | Stress response | Promoter |
| IL8 | **rs2227306** | 4q13-q21 | 3114065 | Stress response | Intron 2 |
| SOD2 | **rs2758331** | 6q25.3 | 2392411 | Stress response | Intron 5 |
| SOD2 | **rs5746105** | 6q25.3 | 2399979 | Stress response | Intron 3 |
| SOD2 | rs4880 | 6q25.3 | 2401213 | Stress response | Exon 1 |
| TNF | rs1800629 | 6p21.3 | 22401282 | Stress response | Promoter |
| TNF | rs2857712 | 6p21.3 | 22400904 | Stress response | Promoter |
| IL2 | rs2069762 | 4q26-q27 | 47873111 | Stress response | Promoter |
| IL12 | rs17875322 | 4q26-q27 | 3552508 | Stress response | 3’ UTR |
| IFNG | rs2069727 | 12q14 | 30691529 | Stress response | 3’ UTR |
| SOD1 | rs4998557 | 21q22.11 | 18696763 | Stress response | Intron 1 |
| SOD1 | rs9967983 | 21q22.11 | 18699353 | Stress response | Intron 2 |
| SOD1 | rs12626475 | 21q22.11 | 18704800 | Stress response | 3’ near gene |
| SOD3 | rs2536512 | 4p15.3-p15.1 | 15476673 | Stress response | Exon 3 |
| SOD3 | rs2855262 | 4p15.3-p15.1 | 15477334 | Stress response | 3'UTR |
| SOD3 | rs2284659 | 4p15.3-p15.1 | 15470155 | Stress response | 5’ near gene |
| PRKCB1 | **rs195990** | 16p11.2 | 15259464 | Stress response | Intron 2 |
| PRKCB1 | **rs198145** | 16p11.2 | 15515694 | Stress response | Intron 16 |
| PRKCA | **rs17633401** | 17q22-q23.2 | 23030104 | Stress response | Intron 1 |
| PRKCA | **rs1877848** | 17q22- | 23243284 | Stress response | Intron 3 |

| | | | | | |
|---|---|---|---|---|---|
| | | q23.2 | | | |
| PRKCA | **rs721429** | 17q22-q23.2 | 23420646 | Stress response | Intron 8 |
| PRKCA | **rs4381631** | 17q22-q23.2 | 23495184 | Stress response | Intron 13 |
| SIRT6 | rs352496 | 19p13.3 | 4126252 | Cell cycle regulation | Promoter |
| ARNTL | rs2279287 | 11p15 | 12085726 | Cell cycle regulation | Promoter |
| ARNTL | rs1982350 | 11p15 | 12137372 | Cell cycle regulation | 5’ UTR |
| ARNTL | rs3789327 | 11p15 | 12172557 | Cell cycle regulation | Intron 5 |
| ARNTL | rs2278749 | 11p15 | 12185119 | Cell cycle regulation | Intron 11 |
| CLOCK | rs1801260 | 4q12 | 3641252 | Cell cycle regulation | 3’ UTR |
| PER1 | rs2253820 | 17p13.1 | 7645518 | Cell cycle regulation | Exon 17 |
| PER2 | rs2304672 | 2q37.3 | 5119343 | Cell cycle regulation | 5’ UTR |
| PER3 | rs10462020 | 1p36.23 | 2418050 | Cell cycle regulation | Exon 15 |
| MBD1 | rs125555 | 18q21 | 29289281 | Cell cycle regulation | Exon 10 |
| MBD2 | rs7614 | 18q21 | 33170346 | Cell cycle regulation | 3’ UTR |
| MBD2 | rs603097 | 18q21 | 33242208 | Cell cycle regulation | Promoter |
| TGFB1 | **rs8179181** | 19q13.1 | 14106424 | Cell cycle regulation | Intron 6 |
| TGFB1 | **rs4803455** | 19q13.1 | 14119727 | Cell cycle regulation | Intron 3 |
| TGFB1 | **rs2241715** | 19q13.1 | 14125104 | Cell cycle regulation | Intron 2 |
| TGFB1 | rs1800471 | 19q13.1 | 14127094 | Cell cycle regulation | Exon 1 |
| TGFB1 | rs1982073 | 19q13.1 | 14127139 | Cell cycle regulation | Exon 1 |
| APE1 | **rs3136820** | 14q11.2 | 2197 | Cell cycle regulation | Exon 5 |
| ATM | **rs664677** | 11q22-q23 | 11705598 | Cell cycle regulation | Intron 22 |
| ATM | **rs3092859** | 11q22-q23 | 11705872 | Cell cycle regulation | Exon 24 |
| ATM | **rs1801516** | 11q22-q23 | 11737878 | Cell cycle regulation | Exon 39 |
| ATM | **rs611646** | 11q22-q23 | 11739513 | Cell cycle regulation | Intron 38 |
| ATM | **rs227040** | 11q22-q23 | 11785807 | Cell cycle regulation | Intron 60 |

| | | | | | |
|---|---|---|---|---|---|
| ATR | **rs10804682** | 3q22-q24 | 48729680 | Cell cycle regulation | Intron 25 |
| ATR | **rs9855919** | 3q22-q24 | 48729735 | Cell cycle regulation | Intron 25 |
| ATR | rs2227928 | 3q22-q24 | 48776758 | Cell cycle regulation | Exon 4 |
| BAX | rs4645878 | 19q13.3-q13.4 | 21726128 | Cell cycle regulation | Promoter |
| BAX | **rs1805419** | 19q13.3-q13.4 | 21727294 | Cell cycle regulation | Intron 4 |
| CCND1 | **rs603965** | 11q13 | 322034 | Cell cycle regulation | Exon 4 |
| CCND1 | **rs649392** | 11q13 | 324992 | Cell cycle regulation | Intron 6 |
| CDKN1B | **rs34330** | 12p13.1-p12 | 5629669 | Cell cycle regulation | 5’ UTR |
| CDKN1B | **rs2066827** | 12p13.1-p12 | 5630073 | Cell cycle regulation | Exon 2 |
| CDKN1B | **rs34329** | 12p13.1-p12 | 5632207 | Cell cycle regulation | Intron 4 |
| CDKN2A | rs3088440 | 9p21 | 21958159 | Cell cycle regulation | 3’ UTR |
| CDKN2A | rs11515 | 9p21 | 21958199 | Cell cycle regulation | 3’ UTR |
| CDKN2A | **rs3731239** | 9p21 | 21964218 | Cell cycle regulation | Intron 2 |
| CDKN2A | **rs3731211** | 9p21 | 21976847 | Cell cycle regulation | Intron 2 |
| EGF | rs4444903 | 4q25 | 35382256 | Cell cycle regulation | 5’ UTR |
| EGF | **rs10470911** | 4q25 | 35413417 | Cell cycle regulation | Intron 5 |
| EGF | **rs929446** | 4q25 | 35431490 | Cell cycle regulation | Intron 9 |
| EGF | **rs7692976** | 4q25 | 35459713 | Cell cycle regulation | Intron 19 |
| EGF | **rs2298999** | 4q25 | 35460053 | Cell cycle regulation | Intron 19 |
| EGF | **rs4698803** | 4q25 | 35462573 | Cell cycle regulation | Exon 20 |
| EGF | **rs9991904** | 4q25 | 35470725 | Cell cycle regulation | Intron 22 |
| EGF | **rs6533485** | 4q25 | 35475709 | Cell cycle regulation | Intron 23 |
| ERBB2 | **rs2952155** | 17q21.1 | 1586014 | Cell cycle regulation | Intron 4 |
| ERBB2 | **rs1801200** | 17q21.1 | 1603884 | Cell cycle regulation | Exon 17 |

| | | | | | |
|---|---|---|---|---|---|
| ERBB2 | **rs1058808** | 17q21.1 | 1608333 | Cell cycle regulation | Exon 31 |
| ERCC1 | rs3212986 | 19q13.32 | 18180954 | Cell cycle regulation | 3'UTR |
| ERCC1 | **rs3212955** | 19q13.32 | 18191714 | Cell cycle regulation | Intron 5 |
| ERCC1 | **rs11615** | 19q13.32 | 18191871 | Cell cycle regulation | Exon 5 |
| ERCC1 | rs3212948 | 19q13.32 | 18192580 | Cell cycle regulation | Intron 3 |
| ERCC2 | **rs13181** | 19q13.3 | 18123137 | Cell cycle regulation | Exon 23 |
| ERCC2 | **rs1799787** | 19q13.3 | 18124362 | Cell cycle regulation | Intron 20 |
| ERCC2 | **rs3916874** | 19q13.3 | 18125144 | Cell cycle regulation | Intron 18 |
| ERCC2 | **rs238415** | 19q13.3 | 18125453 | Cell cycle regulation | Intron 18 |
| ERCC2 | **rs50872** | 19q13.3 | 18130667 | Cell cycle regulation | Intron 13 |
| ERCC2 | **rs50871** | 19q13.3 | 18130733 | Cell cycle regulation | Intron 13 |
| ERCC2 | **rs1799793** | 19q13.3 | 18135477 | Cell cycle regulation | Exon 10 |
| ERCC2 | **rs238406** | 19q13.3 | 18136527 | Cell cycle regulation | Exon 6 |
| ERCC5 | **rs2296147** | 13q33 | 16588051 | Cell cycle regulation | 5' UTR |
| ERCC5 | **rs1998876** | 13q33 | 16590719 | Cell cycle regulation | Intron 2 |
| ERCC5 | **rs4771436** | 13q33 | 16591696 | Cell cycle regulation | Intron 2 |
| ERCC5 | **rs1047768** | 13q33 | 16594193 | Cell cycle regulation | Exon 2 |
| ERCC5 | **rs17655** | 13q33 | 16617678 | Cell cycle regulation | Exon 15 |
| ERCC5 | **rs873601** | 13q33 | 16618013 | Cell cycle regulation | 3' UTR |
| GADD45A | **rs532446** | 1p31.2 | 2441 | Cell cycle regulation | Intron 3 |
| MDM2 | **rs1625525** | 12q14.3-q15 | 31356137 | Cell cycle regulation | Intron 5 |
| NBS1 | **rs12680687** | 8q21 | 4169561 | Cell cycle regulation | Intron 14 |
| NBS1 | **rs709816** | 8q21 | 4185884 | Cell cycle regulation | Exon 10 |
| NBS1 | rs1805794 | 8q21 | 4208652 | Cell cycle regulation | Exon 5 |
| OGG1 | **rs2072668** | 3p26.2 | 9738140 | Cell cycle regulation | Intron 5 |
| OGG1 | rs1052133 | 3p26.2 | 9738773 | Cell cycle regulation | Exon 7 |

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| TP53 | rs1625895 | 17p13.1 | 7175464 | Cell cycle regulation | Intron 6 |
| TP53 | rs1042522 | 17p13.1 | 7176821 | Cell cycle regulation | Exon 4 |
| CYP2B6 | **rs2054675** | 19q13.2 | 13763973 | Metabolism of xenobiotics | Promoter |
| CYP2B6 | **rs1987236** | 19q13.2 | 13777251 | Metabolism of xenobiotics | Intron 1 |
| CYP2B6 | **rs1042389** | 19q13.2 | 13792371 | Metabolism of xenobiotics | 3’ UTR |
| CYP2B6 | **rs1552219** | 19q13.2 | 13793592 | Metabolism of xenobiotics | 3’ UTR |
| CYP2B6 | **rs434606** | 19q13.2 | 13800223 | Metabolism of xenobiotics | 3’ UTR |
| CYP2D6 | **rs769258** | 22q13.1 | 1650 | Metabolism of xenobiotics | Exon 1 |
| CYP2D6 | **rs1065852** | 22q13.1 | 1719 | Metabolism of xenobiotics | Exon 1 |
| CYP2D6 | **rs28371703** | 22q13.1 | 2593 | Metabolism of xenobiotics | Exon 2 |
| CYP2D6 | **rs28371704** | 22q13.1 | 2603 | Metabolism of xenobiotics | Exon 2 |
| CYP2D6 | **rs28371705** | 22q13.1 | 2616 | Metabolism of xenobiotics | Exon 2 |
| GSTA3 | **rs494334** | 6p12.1 | 43618889 | Metabolism of xenobiotics | 3’ UTR |
| GSTA3 | **rs3818234** | 6p12.1 | 43621357 | Metabolism of xenobiotics | Intron 4 |
| GSTA3 | **rs523605** | 6p12.1 | 43635838 | Metabolism of xenobiotics | Promoter |
| GSTA4 | rs405729 | 6p12.1 | 43701012 | Metabolism of xenobiotics | 3’ UTR |
| GSTA4 | **rs673197** | 6p12.1 | 43702488 | Metabolism of xenobiotics | Intron 5 |
| GSTA4 | **rs2274760** | 6p12.1 | 43708386 | Metabolism of xenobiotics | Intron 3 |

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| GSTA4 | **rs508078** | 6p12.1 | 43710167 | Metabolism of xenobiotics | Intron 2 |
| GSTA4 | **rs182623** | 6p12.1 | 43720089 | Metabolism of xenobiotics | Promoter |
| GSTZ1 | **rs2111699** | 14q24.3 | 58788350 | Metabolism of xenobiotics | Intron 1 |
| GSTZ1 | **rs2270422** | 14q24.3 | 58792577 | Metabolism of xenobiotics | Intron 2 |
| GSTZ1 | **rs2270423** | 14q24.3 | 58795391 | Metabolism of xenobiotics | Intron 3 |
| GSTZ1 | **rs1017186** | 14q24.3 | 58797722 | Metabolism of xenobiotics | 3’ UTR |
| HNMT | **rs3100701** | 2q22.1 | 7339358 | Metabolism of xenobiotics | Intron 2 |
| HNMT | **rs1020678** | 2q22.1 | 7351816 | Metabolism of xenobiotics | Intron 2 |
| HNMT | **rs1455158** | 2q22.1 | 7382647 | Metabolism of xenobiotics | 3’ UTR |
| NAT1 | **rs1057126** | 8p22 | 5925572 | Metabolism of xenobiotics | 3’ UTR |
| NAT1 | **rs15561** | 8p22 | 5925579 | Metabolism of xenobiotics | 3’ UTR |
| NAT2 | **rs1799929** | 8p22 | 6102922 | Metabolism of xenobiotics | Exon 1 |
| NAT2 | **rs1799930** | 8p22 | 6103031 | Metabolism of xenobiotics | Exon 1 |
| NNMT | **rs2244175** | 11q23.1 | 17724937 | Metabolism of xenobiotics | Promoter |
| NNMT | **rs2852432** | 11q23.1 | 17726420 | Metabolism of xenobiotics | Promoter |
| NNMT | **rs1941404** | 11q23.1 | 17731454 | Metabolism of xenobiotics | Intron 2 |
| NNMT | **rs11214938** | 11q23.1 | 17735076 | Metabolism of xenobiotics | Intron 2 |

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| NNMT | **rs2852425** | 11q23.1 | 17744923 | Metabolism of xenobiotics | Intron 2 |
| NQO1 | **rs2965757** | 16q22.1 | 23352045 | Metabolism of xenobiotics | 3’ UTR |
| NQO1 | **rs10517** | 16q22.1 | 23357959 | Metabolism of xenobiotics | 3’ UTR |
| NQO1 | **rs1800566** | 16q22.1 | 23359344 | metabolism of xenobiotics | Exon 6 |
| NQO1 | **rs2917670** | 16q22.1 | 23373162 | Metabolism of xenobiotics | Intron 1 |
| PON1 | **rs854544** | 7q21.3 | 20157667 | Metabolism of xenobiotics | 3’ UTR |
| PON1 | **rs854548** | 7q21.3 | 20160096 | Metabolism of xenobiotics | 3’ UTR |
| PON1 | **rs1157745** | 7q21.3 | 20175314 | Metabolism of xenobiotics | Intron 4 |
| PON1 | **rs854556** | 7q21.3 | 20179199 | Metabolism of xenobiotics | Intron 3 |
| PON1 | **rs757158** | 7q21.3 | 20189804 | Metabolism of xenobiotics | Promoter |
| PON2 | **rs3735586** | 7q21.3 | 20269858 | Metabolism of xenobiotics | Intron 7 |
| PON2 | **rs2375005** | 7q21.3 | 20271152 | Metabolism of xenobiotics | Intron 6 |
| PON2 | **rs2299263** | 7q21.3 | 20274687 | Metabolism of xenobiotics | Intron 5 |
| PON2 | **rs1639** | 7q21.3 | 20278126 | Metabolism of xenobiotics | Intron 3 |
| SULT1A1 | **rs4149396** | 16p12.1 | 19930593 | Metabolism of xenobiotics | Exon 7 |
| SULT1A1 | **rs2411453** | 16p12.1 | 19945100 | Metabolism of xenobiotics | Intron 2 |
| SULT1A2 | **rs762634** | 16p12.1 | 19916414 | Metabolism of xenobiotics | Exon 10 |

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| SULT1A2 | rs1059491 | 16p12.1 | 19916734 | Metabolism of xenobiotics | Exon 8 |
| SULT1A2 | **rs3743963** | 16p12.1 | 19917765 | Metabolism of xenobiotics | Intron 6 |
| DRD2 | rs1800497 | 11q23 | 16833244 | Neuroactive ligand-receptor interaction pathway | 3’ UTR |
| DRD2 | rs1079597 | 11q23 | 16858702 | Neuroactive ligand-receptor interaction pathway | Inton 1 |
| DRD2 | rs1799732 | 11q23 | 16908669 | Neuroactive ligand-receptor interaction pathway | 5’ UTR |
| DRD4 | rs936461 | 11p15.5 | 513484 | Neuroactive ligand-receptor interaction pathway | 5’ UTR |
| DRD4 | rs4987059 | 11p15.5 | 513547 | Neuroactive ligand-receptor interaction pathway | Promoter |
| DRD4 | rs3758653 | 11p15.5 | 513581 | Neuroactive ligand-receptor interaction pathway | Promoter |
| HTR2A | rs6306 | 13q14-q21 | 28451462 | Neuroactive ligand-receptor interaction pathway | Promoter |
| HTR2A | rs6311 | 13q14-q21 | 28451479 | Neuroactive ligand-receptor interaction pathway | Promoter |
| NTRK1 | rs6339 | 1q21-q22 | 7339301 | Neuroactive ligand-receptor interaction pathway | Exon 15 |
| NTRK1 | rs6337 | 1q21-q22 | 7339350 | Neuroactive ligand-receptor interaction pathway | Exon 15 |
| POMC | rs1042571 | 2p23.3 | 4199820 | Neuroactive ligand-receptor interaction pathway | 3’ UTR |

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| POMC | rs7566506 | 2p23.3 | 4235406 | Neuroactive ligand-receptor interaction pathway | 5’ UTR |
| SLC6A3 | rs2652511 | 5p15.3 | 121756 | Neuroactive ligand-receptor interaction pathway | 5’ UTR |
| GH1 | **rs2070680** | 17q24.2 | 20638999 | INS/IGF-1/GH | 3’ UTR |
| GH1 | rs2854184 | 17q24.2 | 20650004 | INS/IGF-1/GH | 5’ near gene |
| GH1 | rs2727308 | 17q24.2 | 20641637 | INS/IGF-1/GH | 3’ near gene |
| GHR | **rs11949751** | 5p13-p12 | 42542342 | INS/IGF-1/GH | Intron 2 |
| GHR | **rs4292454** | 5p13-p12 | 42596002 | INS/IGF-1/GH | Intron 2 |
| JAK2 | rs7849191 | 9p24 | 4978761 | INS/IGF-1/GH | Intron 2 |
| JAK2 | rs7037207 | 9p24 | 5033156 | INS/IGF-1/GH | Intron 4 |
| JAK2 | **rs3780378** | 9p24 | 5102288 | INS/IGF-1/GH | Intron 22 |
| SOCS1 | rs4780355 | 16p13.13 | 2660937 | INS/IGF-1/GH | 3’ near gene |
| SOCS1 | rs193779 | 16p13.13 | 2664044 | INS/IGF-1/GH | 5’ near gene |
| IGF1 | rs12821878 | 12q23.2 | 26349857 | INS/IGF-1/GH | Intron 2 |
| IGF1 | rs7136446 | 12q23.2 | 26320705 | INS/IGF-1/GH | Intron 2 |
| IGF1 | rs35767 | 12q23.2 | 26357759 | INS/IGF-1/GH | promoter |
| IGF1 | rs6214 | 12q23.2 | 26275759 | INS/IGF-1/GH | 3’ UTR |
| IGF1R | **rs874305** | 15q26.3 | 646096 | INS/IGF-1/GH | Intron 1 |
| IGF1R | **rs2018860** | 15q26.3 | 708561 | INS/IGF-1/GH | Intron 2 |
| IGF1R | **rs8037002** | 15q26.3 | 896014 | INS/IGF-1/GH | Intron 5 |
| IGF1R | **rs2229765** | 15q26.3 | 928076 | INS/IGF-1/GH | Exon 16 |
| GAP | rs388340 | 5q13.3 | 37179178 | INS/IGF-1/GH | Intron 1 |
| GAP | rs3752862 | 5q13.3 | 37228331 | INS/IGF-1/GH | Intron 5 |
| GAP | rs16902632 | 5q13.3 | 37249171 | INS/IGF-1/GH | Intron 9 |
| AKT1 | rs2494732 | 14q32.32 | 86240237 | INS/IGF-1/GH | Intron 11 |
| AKT1 | rs2498802 | 14q32.32 | 86235487 | INS/IGF-1/GH | 3' near gene |

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| PDK1 | rs758319 | 16p13.3 | 2547941 | INS/IGF-1/GH | Exon 2 |
| PDK1 | rs3087784 | 16p13.3 | 2587914 | INS/IGF-1/GH | 3’ UTR |
| PDK1 | rs1005273 | 16p13.3 | 2585966 | INS/IGF-1/GH | Intron 12 |
| PI3K | **rs1445760** | 5q13.1 | 18187374 | INS/IGF-1/GH | Intron 9 |
| PI3K | rs3730089 | 5q13.1 | 18182506 | INS/IGF-1/GH | Exon 2 |
| PI3K | **rs10940160** | 5q13.1 | 18157585 | INS/IGF-1/GH | Intron 1 |
| PI3K | rs6890202 | 5q13.1 | 18165320 | INS/IGF-1/GH | Intron 3 |
| BAD | rs671976 | 11q13.1 | 9351824 | INS/IGF-1/GH | Intron 1 |
| BAD | rs604203 | 11q13.1 | 9322730 | INS/IGF-1/GH | 3’ near gene |
| FOXO 3A | rs2253310 | 6q21 | 13058022 | INS/IGF-1/GH | Intron 1 |
| FOXO 3A | rs2802288 | 6q21 | 13065644 | INS/IGF-1/GH | Intron 1 |
| FOXO 3A | rs4946935 | 6q21 | 13170171 | INS/IGF-1/GH | Intron 2 |
| FOXO 1A | rs2701896 | 13q14.1 | 22107892 | INS/IGF-1/GH | 3’ near gene |
| FOXO 1A | rs2755209 | 13q14.1 | 22117804 | INS/IGF-1/GH | Intron 1 |
| FOXO 1A | rs17061503 | 13q14.1 | 22226758 | INS/IGF-1/GH | 5’ near gene |
| AFX | rs12013673 | Xq13.1 | 8635087 | INS/IGF-1/GH | Intron 1 |
| AFX | rs5980742 | Xq13.1 | 8639619 | INS/IGF-1/GH | Intron 2 |
| AFX | rs5980741 | Xq13.1 | 8632407 | INS/IGF-1/GH | 5’ near gene |
| MTOR | rs2275527 | 1p36.2 | 5728013 | INS/IGF-1/GH | Exon 39 |
| MTOR | rs2261434 | 1p36.2 | 5707498 | INS/IGF-1/GH | Intron 54 |
| MTOR | rs1770345 | 1p36.2 | 5751947 | INS/IGF-1/GH | Intron 30 |
| RAPTOR | **rs7209040** | 17q25.3 | 930833 | INS/IGF-1/GH | Intron 1 |
| RAPTOR | **rs12949279** | 17q25.3 | 961950 | INS/IGF-1/GH | Intron 1 |
| RAPTOR | **rs7208502** | 17q25.3 | 1073867 | INS/IGF-1/GH | Intron 3 |
| RAPTOR | **rs2292639** | 17q25.3 | 1319494 | INS/IGF-1/GH | 3’ near gene |
| RAPTOR | rs1062935 | 17q25.3 | 1343396 | INS/IGF-1/GH | 3’ UTR |
| PGC1a | rs8192678 | 4p15.1 | 14491020 | INS/IGF-1/GH | Exon 8 |

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| PGC1a | rs12650562 | 4p15.1 | 14476545 | INS/IGF-1/GH | Intron 12 |
| PGC1a | rs2946385 | 4p15.1 | 14561681 | INS/IGF-1/GH | Intron 2 |
| PGC1a | rs4235308 | 4p15.1 | 14539770 | INS/IGF-1/GH | Intron 2 |
| PPARa | rs135539 | 22q13.31 | 1829973 | INS/IGF-1/GH | Intron 1 |
| PPARa | rs5766741 | 22q13.31 | 1875896 | INS/IGF-1/GH | Intron 3 |
| PPARa | rs9626814 | 22q13.31 | 1907960 | INS/IGF-1/GH | 3’ UTR |
| LEP | rs13228377 | 7q31.3 | 53063520 | INS/IGF-1/GH | 5’ near gene |
| LEP | rs12706832 | 7q31.3 | 53070715 | INS/IGF-1/GH | Intron 1 |
| LEP | rs3828942 | 7q31.3 | 53077881 | INS/IGF-1/GH | Intron 2 |
| LEP | rs11761556 | 7q31.3 | 53080645 | INS/IGF-1/GH | 3’ UTR |
| LEPR | rs12145690 | 1p31 | 35858932 | INS/IGF-1/GH | Inton 1 |
| LEPR | rs1137100 | 1p31 | 36008360 | INS/IGF-1/GH | Exon 4 |
| LEPR | rs1137101 | 1p31 | 36030432 | INS/IGF-1/GH | Exon 6 |
| LEPR | rs6688776 | 1p31 | 36040096 | INS/IGF-1/GH | Intron 10 |
| AMPK | rs12517210 | 5p12 | 40737197 | INS/IGF-1/GH | Intron 8 |
| AMPK | rs837101 | 5p12 | 40746456 | INS/IGF-1/GH | Intron 3 |
| AMPK | rs249429 | 5p12 | 40754996 | INS/IGF-1/GH | Intron 1 |
| AMPK | rs466108 | 5p12 | 40769503 | INS/IGF-1/GH | Intron 1 |
| ACC | rs829152 | 17q21 | 1007776 | INS/IGF-1/GH | Intron 1 |
| ACC | rs2302800 | 17q21 | 901514 | INS/IGF-1/GH | Intron 10 |
| ACC | rs732770 | 17q21 | 714789 | INS/IGF-1/GH | 3’ near gene |
| ACC | rs11659129 | 17q21 | 831441 | INS/IGF-1/GH | Intron 34 |
| CPT1A | rs11228372 | 11q13.2 | 13902373 | INS/IGF-1/GH | Intron 1 |
| CPT1A | rs4930248 | 11q13.2 | 13871699 | INS/IGF-1/GH | Intron 6 |
| CPT1A | rs17610395 | 11q13.2 | 13868123 | INS/IGF-1/GH | Exon 8 |
| INS | rs7924316 | 11p15.5 | 960688 | INS/IGF-1/GH | 3’ near gene |
| INS | rs2070762 | 11p15.5 | 973576 | INS/IGF-1/GH | 3’ near gene |

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| INS | rs3842748 | 11p15.5 | 968636 | INS/IGF-1/GH | Intron 2 |
| INSR | rs11667110 | 19p13.3-p13.2 | 7076609 | INS/IGF-1/GH | Intron 13 |
| INSR | rs8103483 | 19p13.3-p13.2 | 7085374 | INS/IGF-1/GH | Intron 11 |
| INSR | rs12971499 | 19p13.3-p13.2 | 7154282 | INS/IGF-1/GH | Intron 2 |
| INSR | rs1896639 | 19p13.3-p13.2 | 7143846 | INS/IGF-1/GH | Intron 2 |
| IRS-1 | rs17208239 | 2q36 | 77807113 | INS/IGF-1/GH | 3’ near gene |
| IRS-1 | rs1801276 | 2q36 | 77871337 | INS/IGF-1/GH | Exon 1 |
| IRS-1 | rs1820841 | 2q36 | 77856876 | INS/IGF-1/GH | Intron 2 |
| PI3K | rs870995 | 3q26.3 | 85408156 | INS/IGF-1/GH | Intron 1 |
| PI3K | rs1607237 | 3q26.3 | 85445447 | INS/IGF-1/GH | Intron 20 |
| PI3K | rs1568205 | 3q26.3 | 85430949 | INS/IGF-1/GH | Intron 9 |
| GLUT4 | rs2654185 | 17p13 | 6780710 | INS/IGF-1/GH | 5’ near gene |
| GLUT4 | rs5418 | 17p13 | 6782440 | INS/IGF-1/GH | 5’ UTR |
| GLUT4 | rs5435 | 17p13 | 6784471 | INS/IGF-1/GH | Exon 4 |
| GLUT4 | rs35198331 | 17p13 | 6784154 | INS/IGF-1/GH | Exon 3 |
| SHC1 | rs4845401 | 1q21 | 5431948 | INS/IGF-1/GH | Intron 3 |
| SHC1 | rs1050947 | 1q21 | 5425860 | INS/IGF-1/GH | 3' UTR |
| SHC1 | rs6661212 | 1q21 | 5437051 | INS/IGF-1/GH | Intron 1 |
| GRB2 | rs2053158 | 17q24-q25 | 7319731 | INS/IGF-1/GH | Intron 1 |
| GRB2 | rs959260 | 17q24-q25 | 7295694 | INS/IGF-1/GH | Intron 2 |
| GRB2 | rs7219 | 17q24-q25 | 7241640 | INS/IGF-1/GH | 3’ UTR |
| SOS | rs1059310 | 2p21 | 18028570 | INS/IGF-1/GH | 3’ near gene |
| SOS | rs2888586 | 2p21 | 18063222 | INS/IGF-1/GH | Intron 9 |
| SOS | rs963828 | 2p21 | 18150643 | INS/IGF-1/GH | Intron 1 |
| HRAS | rs12628 | 11p15.5 | 474242 | INS/IGF-1/GH | Exon 2 |

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| HRAS | rs4963176 | 11p15.5 | 478532 | INS/IGF-1/GH | 3’ near gene |
| HRAS | rs12577324 | 11p15.5 | 469967 | INS/IGF-1/GH | Downstream |
| RAF1 | rs15997 | 3p25 | 12564070 | INS/IGF-1/GH | 3' near gene |
| RAF1 | rs1532533 | 3p25 | 12575706 | INS/IGF-1/GH | Intron 10 |
| RAF1 | rs11705805 | 3p25 | 12602370 | INS/IGF-1/GH | Intron 1 |
| RAF1 | rs3821611 | 3p25 | 12645208 | INS/IGF-1/GH | Intron 1 |
| MAP2K1 | rs1549854 | 15q22.1-q22.33 | 37487054 | INS/IGF-1/GH | Intron 1 |
| MAP2K1 | rs1432442 | 15q22.1-q22.34 | 37509588 | INS/IGF-1/GH | Intron 1 |
| MAP2K1 | rs7181936 | 15q22.1-q22.35 | 37538228 | INS/IGF-1/GH | Intron 5 |
| ERK1 | rs7542 | 16p11.2 | 21438919 | INS/IGF-1/GH | Exon 8 |
| ERK1 | rs11865086 | 16p11.2 | 21443572 | INS/IGF-1/GH | Intron 2 |
| ERK1 | rs12444415 | 16p11.2 | 21431424 | INS/IGF-1/GH | 5’ near gene |
| SHP12 | rs11066301 | 12q24 | 3440881 | INS/IGF-1/GH | Intron 1 |
| SHP12 | rs11066320 | 12q24 | 3475924 | INS/IGF-1/GH | Intron 6 |
| SHP12 | rs7313360 | 12q24 | 3503044 | INS/IGF-1/GH | Intron 13 |
| IFG2 | rs3213221 | 11p15.5 | 944285 | INS/IGF-1/GH | Intron 1 |
| IFG2 | rs734351 | 11p15.5 | 943454 | INS/IGF-1/GH | Intron 2 |
| IFG2 | rs680 | 11p15.5 | 940875 | INS/IGF-1/GH | 3’ near gene |
| IGFBP1 | rs4619 | 7p13-p12 | 45421627 | INS/IGF-1/GH | Exon 4 |
| IGFBP3 | rs2132571 | 7p13-p12 | 45450632 | INS/IGF-1/GH | 5’ near gene |
| IGFBP3 | rs2453839 | 7p13-p12 | 45442531 | INS/IGF-1/GH | Intron 5 |
| IGFALS | rs3751893 | 16p13.3 | 1782210 | INS/IGF-1/GH | Exon 2 |
| IGFALS | rs17559 | 16p13.3 | 1781034 | INS/IGF-1/GH | Exon 2 |
| IGFALS | rs2230053 | 16p13.3 | 1780854 | INS/IGF-1/GH | Exon 2 |