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Down Syndrome: Neuropsychological phenotype and mitochondrial DNA

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ABBREVIATIONS

Aβ	β-amyloid
AD	Alzheimer's Disease
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
ATPase	ATP synthase
BACE2	β-site APP-cleaving 2 enzyme
BF	Bromophenol Blue
CBS	Cystathionin β-synthase
CNS	Central Nervous System
CO	Cytochrome c Oxidase
CPEO	Chronic Progressive External Ophthalmoplegia
CR	Control Region
CSB	Conserved Sequence Block
D-loop	Displacement Loop
DS	Down Syndrome
DSCR	Down Syndrome critical Region
dsDNA	Double-Strand DNA
DSM	Down Syndrome Mother
DSS	Down Syndrome Sibling
DYRK1A	Dualspecificity Tyrosine-(Y)-Phosphorylation Kinase 1A
ETC	Electron Transport Chain
GARS–AIRS–GART	Complex Glycinamide Ribonucleotide Synthase–Aminoimidazole Ribonucleotide Synthase–Glycinamide Formyl Transferase
GATA 1	Globin Transcription Factor 1
Hsa21	Human Chromosome 21
HSP	Heat Shock Protein
HVS	Hypervariable Sequence
ID	Intellectual Disability
IQ	Intelligence Quotient
KSS	Kearns-Sayre Syndrome
LDL	Low-Density Lipoprotein
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MERRF	Myoclonic Epilepsy and Ragged Red Fiber
miRNA	Micro-RNA
mtDNA	Mitochondrial DNA
mtTFA	Mitochondrial Transcription Factor A

ND	NADH Dehydrogenase
nDNA	Nuclear DNA
O_H	Origin of H-strand Replication
O_L	Origin of L-strand Replication
OS	Oxidative Stress
OXPHOS	Oxidative Phosphorylation
PARP	Poly (ADP-ribose) Polymerase
P_H	H-strand Promoter
P_L	L-strand Promoter
RCAN1	Regulator of Calcineurin 1
REST	RE1-Silencing Transcription Factor
ROS	Reactive Oxygen Species
RRF	Red Ragged Fibers
SOD-1	Cu^{2+}/Zn^{2+} Superoxide Dismutase
XC	Xilene Cyanol
YBP	Years Before Present

PREFACE

Down Syndrome is the most known autosomal trisomy, due to the presence in three copies of chromosome 21. Many studies were designed to identify phenotypic and clinical consequences related to the triple gene dosage. However, the general conclusion is a senescent phenotype; in particular, the most features of physiological aging, such as skin and hair changes, vision and hearing impairments, thyroid dysfunction, Alzheimer-like dementia, congenital heart defects, gastrointestinal malformations, immune system changes, appear in DS earlier than in normal age-matched subjects.

The only established risk factor for the DS is advanced maternal age, responsible for changes in the meiosis of oocytes, in particular the meiotic nondisjunction of chromosome 21. In this process mitochondria play an important role since mitochondrial dysfunction, due to a variety of extrinsic and intrinsic influences, can profoundly influence the level of ATP generation in oocytes, required for a correct chromosomal segregation.

The aim of this study is to analyze the mtDNA and APOE genotypes of DS subjects and compare with their mothers and siblings of age-gender comparable, in order to identify any differences that might justify the early neurocognitive decline observed in DS.

Chapter 1

DOWN SYNDROME

1. The Down Syndrome: epidemiological aspects

Down Syndrome (DS), or trisomy 21, is the most common and known chromosomal anomaly, caused by the presence in three copies of chromosome 21 (Hsa21) or, more rarely, parts of it, following a meiotic non-disjunction during ovogenesis. The origin of the supernumerary chromosome 21 is maternal in 93% of cases, while in 7% of cases is due to nondisjunction of the chromosome of paternal origin.

In 95% of cases, trisomy 21 is in free form, which is the presence of the entire surplus Hsa21 not translocated to other chromosomes; in most cases, the trisomy 21 is homogeneous, i.e. it is present in all cells analyzed, while in 3,5% of patients trisomy 21 is in the mosaic, only present in some cells. In 4% of cases, the trisomy 21 is due to a translocation of Hsa21 on another chromosome (Robertsonian translocation), and finally in 1% of patients trisomy 21 is partial. From the clinical point of view the effect is the same, while the reproductive consequences (risk of recurrence) for the parental couple are related to the type of cytogenetic trisomy 21. Therefore for the diagnosis of DS is needed the presence of trisomy 21 in association with the clinical picture.

The estimated incidence of DS is approximately 1/700-1/1000 live births, with a slight predominance in males [Canfield et al. 2006]: the annual number of children born with DS appears to be decreasing, mainly because of the voluntary interruption of pregnancy (VIP); VIP logs show a progressive increase in the years of voluntary terminations for DS and it is estimated that the percentage of abortions after diagnosis of DS is about 58%.

The only established risk factor for the DS is advanced maternal age, responsible for changes in the process of meiosis (M) of oocytes, in particular the meiotic nondisjunction of Hsa21 [Ghosh et al. 2009]; during both the phase I (MI) and the phase II (MII), the error rate varies according to maternal age: they are lower among mothers aged <19 and ≥ 40 years and higher in the intermediate group and, in women aged ≥ 40 years, the MII nondisjunction is more common [Allen et al. 2009].

Trisomy 21 results in a high risk of spontaneous abortion and neonatal morbidity and mortality: in the period between the time of chorionic villus sampling and the period of gestation, the percentage of miscarriages is 43%, while between the time of amniocentesis and the term of gestation is 23% [Morris et al. 1999]. The literature has shown a correlation between maternal age and a directly proportional

incidence of spontaneous abortion of fetuses with DS, with the increasing of the incidence of spontaneous abortions associated to the increasing maternal age (from 23% for mothers at the age of 25 years to 45% for those 45 years) [Savva et al. 2006].

In recent years the quality and life expectancy of patients with DS were significantly improved, thanks to continued progress in medical treatment: in particular has increased survival in early childhood, following the improvement of cardiac surgery, and most people with DS reach over 60 years of age; life expectancy for males is greater of about 3.3 years compared to females; life extension, however, lead to an increase of percentage of diseases associated with aging [Glasson et al. 2002].

2. The Down Syndrome: cytogenetic aspects

Many studies were designed to identify phenotypic and clinical consequences related to the triple gene dosage due to trisomy 21.

Chromosome 21 has been completely sequenced in the late '90s [Hattori et al. 2000]. The genes of Hsa21 so far identified are more than 400 [Gardiner et al. 2006], 20-50 of which are located at the terminal part of the long arm (21q22.2), the so-called Down Syndrome Critical Region (DSCR). Although this number is high, it is not an intractable one, and the number of genes that are involved in DS is likely to be considerably less than their total number on the chromosome. The main question is whether the DS phenotype is determined by only a few genes with a major phenotypic effect or whether the phenotype results from the interaction of several genes of modest effect. Based on studies in animal models, there is reason to hope that the former might be the case.

Furthermore DS is a disorder of gene dosage in which the genes that are involved are normal and their gene products are also normal. The genetic abnormality is, therefore, quantitative rather than qualitative and involves the production of increased amounts of the products of the genes on chromosome 21. The simplest expectation was that the degree of overexpression of the unbalanced gene would be about 50% [Hattori et al. 2000] and it now seems to be generally true [Amano et al. 2004, Dauphinot et al. 2005]. This raises the question of how such a relatively modest increase in gene expression can have such deleterious consequences. The genes in triplicate Hsa21 have an effect on the entire integrated genome and affect the expression of many other genes, activating or inhibiting, depending on the individual genetic constitution (genetic polymorphism): this could explain the variability of phenotypic expression and diversity disease in individual patients with DS.

Finally trisomy for functional non-protein-coding DNA elements might be involved in some of the abnormal phenotypes. Comparative analysis of human with other vertebrate genomes revealed the presence of conserved regions that are not part of the annotated genes [Dermitzakis et al. 2005]. The

sequence conservation strongly indicates that these elements are functional, but the precise function of each one of them is unknown. It is however possible that three copies of some of the conserved elements contribute to the DS pathology.

3. The Down Syndrome: pathogenic aspects

Similar to almost all conditions that result from chromosome imbalance, DS affects multiple systems and produces both structural and functional defects [Epstein, 2001]. In addition, the evolution of the phenotype has a temporal dimension so the issues concerning affected individuals, their families and society change with age. A chronology of the major components of the DS phenotype is presented in **Table 1**.

At birth	Infancy and childhood	Adulthood
Structural		
Dysmorphic features[a]	Growth retardation and obesity	
Congenital heart disease		
Duodenal stenosis or atresia		
Imperforate anus		
Hirschprung disease		
Central nervous system		
Hypotonia	Developmental and mental retardation	Decrease in cognitive function
	Decreased sensitivity to pain	Alzheimer disease
Immune and hematopoietic systems		
Transient myeloproliferative disorder	Leukemia	
	Immune defects and/or infection	
Other	Thyroid dysfunction	Male sterility
		Reduced longevity

[a]The dysmorphic features include (in order of frequency with which they are noted): upslanting palpebral fissures (82%), loose skin on the nape of the neck, narrow palate, brachycephaly, flat nasal bridge, wide gap between the first and second toes, short broad hands, short neck, abnormal teeth, epicanthic folds, short and incurved fifth fingers, open mouth with down turned corners and protruding tongue, transverse palmar creases, and folded or dysplastic ears (50%) [7].

Table 1: Major components of the phenotype of Down syndrome (Source: *"The challenge of Down syndrome"*, Antonarakis et al, 2006)

The clinical diagnosis of DS can be made at birth on the basis of the presence of marked hypotonia and a constellation of minor dysmorphic features that involve all parts of the body but are especially visible in the craniofacies, hands and feet. Nevertheless, these dysmorphic features have little effect on function. Anyhow, there are two groups of major congenital abnormalities associated with DS, congenital heart disease and several types of gastrointestinal-tract obstruction or dysfunction, that can cause morbidity or even death if untreated.

In recent years considerable progress has been made in understanding and explanation of the genetic mechanisms underlying the main phenotypic characteristics of DS: developmental abnormalities, susceptibility to certain diseases, disability. In particular, through an algorithm to identify non-coding RNAs, five microRNAs (miRNAs) have been identified on Hsa21 [Kuhn et al. 2008, Sethupathy et al. 2007]. Micro-RNAs regulate the expression of other genes [Bartel, 2004], and their role in DS is not fully understood.

The five miRNAs are: miR-99a, let-7c, miR-125b-2, miR-155, and miR-802; the overexpression of these miRNAs, in the presence of trisomy 21, results in reduced expression of the encoded protein (down-regulation effect), at fetal heart and nervous tissue, resulting in the heart and brain abnormalities characteristic of DS [Kuhn et al. 2008].

Moreover, a recent paper has suggested that trisomy of the Hsa21 genes, dualspecificity tyrosine-(Y)-phosphorylation-regulated kinase 1A (DYRK1A) and regulator of calcineurin 1 (RCAN1), may have an impact on the development of multiple tissues [Arron at al. 2006]. DYRK1A is a priming kinase that facilitates the further phosphorylation of numerous proteins by other kinases (**Figure 1**) [de Graaf et al. 2004 and 2006; Adayev et al. 2006; Kim et al. 2006; Ryoo et al. 2007 and 2008; Huang et al. 2004; Woods et al. 2001; Aranda et al. 2008; Gwack et al. 2006] and that is up-regulated in a number of tissues from people with DS [Dowjat et al. 2007; Liu et al. 2008]. RCAN1 is a regulator of the protein phosphatase calcineurin [Fuentes et al. 2000]. Crabtree and colleagues hypothesized that trisomy of these two genes may act synergistically to alter signalling via the NFAT family of transcription factors [Arron et al. 2008].

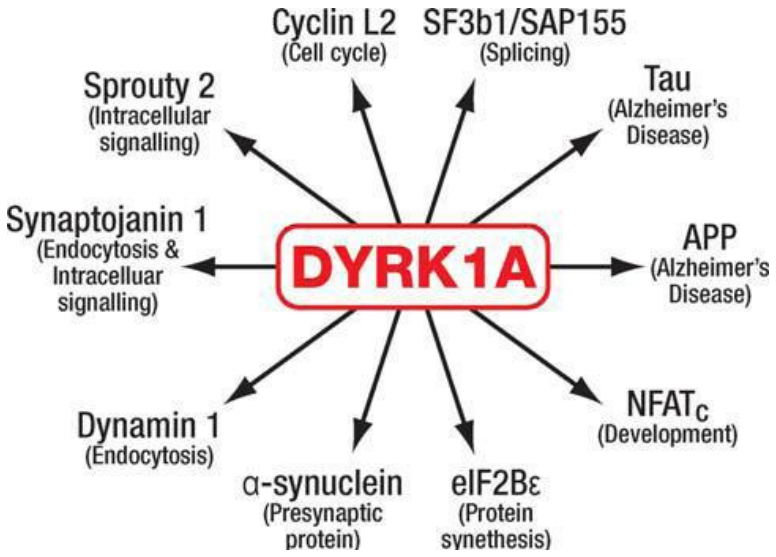


Figure 1: Dualspecificity tyrosine-(Y)-phosphorylation-regulated kinase 1A (DYRK1A) (Source: "*Down syndrome – recent progress and future prospects*", Wiseman et al. 2009)

In an independent study, increased DYRK1A gene dosage decrease the expression level of RE1-silencing transcription factor (REST) [Canzonetta et al. 2008], which is required both to maintain pluripotency and to facilitate neuronal differentiation; so a perturbation in REST expression may alter the development of many cell types. Indeed, over expression of DYRK1A in some animal models is associated with a number of phenotypes, including heart defects and abnormal learning and memory (**Figure 2**) [Arron et al. 2006; Ryoo et al. 2007; Altafaj et al. 2001; Martinez et al. 2004; Ahn et al. 2006]. However, not all

animal models that over-express DYRK1A exhibit these defects, suggesting that the outcome of DYRK1A trisomy could be affected by polymorphisms or differences in the expression of other genes [Olson et al. 2004].

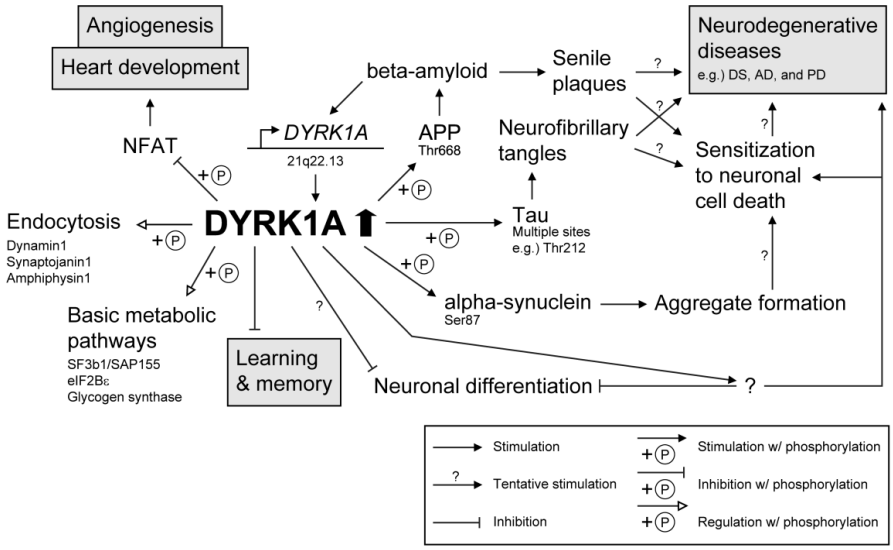


Figure 2: Main roles of DYRK1A in various cellular processes and their correlation with the neurodegenerative phenomena taking place in Down syndrome (Source: *"Two key genes closely implicated with the neuropathological characteristics in Down syndrome: DYRK1A and RCAN1."*, Park et al. 2009)

3.1 Phenotypic variability

Almost every aspect of the phenotype of DS is subject to a high degree of variability [Antonarakis and Epstein, 2006]. No two patients are likely to have exactly the same combination of features and none of the minor dysmorphic features is present in >80% of the cases. Nevertheless, even when superimposed upon familial features, the overall pattern of minor anomalies is consistent and distinct, and enables clinical recognition [Epstein, 2001].

Furthermore, life expectancy of DS individuals has been rapidly increasing over the past 20 years, with a doubling of the median age of death in white populations (up to 50 years in 1997), even if environmental effects that result from social disparities seem to have deprived various ethnic groups of similar benefits [Yang et al. 2002, Leonard et al. 2000].

3.2 Congenital Heart Defects and Gastrointestinal System Malformations

Approximately half of all children with DS are born with a significant congenital heart defect (CHD), the most common of which is an atrioventricular septal defect (AVSD) [Freeman et al., 1998].

Typically developing children with CHD have been shown to have neurocognitive and psychomotor deficits [Malec et al. 1999]. For instance, school-aged children with hypoplastic left heart syndrome have a mean full-scale IQ of 86, which is below the population normative value, approximately one standard deviation [Mahle et al. 2006]. Visootsak et al. (2011) have documented that there may be possible developmental differences in children with DS+AVSD compared to children with DS without CHDs. Issues that remain largely unknown in children with DS+AVSD are the magnitude of their cognitive, language, motor, social, and adaptive deficits; the patient-related characteristics and perioperative factors may influence their neurodevelopmental outcomes and the effect of home environmental variables on their development.

Gastrointestinal defects are second to heart defects among the common serious structural birth defects associated with DS [Fabia and Drolette, 1970; Kallen et al. 1996] and atresia/stenosis of the small intestine were most common [Cleves et al. 2007].

3.3 Immune and Hematopoietic Systems

In addition to the effects on function of the heart and gastrointestinal system, trisomy 21 also produces many changes in cellular function [Epstein, 2001]. Among these, those involving the immune and hematopoietic systems. The impact of infection has been greatly reduced with the availability of immunizations for various viral and bacterial pathogens, improved hygiene and antibiotics, however infection is still a major cause of death in DS at any age [Hill et al. 2003], and has been suggested that this susceptibility to infection depends on abnormal maturation of the thymus and impaired function of T lymphocytes [Philip et al. 1986, Murphy et al. 1995]. Franceschi et al. (1978) found a decreased percentage of T lymphocytes and an increased percentage of “null cells” (non-T, non-B lymphocytes), so the immunologic defect seems to be a congenital feature of DS, and the antigen overloading could worsen the intrinsic immunological derangement and lead to a stress-immunodeficiency. In particular subjects with DS showed normal responsiveness in allogeneic mixed lymphocyte reactions, but their response to phytohemagglutinin and in autologous mixed lymphocyte reactions were severely impaired; moreover, the blood concentration of serum thymic factor in the majority of DS subjects was much lower than that found in age-matched healthy controls [Franceschi et al. 1981].

Furthermore, infants with DS develop acute leukemia, most commonly acute megakaryoblastic (M7) leukemia, with a frequency twenty times higher than that in the normal population [Hitzler et al. 2003]. This is often preceded by a neonatal leukemoid reaction (transient myeloproliferative disorder), which might be a

form of transient leukemia. A unique non-activating mutation in the GATA binding protein 1 (GATA1) gene (on the X chromosome) has been found in association with both the leukemia and the leukemic reaction [Hitzler et al. 2003, Wechsler et al. 2002], and children with DS seem to have a better prognosis and require less chemotherapy than children without DS [Gamis, 2005].

3.4 Central Nervous System

The trisomic state hits the Central Nervous System (CNS), which results vulnerable throughout life. The brain is grossly normal with the exception of some degree of hypoplasia of the cerebellum [Aylward et al. 1997]. Nevertheless, perhaps the most frequent sign of DS is a central hypotonia, followed by delayed cognitive development in infancy and childhood leading to mild to moderate mental retardation, and a loss of cognitive abilities in adulthood and the development of Alzheimer's disease (AD) in later years [Busciglio et al. 2002].

The literature shows that the 8-13,6% of individuals with DS develop epilepsy, [Pueschel et al. 1991; Goldberg-Dtern et al. 2001], that cognitive development is in the range of mental retardation (usually grade medium-mild) [Brown et al. 1990] and that there is a frequency of autistic disorder, 7-11% [Kent et al. 1999; Kroeger and Nelson, 2006].

In general, the stages of development follow the normal sequence, with a particular deficit in the production of language [Chapman et al. 1998]; in childhood have been reported behavioral disorders such as attention deficit hyperactivity disorder (6.1% of cases), oppositional defiant disorder conduct (5.4%) or aggressive behavior (6.5%) while it can take as an adult major depression (6.1%) or aggressive behavior (6.1%) [Myers and Pueschel, 1991].

All domains of intellectual function do not have uniformly cognitive impairment, and there are both strengths and weaknesses in intellectual ability [Nadel 2003]. The forms of learning that depend on the hippocampus are particularly affected, and there is also evidence for impairment of prefrontal cortex and cerebellar functions [Nadel 2003]. Speech and articulation are particularly affected. Numerous anatomical, biochemical and physiological studies have shed little light on the basis for cognitive impairment [Epstein, 2001]. Several years ago have been observed morphological abnormalities of the dendritic spines, which are the sites of synapse formation, and this originally non-specific finding has been considerable reappraised with the discovery of similar abnormalities in the brains of Ts65Dn mice [Belichenko et al. 2004]. Hippocampal neurons in Ts65Dn brains also exhibit major abnormalities in long-term potentiation (LTP) and long-term depression (LTD), which are electrophysiological phenomena associated with learning and memory [Kleschevnikov et al. 2004]. The finding of similar abnormalities in the brains of people with DS might increase the understanding of the basis of cognitive impairment in DS and might provide potential therapeutic targets [Antonarakis and Epstein, 2006]. Moreover, another abnormality in the Ts65Dn mouse

brain might be directly relevant to humans, i.e. the progressive loss and atrophy of cholinergic neurons in the basal forebrain [Salehi et al. 2006; Granholm et al. 2000], a neuronal population that is particularly vulnerable in AD.

The diagnosis of dementia in DS requires an understanding of baseline cognitive and emotional functioning in the disorder. For example, most healthy people with DS prior to the onset of dementia would score below detectable levels on the Mini Mental State Examination, a screening test for dementia administered to risk individuals in the general population. In the child and adult with DS, measures of IQ, general cognitive functioning, and mental age are at least two standard deviations below the norm and these global anchor measures appear to exceed performance in language, phonology, and memory [Miller, 1988]. Auditory verbal memory is selectively impaired beyond global baseline functioning [Varnhagen et al., 1987]. These findings can become confounding factors for the determination of dementia in DS and have led to a consensus statement for testing individuals at risk [Aylward et al., 1997]. One alternative to traditional direct testing of adults with developmental disabilities involves looking to sources other than the patient for information [Burt et al., 1998].

Chapter 2

DOWN SYNDROME AND ACCELERATED AGING

1. Introduction

In the previous chapter we described the cytogenetic and pathogenic aspects of DS in which multiple major aspects of the senescent phenotype appear. In other words, it has been shown that most features of physiological aging appear in DS earlier than in karyotypically normal age-matched subjects.

During the last two generations there was a highly significant change in the survival of people with DS. In fact, while in the 1940s, the average life expectancy for individuals with DS was 12 years [Penrose, 1949], individuals with DS now enjoy life expectancies into their 60s [Bittles and Glasson, 2004] thanks to medical breakthroughs and improvements in services. Along with this longer life expectancy comes a larger population of adults with DS who display premature age-related changes in their health status [Esbersen, 2010]. Indeed, adults with DS experience much earlier than non trisomic people a series of age related phenomena such as Alzheimer-like dementia, altered free radical metabolism and impaired mitochondrial function that lead to neuronal degeneration [Lott et al. 2001; Busciglio et al. 2002 and 2007; Haan et al. 2003], skin and hair changes, early onset menopause, visual and hearing impairments, adult onset seizure disorder, thyroid dysfunction, diabetes, obesity, sleep apnea and musculoskeletal problems [Esbensen, 2010]. Therefore, DS has been often defined as an accelerated aging syndrome, even though aging is not so premature in DS as in other syndromes such as Hutchinson-Gilford progeria or Werner's Syndrome. A more detailed discussion on the main aspects of DS premature aging is reported below.

1.1 Skin and Hair changes

Adults with DS experience a number of dermatological and autoimmune symptoms characteristic of accelerated aging, such as hair loss, premature graying of the hair and wrinkling of the skin [Lott et al. 1982]. Further, sun-exposure may have a greater effect on skin wrinkling for individuals with DS than for the general population, contributing to the appearance of premature skin aging in adults with DS [Brugge et al. 1993]. Significant differences in the skin and chronological age of the individual have been found in post-mortem examinations of the skin of adults with DS [Edwards, 1978].

Alopecia areata (the loss of hair) affects between 6% to 18.4% of adults with DS [duVivier & Munro, 1975; Prasher, 1994b]. It is thought that this elevated rate of condition in adults with DS may be related to

immunological deficiency in thymus dependent function [Carter & Jegasothy, 1976]. Other skin conditions common in adults with DS include atopic dermatitis, fungal infections, seborrhoeic dermatitis, and xerosis, affecting 34% to 39% of adults [Kerins et al. 2008; Prasher, 1994b; Roizen & Patterson, 2003].

1.2 Vision and Hearing Impairments

Visual impairments (44-71%) and eye abnormalities are common among aging adults with DS [Gardiner, 1967; Jacobson, 1988; McCarron et al. 2005]. Among visual impairments cataracts (11-33%), strabismus (23-37%), refraction problems (30-34%) and keratoconus (15%) are common ophthalmological problems [Aitchison, et al. 1990; Prasher, 1994b; Van Allen et al. 1999; Van Buggenhout et al., 1999).

Age-related hearing loss is more common among adults with DS compared to the general population, and appears to have an earlier age of onset. High frequency sensorineural hearing impairments (such as presbycusis) in adults with DS onset about 20 to 30 years earlier than in their peers with intellectual disability (ID), and about 30 to 40 years earlier than in the general population [Buchanan, 1990]. Rates of hearing loss among adults with DS range from 12 to 72% [Howells, 1989; Prasher, 1994b; Van Buggenhout et al., 1999], and may depend on the nature of the hearing assessment.

1.3 Seizure disorder

Seizure increases with age for individuals with DS, especially for individuals suffering from comorbid dementia. One early estimate of the rate of seizures was 12.2% for adults with DS over the age of 55 and 15.8% for adults over the age of 60 [Veall, 1974]. A lifespan study of individuals with DS found that approximately 8% of individuals with DS suffer from seizures, of which 40% experienced seizures after the age of 20 [Pueschel et al. 1991]. This higher rate of seizures may be related to the gene for myoclonus epilepsy being mapped to chromosome 21, even if this form of seizure is more commonly found in children and adolescents [Hattori et al. 2006]. In fact, older patients with DS typically have tonic-clonic, complex partial or simple partial seizures [Pueschel et al. 1991]. Alternatively, structural abnormalities and biochemical aberrations of the CNS in adults with DS may in part be responsible for increased seizure frequency [Pueschel et al. 1991].

1.4 Thyroid dysfunction

The risk for thyroid disease increases with age in individuals with DS [Korsager et al. 1978). Approximately 35-40% of adults with DS have abnormal thyroid function, although only 7-8% have active hypothyroidism [Dinani & Carpenter, 1990; Prasher, 1994a; Prasher, 1994b]. Also comparable to seizure findings, the rate of thyroid disease in adults with DS is greater than that found in the general population [Coleman, 1994].

1.5 Immune system changes

In DS have been found clinical manifestations and biochemical markers indicative of the "early aging", i.e. the production of autoantibodies and the development of autoimmune diseases. Kusters et al. (2009) hypothesized that the immune system in DS is intrinsically deficient from the very beginning, and not simply another victim of a generalized process of precocious aging.

In DS patients are observed multiple immunological abnormalities, including alterations in subpopulations of lymphocytes, disorders of the cells of immune system, cancer cell growth and production of autoantibodies; they are more frequently age-related abnormalities and part of the "early aging" framework [Cossarizza et al. 1990]. Another characteristic of DS subjects is represented by the progressive expansion of the non-functional natural killer, resulting in a deficiency of natural killer activity itself [Cossarizza et al. 1991].

1.6 CNS changes

In DS subjects, the deposition of amyloid protein is first observed in the second decade of life [Rumble et al. 1989], and the full pathology of AD seems to be invariably present from 35 years of age onwards – thirty to forty years earlier than in the normal population. Nevertheless, the age-associated decrease in cognitive function observed in DS subjects is still a matter of study, as its features and molecular mechanisms are largely not elucidated.

Patients with AD in the general population and DS were been evaluated by informant measures of language and emotional functioning [Nelson et al., 1995]. The early presentation of dementia in DS seems to be characterized by depression, indifference, and pragnosia (socially deficient communication). These variables reflect emotional change and pragmatic language functioning and they correlate highly with the presence of pathological release reflexes on the neurological examination and the presence of cortical atrophy on the MRI scans of demented patients with DS. Taken together, these factors may be considered a sign of prefrontal lobe dysfunction in DS that causes inattentiveness, heightened threshold to stimulation, apathy, decomposition of gait, labile mood and sphincteric incontinence.

2. Alzheimer's disease in Down Syndrome

The ability to detect dementia in DS population is hampered by developmental differences as well as the sensitivity of existing test tools. Despite the apparent clinical heterogeneity in aged individuals with DS, age-associated neuropathology is a consistent feature [Lott and Head, 2001].

The first description of the link between DS and AD is found in an English publication by Jervis (1948) who described three individuals with DS (>40 years) who had intellectual deterioration, neuronal cells loss, senile plaques and neurofibrillary tangles. Neuropathological evidence of the association between DS and AD was described by many scientists [Struwe, 1929; Bertrand and Koffas, 1949]. In the subsequent three decades numerous reports have shown that the brain lesions of AD in DS, visualized by light and electron microscopy, were similar, if not identical, to the findings of AD in the general population [Lott, 1982, 1986 and 1992]. In 1972, Malamud published a monograph characterizing the age-dependency of AD in DS and reported that the microscopic characteristics of AD were ubiquitous in brain samples of individuals with DS over age 40 years.

2.1 APP gene

The two neuropathological features necessary for a diagnosis of AD are neurofibrillary tangles and senile plaques [Kachaturian et al., 1985; Mirra et al., 1991; National Institute on Aging, 1997], of which the principal component is β-amyloid (Aβ), a hydrophobic 39 to 43 amino-acid peptide which can adopt a β-pleated sheet configuration. Aβ is derived from the proteolytic processing of a longer amyloid precursor protein (APP), a member of a large family of 70-kDa transmembrane glycoproteins derived from alternative splicing of precursor mRNA [Selkoe, 1996]. APP gene is located on chromosome 21 (21q21) so, in DS, it is over-expressed and triplicated in virtually all forms of the disorder [Tanzi et al., 1987; Kang et al., 1987]. This over-expression of APP probably accounts for the early deposition of Aβ in the brains of children with DS as early as eight years [Leverenz and Raskind, 1998]. By age of thirty years, Lemere et al. (1996) found Aβ deposition in up to fifty percent of brain from individuals with DS. Therefore Aβ accumulation appears to occur in an age dependent manner in DS and the syndrome could be a valuable model system for understanding senile plaque progression.

Aβ is a long-lived peptide in which aspartic acid and asparagine at positions 1, 7, 23, and 27 are susceptible to chemical damage by nonenzymatic processes such as racemization, isomerization, and deamidation. The spontaneous post-translational modifications of these amino acids affect critical cellular and molecular events during aging by interfering with protein function.

Several studies report the presence of spontaneously modified, and thus older plaque deposits in AD and DS brain [Fonseca et al., 1999; Azizeh et al., 2000]. The authors observed the distribution of longed

lived senile plaques in DS brain, using affinity-purified polyclonal antibodies for post translationally modified Aβ (with either a racemized or isomerized aspartic acid at position 7). The immuno-positive plaques were initially found in clusters within the superficial layers of the frontal and entorhinal cortex but, with advancing age, increasing numbers appeared in deeper layers. These findings suggest that there is a progression of Aβ deposition from superficial to deeper cortical layers. Previous reports of senile plaque pathogenesis in the brains of individuals with DS demonstrate that Aβ initially appears as clusters within supragranular cortical regions [Armstrong and Smith, 1994; Hof et al., 1995]. Moreover, the findings suggest that the earliest deposition of Aβ may involve the frontal cortex which, in turn, may account for the frontal lobe symptomatology which characterizes the earliest stages of dementia in DS.

Once initial Aβ or neurofibrillary tangle pathology has appeared in the DS brain, Aβ deposits, neurofibrillary tangles, and cognitive decline in individuals with DS after age 35–40 years increase exponentially [Coyle et al., 1986; Mann et al., 1988; 1989; Mann et al., 1992; Wisniewski and Wegiel, 1992; Armstrong et al., 1994; Hof et al., 1995; Hyman et al., 1995; Leverenz et al., 1998]. Prevalence studies of dementia in DS have shown a clinical correlation of the neuropathological changes and only eight percent of individuals between 35–49 years showed cognitive decline with the prevalence rising to over seventy-five percent above age sixty years [Lai and Williams, 1989]. Thus, although the deposition of amyloid appears to be linear throughout the lifespan in DS, additional factors seem to be involved in the marked increase in both neuropathology and clinical dementia in the fourth and fifth decades.

Inflammatory factors may also play a significant role in the development or acceleration of AD pathology in DS. For example, the levels of interleukin-1, a proinflammatory cytokine, and S100β increase in the brain [Griffin et al., 1989]. Activation of the proinflammatory classical complement cascade in association with the development of amyloid pathology contribute to strengthen the role of inflammatory processes possibly accelerating DS pathology [Lott and Head, 2001].

2.2 APOE gene

In a meta-analysis, Deb et al. (2000) concluded that demented adults with DS showed a significantly higher frequency of ε4 allele of the apo-lipoprotein gene compared to nondemented adults.

Apolipoprotein E (APOE) is a polymorphic protein encoded by a gene located on human chromosome 19q13.2; it has a central role in plasma lipoprotein metabolism and cholesterol homeostasis for its ability to interact with the LDL receptor and the LDL receptor-related protein; APOE is the major apolipoprotein in brain, its production and accumulation are increased in CNS disorders and in peripheral nerve injury [Weisgraber et al. 1994].

There are three common alleles of the APOE gene, indicates as APOE ε2, ε3, ε4. Numerous report have established that the frequency of the ε4 allele increases in late-onset familial and in sporadic AD

[Corder et al. 1993; Tsai et al. 1994; Saunders et al. 1993]; yet, APOE ε4 allele must not be considered as a genetic mutation causing AD, but rather as a susceptibility gene or risk factor for earlier onset. In fact, reactions of immunoreactivity has been shown APOE within senile plaques [Schmechel et al. 1993], suggesting the possible role of APOE as a pathological "chaperon protein" involved in insoluble amyloid formation. It has been also demonstrated that the development of pathologic changes in DS cases does not always parallel that observed in AD patients; in particular amyloid accumulation represents a major and early feature of DS but it is not considered a critical factor in the development of the dementia that occurs in most DS patients only after 50 years [Wisniewski et al. 1995].

Del Bo et al. (1997) have observed that DS patients do not differ from the general population in terms of the ε4 allele's frequency; in agreement with this evidence, no significant differences in ε4 allele frequency between AD-DS cases and DS controls have been detected [Van Gool et al. 1995].

A previous report on DS patients described an association between longevity and the absence of clinical evidence of dementia [Royston et al. 1994], suggesting a modulation of the clinical phenotype of DS by genes on chromosomes other than chromosome 21.

How APOE4 protein can contribute to quicken the progression of mental retardation is unknown, but it is important to underline that a single amino acid difference between APOE3 and APOE4 isoforms is able to produce a remarkable biological effect on the expression of mental decline. Specifically, data presented by Del Bo et al. (1997) show a significant inverse correlation between full scale IQ values and age in the subgroup of DS patients having at least one APOE ε4 allele.

Anomalies of the developmental processes and metabolic alterations due to partial or total gene dosage of chromosome 21 may affect cortical neuronal function in DS subjects. In particular, the triplication of chromosome 21 could induce the overexpression of other proteins other than β-amyloid, such as CuZn-superoxide dismutase, S100 β protein and the glutamate receptor subunit GluR5, which might participate actively in the accelerated degenerative process observed in DS patients [Dickinson and Singh, 1993; Becker et al. 1993].

2.3 Oxidative stress

Individuals with DS share a common predisposition towards oxidative stress (OS). Oxidative stress results from an imbalance in the metabolism of free radicals such as reactive oxygen species (ROS) and it is thought to have a direct role in the development of neuropathological changes of AD in DS [Kedziora and Bartosz, 1988; Busciglio et al., 1998].

The most susceptible target of ROS are mitochondria and mitochondrial control region mutations occur both in demented individuals with DS and in those with AD in the general population [Coskun et al. 2010]. Mitochondrial membrane potentials measured in blood mononuclear cells from individuals with DS

are more susceptible to damaging agents than controls [Roat et al. 2007]. Oxidative stress is significant even in fetuses with DS, as suggested by functional genomic analysis of amniotic fluid cell-free mRNA [Slonim et al. 2009]. While oxidative stress and antioxidant systems are involved in the cognitive dysfunction associated with the pre-demented state in DS, the relationship to genes and gene products is not yet clear [Strydom et al. 2009]. Oxidative stress seems to start before birth because the glycation end products, associated with cellular oxidation, increased in brains from fetuses with DS [Odetti et al. 1998].

Oxidative stress is also increased in mouse models for DS. In mouse the trisomy 16, which has genetic homology to human chromosome 21, is associated to decreased respiration at the level of Complex I in mitochondria [Bambrick and Fiskum, 2008] and to increased of lipid peroxidation, a measure of oxidative stress, in the brain [Ishihara et al., 2010]. In this model oxidative stress has been identified even as a possible therapeutic target [Gardiner, 2010]. In fact, alpha-tocopherol, an antioxidant, delayed the onset of cognitive and morphological abnormalities in the Ts65Dn mouse [Lockrow et al., 2009] and reduced the oxidation state of S100β protein and subsequently its influence on the neuroinflammatory process [Bialowas-McGoey et al., 2008].

However, not all investigators agree on the importance of oxidative stress factors in DS. For example, Hayn et al. (1996) found no abnormalities in brain concentration of SOD-1, glutathione peroxidase, malondiadehyde, or o-tyrosine in DS compared to controls, although the level of choline acetyltransferase was reduced (in keeping with the cholinergic deficit). Likewise there appears to be evidence against a damage to nuclear DNA isolated from cortical brain areas in DS and induced by the increased ROS [Seidl et al., 1997].

Head et al. (2000) suggest that oxidation of Aβ is an early event in neuritic plaque biogenesis. The distribution of oxidatively modified Aβ in the entorhinal and frontal cortex in DS, AD, and canine brain, oxidized Aβ was observed within a subset of diffuse plaques and the cores of neuritic plaques. Confocal studies indicated that oxidized Aβ deposits were within activated microglia, suggesting that this oxidative modification may seed the formation of neuritic and cored plaques. Oxidative modifications of amyloid lead to more highly aggregated and insoluble deposits suggesting that this is a potential mechanism by which amyloid pathology is initiated and propagated in DS with AD.

These studies suggest that oxidative damage and amyloid accumulation interact and thus administration of an antioxidant on a chronic basis may delay the start of dementia in DS or slow its course [Lott and Head, 2001].

Figure 1 summarizes the relationship between oxidative stress, Aβ deposition, and neurodegeneration in AD. It is supposed that oxidative stress may exacerbate Aβ aggregation [Dyrks et al. 1992], while fibrillar Aβ may increase intraneuronal generation of free radicals [Behl et al. 1994] and trigger an inflammatory reaction causing further oxidative damage [Pachter, 1997].

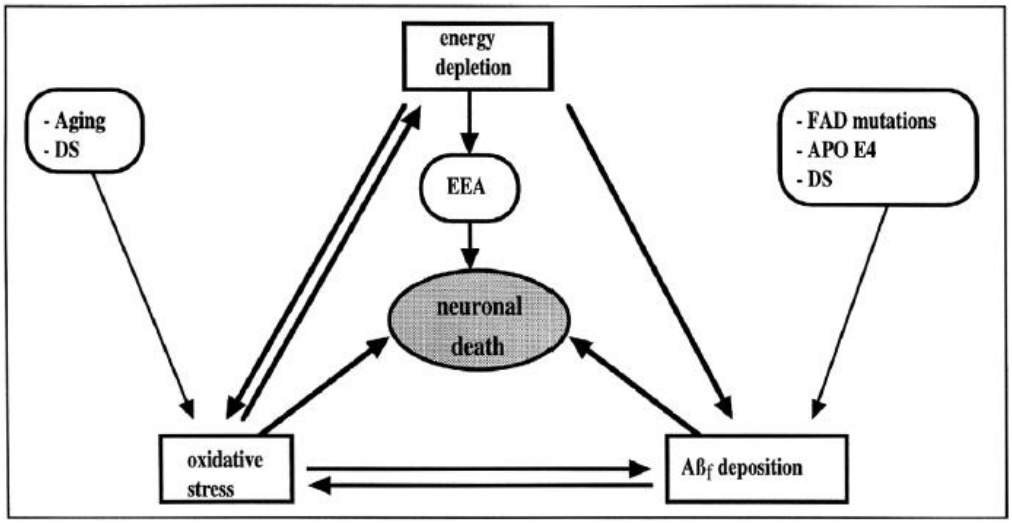


Figure 1: Mechanisms of neuronal death in Alzheimer's disease (Source: "*Stress, Aging, and Neurodegenerative Disorders"*, Busciglio et al. 1998)

Aging, DS or gene mutations associated with familial AD might be considered predisposing factors which contribute by either increasing oxidative stress [Carney J and Carney A, 1994] or Aβ production and deposition [Selkoe, 1997], and impair mitochondrial function, resulting in energy depletion followed by further neuronal degeneration [Flint Beal, 1992]. Finally, impaired cellular energy metabolism increases the amyloidogenic processing of the Aβ precursor protein [Gabuzda et al. 1994] potentially leading to increased Aβ production. In this context, neuronal death would be caused by the simultaneous action of oxidative stress, Aβ cytotoxicity and excitatory amino acids cytotoxicity.

Combined therapeutic intervention to prevent the neurodegenerative process can involve antioxidant strategies, treatments designed to inhibit Aβ deposition and/or fibrillization, protection of mitochondrial function and energy production, and prevention of the inflammatory and excitotoxic processes [Busciglio et al. 1998].

2.4 The "gene-dosage" and the "two-hit" hypothesis

The **"gene dosage effect"** hypothesis holds that the dosage imbalance of a specific individual gene or a small group of genes is responsible for the specific individual DS traits [Korenberg et al. 1990; Reeves et al. 2001]. Overexpression of the encoded proteins by Chr 21 leads to overconsumption of their substrates and overproduction of their metabolic end-products. The best-documented gene products of this phenomenon are the amyloid precursor protein (APP) and the cytoplasmic enzyme Cu^{2+}/Zn^{2+} superoxide dismutase (SOD-1) (**Figure 2**), both of which are responsible for the regulation of reactive oxygen species (ROS) homeostasis [Schuchmann and Heinemann, 2000], which may be a fundamental factor in the development of oxidative stress (OS), preceding the signature pathology by decades and leading to neuronal death and disease progression. Additionally, on the long arm of Chr 21 (21q) there is the DS

critical/candidate region (DSCR) (q22.3), which includes the gene of cystathionin β-synthase (CBS), the gene for the S100β calcium-binding protein and that for the β -site APP-cleaving 2 enzyme (BACE2) (**Figure 2**). Another gene contained by 21q is the gene coding for the trifunctional enzyme complex glycinamide ribonucleotide synthase–aminoimidazole ribonucleotide synthase–glycinamide formyl transferase (GARS–AIRS–GART) (q22.1) (**Figure 2**), which catalyzes certain steps of *de novo* purine synthesis [Capone, 2000]. However, not all proteins whose genes are encoded on this chromosome display overexpression, suggesting that the DS phenotype cannot be explained simply by the "gene dosage effect" [Cheon, 2003].

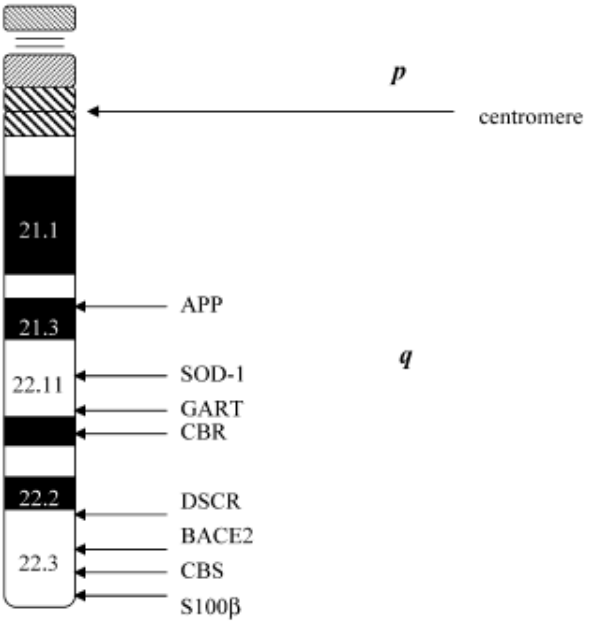


Figure 2: Chromosome 21: localization of the most important AD and DS related genes (Source: "*Oxidative stress: A bridge between Down's syndrome and Alzheimer's disease*", Zana et al. 2007)

The **"two-hit"** hypothesis for AD has been recently proposed. Initiating event can independently be either OS or alterations in mitotic signaling, but both processes are necessary and sufficient to propagate AD pathogenesis [Zhu et al. 2004]. According to this model, the first insult may leave neurons very vulnerable to an additional insult. In particular, it has been suggested that susceptible neurons (under stress or bearing mutations) devote their compensatory potential to adjusting to current stimuli and thereby lose the capability of further adaptation needed to respond to other insults in the future. Therefore it seems to be that the OS alone is a necessary, but not sufficient, event leading to AD [Zana et al. 2007].

3. Theories on aging

Several theories have been proposed to account for the premature aging observed in adults with DS.

In recent decades, the study of aging has expanded rapidly. A number of theories (**Table 1**) have been generated by biological, epidemiologic and demographic data, in order to identify a cause or process to explain aging and its inevitable consequence, death [Weinert and Timiras, 2003]. However, in recent years, the view of aging as an extremely complex, multifactorial process has replaced the search for a single cause of aging, such as a single gene or the decline of a key body system [Kowald and Kirkwood, 1996]. Several processes may interact simultaneously and may operate at many levels of functional organization [Franceschi et al, 2000a]. Similarly, different theories of aging are not mutually exclusive and may adequately describe some or all features of the normal aging process alone or in combination with other theories.

Biological Level/Theory	Description
Evolutionary	
Mutation accumulation*	Mutations that affect health at older ages are not selected against.
Disposable soma*	Somatic cells are maintained only to ensure continued reproductive success; after reproduction, soma becomes disposable.
Antagonistic pleiotropy*	Genes beneficial at younger age become deleterious at older ages.
Molecular	
Gene regulation*	Aging is caused by changes in the expression of genes regulating both development and aging.
Codon restriction	Fidelity/accuracy of mRNA translation is impaired due to inability to decode codons in mRNA.
Error catastrophe	Decline in fidelity of gene expression with aging results in increased fraction of abnormal proteins.
Somatic mutation	Molecular damage accumulates, primarily to DNA/genetic material.
Dysdifferentiation	Gradual accumulation of random molecular damage impairs regulation of gene expression.
Cellular	
Cellular senescence-Telomere theory*	Phenotypes of aging are caused by an increase in frequency of senescent cells. Senescence may result from telomere loss (replicative senescence) or cell stress (cellular senescence).
Free radical*	Oxidative metabolism produces highly reactive free radicals that subsequently damage lipids, protein and DNA.
Wear-and-tear	Accumulation of normal injury.
Apoptosis	Programmed cell death from genetic events or genome crisis.
System	
Neuroendocrine*	Alterations in neuroendocrine control of homeostasis results in aging-related physiological changes.
Immunologic*	Decline of immune function with aging results in decreased incidence of infectious diseases but increased incidence of autoimmunity.
Rate-of-living	Assumes a fixed amount of metabolic potential for every living organism (live fast, die young).

Table 1: Classification of the most important theories of aging (Source: *"Invited Review: Theories of aging"*, Weinert and Timiras, 2003)

3.1 The evolutionary theories

Evolutionary theories argue that aging results from a decline in the force of natural selection. Since evolution acts primarily to maximize reproductive fitness in an individual, longevity is a trait to be selected only if it is beneficial for fitness. Life span is, therefore, the result of selective pressures and may have a large degree of plasticity within an individual species, as well as among species. The evolutionary theory was first formulated in the 1940s based on the observation that Huntington's disease, a dominant lethal mutation, remained in the population even though it should be strongly selected against [Haldane, 1941]. This observation inspired the ***Mutation Accumulation Theory*** of aging, which suggests that detrimental, late-acting mutations may accumulate in the population and ultimately lead to pathology and senescence [Medawar, 1952]. Life span is largely a function of survivability and reproductive strategy in a competitive

environment, so it is species-specific. Consequently, organisms that die primarily from predation and environmental hazards will evolve a life span optimized for their own particular environment. The observation that organisms can age in a natural environment [Loison et al. 1999] indicates that extending life span can be beneficial to fitness, but there are other considerations that might necessitate sacrificing longevity for reproductive fitness. This concept is the basic idea of the ***Disposable Soma Theory*** of aging which argues that the somatic organism is effectively maintained only for reproductive success; afterward it is disposable. In other words, somatic maintenance, in particular, longevity, has a cost; the balance of resources invested in longevity vs. reproductive fitness determines the life span.

The concept of an evolutionary tradeoff is also essential in the ***Antagonistic Pleiotropy Theory***, in addition to the Disposable Soma Theory. The first suggests that some genes may be selected for beneficial effects early in life and yet have unselected deleterious effects with age, thereby contributing directly to senescence; the second explains why we live for a certain period of time but does not postulate the specific cause of aging.

3.2 The Network Theory

In 1989 Franceschi proposed that aging was directly controlled by a network of cellular and molecular defence mechanisms, so called the ***Network Theory*** of aging [Franceschi, 1989]. The aim of this theory was to combine suggestions deriving from evolutionary theories of aging [Kirkwood, 1977; Kirkwood and Holliday, 1979] with data emerging from cellular and molecular biology of aging. Indeed, cells are constantly exposed to a variety of internal and external stressors, which are potentially dangerous for the maintenance of cell functional integrity. Such stressors are very diverse and include different physical (UV and gamma radiation, heat), chemical (components of the body and products of metabolism, such as oxygen free radicals and reducing sugars), and biological agents (viruses). In the course of evolution, however, a number of mechanisms have emerged, such as DNA repair systems, production of heat shock proteins (HSP), antioxidant defense systems either enzymatic or non-enzymatic, activation of poly (ADP-ribose) polymerase (PARP), allowing the cell to overcome these potentially harmful agents (**Figure 3**).

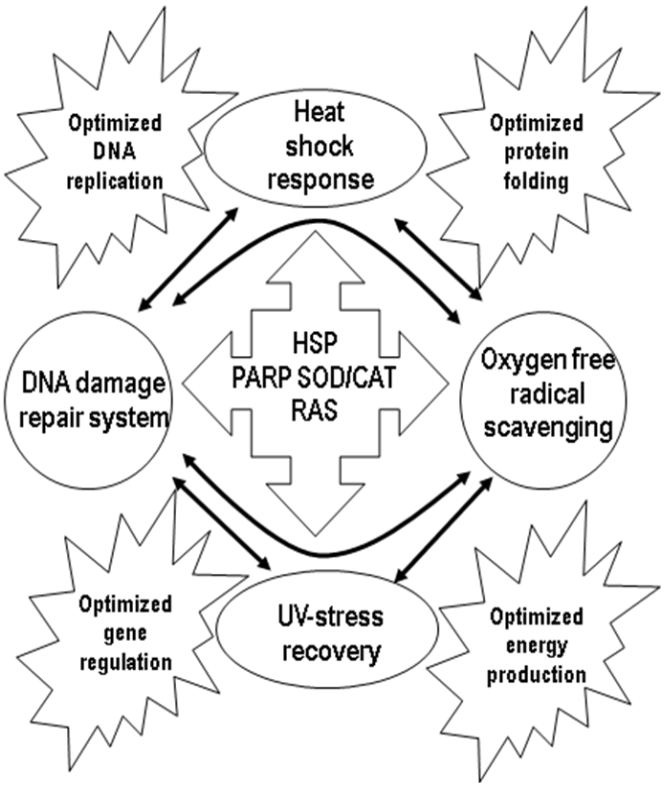


Figure 3: The "anti-aging" cellular defense network (Source: "*Inflamm-aging. An evolutionary perspective on immunosenescence",* Franceschi et al, 2000b)

A failure of these mechanisms does not allow the cell to maintain its homeostasis and this fact coincides with cell senescence (aging at the cellular level). However, the persistence of factors involved in these defense mechanisms, such as Hsp70 and catecholamines, is highly toxic, so the strategy is to maintain long-term survival stress responses in a fixed range, beyond which the ability to survival decades because of insufficient responses or, conversely, because of excessive responses [Franceschi et al, 2000a].

3.3 The Remodeling Theory

Over the years has become increasingly important the question of what was the contribution of the immune system to longevity [Franceschi et al, 1995; Franceschi and Cossarizza, 1995]. From this need was born the idea of studying healthy centenarians as a model of physiological aging and immunosenescence.

The ***Immunosenescence*** is the net result of continuous adaptation to changes in the body that are deteriorating over time. The body's resources are continuously optimized, and immunosenescence is a dynamic process, which includes a loss that is a purchase. Centenarians in good health are considered as

the model for excellence for the study of aging as they have the best ability to adapt to damaging agents and particularly harmful to stress [Franceschi et al, 1995].

One of the main feature of the aging process appears to be a progressive pro-inflammatory state given by continuous antigenic challenge, resulting by chronic exposure to antigens. This process has been defined ***Inflammaging*** [Franceschi et al, 2000b] and is characterized by a dichotomy in which the evolutionary innate immunity is largely preserved or even activated and provides a background for the biological susceptibility to age-related diseases. In particular, the beneficial effects of inflammation, aimed at the neutralization of harmful agents at early and adult life, may become detrimental in later life. Moreover, the absence of robust (protective and predisposing) gene variants and/or the presence of weak (or frail) genetic variants are probably necessary to develop age-related diseases and organ-specific with an inflammatory pathogenesis, such as atherosclerosis, Alzheimer's disease, osteoporosis, and diabetes. Thus a model for the genetic basis of inflammaging can be proposed, in which apolipoprotein E is assumed to represent a prototypical frailty gene and IL-6/IL-10 to represent prototypical pro-inflammatory and anti-inflammatory genes (**Figure 4**). According to this model, the individual's genetic make-up is responsible for different trends of the age-related increase in inflammaging (the slope of inflammaging) [Franceschi and Bonafè, 2003].

Genetics of inflammaging and frailty (the DDD paradigm)

The depicted model suggests that the threshold over which disability, disease and death (DDD) occur can be reached at different rates in different individuals, according to interactions between genes playing a major role in inflammaging. The apolipoprotein E (APOE) e4+ and e4− genotypes are taken as genetic markers dictating low (blue; APOE e4+) and high (green; APOEe4−) thresholds beyond which DDD ensue. Functional polymorphisms of IL-6 and IL-10 dictate the rate of inflammaging (slope) and thus the point (age) at which the DDD threshold is intercepted.

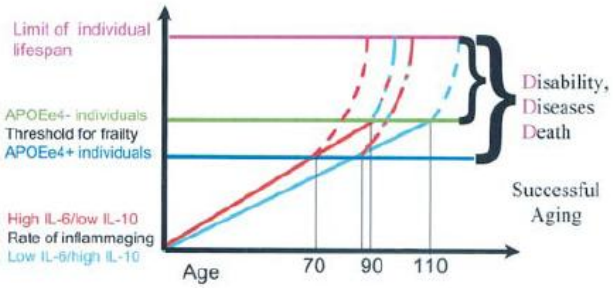


Figure 4: (Source: "*Centenarians as a model of healthy aging*", Franceschi and Bonafè, 2003)

Thus, inflammaging could be the ultimate proof that the beneficial effect of the defense system network (innate immunity, stress, and inflammation), devoted to the neutralization of dangerous/harmful agents early in life and in adulthood, turns out to be detrimental late in life, in a period largely not foreseen by evolution. Thus, a tradeoff between early beneficial effects and late negative outcomes can occur at the genetic and molecular level. Similar phenomena have been predicted by evolutionary geneticists who proposed the theory of antagonistic pleiotropy [Franceschi et al, 2000b].

3.4 mtDNA damage and the mitochondrial theory of aging

Among all aging theories, the ***Freee Radical Theory*** is one of the most gained and discussed (**Figure 5**).

TABLE 1. HYPOTHESES ON THE ROLE OF MITOCHONDRIA IN AGING
(AUTHOR, YEAR, AND PROPOSED CAUSE OF AGING)

HARMAN (1972): Free radical damage to mitochondria (of all cell types), including their DNA.
MIQUEL ET AL. (1980): Oxygen stress resulting in oxyradical-induced inactivation of the mtDNA of fixed-postmitotic (irreversibly differentiated) cells, with resulting bioenergetic decline.
FLEMING ET AL. (1982): Irreversible injury to mtDNA.
MIQUEL AND FLEMING (1986): Intrinsic mitochondrial mutagenesis in terminally differentiated cells.
RICHTER (1988): Intranuclear accumulation of mitochondrial DNA fragments.
LINNANE ET AL. (1989): Accumulation of mitochondrial mutations (and cytoplasmic segregation of these mutations), with bioenergetic decline.
MIQUEL (1991): Mitochondrial genetic and membrane injury linked to the loss of regenerative mechanisms in irreversibly differentiated cells

Modified from Miquel (1991).

Figure 5: (Source: "*An update of the oxygen stress-mitochondrial mutation theory of aging: genetic and evolutionary implications*", Miquel J, 1998)

It has been proposed for the first time by Harman in 1957 and explains how aging is strictly associated with high level of free radicals, chemical species with a free electron in their external orbital and, for this reason, they are extremely reactive and instable. An increasing speed of producing reactive oxygen species (ROS), a decline of antioxidant defense system and a low efficiency in repairing damaged molecules are conditions related to oxidative stress, which could interact with aging.

Although the available evidence strongly suggests that mitochondria play a role in this process, there appears to be a wide range of opinions as to the exact nature of the involvement of mitochondria in aging. The ***Mitochondrial Theory***, proposed by Miquel in 1980, is considered an extension of the free radical theory. As it is commonly known, nDNA is protected by histone proteins and various repair enzymes, which minimizes damage to nDNA from free radicals/oxidants. mtDNA is organized into complexes called nucleoids, composed of proteins belonging to the mtDNA replisome that provide protection from oxidative stress and repair of damages [Chen and Butow, 2005]; nevertheless, the proximity to the main source of ROS, i.e. the electron transport chain (ETC), leads to a dramatically higher rate of damage with respect to nDNA. The commonest form of free radical damage to mtDNA molecules is the production of 8-OHdG, an oxidized guanine base. Even in young (3 month old) rats, the level of 8-OHdG is already 16 times higher in mtDNA than nDNA [Richter et al. 1995]. Since mtDNA damage accumulates during the life of an individual, the functionality of the ETC enzyme complexes that produce ATP and that are in part encoded by mtDNA,

decrease dramatically and gradually produces a cellular energy crisis. The system is not capable to keep the equilibrium and it leads to cellular aging and afterward to organism aging. The dramatic mutation rate causes defective structures formation in the respiratory chain and consequently a defective functionality which creates a rising in ROS production. This process is the so-called vicious cycle, which is the basis of the mitochondrial theory of aging, and in which somatic mutations of mtDNA engender respiratory chain dysfunction, enhancing the production of DNA-damaging oxygen radicals.

When a new mtDNA mutation arises in a cell, a mixed intracellular population of mtDNAs is generated (wild-type and mutated mtDNAs), a state known as eteroplasmy. The mutant and normal molecules are randomly distributed into the daughter cells and as a consequence of this replicative segregation, the proportion of mutant and normal mtDNAs can drift toward an homoplasmic condition (all mutant or all wild type).

Furthermore, it has been observed that in order to compensate the energy deficiency, mtDNA copy number is preferentially, clonally, amplified within cells with defective mitochondria [Coskun et al. 2003]. This may be a consequence of the nucleus attempting to compensate for the energy deficiency by providing the cell with more mitochondria and more mtDNA. As mutant mtDNA can have a replicative advantage, cells can be enriched in mutant mtDNA by genetic drift. As the percentage of mutant mtDNAs increases, the mitochondrial energetic output declines, ROS production increases, and the propensity for apoptosis increases. As cells are progressively lost through apoptosis, tissue function declines, ultimately leading to aging of the organism. Thus according to this theory the accumulation of mutant mtDNA creates the aging clock (**Figure 6**).

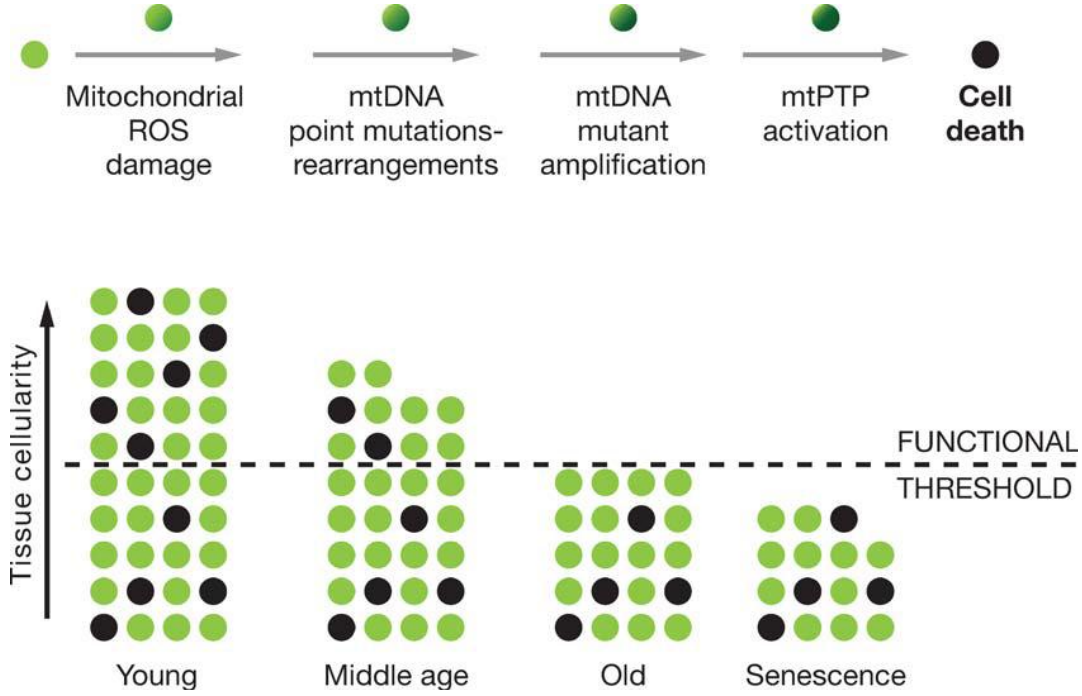


Figure 6: Aging model. The dot line shows the minimum number of cells for the tissue to function normally. In black are die cells in a mitochondrial-mediate process. In green cells with optimal function. (Source: *"A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine"*, Wallace DC, 2005).

3.5 Mitochondrial dysfunction and DS

Probably the most accredited theory to explain premature aging in DS subjects is the ***Mitochondrial Dysfunction***. Oxidative stress in DS has been documented in convincing epidemiological, in vitro and in vivo, data. For example, the level of malondialdehyde (MDA), a biomarker of lipid peroxidation, is elevated by 30–60% in the brain, erythrocytes, fibroblasts and urine and the level of 8-hydroxy-20-deoxyguanosine, a biomarker of oxidative damage to DNA, is increased by 70% [Anneren and Epstein 1987; Brooksbank and Balazs, 1984; Bras et al. 1989; Jovanovic et al. 1998; Busciglio and Yankner, 1995]. The mitochondrial respiratory system is the most important intracellular source of ROS and free radicals, and mitochondria have a central role in different types of apoptosis. Numerous studies have demonstrated that the accumulation of mitochondrial DNA (mtDNA) mutations is a major contributor to degenerative diseases and human aging.

Busciglio and co-workers have shown that the level of intracellular ROS is increased 3- to 4-fold in DS neurons, with substantially elevated levels of lipid peroxidation that precede neuronal death [Busciglio and Yankner, 1995]. The source of these ROS is unknown, but it was shown that free radical scavengers were able to protect the neurons from cell death. The group also showed that in DS astrocytes there is a defect in AβPP processing that might either be the cause or effect of the observed mitochondrial

dysfunction. However, inhibition of mitochondrial function in normal cells induced similar abnormalities of AβPP processing, implying that mitochondrial dysfunction in DS cells is a cause of the AβPP processing defect.

Arbuzova (1996 and 1998) has suggested that mtDNA mutations have a role in the pathogenesis of DS. Apart from helping to explain free radical damage and development of AD, the presence of mtDNA mutations could explain the association of DS with premature aging and diabetes, which can result from mtDNA mutations [Wallace, 1999]. Furthermore mtDNA is almost entirely of maternal origin, as is the extra chromosome 21 in the majority of DS cases, and examination of pedigrees from families with aneuploidy recurrence indicates cytoplasmic inheritance of a risk factor [Arbuzova et al. 2001].

Mutations in mtDNA may cause an increase in the generation of free-radicals and reduce ATP levels. This, in turn, could affect the synaptonemal complex, chromosome segregation and division spindle, and could alter recombination, because the enzymes participating in recombination and DNA repair are ATP-dependent [Strick et al. 2000; Schar et al. 1997], leading to aneuploidy. Animal studies support the notion of the influence of mtDNA mutations on the meiotic apparatus. For example, the Dip 1 mutation in mice, which produces a high incidence of ovulated diploid oocytes, is carried by the mitochondria [Beerman et al. 1988]. It is notable that an impairment of the function of the oxidant–antioxidant system has been found in DS mothers and mtDNA sequencing in a donor of the extra chromosome 21 has identified a number of novel mutations all causing amino acid changes [Arbuzova, 1998].

Theoretically, both point mutations and micro-deletions could induce chromosomal non-disjunction. Arbuzova et al. (2002) have speculated that, rather than a single base change being responsible, several deleterious mutations decrease mitochondrial function in a different way but enough powerful to affect meiosis.

Chapter 3

MITOCHONDRIAL GENETICS

1. Introduction

It has been suggested that the risk of Down syndrome is closely linked to maternal age. In the past the association of Down syndrome with age was explained by relaxation of selection against trisomic fetuses in older women, but recently there have been suggestions that it is due to meiotic error caused by the aging of oocytes [Ghosh et al. 2009]. However, the exact mechanism has not yet been determined.

Mitochondria play an important role in meiosis of mammalian oocytes. In an experiment with mouse oocytes, it has been shown that mitochondria translocate to the perinuclear region during formation of the first metaphase spindle and disperse subsequently during the release of the first polar body. These rearrangements of mitochondria are regarded as essential for the specification of localized activities of ATP at the meiotic spindle and in order to facilitate development, a large supply of ATP is required [Van Blerkom et al. 1984, 1995, 1997, 1998]. According to Van Blerkom et al. (1998), mitochondrial dysfunction is due to a variety of intrinsic and extrinsic influences and it can profoundly influence the level of ATP generation in oocytes and early embryos; this in turn may result in aberrant chromosomal segregation or developmental arrest [Hsieh et al. 2001]. Chromosomal movements during meiosis are directed by microtubule assembly within the spindle. According to Battaglia et al. (1996), the spindle exhibited abnormal tubulin placement and one or more chromosomes were displaced from the metaphase plate during the second meiotic division in 79% of oocytes in an older age group under investigation. In contrast, only 17% of the oocytes from a younger age group exhibited aneuploidy. This indicates that regulatory mechanisms responsible for the assembly of the meiotic spindle are significantly altered in older women, leading to a higher prevalence of aneuploidy [Battaglia et al. 1996]. By inhibiting mitochondrial function in mice with chloramphenicol, Beermann and Hansmann (1986) demonstrated that defective mitochondrial function interferes with ordered chromosome segregation during the first meiotic division [Beerman et al. 1986 and 1988].

Mitochondrial dysfunction leads to oxidative damage and apoptosis, hypoxia, and deletion or point mutations in the mitochondrial genome; especially in oocytes of older women, these adverse influences may contribute to reduced mitochondrial function in the human female gamete. Linnane et al. (1989) speculated that the accumulation of mitochondrial DNA (mtDNA) mutations in oocytes result in a reduction of gene expression and since mtDNA can only be inherited through the maternal line, mitochondrial gene

expression measured in amniocytes, a fetal tissue, might reflect mitochondrial gene expression during meiotic division in oocytes, causing organ dysfunction.

As we seen so far, mitochondria are organelles very important for the cells. They are considered the "cellular power plants", because in them occur the biochemical processes, the most important of which is oxidative phosphorylation (OXPHOS), that provide the energy to the cells they need for all their vital functions. Therefore, as already explained in the previous chapter, mitochondria and mtDNA might be heavily involved in aging, but mtDNA is characterized, as well as somatic mutations, by a peculiar genetic, which will be descrided later.

2. Mitochondrial DNA

Human mtDNA is a circular double-stranded molecule that is 16569 bp long (**Figure 1**).

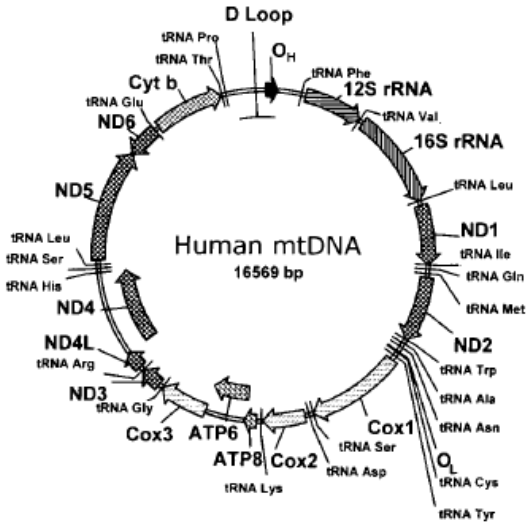


Figure 1 Map of human mtDNA
O_H and O_L, origins of heavy and light strand replication respectively; ND1–ND6, NADH dehydrogenase (ETC complex I) subunits 1–6; Cox1–Cox3, cytochrome oxidase subunits 1–3 (ETC complex IV); ATP6 and ATP8, subunits 6 and 8 of mitochondrial ATPase (complex V); Cyt b, cytochrome *b* (complex III).

Figure 1: (Source: "*Mitochondrial DNA and aging*", Alexeyev et al. 2004)

It encodes 2 rRNAs, 22 tRNAs and 13 polypeptides, of which seven are components of complex I (NADH dehydrogenase), three are components of complex IV (cytochrome *c* oxidase), two are subunits of complex V (ATP synthase) and cytochrome *b* (a subunit of complex III) [Anderson et al. 1981]. The inheritance of mtDNA is exclusively maternal and mtDNA is present in one to several thousand copies per

cell [Takamatsu et al. 2002] and is "encapsulated" into mitochondria at 1–11 copies per mitochondrion with the mean being two genomes per organelle [Cavelier et al. 2000]. The two mtDNA strands can be separated by denaturing caesium chloride gradient centrifugation [Kasamatsu and Vinograd, 1974]. In the heavy strand (purine-rich) is encoded most of the information, i.e. two rRNAs, 14 tRNAs and 12 polypeptides. The light strand (pyrimidine-rich) contains genetic information for only one polypeptide and eight tRNAs. Mitochondrial genes have no introns, some of which overlap and, in some instances, termination codons are not encoded, but are generated post-transcriptionally by polyadenylation [Ojala et al. 1981]. Intergenic sequences are absent or limited to a few bases. MtDNA maintenance and transcription is totally dependent by nuclear-encoded proteins. In fact, the mitochondrial proteome consists of an estimate of 1500 polypeptides [Taylor et al. 2003] of which only 13 are encoded by its own DNA (**Figure 2**).

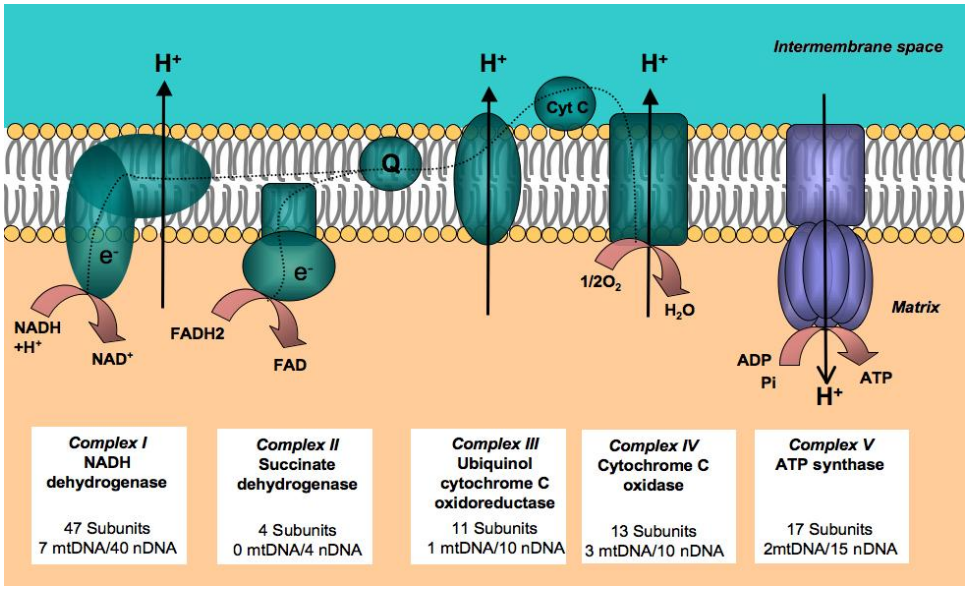


Figure 2: Mitochondrial respiratory chain (Source: "*Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis*", Bellance et al. 2009)

MtDNA replication is conducted by the heterodimeric DNA polymerase γ [Lim et al. 1999]. Replication occurs bidirectionally, initiated at two spatially and temporally distinct origins of replication, O_H and O_L, for the heavy and light strand origins of replication respectively [Taanman, 1999]. The mitochondrial proteome is dynamic, i.e. both the precise polypeptide composition and the relative abundance of a given polypeptide may vary in mitochondrial proteomes of different tissues as well as in the same tissue over time [Mootha et al. 2003]. In the same cell may coexist wild-type (normal) and mutated mtDNA, a condition called heteroplasmy [Wallace, 1992]. However, there are highly conserved regions, while other regions show a very high variability.

The overall mutation rate is ten to twenty times higher than the nuclear genome and most of the replacements that have been found in mitochondrial DNA are point mutations, with a strong preponderance

of transitions (substitutions purine→purine or pirimidine→pirimidine) respect to transvertions (substitutions purine→pirimidine or vice versa). Moreover mtDNA is located in the mitochondrial matrix, near the mitochondrial respiratory chain, that is a potent source of DNA damaging free radicals. Particularly, the control region (CR), also called displacement loop (D-loop, nt 16024-576) [Taanman, 1999], is composed of 1121 nucleotides, that are the most sensitive to mutagenesis [Chinnery et al, 1999]. The CR includes the L- and H-strand promoters (P_L and P_H), their mitochondrial transcription factor A (mtTFA) binding sites, the downstream conserved sequence blocks (CSB) I, II, and III, and the origins of H-strand replication (O_{H1} and O_{H2}) [Shadel and Clayton, 1997] (**Figure 3**). In this particular trait three hypervariable sequences are present, called HVSI, HVSII and HVSIII, characterized by a high level of mutation, higher than whole mtDNA variability, showing a high number of hotspot [Malyarchuk and Rogozin, 2004].

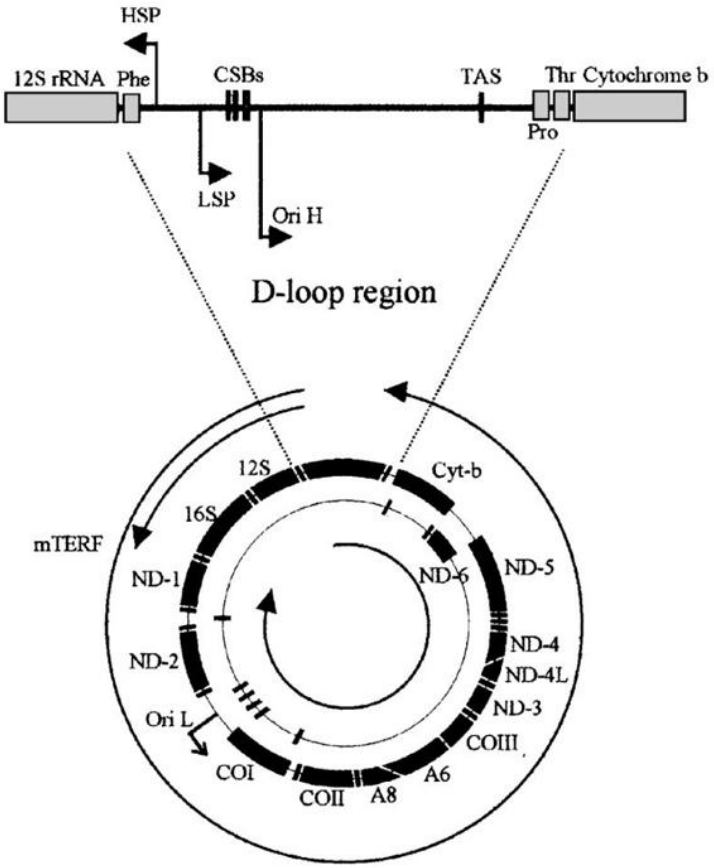


Figure 3: Schematic representation of the D-loop region (Source: *"Does the mitochondrial transcription-termination complex play an essential role in controlling differential transcription of mitochondrial DNA?"*, Selwood et al. 2000)

In addition, mtDNA has a high mutation fixation rate, which explains the high level of mtDNA substitutions; moreover their relative stability make the d-loop the ideal candidate for the identification and

classification of mitochondrial haplogroups, i.e. groups of related individual mtDNA sequences (haplotypes) [Kivisild et al, 2005; Torroni et al, 2006].

3. MtDNA variability

As a direct consequence of the mtDNA features, there are two levels of mtDNA variability: an hereditary or inter-individual variability, and a somatic or intra-individual variability.

Because of solely maternal inheritance, mtDNAs can only evolve by the sequential accumulation of mutations along radiating maternal lineages and the number of mutation, that differentiates the mtDNA of an individual from that of his ancestor, can be used as a molecular clock, providing a useful tool to the phylogenetic reconstruction.

This means that the human mtDNA is a molecular archive of the history and migration of women who have passed on to subsequent generations. If a mtDNA mutation arises and mutation is beneficial in a particular environment, it and its descendants will increase in frequency in that environment. Thus, different subsets of the variation in mtDNA tend to be confined to different regions and different human populations [Torroni et al. 2006].

3.1 Haplogroups

Phylogeographic studies of the human mtDNAs have revealed a remarkable correlation between mtDNA lineages and the geographic origins of indigenous populations. These regional mtDNA lineages are known as haplogroups. The various regional haplogroups form the branches on a single human dichotomous mtDNA phylogenetic tree, generated by the accumulation of sequential mtDNA mutations on radiating maternal lineages. The human mtDNA tree is rooted in Africa and specific branches were radiated into different geographic regions, probably constrained by the climatic zones [Cann et al. 1987, Johnson et al. 1983, Merriwether et al. 1991, Wallace et al. 1999] (**Figure 4**).

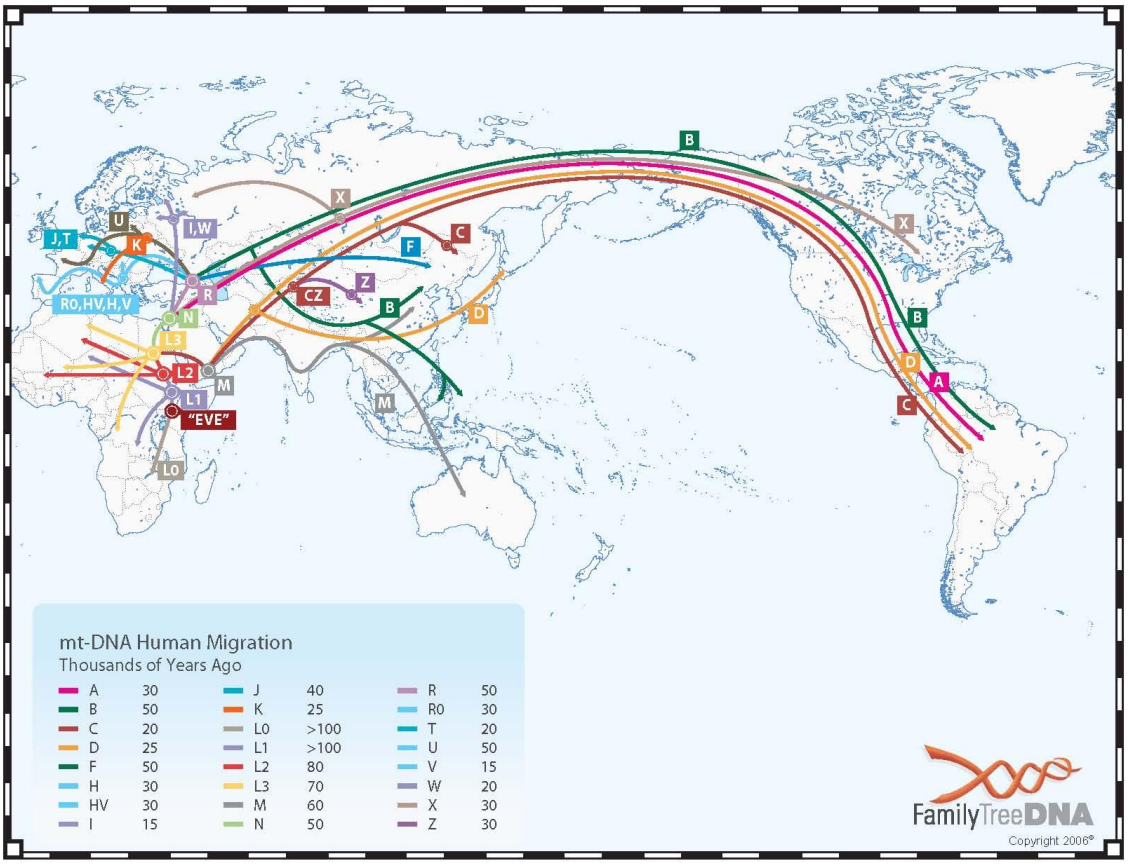


Figure 4: Diagram outlining the migratory history of the human mtDNA haplogroups (Source: *http://www.familytreedna.com*)

African mtDNAs are the most diverse and thus most ancient, with an overall age of about 150,000 to 200,000 years before present (YBP). African mtDNAs fall into four major haplogroups: L0 (oldest), L1, L2, and L3 (youngest). L0, L1, and L2 represent about 76% of all sub-Saharan African mtDNAs and are defined by a *Hpa*I restriction site at np 3592. In northeastern Africa, two mtDNA lineages, M and N, arose from L3 about 65,000 YBP. These mtDNA lineages left sub-Saharan Africa and radiated into Eurasia to give all of the Eurasian mtDNAs. In Europe, haplogroups L3 and N gave rise to haplogroups H (about 45% of European mtDNAs), T, U, V, W, and X (about 2%) as well as I, J (about 9%), and K (Uk). Europeans separated from Africans about 40,000–50,000 YBP. In Asia, lineages M and N radiated to give rise to, starting from N, haplogroups A, B, F and from M, haplogroups C, D, G [Wallace et al. 1999].

As Asians migrated northeast into Siberia, haplogroups A, C, D became progressively enriched, such that they became the predominant mtDNA lineages in the indigenous peoples of extreme northeastern Siberia, Chukotka. When the Bering land bridge appeared, about 20,000 to 30,000 YBP, people harboring these mtDNA haplogroups were in a position to migrate into the New World, where they founded the Paleo-Indians. After the land bridge submerged, haplogroup G arose in Central Asia and moved into northeastern Siberia to populate the area around the Sea of Okhotsk. Later, a migration

carrying haplogroup B started from eastern Central Asia and moved along the coast to the New World, by-passing Siberia. Haplogroup B then mixed with A, C, and D in southern North America, Central America, and northern South America to generate the Paleo-Indians. In addition, haplogroup X was brought to the New World in a migration that took place about 15,000 YBP. These immigrants settled in the Great Lakes region, and haplogroup X mtDNAs are found in 25% of the Ojibwa mtDNAs today. Since haplogroup X is found primarily in northeastern Europe, it has been speculated that an ancient European migration carrying this haplogroup might also have contributed to the Paleo-Indian populations, perhaps bringing the progenitors of the Clovis lithic culture to the Americas [Wallace et al. 1999].

Later migrations from northeastern Siberia, carrying a modified lineage of haplogroup A, founded the Na-Dené populations about 9500 YBP. More recently, immigrants from Siberia bearing derived lineages of haplogroups A and D moved along the Arctic Circle to found the Eskimos and Aleuts [Wallace et al. 1999].

Analysis of the mtDNA nucleotide variants revealed three different categories of variants: (*a*) neutral, including synonymous and weakly conserved nonsynonymous amino acid substitutions; (*b*) deleterious, altering highly conserved amino acids but located at the tips of the branches of the tree, indicating they are recent; and (*c*) adaptive, altering highly conserved amino acids but located within the internal branches of the tree, indicating that they are ancient. It is supposed that the mutations in class (*c*) are adaptive because they alter highly conserved amino acids, yet they have persisted in the face of intense purifying selection for tens of thousands of years. Hence, as these variants are not neutral or deleterious, they must be adaptive [Wallace, 2005].

3.2 Point mutations of mtDNA

Because of the proximity of the respiratory chain, source of ROS, and the limited ability to correct errors of the enzyme deputy to the replication of mtDNA, polymerase γ [Kaguni 2004; Kujoth et al. 2005; Johnson et al. 2001], the polymorphisms and single base mutations in mtDNA are very frequent.

The identification of systemic diseases caused by mtDNA mutations supported the hypothesis that mtDNA mutations might be a key factor in aging and age related degenerative diseases [Wallace et al. 2005]. Pathogenic mtDNA mutations fall into three categories: rearrangement mutations [Holt et al. 1988], polypeptide gene missense mutations [Wallace et al. 1988a], and protein synthesis (rRNA and tRNA) gene mutations [Shoffner et al. 1990, Wallace et al. 1988b]. The number of mtDNA-associated diseases identified increased quickly, hence it is thought that mitochondrial diseases commonly have a delayed onset and progressive course and that they result in the same clinical problems as observed in age-related diseases and in the elderly. Clinical manifestations that have been linked to mtDNA mutations affect the brain, heart, skeletal muscle, kidney, and endocrine system, the same tissues affected in aging. Specific symptoms include dementias, cardiovascular disease, muscle weakness, forms of blindness, deafness,

movement disorders, renal dysfunction, and endocrine disorders including diabetes [DiMauro and Schon, 2003; Wallace, 1999; Wallace and Lott, 2002; Wallace et al, 2001; http://www.mitomap.org].

3.2.1 Pathogenic mtDNA rearrangement mutations

MtDNA rearrangement mutations can be either inherited or spontaneous. Inherited mtDNA rearrangement mutations are primarily insertions. The first inherited insertion mutation identified caused maternally inherited diabetes and deafness [Ballinger et al. 1992 and 1994].

Spontaneous rearrangement mutations, above all deletions, generally result in a related spectrum of symptoms, irrespective of the position of the deletion end points. This is because virtually all deletions remove at least one tRNA and thus inhibit protein synthesis [Moraes et al. 1989]. Therefore the nature and severity of the symptoms from mtDNA deletion rearrangements is not a consequence of the nature of the rearrangement, but rather of the tissue distribution of the rearranged mtDNAs. Of all the deletions, the most often found, and that removes all or part of 7 of the 13 proteins encoded by mtDNA and 5 of the 22 tRNA genes, regardless of phenotype, size 4977 bp and is commonly referred to as “common deletion”, which seems to be flanked by direct repeats of 13 bp [Gadaleta et al. 1992; Lee et al. 1997]. A lethal childhood pancytopenia, known as Pearson marrow pancreas syndrome, is caused by widely disseminated rearrangements that prevent bone marrow stem cells from proliferating. Mitochondrial myopathy with ragged red fibers (RRF), instead, results by widely distributed mtDNA rearrangements that spare the bone marrow. Mitochondrial myopathy is frequently associated with ophthalmoplegia and ptosis, which is referred to as chronic progressive external ophthalmoplegia (CPEO) and, when the case is more severe, with multisystem involvement, it is the Kearns-Sayre syndrome (KSS) [Wallace and Lott, 2002].

3.2.2 Pathogenic mtDNA missense mutations

Missense mutations are substitutions of a single base that give rise to a codon encoding a different amino acid. Missense mutations in mtDNA polipeptide genes can also result in an array of clinical manifestations. For example, a mutation at np 8993 (T>G), in the mtDNA ATP6 gene, is associated with neurogenic muscle weakness, ataxia, and retinitus pigmentosum (NARP), when present at lower percentages of mutant [Holt et al. 1990], and lethal childhood Leigh syndrome when present at higher percentages of mutant [Tatuch et al. 1992]. This mutation causes a marked inability of the ATP synthase to utilize the electrochemical gradient to make ATP [Trounce et al. 1994] with a consequent increase in mitochondrial ROS production [Mattiazzi et al. 2004].

Progressive muscle weakness has been increasingly linked to missense and nonsense mutations in the cytb gene [Andreu et al. 1999; Dumoulin et al. 1996]. Rare nonsense or frameshift mutants in COI have been associated with encephalomyopathies [Bruno et al. 1999; Comi et al 1998].

Missense mutations in complex I genes have been linked to Leigh syndrome [Solano et al. 2003], generalized dystonia and deafness [Jun et al. 1994], and to Leber hereditary optic neuropathy (LHON), a form of midlife, sudden-onset blindness [Brown et al. 2000 and 2001; Wallace et al. 1988]. LHON is a particular disease because the mutations in ND genes produce very different conserved amino acids changes, resulting in varying severities of complex I defects [Brown et al. 2000]. This anomaly has been explained by the discovery that the mildest LHON mtDNA mutations, particularly those in ND6 at np 14484 and ND4L at np 10663, are usually found on a particular mtDNA background, the European mtDNA lineage J [Brown et al. 1997 and 2001]. Lineage J mtDNAs have been found to harbor mtDNA missense mutations that partially uncouple OXPHOS, thus exacerbating the ATP defect of the pathogenic mutations [Ruiz-Pesini et al. 2004].

3.2.3 Pathogenic mtDNA proteins synthesis mutations

Base substitutions in mtDNA protein synthesis genes can also result in multisystem disorders with a wide range of symptoms, including mitochondrial myopathy, cardiomyopathy, dementia, diabetes, intestinal dysmotility, deafness, mood disorders, movement disorders.

The most common mtDNA protein synthesis mutation is at np 3243 (A>G) in the tRNALeu gene [Goto et al. 1990]. This mutation has a wide variability in its clinical manifestations. When present at relatively low levels (10%–30%) in the patient's blood, the patient may manifest only type II diabetes with or without deafness [van den Ouweland et al. 1994]. By contrast, when the A3243G mutation is present in >70% of the mtDNAs, it does not cause diabetes, but instead causes the MELAS syndrome, with more severe symptoms such as short stature, cardiomyopathy, CPEO, and mitochondrial encephalomyopathy, lactic acid and stroke-like episodes [Goto et al. 1990].

Moreover Shoffner et al. (1990) and Wallace et al. (1988) have seen that, in myoclonic epilepsy and ragged red fiber (MERRF) disease, the level of mutant heteroplasmy of the tRNALys np 8344 A to G mutation plus the age of the patient influence the severity of the clinical symptoms.

3.3 A special case: C150T

MtDNA mutations can influence the proper functioning of the cell and the energy state, and result in a variability in the disease incidence.

Mutations localized in the non-coding control region (CR) require a separate argument to justify their presence. In fact, these mutations do not produce defective polypeptides and therefore do not act directly on the respiration affecting the ATP/heat production or the ROS production. Even so the potential importance of these mutations is even higher, since they may act on the regulation of the mtDNA gene expression.

Michikawa et al. (1999) reported that some mutations in the CR, as T414G, T408A, A189G, accumulate with age in specific tissues, and most recently a C150T mutation has been identified in lymphocytes of centenarians and twins [Zhang et al. 2003] (**Figure 5**). The C150T seems to be particularly interesting because it is localized next to one of the main origins of mtDNA H-strand replication (situated in position 151). Zhang et al. (2003) proposed that the mutation conferred some mtDNA replicative advantages, but they could only demonstrate that it shifted the position of the origin from 151 to 149. On the contrary, Fish et al. (2004) have observed that the C150T mutation inactivates the origin close to it, while the mtDNA tries to compensate by enhancing the activity of the origin in position 191. So, a decrease of activity for the origin near 150 could actually justify more easily the association with longevity from the point of view of performances/life span. In this case, less mtDNA could be related to a lower respiratory activity, granting a slightly lower physical performances and a concomitant lower oxidative stress at the cellular level. However, such a speculation has to take into account the recent hypothesis that the "real origin", which generates complete mtDNA chains would be in position 57, while other origins previously known as reserves would have a role in forming the 7S DNA [Fish et al. 2004].

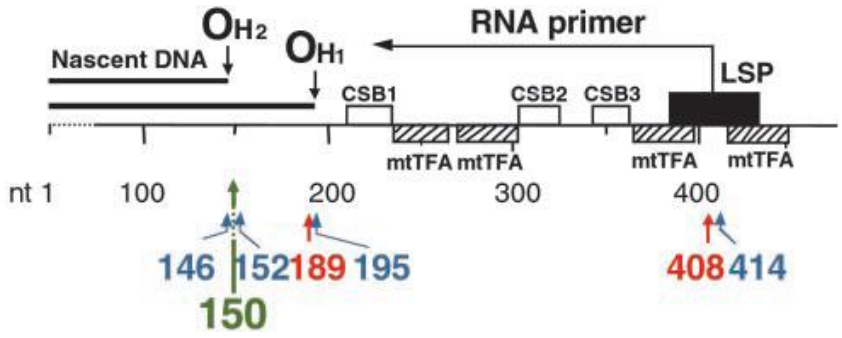


Figure 5: Position of the C150T mutation in the D-loop and other tissue-specific aging-dependent somatic mutations (Source: "*Strikingly higher frequency in centenarians and twins of mtDNA mutation causing remodeling of replication origin in leukocytes*", Zhang et al. 2003)

Zhang et al. (2003) argue that the somatic event in or near the 150 position could lead to a general remodeling of the entire system of replication, probably controlled by the nucleus. Therefore this remodeling could accelerate mtDNA replication, to compensate the oxidative damage of the same DNA and its functional deterioration occurring in old age. The age-dependent accumulation of point mutations with age, previously identified in fibroblasts and skeletal muscle in critical sites for mtDNA replication, can be considered part of the remodeling.

The data also showed that C150T variant causes a remodelling of the replication origin at position 151 and can be either inherited (polymorphism) or somatically acquired (mutation). It also may cause an

activity decreasing. Coskun et al. (2003) suggested that mtDNA haplogroups are likely not neutral and that C150T variant associated with haplogroup J [De Benedictis et al, 1999] might have changed oxidative phosphorylation efficiency (OXPHOS) by reducing the activity. Moreover J haplogroup is associated to the presence of at least four mutations close to replication origins, including C150T [Niemi et al., 2005]. Such observation suggests that functional mutations in the CR may interact with mtDNA haplogroups and indirectly support the traditional model of replication against the new symmetric model, which reduces O_H to a simple point of arrest [Raule et al. 2007].

The result of heteroplasmic mutations depends on several factors, including type and location of the variation, replication rate of the cell, and also chance since the mutant molecules can be randomly lost as a consequence of mitochondria replicative segregation. In any case, since mutations are stochastic events, mtDNA **heteroplasmy** tends to increase with age.

Usually, a low level of heteroplasmy does not impair mitochondrial function, but once the level of mutant mtDNA exceeds a certain threshold, OXPHOS dysfunction may arise [DiMauro et al. 2003 and 2005]. The cell tries to cope with such a stressful condition by increasing OXPHOS, and therefore producing ROS, in a vicious circle that may become lethal to the cell itself.

In general, the age-related accumulation of mtDNA somatic mutations leads to a decline in mitochondrial function, which contributes to aging and degenerative diseases [Linnane et al. 1989; Wallace, 2005]. In fact, most of the literature on mtDNA somatic mutations reports data on the role played by mtDNA heteroplasmy on age-related diseases.

The study by Zhang et al. (2003) is of great value because it indicates a possible beneficial effect on longevity by a mtDNA somatic mutation able to restore the mitochondrial replication machinery. Therefore, it seemed worthwhile to further investigate possible links between mtDNA CR heteroplasmy and longevity. Since longevity shows clear patterns of familiarity [Cournil and Kirkwood, 2001; Hjelmborg et al. 2006], the study of such a heteroplasmy in relatives of centenarians may help to clarify the role of mtDNA somatic variability in longevity. In particular, Rose et al. (2007 and 2010) argue that the patterns of mtDNA CR heteroplasmy do not differ between centenarians and their descendants, but differ between relatives of centenarians and age-matched controls. These results show that mtDNA CR heteroplasmy cannot be accounted for only by to age-related stochastic mutations. What is more, the finding that mtDNA CR heteroplasmy is greater in descendants of centenarians than in age-matched controls suggests a beneficial role of mtDNA heteroplasmy for attaining longevity. In fact, several data show that the offspring of centenarians have a better chance to attain longevity than the general population [Gudmundsson et al. 2000; Atzmon et al. 2005]. Recent findings suggest that at least some aspects of the above theory require reconsideration [Sato et al. 2006]. In fact, a key for explaining the paradox that mtDNA heteroplasmy could

be beneficial for longevity may be the new emerging concept of mitochondria complementation, which suggests that human cells are protected from mitochondrial dysfunction by complementation of mtDNA products in fused mitochondria [Ono et al. 2001]. The beneficial effect of complementation may be enhanced by efficient mtDNA replication, as provided by CR mutations which introduce alternative replication sites. In fact, multiple replication origins falling in D-loop region could play a major role in accelerating mtDNA synthesis to satisfy developmental, physiological, or aging-related demands [Fish et al. 2004]. However, neither the replicative advantage of some variants nor the mitochondrial complementation can explain, by themselves, the heteroplasmy patterns. By contrast, it is likely that the interplay among new replication origins, mitochondrial complementation and nuclear factors might provide an advantage for pursuing longevity by counteracting age-related mitochondrial damages [Rose et al. 2007]. In this frame, the subjects who are genetically predisposed to mtDNA CR heteroplasmy would be clearly favoured in the demographic selection [Perls et al. 2002].

Chapter 4

AIM OF THE STUDY

As discussed in the previous chapters, Down Syndrome (DS) is a genetic disorder in which multiple major aspects of the senescent phenotype occur much earlier than in karyotypically normal age-matched subjects.

In particular, in DS there is an acceleration of aging process of the Central Nervous System, with an early decline in the already reduced cognitive ability similar to that seen in Alzheimer's disease (AD). The trisomy 21 also leads to a dose-dependent increase in the production of the amyloid protein precursor (APP), as the gene for APP is located on chromosome 21 and, consequently, in the production of amyloidogenic fragments causing an early and predominant formation of senile plaques, typical of AD. It is known that oxidative damage and neuroinflammation interact with each other, accelerating the progress of the disease particularly in persons with Down syndrome over 40 years showing that inflammation is a driving force in the neuropathology of AD in subjects with Down syndrome. In addition, some genes and some allelic variants are associated with age-related neurodegeneration and the AD. In particular, APOE ε4 allele is recognized as a risk factor for the AD.

The aim of this study is to analyze an integrated set of molecular genetic parameters (sequencing of the entire mtDNA, heteroplasmy of the control region of mtDNA, haplotypes of APOE), in order to understand whether they are associated to early neurocognitive decline in the DS and consequently if they can be considered risk factors for such a decline. In particular, the study will aim to:

a. sequencing the entire mtDNA (16,569 bp) in all DS and in their mothers (DSM) and brother/sister (DSS) to identify specific mtDNA mutations;
b. quantify the level of heteroplasmy around the position 150 of the D-loop, previously associated with longevity;
c. genotype all subjects DS, mothers and brothers for APOE ε4 allele, known neurocognitive risk factor;
d. correlate genetic data with the cognitive status of these people, that has been carefully assessed by using a battery of tests designed for people with learning disabilities.

Chapter 5

MATERIALS AND METHODS

1. Samples

Taking into account that the mtDNA is inherited only from the mother in both boys and girls, maternal mtDNA becomes a reference and a standard (and therefore the better control) to detect any differences or mutations that have occurred and have accumulated with age in the mtDNA of the son/daughter with DS.

Any collection of samples of brother or sister of the DS subjects (with no DS) represents a further check on the presence of mutations in mtDNA. Whatever the sex of the subject in question is not relevant because only the mother is responsible for inheritance of mtDNA.

For this reason, the study population is represented by the following groups of subjects (141 subjects in total):

- 53 subjects affected by Down Syndrome (DS);
- the mother of each subject DS (DSM), for a total of 47 subjects;
- the siblings of each subject DS (DSS) of comparable age/gender, for a total of 41 subjects.

2. General protocol

All subjects were recruited through the collaboration of Department of Experimental Pathology (University of Bologna), Institute of Pediatric Medical and Surgical Science of S.Orsola-Malpighi Hospital (University of Bologna) and the organization CEPS (Emilian Center Onlus Social Problems for Trisomy 21) of Bologna.

Were excluded subjects with an acute pathology in place; patients with hepatic, renal, or cardiac deficiency; patients with associated mitochondrial disease (extremely rare situation); patients who have taken two months before the recruitment multivitamin or other antioxidants.

For each subject, given informed consent and by using appropriate questionnaires, with a section reserved to the mother and brother, were collected the following data:

- recent and remote medical history, with particular attention to the presence of the diseases most commonly found in the DS and its treatment programs;

- socio-economic history, with particular focus on the social and work integration of DS subject and to the care pathways in the area;
- auxologic parameters, more specifically weight (P), height (H), head circumference (CC), abdominal circumference (AC), body mass index (BMI) and their percentiles;
- neurological exam, neuropsychological and neuropsychiatric evaluation of the cognitive and language in relation to different ages, through the use of ladders and specific tests;
- compilation of the questionnaire DSQIID (Dementia Screening Questionnaire for Individuals with Intellectual Disabilities) to identify predictors of dementia;
- blood sampling (10-12 cc) to assess not only the main biochemical parameters commonly measured in the follow-up of the DS, but also specific biomarkers of antioxidant capacity and levels of oxidative stress and to extract the total DNA, on which three molecular studies were carried out, in particular complete sequencing of mtDNA, D-loop heteroplasmy, APOE genotyping.

3. Purification of DNA

The QIAamp 96 DNA Blood Kit (QIAGEN) was used for DNA extraction from whole blood. The kit combines the selective binding properties of a silica-gel membrane with a high-throughput 96-well format and allows simultaneous processing of up to 192 samples. The procedure is the following (**Figure 1**):

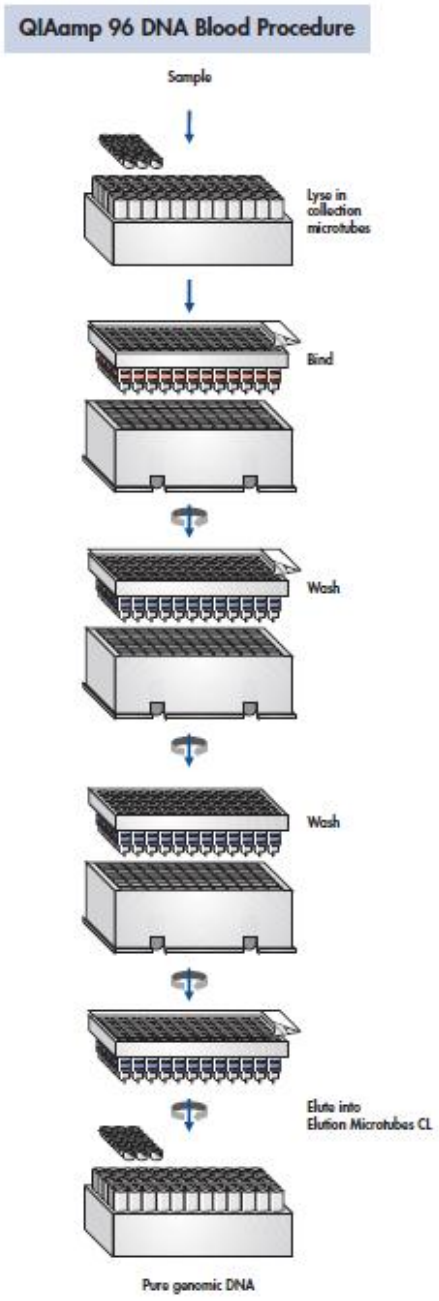


Figure 1: procedure for the purification of DNA

- Pipet 20 µl QIAGEN Protease stock solution into the bottom of the collection microtubes.
- Add 200 µl whole blood to the collection microtubes by touching the insides of the tubes without wetting the rims.
- Add 200 µl Buffer AL to each sample, taking care not to wet the rims of the collection microtubes. Seal the tubes using the caps for collection microtubes.
- Cover the rack with the plastic cover supplied, and mix thoroughly by shaking vigorously for 15 s. For efficient lysis, it is essential that the samples and Buffer AL are mixed immediately and

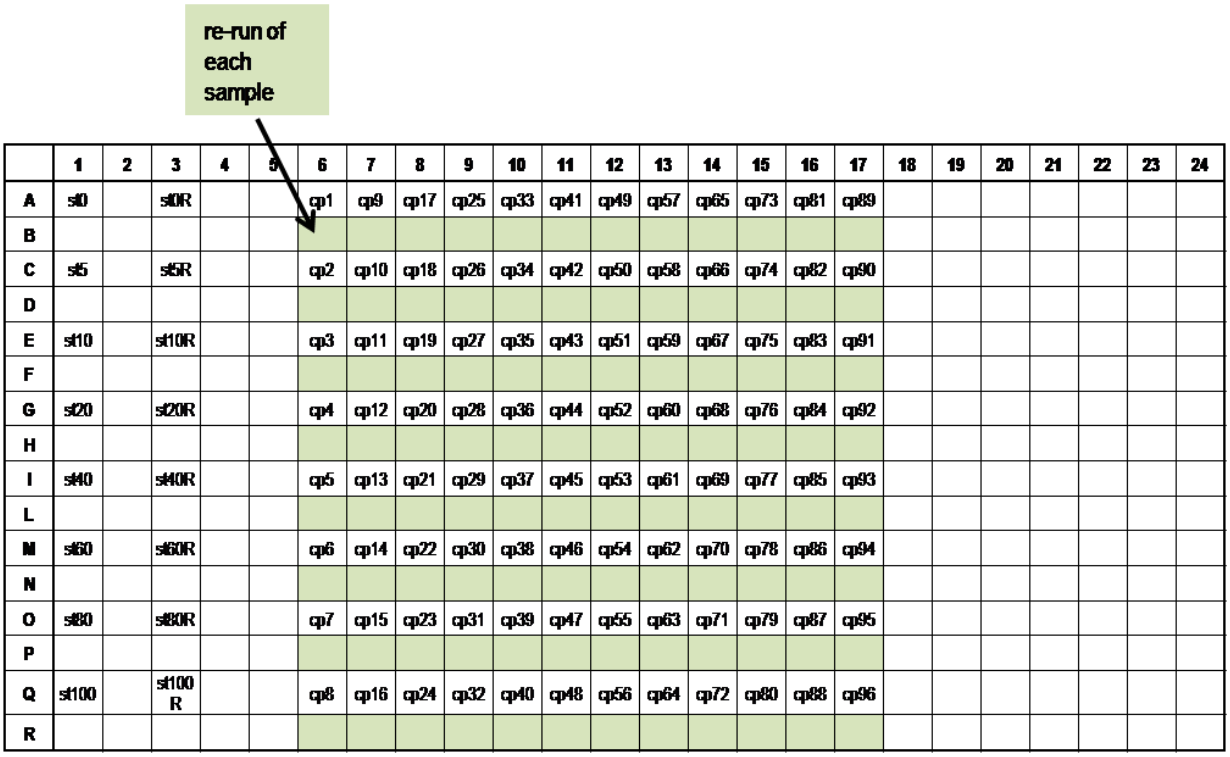
thoroughly to yield a homogeneous solution. Hold the racked collection microtubes with both hands and shake up and down vigorously.

- Centrifuge briefly at 3000 rpm to collect any solution from the caps.
- Incubate at 70°C for at least 10 min in an incubator or oven.
- Centrifuge briefly at 3000 rpm to collect any lysate from the caps
- Remove the caps and add 200 µl ethanol 100% to each tube.
- Seal the tubes using new caps for collection microtubes. Shake vigorously for 15 s.
- Centrifuge briefly at 3000 rpm to collect any solution from the caps
- Place QIAamp 96 plate on top of an S-Block.
- Carefully apply the mixture (620 µl per collection microtube) to the QIAamp 96 plate
- Seal the QIAamp 96 plate with an AirPore Tape sheet. Centrifuge at 4500 rpm for 6 min.
- Remove the tape. Carefully add 500 µl Buffer AW1 to each well.
- Seal the QIAamp 96 plate with a new AirPore Tape sheet. Centrifuge at 4500 rpm for 4 min.
- Remove the tape. Carefully add 500 µl Buffer AW2 to each well.
- Centrifuge at 4500 rpm for 25 min at 40°C. The heat generated during centrifugation ensures evaporation of residual ethanol in the sample (from Buffer AW2) that might otherwise inhibit downstream reactions.
- Place the QIAamp 96 plate on top of a rack of elution microtubes.
- To elute DNA, add 150 µl Buffer AE, equilibrated to room temperature, to each well. Seal the QIAamp 96 plate with a new AirPore tape sheet and incubate for 1 min at room temperature. Centrifuge at 4500 rpm for 7 min.

4. Quantification of DNA

The extracted DNA was quantified with Quant-iT™ dsDNA Broad Range Assay Kit, using the PicoGreen®, an ultra sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution.

Before starting the experiment, it is necessary to prepare a working solution of the Quant-iT™ dsDNA BR reagent by making a 200-fold dilution of the concentrated DMSO solution in TE. For example, to prepare enough working solution to assay 96 samples in a 12 mL final volume, add 60 µL Quant-iT™ dsDNA BR reagent to 12 mL TE. The kit provides pre-diluted DNA standards, which are λ DNA standards with a concentration of 0, 5, 10, 20, 40, 60, 80, and 100 ng/µL. Ninety-six samples are quantified in a 384-well plate, because each sample is quantified in double (**Figure 2**), and into each well are dispensed 48 µL of working solution and 2 µL of each unknown DNA sample.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	st0		st0R			cp1	cp9	cp17	cp25	cp33	cp41	cp49	cp57	cp65	cp73	cp81	cp89							
B																								
C	st5		st5R			cp2	cp10	cp18	cp26	cp34	cp42	cp50	cp58	cp66	cp74	cp82	cp90							
D																								
E	st10		st10R			cp3	cp11	cp19	cp27	cp35	cp43	cp51	cp59	cp67	cp75	cp83	cp91							
F																								
G	st20		st20R			cp4	cp12	cp20	cp28	cp36	cp44	cp52	cp60	cp68	cp76	cp84	cp92							
H																								
I	st40		st40R			cp5	cp13	cp21	cp29	cp37	cp45	cp53	cp61	cp69	cp77	cp85	cp93							
L																								
M	st60		st60R			cp6	cp14	cp22	cp30	cp38	cp46	cp54	cp62	cp70	cp78	cp86	cp94							
N																								
O	st80		st80R			cp7	cp15	cp23	cp31	cp39	cp47	cp55	cp63	cp71	cp79	cp87	cp95							
P																								
Q	st100		st100 R			cp8	cp16	cp24	cp32	cp40	cp48	cp56	cp64	cp72	cp80	cp88	cp96							
R																								

Figure 2: preparation of the 384-well plate for the quantification of DNA

The entire protocol has been automated with the MICROLAB STAR workstation (Hamilton Robotics), equipped with 16 channels and able to arrange all the operations, reducing time and manual errors.

After dispensing standards and samples, there is a 5 minutes of incubation at room temperature and protected from light, then we proceeded to read with fluorimetric λ excitation of 480 nm and λ emission at 520 nm using a fluorometer Synergy™ HT (Bio-Tek Instruments, Winooski, Vermont; software KC-4). The concentration of the samples was extrapolated from the intensity of fluorescence for each sample reported on the standard curve, constructed with the given dilution of standard DNA, after subtraction of white to each reading.

5. Complete sequencing of mtDNA

The general scheme for the complete sequencing of mtDNA is as follows:

- Amplification of mtDNA (16569 bp) with 46 pairs of primers
- Agarose gel electrophoresis
- PCR clean-up reaction using ExoSAP-IT®
- Sequencing reaction
- Sequencing clean-up reaction

- ❖ Sequences analysis with SeqScape v2.5 software (Applied Biosystems) in order to compare each sequence with the Cambridge Reference Sequence and to attribute a subhaplogroup

PCR amplification

Forty-six pairs of primers (See Appendix), which are specific for the mtDNA and possessing at 5' the M13 universal primer useful for the following sequencing reaction, are used for complete mtDNA amplification reaction.

Amplification is performed in a 25 µl volume. Each amplification contains at final concentration:

REAGENTS	[STOCK]	[FINAL]	VOLUME (µl)
Buffer	10X	1X	2,5
$MgCl_2$	50 mM	1,5 mM	0,75
dNTPs	100 mM	250 µM	0,5
Taq	5U/µl	0,06U/µl	0,3
Primers	50 µM	1,25 µM	4
DNA	60 ng/µl	15 ng	0,4
ddH_2O	/	/	16,55
FINAL VOLUME			**25**

The PCR cycling profile (using Biometra T1 Thermocycler) is as follows:

Temperature	Time	Cycle number
95°C	3 min	1
95°C 60°C 68°C	40 sec 40 sec 2 min	30
68°C	5 min	1

Agarose gel electrophoresis

It is very important to verify the amplification on an agarose gel 1.5%. Materials we need are:

- 1.5% agarose gel made up with standard 1X TBE (see Appendix)
- Gel red solution if this has not been pre-incorporated into the gel
- Tracking dye (Bromophenol Blue + glycerol)

In each well pipet 1.2 µl of the PCR reactions and 8.8 µl (6.2 µl H_2O and 2.6 µl BF6X) to reach a volume of 10 µl. Load in a well a marker which contains a mix of known fragments and used to compare our bands. Run the gel at 150 V for half an hour.

PCR clean-up

The clean-up reaction is important because it purify PCR products from primers or nucleotides not incorporated. For PCR clean-up add 2 µl of Exosap-IT® (directly to PCR product) each 5 µl of PCR product (**Figure 3**). We experimented that only 1 µl of ExoSAP each 25 µl PCR product is sufficient and it allows to reduce costs. ExoSAP-IT® (USB, PN 78200) is a mix containing two enzymes: an exonuclease (ExoI) that degrades residual single-stranded primers and any extraneous single-stranded DNA produced by PCR, and a shrimp alkaline phosphates (SAP) that hydrolyzes remaining dNTPs from the PCR mixture which could interfere with the sequencing reaction.

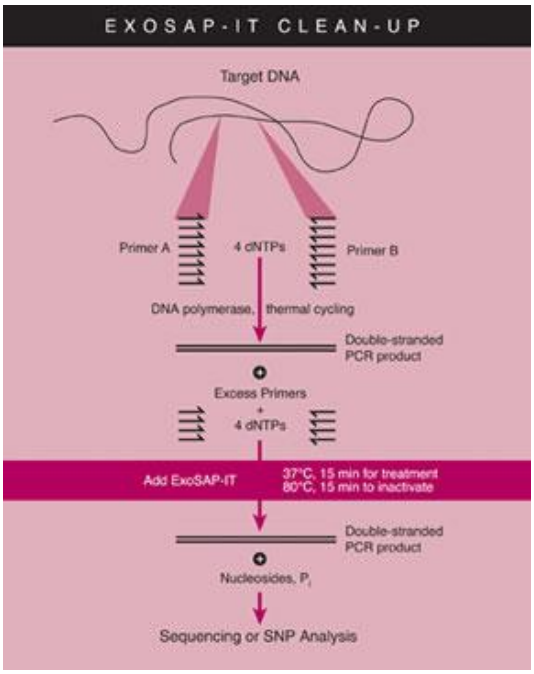


Figure 3: PCR clean-up

The PCR cycling profile (using Biometra T1 Thermocycler) is as follows:

1. activation of enzyme at 37°C for 30 min
2. inactivation at 80°C for 15 min

Sequencing reaction

The laboratory method involves the use of the sequencing kit developed by Applied Biosystems, which includes the BigDye Terminator v.3.1 Ready Reaction Cycle Sequencing Kit, a reaction mixture containing: Taq polymerase, $MgCl_2$, dNTPs and ddNTPs, and universal primers M13 Forward (5'-TGTAAAACGACGGCCAGT-3') or M13 Reverse (5'-CAGGAAACAGCTATGACC-3'), for two parallel amplifications, each using one of the two primers.

The reaction is performed in a 10 µl volume. Each reaction contains at final concentration:

REAGENTS	[STOCK]	[FINAL]	VOLUME (µl)
Buffer 5X (Applied Biosystems)	5X	0.8X	1.6
Big Dye Terminator v.3.1 (Applied Biosystems)	8 µl in a total volume of 20 µl per reaction	1 µl	0.8
Primers M13 Forward or M13 Reverse	100 µM	0.32 µM	1
Cleaned-up PCR product	/	/	2
Water	/	/	4,6
Final volume		**10**	

The thermal cycling profile is as follows:

Temperature	Time	Cycle number
96°C	1 min	1
96°C 50°C 60°C	10 sec 5 sec 4 min	25

The BigDye Terminator reagent contains a mix of nucleotides. Free bases that match the template sequence can attach to the new strand's growing (3') end, but among all the free bases swimming in the solution, there are a few having a chemical fluorescent part, i.e. dye. When the coloured bases attach to the growing strand, the extra chemical part keeps the new DNA strand from growing any further. A different coloured dye is attached to each of the four kinds of bases.

A completed sequencing reaction contains an array of coloured DNA fragments. The shortest are the length of the primer plus one coloured base. The longest fragments are usually between 500 and 800 bases long, which is when the sequencing reaction runs out of steam.

Sequencing clean-up reaction

To perform the sequencing clean-up reaction, it is important to add in each well of the sequencing 96-plate the following reagents:

- 2 µl of sodium acetate (NaAc) (See Appendix);
- 50 µl Ethanol 100% stored at –20°C, in order to precipitate sequenced fragments;
- Incubate at room temperature for 10 min;
- Spin the plate at 1100 xg for 40 min;
- Remove cover and invert onto paper towel and spin at 100xg for no more than 1 min
- 70 µl Ethanol 70% stored at –20°C
- Spin at 1100 xg for 14 min;

- Remove cover and invert onto paper towel and spin at 100xg for no more than 1 min;
- Store the plate at 37°C for at least 1h in order to evaporate all the ethanol;
- Resuspend all wells with 10µl Hi Di Formamide (denaturing agent which allows to maintain a denaturing state for capillary elechtrophoresis);
- Denaturing at 95°C for 2 min.

5.1 Analysis at ABI3730 sequencer

The sample are then loaded on the automatic sequencer (ABI3730, 48 capillaries). Inside the sequencer the separation of the fragments depends on their molecular weight, process called *capillary electrophoresis*. The sequencing machine sets up an electric field; all the DNA moves down through a porous gel toward the positive charge. Shorter fragments of DNA move more quickly through the matrix of the gel than larger fragments do (**Figure 4**).

In the sequencing machine, a laser excites the fluorescent dyes, and a camera detects the lights that the excited dyes emit. One by one, the sequencing machine reads the DNA molecules passing down the gel, and sends the information to a computer. Each nucleotide have a different emission spectrum allowing to identify different bases.

A computer program integrates the information from individual sequencing reactions. It spots where fragments overlap, to puzzle the pieces back together.

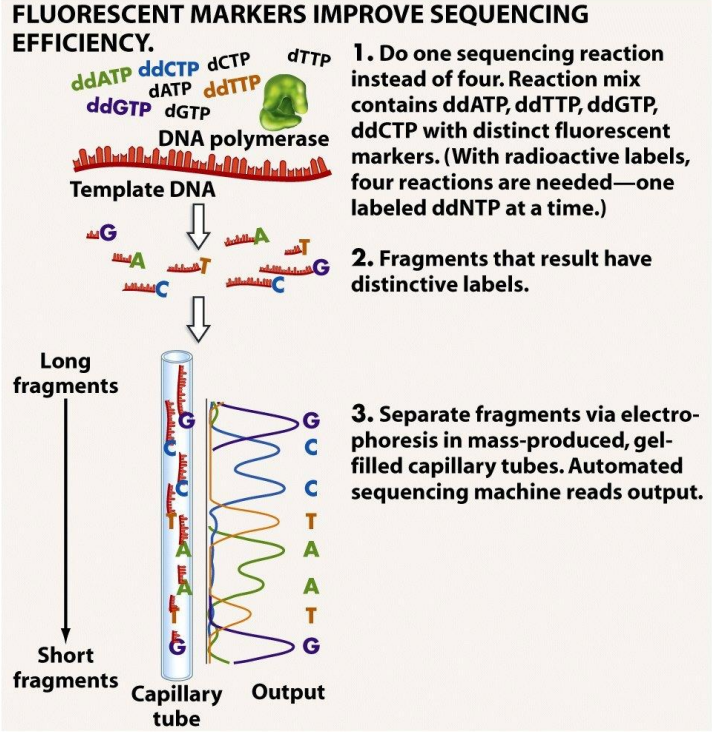


Figure 4: principle of sequencing function. The sequencing fragments obtained pass through a laser, while a system provides to record the energy emitted by the excited fluorescent molecule.

Many overlapping sequencing reads are needed to reveal the uninterrupted sequence of the original stretch of DNA. Some stretches of DNA are easier to read and need to be sequenced a little less often to get high-quality sequence. Some stretches need to be analyzed more exhaustively to get finished high-quality sequence.

The sequencer gives data in electropherogram form, through a file (.ab1) that shows four different peaks (green for adenine, red for timine, blue for cytosine and black for guanosine) and the height of each peak depends on the detected intensity.

We can read files .ab1 with the software SeqScape v2.5 (Applied Biosystems) which allows the alignment, the assembling of the overlapping fragments and a comparison with the Cambridge Reference Sequence (**Figure 5**).

Figure 5: Image of an analysis with Seqscape. The Cambridge reference sequence is in brown, coloured peak represent all the four nitrogenous bases and the green bars indicates the quality of the peak relative to the background noise.

5.2 Automated procedure

We have automated the amplification reaction with the MICROLAB STAR workstation, equipped with 16 channels and able to arrange all the operations, reducing time and manual errors. The platform can prepare reactions for 4 different samples, can distribute the reagent mix (manually prepared) in each well and the 46 different primers stored in plates. (**Figure 6**).

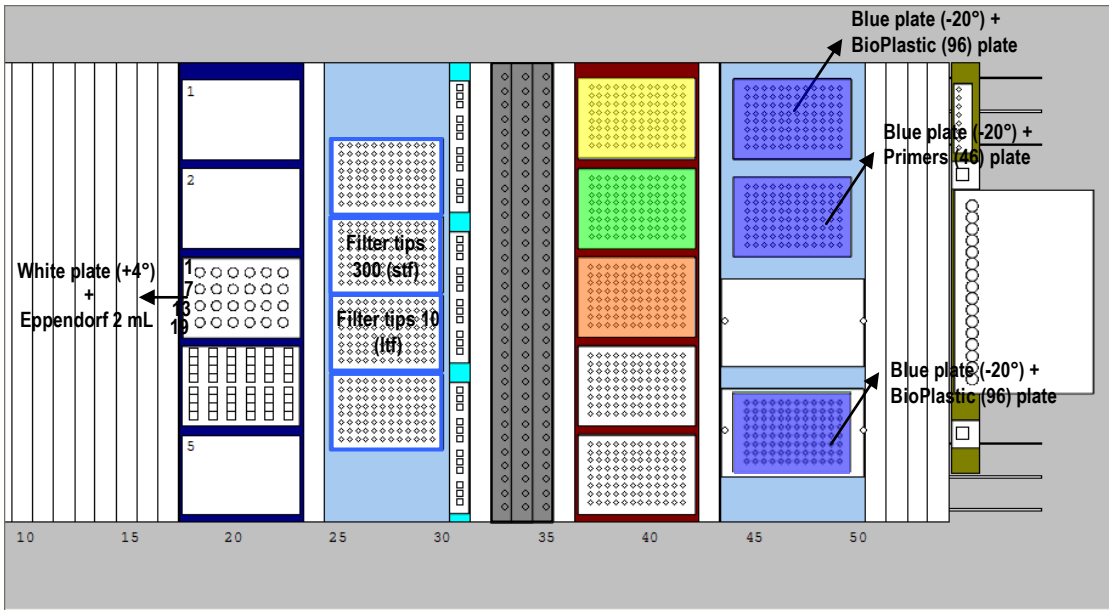


Figure 6: robotic platform and the localization of all components and reagents.

6. Assignment of subhaplogroups

The complete sequencing of mtDNA allowed us to assign to each sample its haplogroup, following Phylotree (http://www.phylotree.org/) [van Oven and Kayser, 2009], a phylogenetic tree of global human mtDNA variation (**Figure 7**), based on both coding and control region mutations, and including the most updated haplogroup nomenclature. This mtDNA tree is meant as a framework for evolutionary anthropologists, medical geneticists, genealogists and forensic geneticists. The tree is updated at least every six months with data related to new published complete mtDNA sequences. This information about haplogroup classification allows us to confirm the kinship between DS, DSM and DSS.

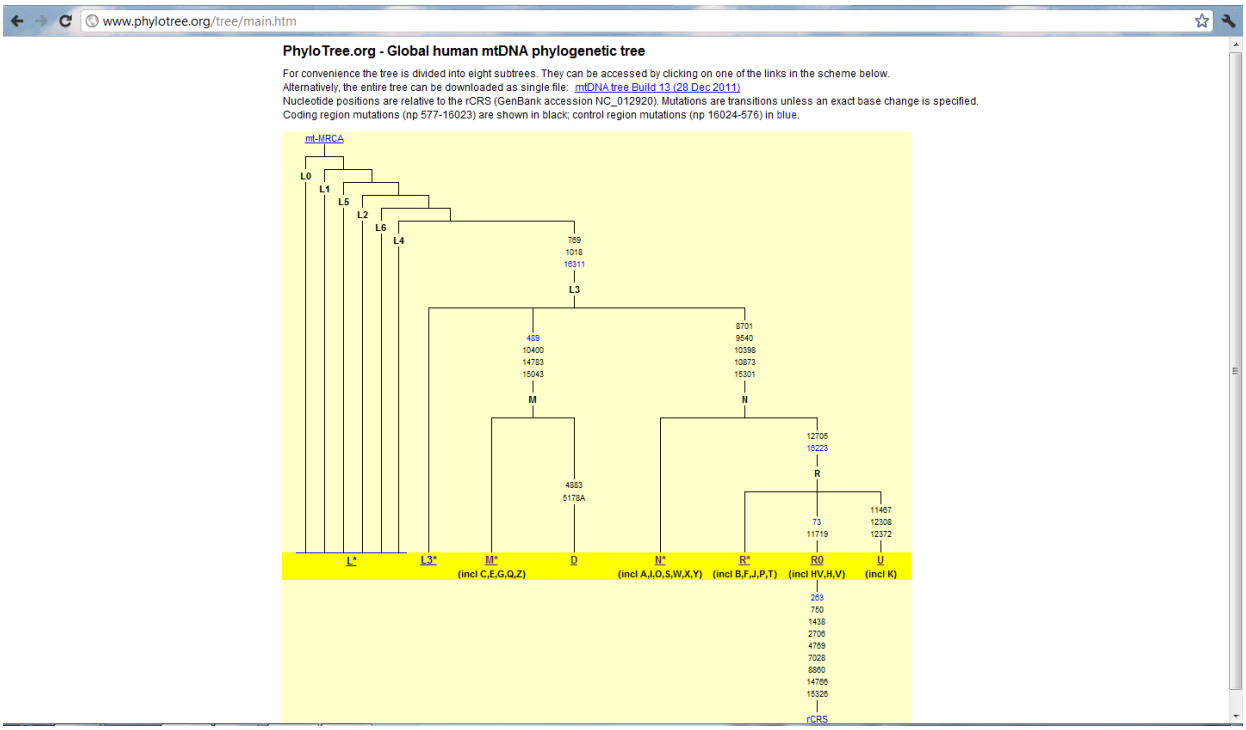


Figure 7: phylogenetic tree of the Phylotree reference database

7. Complete sequencing of mtDNA by Long-PCR

PCR amplification

The protocol for the complete sequencing of mtDNA was implemented from another protocol, which included the use of a new taq, the Expand Long Range Taq (Roche), and of only four primers (2 forward primers and 2 reverse primers) for the amplification of entire mtDNA. In fact this taq is optimized to efficiently amplify large genomic DNA fragment from 5 kb to 25 kb in combination with a threefold higher fidelity than Taq DNA polymerase.

Amplification was performed in a 25 µl volume, using the following pairs of primers:

- LONG FW1 (5'-ACATAGCACATTACAGTCAAATCCCTTCTCGTCCC-3') and LONG RV1 (5'-ATTGCTAGGGTGGCGCTTCCAATTAGGTGC-3');
- LONG FW2 (5'-TCATTTTTATTGCCACAACTAACCTCCTCGGACTC-3') and LONG RV2 (5'-CGTGATGTCTTATTTAAGGGGAACGTGTGGGCTAT-3').

Each amplification contains at final concentration:

REAGENTS	[STOCK]	[FINAL]	VOLUME (µl)
Buffer with $MgCl_2$	5X	1X	5
dNTPs mix	10 mM/each	500 µM	1,25
Primers	10 µM	0,3 µM	0,75
DNA	30 ng/µl	300 ng	3
Taq	5U/µl	3,5U/µl	0,35
ddH_2O	/	/	13,9
FINAL VOLUME			**25**

The touchdown PCR cycling profile (using Biometra T1 Thermocycler) is as follows:

Temperature	Time	Cycle number
92°C	2 min	1
92°C 60°C 68°C	10 sec 30 sec 13 min	9
92°C 60°C 68°C	10 sec 30 sec 13 min (with an increment of 10 sec per cycle)	19
68°C	7 min	1

Agarose gel electrophoresis

Amplified products were controlled by 0.8% agarose gel electrophoresis in TBE buffer with gel red staining, using 4 µl of PCR product, 2 µl of BF6X and 1 µl of water.

PCR clean-up

The clean-up reaction is carried out with 2 µl of ExoSAP-IT®, and the PCR cycling profile provides for an activation cycle of enzyme at 37°C for 30 min and an inactivation cycle at 80°C for 15 min.

Sequencing reaction

Also in this case, the laboratory method involves the use of the sequencing kit developed by Applied Biosystems, which includes the BigDye Terminator v.3.1 Ready Reaction Cycle Sequencing Kit. Fifty-five

primers are used, most of which are taken from the amplification primers for complete mtDNA, while fourteen have been redesigned, using the tool http://www.genelink.com/tools/gl-tmres.asp to uniform the T annealing, to be more specific (see Appendix).

8. Analysis of heteroplasmy

We analyzed mtDNA CR heteroplasmy in a 300 bp stretch surrounding the 150 position in total DNA from whole blood of all the subjects recruited. The studied region in the mtDNA (nt 16531-261) was amplified by 5'-AATAGCCCACACGTTCCCCTTA-3' forward primer and 5'-GCTGTGCAGACATTCAATTG-3' reverse primer in a final volume of 30 µl. Each amplification contains at final concentration:

REAGENTS	[STOCK]	[FINAL]	VOLUME (µl)
Buffer	10X	1X	3
$MgSO_4$	50 mM	1,75 mM	2,1
dNTPs	10 mM	0,2 mM	0,6/each
Taq (Transgenomic)	5U/µl	2,5 U/µl	0,3
Primers	10 µM	0,3 µM	0,9/each
DNA	60 ng/µl	80-100 ng	5
ddH_2O	/	/	15,4
FINAL VOLUME			**30**

The touchdown PCR cycling profile (using Biometra T1 Thermocycler) is as follows:

Temperature	Time	Cycle number
95°C	2 min	1
95°C 61,6°C (with a decrement of 0.5°C per cycle) 72°C	30 sec 30 sec 30 sec	14
95°C 54,6°C 72°C	30 sec 30 sec 30 sec	19
72°C	5 min	1

Then the PCR products were controlled by 2% agarose gel electrophoresis in TBE buffer with gel red staining.

All the PCR products were denatured at 95°C for 5 min, followed by a reannealing from 95° to 65° with a rate of 1°C/min inducing heteroduplex formations, and then submitted to DHPLC (Denaturing High Performance Liquid Chromatography). The DHPLC is a method of separation on column by using an acetonitril gradient in conditions of increasing temperature, partially denaturing, corresponding to the

melting temperature of the fragment amplified by PCR. At an optimal temperature, that partially denatures the DNA fragments, more than one peak is present on the chromatogram: one peak is for heteroduplex and one for homoduplex because they have different retention times due to less helical fraction in heteroduplexes (**Figure 8**).

We set up a DHPLC protocol in order to quantify the heteroplasmy. We used the WAVE 3500HT machine with 6.5 x 37 mm DNASep Cartridge (Transgenomic), a mutation detection method with an elution rate of 0,9 mL/min in the column (normal course) with a melting temperature of 60°C, a time shift of 2 minutes. We have set other parameters for the analysis: start time of 2-8, detection threshold 0.01, group separation 0.01, noise factor 2.0. Mismatches were recognised by the appearance of two or more peaks in the elution profile.

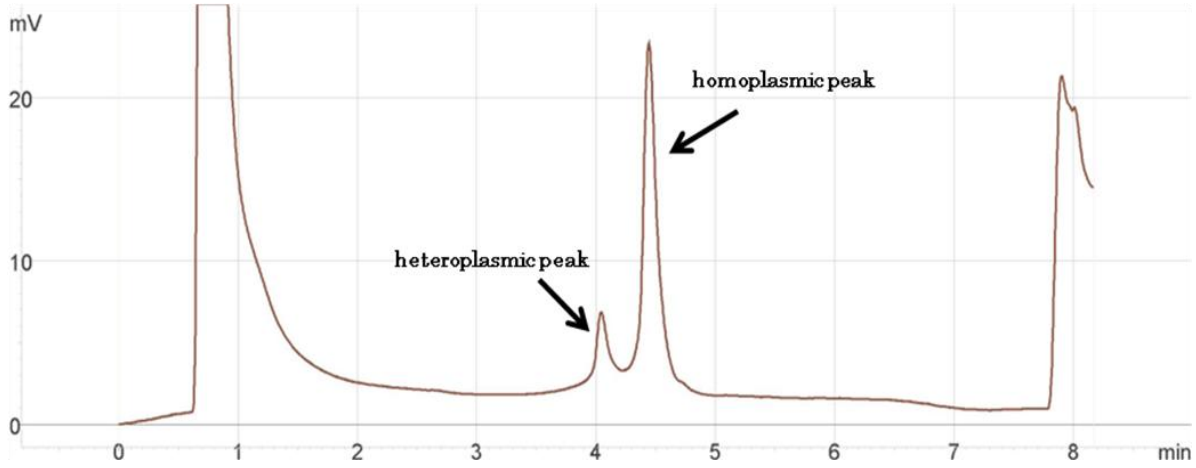


Figure 8: example of chromatogram

8.1 Quantitative analysis

To ensure clean results and homospecies peaks it is necessary to use a DHPLC optimized enzyme, the Optimase DNA polymerase (Transgenomic). This enzyme has the advantage that it does not need additives, which if not eliminated, would cause early column abrasion. Therefore, this Taq should be used to avoid false-positive shoulder peaks in front of the homoduplex peaks. Meierhofer et al (2005) has demonstrated that Optimase DNA polymerase is necessary to produce clear peaks with high amplitude, while other enzymes they tested showed a spectrum of pre-peaks leading to difficultied in detection of low level heteroplasmies.

The phase of chromatogram analysis becomes even more complex in the analysis of mitochondrial DNA because it is present in multiple copies within the same cell and in the same mitochondrion, therefore a mutation in the mtDNA does not lead to the formation of 50:50 ratio of heteroduplex and homoduplex, as it occurs in the genomic DNA. In fact, mutant mtDNA varieties may be present at undetectable low concentrations, and the degree of heteroplasmy may be also tissue-specific. Initially we used two different

methods: the first was the calculation of the integral of heteroplasmic peak areas, by obtaining the equation of a line. The second method was the calculation of the height of heteroplasmic/homoplasmic peaks, obtaining a parabolic relationship [Rose et al, 2007]. In order to built a reference curve, used for estimating the levels of heteroplasmy in our biological samples, plasmids containing the common C150 and the mutant 150T sequences were mixed in different proportions to generate heteroplasmy levels of 0%, 5%, 10%, 20%, 30%, 40%, 50%. Reporting our results (areas or heights) on the reference curve, both of the two methods used seemed not to fit specifically our data.

Finally, the level of heteroplasmy, that is the percentage of the less frequent allele in the heteroplasmic mixture, was calculated as described by Lim et al. (2007). They postulated this universal equation: $\mathbf{y=(A_{HET}/(A_{HET} + A_{HOM}))*100}$, which fits better our data.

It is necessary that the sum of the two heights (homoplasmic and heteroplasmic) is >=20 mV to obtain a sensibility of almost 5%.

9. APOE genotyping

APOE genotyping was performed as previously described [Hixson and Vernier, 1990]. The protocol provides for the PCR amplification of genomic DNA sequence containing APOE gene. The reaction was performed using the following primers: F4 (5'-ACAGAATTCGCCCCGGCCTGGTACACAC-3') and F6 (5'-TAAGCTTGGCACGGCTGTCCAAGGA-3') described by Emi et al. (1988) in a final volume of 30 µl. Each amplification contains at final concentration:

REAGENTS	[STOCK]	[FINAL]	VOLUME (µl)
Buffer	10X	1X	3
$MgCl_2$	50 mM	1,5 mM	0,9
dNTPs	2,5 mM	0,25 mM	3
Primers	10 µM	0,3 µM	1/each
DMSO	100%	10%	3
DNA	60 ng/µl	200 ng	1
Taq	5U/µl	1,5 U/µl	0,3
ddH_2O	/	/	16,8
FINAL VOLUME			**30**

The PCR cycling profile (using Biometra T1 Thermocycler) is as follows:

Temperature	Time	Cycle number
95°C	5 min	1
95°C 63°C 70°C	1 min 1 min 2 min	45
70°C	12 min	1

Amplified products were controlled by 1.5% agarose gel electrophoresis in TBE buffer with gel red staining, using 3 µl of PCR product, 2 µl of XC6X and 5 µl of water. Then amplified products were digested overnight at 37°C with 3.5 U of the restriction enzyme HhaI and visualized via ethidium bromide staining on 4.5% agarose Molecular Screening gel.

The nucleotide substitution, which leads to Arg or Cys at positions 112 and 158 of the APOE gene alters the cleavage site of HhaI: the enzyme acts at GCGC sequences, present at codon Arg residue in position 112 (ε4 allele) and in position 158 (ε3 and ε4 alleles), but is not active on GTGC sequences encoding Cys residues in position 112 in ε3 allele and in positions 112 and 158 in ε2 allele.

The cut to the polymorphic site allows you to assign each sample one of the following genotypes: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4 (**Figure 9**). Except for the band present at 35 bp, which is common to all genotypes, each genotype has a unique combination of HhaI fragments:

- ε2/ε2 contains fragments of 91bp and 81bp, in agreement with the absence of the restriction site for the presence of Cys at position 112 and 158;
- ε3/ε3 contains the fragment of 91bp and 48bp, resulting from cutting at Arg 158;
- ε4/ε4 contains the fragment of 48bp and 72bp, resulting from cutting at Arg 112.

The heterozygous samples include both sets of fragments from each allele [Appel et al. 1995].

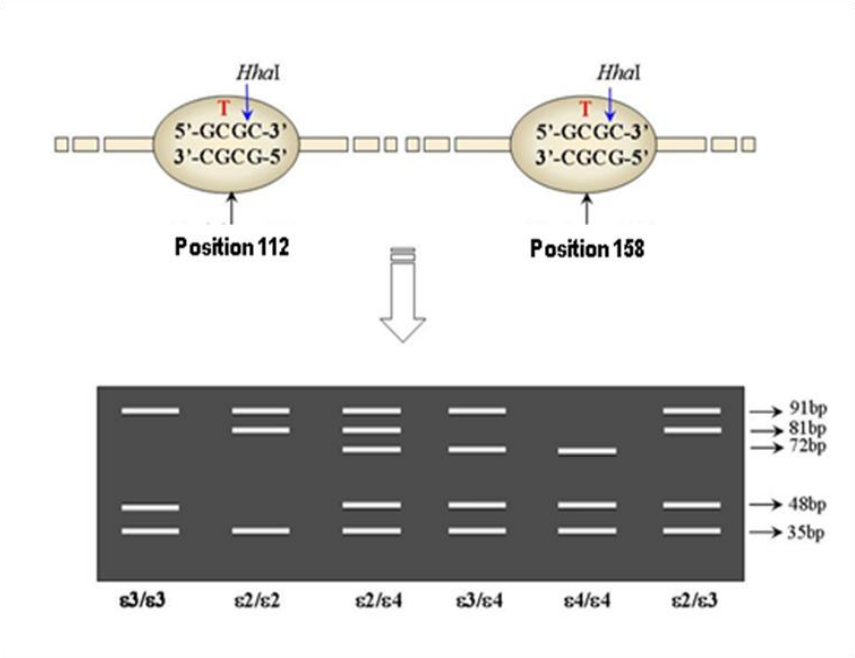


Figure 9: Diagram of the possible genotypes for the polymorphisms of the APOE gene.

10. Statistical analysis

The analysis of the sequences to determine haplogroups and somatic mtDNA variability was carried out by SeqScape software v2.5 (Applied Biosystem).

Mitochondrial sub-haplogroups and genotype frequencies were compared between DS, DSM and DSS subjects using the χ^2 or Fisher-exact test.

The mitochondrial subhaplogroups variability envisages at least 40 subhaplogroups, which have been further grouped into 6 macro-groups considering their frequencies and phylogenetic relationship. The comparison between each of the 6 mtDNA macro-haplogroups in DS, DSM and DSS subjects have been computed by applying Pearson's Chi-squared test with Yates' continuity correction.

Tests for statistical significance were two-sided with = 0.05; effect size for the association was measured as odds ratio (OR) with 95% confidence intervals (CI).

SPSS v.20 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Chapter 6

RESULTS

1. Samples

In this study we analyzed genetic factors that might influence the neurocognitive decline observed in DS. In particular mtDNA has been analyzed, from both a qualitative and a quantitative point of view; the most commonly risk factor for AD, i.e. the APOE ε4 allele, it was also investigated.

The database consists of 141 subjects distributed as follows:

STATUS	N
Down Syndrome (DS)	53
Mothers (DSM)	47
Siblings (DSS)	41

These subjects are distributed this way:

- 37 families in which are present DS, DSM and DSS;
- 10 families where only the DS subject and his/her mother have been recruited;
- 4 families where only the DS subject and his/her brother/sister have been recruited;
- 2 DS subjects, without mother and brother/sister.

Table 1 shows males/females proportion: in our total sample, there is a higher proportion of female (72%) subjects, but at least the DS females equal the DSS females; the reason is that in the database there are only DS patients and their sibling's mothers.

	Males (N=39)			Females (N=102)		
	DS (%)	DSM (%)	DSS (%)	DS (%)	DSM (%)	DSS (%)
N	26 (66.6)	0 (0)	13 (33.4)	27 (26.5)	47 (46)	28 (27.5)
Mean-range	29.6 ± 9.9	0	28.4 ± 9.6	27.0 ± 14.9	56.9 ± 9.5	32.1 ± 14.4
Age-range	16-61	0	13-46	10-70	41-83	12-70

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

The following **Figure 1** reports the total number of males and females, while **Figure 2** represents the age distribution boxplot of participants by status (DS, DSM or DSS).

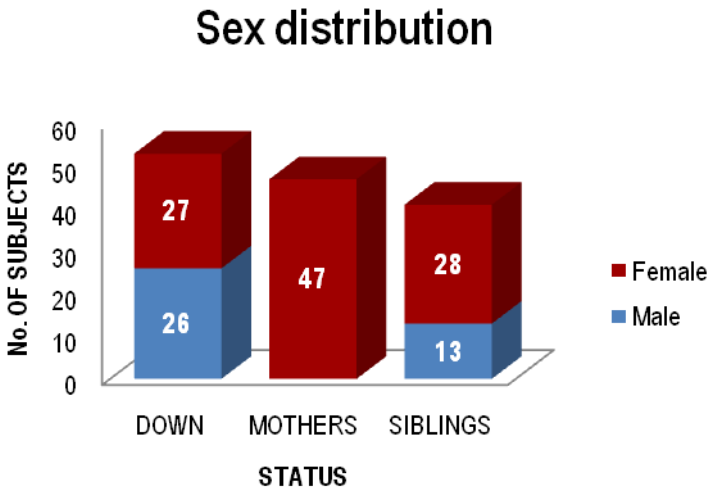


Figure 1: Graphic distribution of DS, DSM and DSS.

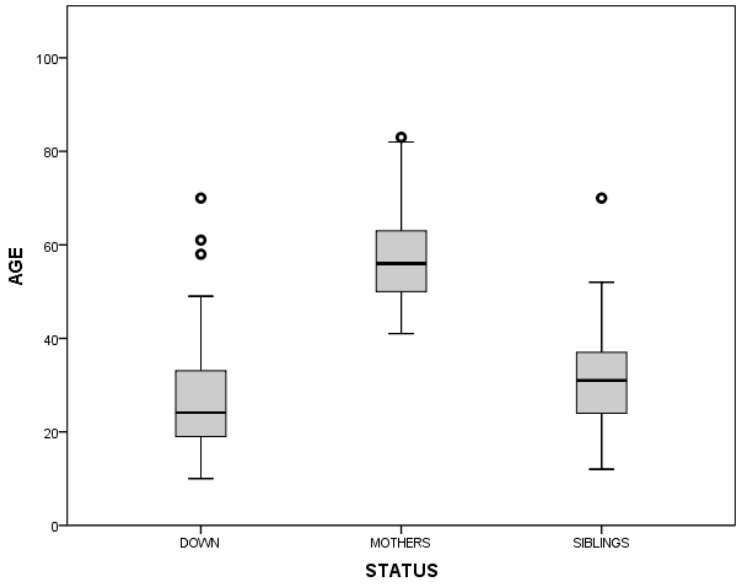


Figure 2: Age distribution boxplot of participants.

2. Distribution of haplogroups

We obtained the complete mtDNA sequences of the subjects recruited using a protocol that provides amplification of entire mtDNA, PCR clean-up reaction using ExoSAP-IT®, sequencing reaction and finally sequences analysis with SeqScape v2.5 software (Applied Biosystems).

The complete mtDNA resequencing allowed us to classify our samples from DS, DSM and DSS into more than 40 haplogroups/sub-haplogroups (see Appendix). Most of these are typical of modern European populations, but a sub-Saharan African (L2a1) mtDNA was also detected. This latter finding is not unexpected, since low frequencies of African haplogroups are not uncommon in populations of southern Europe. However, due to the small number of samples, we have grouped all sequences into macro-haplogroups (**Table 2** and **Figure 3**).

	DS (N=53)			DSM (N=47)			DSS (N=41)		
Macro-haplogroups	**N**	**%**	**SE**	**N**	**%**	**SE**	**N**	**%**	**SE**
HV (1)*	29	54.72	0.0684	27	57.45	0.0721	23	56.09	0.0775
J	2	3.77	0.0262	1	2.13	0.0211	1	2.44	0.0241
K	3	5.66	0.0317	2	4.25	0.0294	2	4.88	0.0336
T	6	11.32	0.0435	5	10.64	0.0450	3	7.32	0.0407
U	12	22.64	0.0575	11	23.40	0.0618	11	26.83	0.0692
Other (2)	1	1.89	0.0187	1	2.13	0.0211	1	2.44	0.0241

Table 2: list of macro-haplogroups with the related frequencies and Standard Error (SE). (1) HV* includes all mtDNAs belonging to haplogroups HV, H and V, (2) Other includes L2a1.

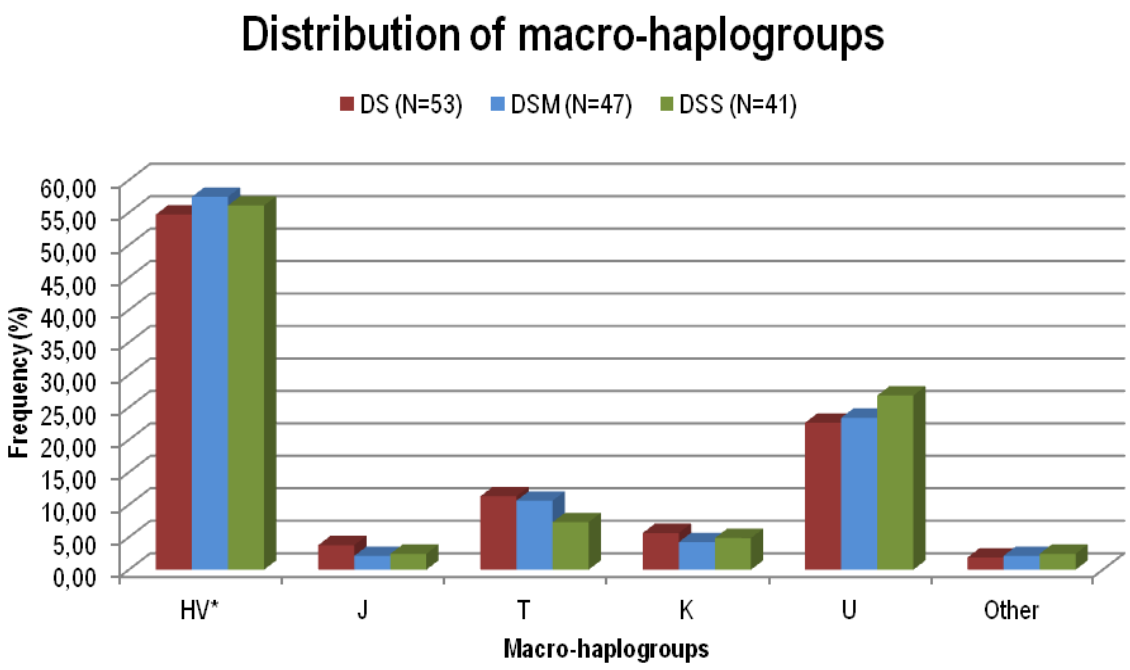


Figure 3: Distribution of macro-haplogroups

The distribution of haplogroups allowed us to confirm the relationship of subjects recruited and to compare our frequencies with those previously reported in literature (**Table 3**) [Richards et al. 2000]; we verified that our results correspond with a good approximation to the reference values referred to populations, with fluctuations attributable at smallness of our database.

Haplogroup	DS	DSM	DSS	Reference (*)
HV	54.72%	57.45%	56.10%	50.4-54.1%
H	43.40%	44.68%	41.46%	44.5-48.2%
V	1.89%	2.13%	2.44%	3.9-5.4%
J	3.77%	2.13%	2.44%	8.3-10.4%
T	11.32%	10.64%	7.32%	7.2-9.2%
U	22.64%	23.40%	26.83%	20.1-23.2%
K	5.66%	4.26%	4.88%	4.9-6.6%
Other (1)	1.89%	2.13%	2.44%	1.4-2.4%

(*) Richards et al. 2000

Table 3: The table shows the haplogrops' frequencies compared with those reported by Richards et al. 2000. (1) Other includes L2a1.

Some differences are present, but none of them reach statistical significance, due to the low number of subjects of our experimental group.

3. Analysis of mtDNA mutations

In order to identify whether the neurocognitive decline of DS subjects could be attributed to specific mutations of mtDNA, the complete mtDNA sequences of 51 DS were analyzed and compared with those of 46 DSM and 41 DSS subjects (**Table 4**). A total of 406 mutated positions were detected in comparison with the Reference Cambridge Sequence and analyzed. Of these, 47 (underlined and colored) were not previously reported in either MITOMAP (www.mitomap.org) or mtDB (www.genpat.uu.se/mtDB), and 19 of them (in bold) are not synonymous and cause an aminoacid change. Moreover, 11 SNPs have an heteroplasmy superior to 25%.

Nucleotide Position[1]	Locus	Nucleotide Change	Aminoacid Change[2]	DS N=51 (%)	DSM N=46 (%)	DSS N=41 (%)
41	D-loop (HVSII)	C>T	non coding	1 (2)	1 (2,2)	0 (0)
53	D-loop (HVSII)	G>A	non coding	1 (2)	1 (2,2)	0 (0)
55	D-loop (HVSII)	T>C	non coding	1 (2)	1 (2,2)	0 (0)
72	D-loop (HVSII)	T>G	non coding	4 (7,8)	4 (8,7)	4 (9,8)
73	D-loop (HVSII)	A>G	non coding	25 (49)	21 (45,7)	19 (46,3)
93	D-loop (HVSII)	A>G	non coding	2 (3,9)	2 (4,3)	1 (2,4)
143	D-loop (HVSII)	G>A	non coding	1 (2)	1 (2,2)	1 (2,4)
146	D-loop (HVSII)	T>C	non coding	6 (11,8)	4 (8,7)	5 (12,2)
150	D-loop (HVSII)	C>T	non coding	7 (13,7)	7 (15,2)	4 (9,8)
151	D-loop (HVSII)	C>T	non coding	5 (9,8)	2 (4,3)	2 (4,9)
152et	D-loop (HVSII)	T+C	non coding	1 (2)	0 (0)	0 (0)
152	D-loop (HVSII)	T>C	non coding	8 (15,7)	11 (23,9)	9 (22)
185	D-loop (HVSII)	G>T	non coding	1 (2)	1 (2,2)	1 (2,4)
189	D-loop (HVSII)	A>G	non coding	2 (3,9)	2 (4,3)	2 (4,9)
195	D-loop (HVSII)	T>C	non coding	11 (21,6)	10 (21,7)	9 (22)
199	D-loop (HVSII)	C>T	non coding	1 (2)	0 (0)	1 (2,4)
204	D-loop (HVSII)	T>C	non coding	1 (2)	1 (2,2)	1 (2,4)
214	D-loop (HVSII)	A>G	non coding	1 (2)	1 (2,2)	1 (2,4)
236	D-loop (HVSII)	T>C	non coding	1 (2)	1 (2,2)	1 (2,4)
239	D-loop (HVSII)	T>C	non coding	2 (3,9)	2 (4,3)	0 (0)
242	D-loop (HVSII)	C>T	non coding	1 (2)	0 (0)	1 (2,4)
249delA	D-loop (HVSII)	del	non coding	1 (2)	1 (2,2)	0 (0)
252	D-loop (HVSII)	T>C	non coding	2 (3,9)	2 (4,3)	2 (4,9)
253	D-loop (HVSII)	C>T	non coding	0 (0)	1 (2,2)	0 (0)
263	D-loop (HVSII)	A>G	non coding	50 (98)	43 (93,5)	40 (97,6)
279	D-loop (HVSII)	T>C	non coding	1 (2)	1 (2,2)	1 (2,4)
295	D-loop (HVSII)	C>T	non coding	2 (3,9)	1 (2,2)	1 (2,4)
296	D-loop (HVSII)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
310	D-loop (HVSII)	T>C	non coding	0 (0)	0 (0)	1 (2,4)
324	D-loop (HVSII)	C>G	non coding	0 (0)	1 (2,2)	0 (0)
456	D-loop (HVSII)	C>T	non coding	3 (5,9)	1 (2,2)	3 (7,3)
462	D-loop (HVSII)	C>T	non coding	1 (2)	0 (0)	1 (2,4)
477	D-loop (HVSII)	T>C	non coding	2 (3,9)	2 (4,3)	1 (2,4)
489	D-loop (HVSII)	T>C	non coding	2 (3,9)	1 (2,2)	1 (2,4)
497	D-loop (HVSII)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
499	D-loop (HVSII)	G>A	non coding	3 (5,9)	3 (6,5)	3 (7,3)
523/524delAC	D-loop (HVSII)	del	non coding	5 (9,8)	6 (13)	6 (14,6)
524+AC	D-loop (HVSII)	ins	non coding	1 (2)	1 (2,2)	2 (4,9)
573+C	D-loop (HVSII)	ins	non coding	1 (2)	1 (2,2)	0 (0)
573+CCC	D-loop (HVSII)	ins	non coding	1 (2)	1 (2,2)	1 (2,4)
709	rRNA 12S	G>A	-	8 (15,7)	5 (10,9)	4 (9,8)
742	rRNA 12S	T>C	-	1 (2)	1 (2,2)	1 (2,4)

750	rRNA 12S	A>G	-	49 (96,1)	44 (95,7)	41 (100)
769	rRNA 12S	G>A	-	1 (2)	1 (2,2)	1 (2,4)
801	rRNA 12S	A>G	-	1 (2)	0 (0)	1 (2,4)
930	rRNA 12S	G>A	-	3 (5,9)	2 (4,3)	1 (2,4)
960delC	rRNA 12S	del	-	1 (2)	0 (0)	1 (2,4)
1018	rRNA 12S	G>A	-	1 (2)	1 (2,2)	1 (2,4)
1075	rRNA 12S	A>G	-	0 (0)	1 (2,2)	0 (0)
1189	rRNA 12S	T>C	-	1 (2)	0 (0)	0 (0)
1438	rRNA 12S	A>G	-	48 (94,1)	45 (97,8)	40 (97,6)
1700	rRNA 16S	T>C	-	2 (3,9)	1 (2,2)	2 (4,9)
1719	rRNA 16S	G>A	-	2 (3,9)	1 (2,2)	1 (2,4)
1721	rRNA 16S	C>T	-	2 (3,9)	2 (4,3)	2 (4,9)
1811	rRNA 16S	A>G	-	6 (11,8)	5 (10,9)	6 (14,6)
1888	rRNA 16S	G>A	-	6 (11,8)	5 (10,9)	3 (7,3)
2083	rRNA 16S	T>C	-	0 (0)	1 (2,2)	1 (2,4)
2158	rRNA 16S	T>C	-	1 (2)	0 (0)	1 (2,4)
2206	rRNA 16S	C>T	-	1 (2)	1 (2,2)	1 (2,4)
2217	rRNA 16S	C>T	-	1 (2)	0 (0)	0 (0)
2259	rRNA 16S	C>T	-	1 (2)	1 (2,2)	1 (2,4)
2308	rRNA 16S	A>G	-	1 (2)	1 (2,2)	1 (2,4)
2392	rRNA 16S	T>C	-	1 (2)	1 (2,2)	0 (0)
2416	rRNA 16S	T>C	-	1 (2)	1 (2,2)	1 (2,4)
2455	rRNA 16S	T>C	-	0 (0)	1 (2,2)	0 (0)
2626	rRNA 16S	T>C	-	1 (2)	1 (2,2)	1 (2,4)
2706	rRNA 16S	A>G	-	29 (56,9)	25 (54,3)	24 (58,5)
2707	rRNA 16S	A>G	-	1 (2)	1 (2,2)	1 (2,4)
2789	rRNA 16S	C>T	-	1 (2)	1 (2,2)	1 (2,4)
2833	rRNA 16S	A>G	-	1 (2)	1 (2,2)	1 (2,4)
2878et	rRNA 16S	G+A	-	0 (0)	1 (2,2)	0 (0)
2983et	rRNA 16S	G+A	-	0 (0)	1 (2,2)	0 (0)
3010	rRNA 16S	G>A	-	8 (15,7)	7 (15,2)	8 (19,5)
3197	rRNA 16S	T>C	-	8 (15,7)	7 (15,2)	7 (17,1)
3348	NADH dehydrogenase subunit 1	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
3391	NADH dehydrogenase subunit 1	G>A	Gly G > Ser S	1 (2)	1 (2,2)	1 (2,4)
3480	NADH dehydrogenase subunit 1	A>G	synonymous	3 (5,9)	2 (4,3)	2 (4,9)
3594	NADH dehydrogenase subunit 1	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
3672	NADH dehydrogenase subunit 1	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
3742	NADH dehydrogenase subunit 1	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
3915	NADH dehydrogenase subunit 1	G>A	synonymous	2 (3,9)	2 (4,3)	0 (0)
3992	NADH dehydrogenase subunit 1	C>T	Thr T > Met M	1 (2)	1 (2,2)	1 (2,4)
4024	NADH dehydrogenase subunit 1	A>G	Thr T > Ala A	1 (2)	1 (2,2)	1 (2,4)
4104	NADH dehydrogenase subunit 1	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
4136	NADH dehydrogenase subunit 1	A>G	Tyr Y > Cys C	1 (2)	1 (2,2)	1 (2,4)
4149	NADH dehydrogenase subunit 1	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)

4188	NADH dehydrogenase subunit 1	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
4216	NADH dehydrogenase subunit 1	T>C	Tyr Y > His H	8 (15,7)	7 (15,2)	4 (9,8)
4227	NADH dehydrogenase subunit 1	A>G	synonymous	1 (2)	1 (2,2)	0 (0)
4227et	NADH dehydrogenase subunit 1	A+G	synonymous	0 (0)	0 (0)	1 (2,4)
4277	tRNA isoleucine	T>C	-	1 (2)	0 (0)	1 (2,4)
4336	tRNA glutamine	T>C	-	1 (2)	1 (2,2)	1 (2,4)
4561	NADH dehydrogenase subunit 2	T>C	Val V > Ala A	1 (2)	1 (2,2)	1 (2,4)
4569et	NADH dehydrogenase subunit 2	G+A	Glu E > Lys K	1 (2)	0 (0)	0 (0)
4580	NADH dehydrogenase subunit 2	G>A	synonymous	1 (2)	1 (2,2)	0 (0)
4592	NADH dehydrogenase subunit 2	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
4640	NADH dehydrogenase subunit 2	C>A	Ile I > Met M	1 (2)	1 (2,2)	1 (2,4)
4646	NADH dehydrogenase subunit 2	T>C	synonymous	2 (3,9)	2 (4,3)	3 (7,3)
4727	NADH dehydrogenase subunit 2	A>G	synonymous	2 (3,9)	2 (4,3)	0 (0)
4732	NADH dehydrogenase subunit 2	A>G	Asn N > Ser S	2 (3,9)	2 (4,3)	2 (4,9)
4745	NADH dehydrogenase subunit 2	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
4769	NADH dehydrogenase subunit 2	A>G	synonymous	49 (96,1)	44 (95,7)	41 (100)
4772	NADH dehydrogenase subunit 2	T>C	synonymous	1 (2)	0 (0)	1 (2,4)
4793	NADH dehydrogenase subunit 2	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
4811	NADH dehydrogenase subunit 2	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
4824	NADH dehydrogenase subunit 2	A>G	Thr T > Ala A	1 (2)	1 (2,2)	0 (0)
4843	NADH dehydrogenase subunit 2	C>T	Thr T > Met M	1 (2)	1 (2,2)	1 (2,4)
4917	NADH dehydrogenase subunit 2	A>G	Asn N-Asp D	6 (11,8)	6 (13)	3 (7,3)
4967	NADH dehydrogenase subunit 2	T>G	Ser S > Arg R	0 (0)	0 (0)	1 (2,4)
5004	NADH dehydrogenase subunit 2	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
5081	NADH dehydrogenase subunit 2	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
5086	NADH dehydrogenase subunit 2	C>T	Thr T > Ile I	1 (2)	1 (2,2)	1 (2,4)
5130	NADH dehydrogenase subunit 2	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
5147	NADH dehydrogenase subunit 2	G>A	synonymous	5 (9,8)	4 (8,7)	2 (4,9)
5153	NADH dehydrogenase subunit 2	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
5187	NADH dehydrogenase subunit 2	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
5231	NADH dehydrogenase subunit 2	G>A	synonymous	1 (2)	0 (0)	0 (0)
5396	NADH dehydrogenase subunit 2	C>T	synonymous	2 (3,9)	2 (4,3)	1 (2,4)
5460	NADH dehydrogenase subunit 2	G>A	Ala A > Thr T	4 (7,8)	3 (6,5)	4 (9,8)
5471	NADH dehydrogenase subunit 2	G>A	synonymous	2 (3,9)	1 (2,2)	2 (4,9)
5495	NADH dehydrogenase subunit 2	T>C	synonymous	2 (3,9)	1 (2,2)	2 (4,9)
5498	NADH dehydrogenase subunit 2	A>G	synonymous	1 (2)	0 (0)	0 (0)
5568	tRNA tryptophan	A>G	-	0 (0)	1 (2,2)	0 (0)
5633	tRNA alanine	C>T	-	1 (2)	1 (2,2)	0 (0)
5656	non-coding nucleotides	A>G	-	1 (2)	1 (2,2)	0 (0)
5824	tRNA tyrosine	G>A	-	1 (2)	1 (2,2)	1 (2,4)
5918	Cytochrome c oxidase subunit I	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
5964	Cytochrome c oxidase subunit I	T>C	synonymous	1 (2)	0 (0)	1 (2,4)
5999	Cytochrome c oxidase subunit I	T>C	synonymous	2 (3,9)	2 (4,3)	3 (7,3)
6040	Cytochrome c oxidase subunit I	A>G	synonymous	1 (2)	0 (0)	1 (2,4)

6047	Cytochrome c oxidase subunit I	A>G	synonymous	2 (3,9)	2 (4,3)	3 (7,3)
6146	Cytochrome c oxidase subunit I	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
6182	Cytochrome c oxidase subunit I	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
6253	Cytochrome c oxidase subunit I	T>C	Met M > Thr T	1 (2)	1 (2,2)	0 (0)
6261	Cytochrome c oxidase subunit I	G>A	Ala A > Thr T	1 (2)	1 (2,2)	1 (2,4)
6392	Cytochrome c oxidase subunit I	T>C	synonymous	1 (2)	1 (2,2)	0 (0)
6413	Cytochrome c oxidase subunit I	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
6663	Cytochrome c oxidase subunit I	A>G	Ile I > Val V	1 (2)	1 (2,2)	1 (2,4)
6776	Cytochrome c oxidase subunit I	T>C	synonymous	3 (5,9)	3 (6,5)	2 (4,9)
6911	Cytochrome c oxidase subunit I	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
6959	Cytochrome c oxidase subunit I	C>T	Gly G > Val V	1 (2)	1 (2,2)	0 (0)
7028	Cytochrome c oxidase subunit I	C>T	synonymous	27 (52,9)	25 (54,3)	24 (58,5)
7076	Cytochrome c oxidase subunit I	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
7175	Cytochrome c oxidase subunit I	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
7193	Cytochrome c oxidase subunit I	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
7256	Cytochrome c oxidase subunit I	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
7269	Cytochrome c oxidase subunit I	G>A	Val V > Met M	1 (2)	0 (0)	0 (0)
7274	Cytochrome c oxidase subunit I	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
7476	tRNA serine 1	C>T	-	1 (2)	1 (2,2)	0 (0)
7492	tRNA serine 1	C>T	-	1 (2)	0 (0)	1 (2,4)
7521	tRNA aspartic acid	G>A	-	1 (2)	1 (2,2)	1 (2,4)
7546	tRNA aspartic acid	T>C	-	1 (2)	1 (2,2)	1 (2,4)
7553	tRNA aspartic acid	A>G	-	0 (0)	0 (0)	1 (2,4)
7598	Cytochrome c oxidase subunit II	G>A	Ala A > Thr T	1 (2)	1 (2,2)	1 (2,4)
7645	Cytochrome c oxidase subunit II	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
7705	Cytochrome c oxidase subunit II	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
7768	Cytochrome c oxidase subunit II	A>G	synonymous	3 (5,9)	3 (6,5)	2 (4,9)
7771	Cytochrome c oxidase subunit II	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
7789	Cytochrome c oxidase subunit II	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
7810	Cytochrome c oxidase subunit II	C>T	synonymous	0 (0)	0 (0)	1 (2,4)
7861	Cytochrome c oxidase subunit II	T>C	synonymous	2 (3,9)	1 (2,2)	2 (4,9)
7873	Cytochrome c oxidase subunit II	C>T	synonymous	1 (2)	1 (2,2)	2 (4,9)
7885	Cytochrome c oxidase subunit II	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
7912	Cytochrome c oxidase subunit II	G>A	synonymous	0 (0)	0 (0)	1 (2,4)
8155	Cytochrome c oxidase subunit II	G>A	synonymous	1 (2)	1 (2,2)	0 (0)
8206	Cytochrome c oxidase subunit II	G>T	Met M > Ile I	1 (2)	2 (4,3)	1 (2,4)
8251	Cytochrome c oxidase subunit II	G>A	synonymous	1 (2)	0 (0)	0 (0)
8269	Cytochrome c oxidase subunit II	G>A	synonymous	1 (2)	0 (0)	1 (2,4)
8280+CCCCCTCTA	non-coding nucleotides	ins	-	1 (2)	1 (2,2)	2 (4,9)
8281delCCCCCTCTA	non-coding nucleotides	del	-	1 (2)	1 (2,2)	0 (0)
8378	ATP synthase F0 subunit 8	A>G	Asn N > Asp D	1 (2)	1 (2,2)	1 (2,4)
8381	ATP synthase F0 subunit 8	A>G	Thr T > Ala A	1 (2)	1 (2,2)	1 (2,4)
8386	ATP synthase F0 subunit 8	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
8412	ATP synthase F0 subunit 8	T>C	Met M > Thr T	1 (2)	0 (0)	1 (2,4)

8431	ATP synthase F0 subunit 8	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
8512	ATP synthase F0 subunit 8	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
8555	ATP synthase F0 subunit 6	T>C	Ile I > Thr T	1 (2)	1 (2,2)	1 (2,4)
8557	ATP synthase F0 subunit 6	G>A	Ala NP > Thr P	1 (2)	0 (0)	1 (2,4)
8642	ATP synthase F0 subunit 6	A>G	Asn N > Ser S	1 (2)	1 (2,2)	1 (2,4)
8697	ATP synthase F0 subunit 6	G>A	synonymous	6 (11,8)	5 (10,9)	3 (7,3)
8701	ATP synthase F0 subunit 6	A>G	Thr T > Ala A	1 (2)	1 (2,2)	1 (2,4)
8803	ATP synthase F0 subunit 6	A>T	Thr T > Ser S	1 (2)	1 (2,2)	1 (2,4)
8818	ATP synthase F0 subunit 6	C>T	synonymous	0 (0)	0 (0)	1 (2,4)
8860	ATP synthase F0 subunit 6	A>G	Thr T > Ala A	50 (98)	45 (97,8)	39 (95,1)
8867	ATP synthase F0 subunit 6	T>C	Ile I > Thr T	1 (2)	1 (2,2)	1 (2,4)
8901	ATP synthase F0 subunit 6	A>G	synonymous	0 (0)	1 (2,2)	0 (0)
8906et	ATP synthase F0 subunit 6	A+G	His H > Arg R	0 (0)	1 (2,2)	1 (2,4)
8938	ATP synthase F0 subunit 6	A>G	Ile I > Val V	1 (2)	1 (2,2)	1 (2,4)
8953	ATP synthase F0 subunit 6	A>G	Ile I > Val V	1 (2)	1 (2,2)	0 (0)
9007	ATP synthase F0 subunit 6	A>G	Thr T > Ala A	1 (2)	1 (2,2)	1 (2,4)
9055	ATP synthase F0 subunit 6	G>A	Ala A > Thr T	2 (3,9)	1 (2,2)	2 (4,9)
9070	ATP synthase F0 subunit 6	T>G	Ser S > Ala A	1 (2)	1 (2,2)	1 (2,4)
9123	ATP synthase F0 subunit 6	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
9201	ATP synthase F0 subunit 6	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
9221	Cytochrome c oxidase subunit III	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
9380	Cytochrome c oxidase subunit III	G>A	synonymous	3 (5,9)	3 (6,5)	1 (2,4)
9438	Cytochrome c oxidase subunit III	G>A	Gly G > Ser S	1 (2)	0 (0)	1 (2,4)
9477	Cytochrome c oxidase subunit III	G>A	Val V > Ile I	8 (15,7)	7 (15,2)	7 (17,1)
9540	Cytochrome c oxidase subunit III	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
9548	Cytochrome c oxidase subunit III	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
9621	Cytochrome c oxidase subunit III	G>A	Ala A > Thr T	1 (2)	1 (2,2)	0 (0)
9656	Cytochrome c oxidase subunit III	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
9664	Cytochrome c oxidase subunit III	A>G	Glu E > Gly G	1 (2)	0 (0)	1 (2,4)
9667	Cytochrome c oxidase subunit III	A>G	Asn N > Ser S	1 (2)	1 (2,2)	1 (2,4)
9677	Cytochrome c oxidase subunit III	C>T	synonymous	0 (0)	1 (2,2)	1 (2,4)
9698	Cytochrome c oxidase subunit III	T>C	synonymous	3 (5,9)	2 (4,3)	2 (4,9)
9716	Cytochrome c oxidase subunit III	T>C	synonymous	2 (3,9)	1 (2,2)	1 (2,4)
9744et	Cytochrome c oxidase subunit III	G+A	Glu E > Lys K	0 (0)	0 (0)	1 (2,4)
9770	Cytochrome c oxidase subunit III	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
9804	Cytochrome c oxidase subunit III	G>T	Ala A > Ser S	1 (2)	1 (2,2)	1 (2,4)
9861	Cytochrome c oxidase subunit III	T>C	Phe F > Leu L	1 (2)	1 (2,2)	1 (2,4)
9863	Cytochrome c oxidase subunit III	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
9932	Cytochrome c oxidase subunit III	G>A	synonymous	1 (2)	1 (2,2)	0 (0)
10101	Cytochrome c oxidase subunit III	T>C	synonymous	1 (2)	1 (2,2)	0 (0)
10115	Cytochrome c oxidase subunit III	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
10143	Cytochrome c oxidase subunit III	G>A	Gly G > Ser S	1 (2)	1 (2,2)	1 (2,4)
10172	Cytochrome c oxidase subunit III	G>A	synonymous	1 (2)	1 (2,2)	0 (0)
10202	Cytochrome c oxidase subunit III	C>T	synonymous	0 (0)	1 (2,2)	0 (0)

10217	Cytochrome c oxidase subunit III	A>G	synonymous	1 (2)	0 (0)	1 (2,4)
10218	Cytochrome c oxidase subunit III	A>G	Lys K > Glu E	0 (0)	1 (2,2)	0 (0)
10320	Cytochrome c oxidase subunit III	G>A	Val V > Ile I	1 (2)	1 (2,2)	1 (2,4)
10365	Cytochrome c oxidase subunit III	G>A	Ala A > Thr T	1 (2)	0 (0)	0 (0)
10398	Cytochrome c oxidase subunit III	A>G	Thr T > Ala A	4 (7,8)	3 (6,5)	3 (7,3)
10463	tRNA arginine	T>C	-	6 (11,8)	5 (10,9)	3 (7,3)
10550	NADH dehydrogenase subunit 4L	A>G	-	3 (5,9)	2 (4,3)	2 (4,9)
10589	NADH dehydrogenase subunit 4L	G>A	synonymous	2 (3,9)	2 (4,3)	0 (0)
10601	NADH dehydrogenase subunit 4L	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
10750	NADH dehydrogenase subunit 4L	A>G	Asn N > Ser S	1 (2)	1 (2,2)	1 (2,4)
10822	NADH dehydrogenase subunit 4	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
10873	NADH dehydrogenase subunit 4	T>C	synonymous	0 (0)	1 (2,2)	1 (2,4)
10907	NADH dehydrogenase subunit 4	T>C	Phe F > Leu L	1 (2)	1 (2,2)	1 (2,4)
10915	NADH dehydrogenase subunit 4	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
10931	NADH dehydrogenase subunit 4	T>C	Ser S > Pro P	1 (2)	0 (0)	1 (2,4)
10954	NADH dehydrogenase subunit 4	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
10993	NADH dehydrogenase subunit 4	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
11009	NADH dehydrogenase subunit 4	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
11167	NADH dehydrogenase subunit 4	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
11251	NADH dehydrogenase subunit 4	A>G	synonymous	8 (15,7)	6 (13)	4 (9,8)
11299	NADH dehydrogenase subunit 4	T>C	synonymous	3 (5,9)	2 (4,3)	2 (4,9)
11332	NADH dehydrogenase subunit 4	C>T	synonymous	2 (3,9)	2 (4,3)	3 (7,3)
11339	NADH dehydrogenase subunit 4	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
11467	NADH dehydrogenase subunit 4	A>G	synonymous	14 (27,5)	12 (26,1)	13 (31,7)
11719	NADH dehydrogenase subunit 4	G>A	synonymous	23 (45,1)	18 (39,1)	18 (43,9)
11812	NADH dehydrogenase subunit 4	A>G	synonymous	5 (9,8)	4 (8,7)	2 (4,9)
11869	NADH dehydrogenase subunit 4	C>A	synonymous	1 (2)	0 (0)	0 (0)
11914	NADH dehydrogenase subunit 4	G>A	synonymous	4 (7,8)	4 (8,7)	3 (7,3)
11935	NADH dehydrogenase subunit 4	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
11944	NADH dehydrogenase subunit 4	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
12007	NADH dehydrogenase subunit 4	G>A	synonymous	1 (2)	0 (0)	1 (2,4)
12061	NADH dehydrogenase subunit 4	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
12193	tRNA histidine	A>G	-	0 (0)	1 (2,2)	0 (0)
12297	tRNA leucine2	T>C	-	1 (2)	1 (2,2)	1 (2,4)
12308	tRNA leucine2	A>G	-	13 (25,5)	12 (26,1)	13 (31,7)
12372	NADH dehydrogenase subunit 5	G>A	synonymous	14 (27,5)	12 (26,1)	13 (31,7)
12396	NADH dehydrogenase subunit 5	T>C	synonymous	1 (2)	0 (0)	1 (2,4)
12397	NADH dehydrogenase subunit 5	A>G	Thr T > Ala A	1 (2)	1 (2,2)	1 (2,4)
12612	NADH dehydrogenase subunit 5	A>T	synonymous	2 (3,9)	1 (2,2)	1 (2,4)
12618	NADH dehydrogenase subunit 5	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
12633	NADH dehydrogenase subunit 5	C>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
12693	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
12705	NADH dehydrogenase subunit 5	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)

12738	NADH dehydrogenase subunit 5	T>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
12744	NADH dehydrogenase subunit 5	C>T	synonymous	1 (2)	1 (2,2)	0 (0)
12757	NADH dehydrogenase subunit 5	T>C	Phe F > Leu L	1 (2)	1 (2,2)	0 (0)
12811	NADH dehydrogenase subunit 5	T>C	Tyr Y > His H	1 (2)	1 (2,2)	1 (2,4)
12858	NADH dehydrogenase subunit 5	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
13015	NADH dehydrogenase subunit 5	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
13308	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	0 (0)	1 (2,4)
13348	NADH dehydrogenase subunit 5	A>G	Met M > Val V	0 (0)	0 (0)	1 (2,4)
13368	NADH dehydrogenase subunit 5	G>A	synonymous	6 (11,8)	5 (10,9)	3 (7,3)
13401	NADH dehydrogenase subunit 5	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
13434	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
13449	NADH dehydrogenase subunit 5	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
13539	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
13590	NADH dehydrogenase subunit 5	G>A	synonymous	2 (3,9)	2 (4,3)	2 (4,9)
13617	NADH dehydrogenase subunit 5	T>C	synonymous	7 (13,7)	7 (15,2)	7 (17,1)
13637	NADH dehydrogenase subunit 5	A>G	Gln Q > Arg R	1 (2)	2 (4,3)	2 (4,9)
13650	NADH dehydrogenase subunit 5	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
13680	NADH dehydrogenase subunit 5	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
13708	NADH dehydrogenase subunit 5	G>A	Ala A > Thr T	2 (3,9)	1 (2,2)	1 (2,4)
13710	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
13722	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
13743	NADH dehydrogenase subunit 5	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
13788	NADH dehydrogenase subunit 5	C>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
13803	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
13827	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
13879	NADH dehydrogenase subunit 5	T>C	Ser S > Pro P	1 (2)	0 (0)	1 (2,4)
13928	NADH dehydrogenase subunit 5	G>C	Ser S > Thr T	1 (2)	1 (2,2)	1 (2,4)
13938	NADH dehydrogenase subunit 5	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
14007	NADH dehydrogenase subunit 5	A>C	Trp W < Cys C	1 (2)	0 (0)	1 (2,4)
14037	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	0 (0)	0 (0)
14094	NADH dehydrogenase subunit 5	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
14139	NADH dehydrogenase subunit 5	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
14167	NADH dehydrogenase subunit 6	C>T	synonymous	3 (5,9)	2 (4,3)	2 (4,9)
14180	NADH dehydrogenase subunit 6	T>C	Tyr Y > Cys C	1 (2)	0 (0)	0 (0)
14182	NADH dehydrogenase subunit 6	T>C	synonymous	3 (5,9)	3 (6,5)	2 (4,9)
14220	NADH dehydrogenase subunit 6	A>G	synonymous	0 (0)	1 (2,2)	1 (2,4)
14233	NADH dehydrogenase subunit 6	A>G	synonymous	5 (9,8)	4 (8,7)	2 (4,9)
14256	NADH dehydrogenase subunit 6	T>C	Ile I > Val V	1 (2)	1 (2,2)	1 (2,4)
14323	NADH dehydrogenase subunit 6	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
14365	NADH dehydrogenase subunit 6	C>T	synonymous	0 (0)	1 (2,2)	1 (2,4)
14410	NADH dehydrogenase subunit 6	G>A	Asp D > Asn N	0 (0)	0 (0)	1 (2,4)
14475	NADH dehydrogenase subunit 6	A>G	synonymous	0 (0)	1 (2,2)	0 (0)
14566	NADH dehydrogenase subunit 6	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
14569	NADH dehydrogenase subunit 6	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)

14582	NADH dehydrogenase subunit 6	A>G	Val V > Ala A	1 (2)	1 (2,2)	1 (2,4)
14620	NADH dehydrogenase subunit 6	C>T	synonymous	2 (3,9)	2 (4,3)	3 (7,3)
14629	NADH dehydrogenase subunit 6	C>T	synonymous	0 (0)	1 (2,2)	1 (2,4)
14696	cytochrome b	A>G	synonymous	1 (2)	1 (2,2)	0 (0)
14766	cytochrome b	C>T	synonymous	23 (45,1)	19 (41,3)	17 (41,5)
14793	cytochrome b	A>G	His H > Arg R	5 (9,8)	4 (8,7)	5 (12,2)
14798	cytochrome b	T>C	Phe F > Leu L	3 (5,9)	2 (4,3)	2 (4,9)
14866	cytochrome b	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
14872	cytochrome b	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
14905	cytochrome b	G>A	synonymous	6 (11,8)	5 (10,9)	3 (7,3)
14944	cytochrome b	C>T	synonymous	1 (2)	1 (2,2)	0 (0)
15088	cytochrome b	C>T	synonymous	1 (2)	1 (2,2)	0 (0)
15139	cytochrome b	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
15191	cytochrome b	T>C	synonymous	1 (2)	1 (2,2)	0 (0)
15204	cytochrome b	T>C	Ile I > Thr T	0 (0)	1 (2,2)	1 (2,4)
15218	cytochrome b	A>G	Thr T > Ala A	3 (5,9)	2 (4,3)	3 (7,3)
14257	cytochrome b	G>A	Asp D > Asn N	1 (2)	1 (2,2)	0 (0)
15301	cytochrome b	G>A	synonymous	1 (2)	1 (2,2)	1 (2,4)
15315	cytochrome b	C>T	Ala A > Val V	1 (2)	1 (2,2)	0 (0)
15323	cytochrome b	G>A	Ala A > Thr T	1 (2)	1 (2,2)	1 (2,4)
15326	cytochrome b	A>G	Thr T > Ala A	49 (96,1)	45 (97,8)	41 (100)
15452	cytochrome b	C>A	Leu L- Ile I	7 (13,7)	6 (13)	2 (4,9)
15454	cytochrome b	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
15458	cytochrome b	T>C	Ser S > Pro P	2 (3,9)	2 (4,3)	1 (2,4)
15497	cytochrome b	G>A	Gly G > Ser S	0 (0)	1 (2,2)	1 (2,4)
15511	cytochrome b	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
15602et	cytochrome b	A+G	Asn N > Asp D	0 (0)	0 (0)	1 (2,4)
15607	cytochrome b	A>G	synonymous	6 (11,8)	5 (10,9)	3 (7,3)
15693	cytochrome b	T>C	Met M > Thr T	2 (3,9)	2 (4,3)	3 (7,3)
15784	cytochrome b	T>C	synonymous	1 (2)	1 (2,2)	1 (2,4)
15789	cytochrome b	C>T	Thr T > Ile I	1 (2)	1 (2,2)	1 (2,4)
15792	cytochrome b	T>C	Ile I > Thr T	1 (2)	1 (2,2)	1 (2,4)
15812	cytochrome b	G>A	Val V > Met M	1 (2)	0 (0)	0 (0)
15817	cytochrome b	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
15829	cytochrome b	A>G	synonymous	1 (2)	1 (2,2)	1 (2,4)
15833	cytochrome b	C>T	synonymous	1 (2)	1 (2,2)	1 (2,4)
15883	cytochrome b	G>A	synonymous	1 (2)	0 (0)	1 (2,4)
15904	tRNA threonine	C>T	-	1 (2)	1 (2,2)	1 (2,4)
15916	tRNA threonine	T>C	-	0 (0)	0 (0)	1 (2,4)
15924	tRNA threonine	A>G	-	2 (3,9)	1 (2,2)	2 (4,9)
15924et	tRNA threonine	A+G	-	1 (2)	0 (0)	0 (0)
15928	tRNA threonine	G>A	-	6 (11,8)	5 (10,9)	3 (7,3)
15930	tRNA threonine	G>A	-	1 (2)	1 (2,2)	1 (2,4)
15944delT	tRNA threonine	del	-	1 (2)	1 (2,2)	1 (2,4)

15948	tRNA threonine	A>G	-	0 (0)	0 (0)	1 (2,4)
16069	D-loop (HVSI)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
16093	D-loop (HVSI)	T>C	non coding	2 (3,9)	2 (4,3)	2 (4,9)
16114	D-loop (HVSI)	C>A	non coding	1 (2)	1 (2,2)	1 (2,4)
16126	D-loop (HVSI)	T>C	non coding	8 (15,7)	6 (13)	4 (9,8)
16129	D-loop (HVSI)	G>A	non coding	1 (2)	1 (2,2)	1 (2,4)
16145	D-loop (HVSI)	G>A	non coding	2 (3,9)	0 (0)	1 (2,4)
16147	D-loop (HVSI)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
16153	D-loop (HVSI)	G>A	non coding	1 (2)	1 (2,2)	0 (0)
16163	D-loop (HVSI)	A>G	non coding	1 (2)	1 (2,2)	0 (0)
16166delA	D-loop (HVSI)	del	non coding	1 (2)	1 (2,2)	1 (2,4)
16169	D-loop (HVSI)	C>T	non coding	0 (0)	0 (0)	1 (2,4)
16172	D-loop (HVSI)	T>C	non coding	2 (3,9)	2 (4,3)	1 (2,4)
16179	D-loop (HVSI)	C>A	non coding	1 (2)	0 (0)	1 (2,4)
16184	D-loop (HVSI)	C>T	non coding	1 (2)	0 (0)	1 (2,4)
16187	D-loop (HVSI)	C>T	non coding	1 (2)	0 (0)	1 (2,4)
16189	D-loop (HVSI)	T>C	non coding	5 (9,8)	4 (8,7)	3 (7,3)
16192	D-loop (HVSI)	C>T	non coding	2 (3,9)	2 (4,3)	2 (4,9)
16193	D-loop (HVSI)	C>T	non coding	1 (2)	1 (2,2)	0 (0)
16213	D-loop (HVSI)	G>A	non coding	1 (2)	1 (2,2)	1 (2,4)
16217	D-loop (HVSI)	T>C	non coding	1 (2)	1 (2,2)	1 (2,4)
16222	D-loop (HVSI)	C>T	non coding	1 (2)	0 (0)	1 (2,4)
16223	D-loop (HVSI)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
16224	D-loop (HVSI)	T>C	non coding	3 (5,9)	2 (4,3)	2 (4,9)
16256	D-loop (HVSI)	C>T	non coding	6 (11,8)	5 (10,9)	6 (14,6)
16261	D-loop (HVSI)	C>T	non coding	2 (3,9)	1 (2,2)	2 (4,9)
16264et	D-loop (HVSI)	C+T	non coding	0 (0)	0 (0)	1 (2,4)
16265	D-loop (HVSI)	A>T	non coding	1 (2)	1 (2,2)	0 (0)
16266	D-loop (HVSI)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
16270	D-loop (HVSI)	C>T	non coding	7 (13,7)	5 (10,9)	6 (14,6)
16274	D-loop (HVSI)	G>A	non coding	1 (2)	1 (2,2)	1 (2,4)
16278	D-loop (HVSI)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
16286	D-loop (HVSI)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
16291	D-loop (HVSI)	C>T	non coding	1 (2)	1 (2,2)	1 (2,4)
16294	D-loop (HVSI)	C>T	non coding	9 (17,6)	8 (17,4)	6 (14,6)
16295	D-loop (HVSI)	C>T	non coding	1 (2)	0 (0)	0 (0)
16295et	D-loop (HVSI)	C+T	non coding	0 (0)	0 (0)	1 (2,4)
16296	D-loop (HVSI)	C>T	non coding	2 (3,9)	2 (4,3)	1 (2,4)
16298	D-loop (HVSI)	T>C	non coding	3 (5,9)	4 (8,7)	4 (9,8)
16304	D-loop (HVSI)	T>C	non coding	6 (11,8)	3 (6,5)	4 (9,8)
16305	D-loop (HVSI)	A>G	non coding	1 (2)	1 (2,2)	1 (2,4)
16309	D-loop (HVSI)	A>G	non coding	1 (2)	1 (2,2)	1 (2,4)
16311	D-loop (HVSI)	T>C	non coding	6 (11,8)	6 (13)	4 (9,8)
16324	D-loop (HVSI)	T>C	non coding	1 (2)	1 (2,2)	0 (0)

16325	D-loop (HVSI)	T>C	non coding	0 (0)	1 (2,2)	1 (2,4)
16336	D-loop (HVSI)	G>A	non coding	1 (2)	1 (2,2)	0 (0)
16343	D-loop (HVSI)	A>G	non coding	1 (2)	1 (2,2)	1 (2,4)
16352	D-loop (HVSI)	T>C	non coding	1 (2)	1 (2,2)	1 (2,4)
16356	D-loop (HVSI)	T>C	non coding	2 (3,9)	2 (4,3)	3 (7,3)
16362	D-loop (HVSI)	T>C	non coding	5 (9,8)	5 (10,9)	3 (7,3)
16390	D-loop (HVSI)	G>A	non coding	2 (3,9)	2 (4,3)	2 (4,9)
16399	D-loop (HVSI)	A>G	non coding	3 (5,9)	2 (4,3)	3 (7,3)
16482	D-loop (HVSI)	A>G	non coding	2 (3,9)	2 (4,3)	0 (0)
16497	D-loop (HVSI)	A>G	non coding	1 (2)	0 (0)	1 (2,4)
16519	D-loop	T>C	non coding	21 (41,2)	21 (45,7)	15 (36,6)
16526	D-loop	G>A	non coding	2 (3,9)	2 (4,3)	2 (4,9)

Table 4: Mutations relative to the revised Cambridge Reference Sequences (rCRS) found in 138 complete mtDNA sequences from DS, DSM and DSS subjects. (1) Novel mutations are underlined and in italics. (2) Mutations resulting in an aminoacid change are in bold.

We found that there are three novel mutations in DS subjects (two of them non synonymous), eight are in DSM subjects (one of them non synonymous) and eight are in DSS subjects (five of them non synonymous) but the comparison is not statistically significant.

We wondered whether in DS subjects there is a specific region of the mtDNA more prone to accumulation of mutations with respect to DSS or DSM subjects. We therefore studied the position of the mutations along the mtDNA genome (**Table 5**) and we found that the mutations accumulate equally in the three groups analyzed, even if the total number of mutations between DS, DSM and DSS subjects is statistically significant.

mtDNA region	N. mutations in DS	N. mutations in DSM	N. mutations in DSS	Fisher-exact test DS-DSM	Fisher-exact test DS-DSS	Fisher-exact test DSM-DSS
D-loop	291	253	229	p=0.9232	p=0.8047	p=0.9186
ND1	25	23	18	p=0.8849	p=0.7583	p=0.6389
ND2	95	82	75	p=0.8759	p=0.829	p=0.9751
ND4L	7	6	4	p=0.9629	p=0.7636	p=0.7555
ND4	73	60	59	p=0.7209	p=0.9968	p=0.7767
ND5	66	57	59	p=0.9263	p=0.6433	p=0.5663
ND6	19	20	18	p=0.9629	p=0.7392	p=0.7452
COI	52	47	46	p=0.9182	p=0.6785	p=0.8318
COII	16	14	16	p=0.9884	p=1	p=0.5849
COIII	40	35	31	p=0.9818	p=0.9037	p=0.901
ATPase6	70	64	57	p=0.8585	p=0.9707	p=0.9253
ATPase8	6	5	6	p=0.9629	p=1	p=0.7664
cyt b	122	110	93	p=0.8898	p=0.7184	p=0.6049
rRNA total	188	166	154	p=0.9743	p=0.9531	p=0.9518
tRNA total	40	35	34	p=0.9818	p=0.9054	p=0.9025
NC(1)	3	3	2	p=1	p=0.8323	p=1
Total	**1113**	**980**	**901**	**p=0.003647**	**p=0.06853**	**p=0.0002313**

Table 5: Number of mutations found along the mtDNA molecule regions in DS, DSM and DSS subjects and Fisher-exact test values in pairs. (1) Non Coding nucleotides.

As expected, D-loop is the region that has more variable sites, following by NADH dehydrogenase, rRNAs, cytochrome b, cytochrome c oxidase, ATPase, tRNAs.

Sporadic mutations (present in one sample only) are found in 15 DS, 16 DSS and 12 DSM subjects (**Table 6**). Even though this difference is not significant (Fisher-exact test, p-value=0.4497), it is very interesting to notice how mutations accumulate in each group. DS subjects have 5 non coding mutations, 5 synonymous mutations and 5 non-synonymous mutations. These mutations, changing the aminoacid sequence, hit NADH dehydrogenase subunits (2 and 6), cytochrome c (subunit I and III) and cytochrome b. At the same time, in DSS subjects we found also 7 non coding mutations, 4 synonymous mutations and 5 non-synonymous mutations, that hit NADH dehydrogenase subunits (2, 5 and 6), cytochrome c (subunit III) and cytochrome b. DSM subjects have, instead, different mutations in different positions: 1 non-synonymous mutation (cytochrome c subunit III), 8 non coding mutations and 3 synonymous mutations.

SPORADIC MUTATIONS			
mtDNA region	**N. mutations in DS**	**N. mutations in DSM**	**N. mutations in DSS**
D-loop	2	2	4
ND1	0	0	1
ND2	3	0	1
ND4L	0	0	0
ND4	1	0	0
ND5	1	0	1
ND6	1	1	1
COI	1	0	0
COII	1	0	2
COIII	1	2	1
ATPase6	0	1	1
ATPase8	0	0	0
cyt b	1	0	1
rRNA total	2	4	0
tRNA total	1	2	3
NC(1)	0	0	0
Total	**15**	**12**	**16**

Table 6: sporadic mutations found along mtDNA molecule regions in DS, DSM and DSS subjects. (1) Non Coding nucleotides.

We have also analyzed the percentage of non-synonymous mutations of each DS subject and compared with their cognitive scores in order to evaluate any significant association. We have found a negative trend, i.e. at increasing the scores of total, verbal and performance IQ decreases the percentage of non-synonymous mutations.

4. Analysis of heteroplasmy

We analyzed mtDNA CR heteroplasmy in a 300 bp strecht (nt 16531-261), surrounding the 150 position.

In order to quantify the levels of heteroplasmy in the biological samples under study, we applied DHPLC to artificial heteroplasmic samples and assembled the reference curve that was used for estimating the levels of heteroplasmy in the biological samples.

The distribution of the levels of heteroplasmy in the three groups (**Figure 4**) shows that most of the samples display 0% of heteroplasmy, 92.45% of DS, 87.23% of DSM and 92.68% of DSS, respectively. Moreover, figure 4 shows that approximately 4% of DS subjects and 2% of both DSM and DSS subjects

have levels of heteroplasmy lower than 2.5%. Levels of heteroplasmy between 2.5%-5% are present only in both DSM and DSS (2.13% and 2.44% respectively), while 2% of both DS and DSS and 8% of DSM have an heteroplasmy between 5%-10%. Finally only one DS subject has a heteroplasmy at 33%.

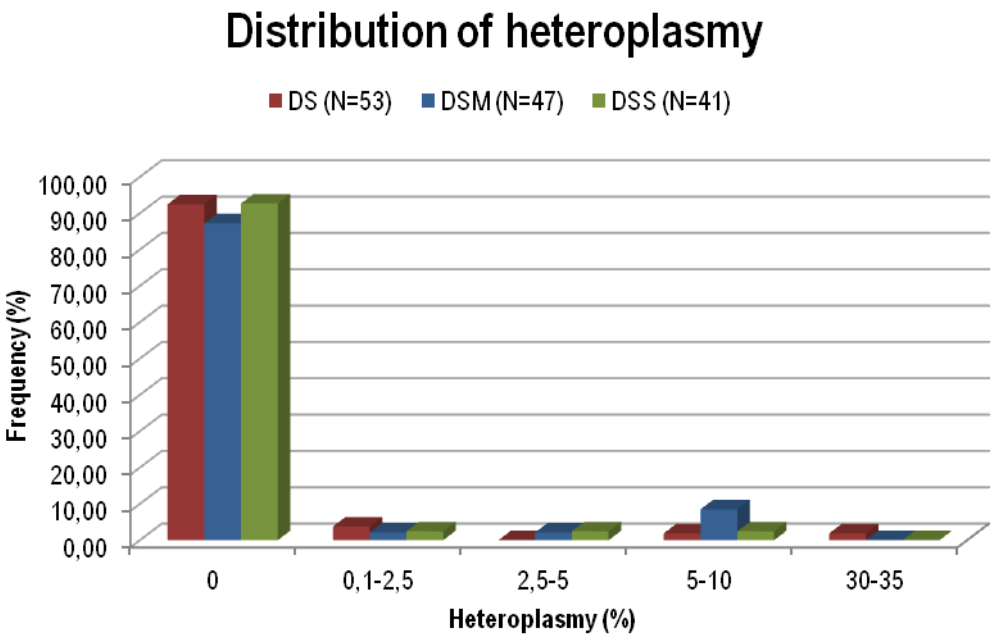


Figure 4: distribution of the levels of heteroplasmy in DS, DSM and DSS subjects.

The number of heteroplasmic samples is so low that any other correlation, such as with mtDNA haplogroups or haplotype, resulted not statistically significant.

5. APOE genotype

We determined the APOE genotype and the corresponding allele frequencies in the three groups under study. As expected APOE ε3/ε3 is the most common genotype in all samples. In particular we found any DS subject with two ε4 allele, 10 (18,87%) with at least one ε4 allele and 43 (81.13%) without ε4 allele; in DSM group we found respectively 0%, 21.28% and 78.72%; in DSS subjects we found respectively 4.88%, 19.51% and 75.61%.

The number of subjects, the respective percentage for each of the six genotypes and the allele frequencies for DS, DSM and DSS, estimated by counting alleles and compared with those previously reported in literature [Mahley and Rall, 2000], are shown in **Table 7**.

Genotype	DS N=53 (%)	DSM N=47 (%)	DSS N=41 (%)	
ε2/ε2	0 (0)	2 (4,26)	0 (0)	
ε2/ε3	6 (11,32)	4 (8,51)	4 (9,76)	
ε3/ε3	37 (69,81)	31 (65,96)	27 (65,85)	
ε2/ε4	0 (0)	2 (4,26)	1 (2,44)	
ε3/ε4	10 (18,87)	8 (17,02)	7 (17,07)	
ε4/ε4	0 (0)	0 (0)	2 (4,88)	
Alleles	**DS N=106 (%)**	**DSM N=92 (%)**	**DSS N=82 (%)**	**Reference (*) (%)**
ε2	6 (5,7)	10 (10,9)	5 (6,1)	6,6
ε3	90 (84,9)	72 (78,3)	65 (79,3)	84,8
ε4	10 (9,4)	10 (10,9)	12 (14,6)	8,2

(*) Mahley and Rall, 2000

Table 7: Comparison of the six different APOE genotypes, and of the allelic frequencies between DS patients and DSM and DSS and compared with those reported by Mahley and Rall, 2000.

The distribution of our sample by group, gender and APOE4 status (**Table 8**) showed no significant difference.

		DS (N=53)	DSM (N=47)	DSS (N=41)
Female		(N=27)	(N=47)	(N=28)
Age (years)	(mean ± SD)	27.0 ± 14.9	56.9 ± 9.5	32.1 ± 14.4
APOE4 status	APOE4+	18.5%	23.4%	28.6%
	APOE4-	81.5%	76.6%	71.4%
Male		(N=26)	(N=0)	(N=13)
Age (years)	(mean ± SD)	29.6 ± 9.9	0	28.4 ± 9.6
APOE4 status	APOE4+	19.2%	0%	15.4%
	APOE4-	80.8%	0%	84.6%

Table 8: Characteristics of the study participants subdivided by group, gender and APOE4 status.

We have also matched the macro-haplogroups with APOE4 status (**Figure 5**) in order to identify the potential sub-haplogroup associated with Down Syndrome. We found no significant differences between the three groups under study.

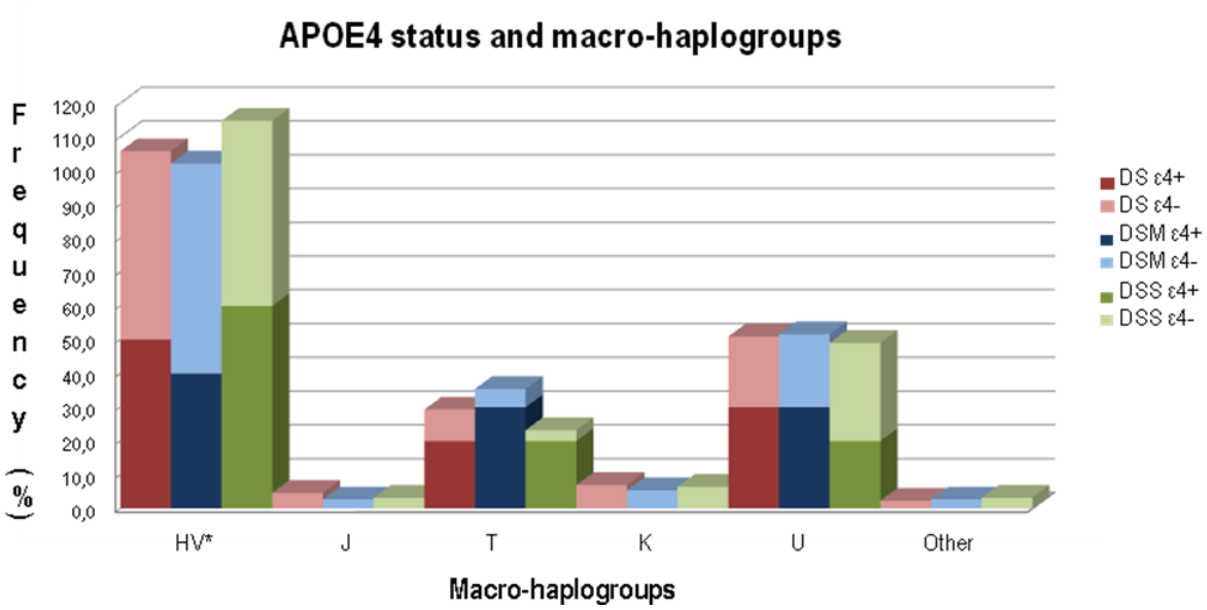


Figure 5:Distribution of macro-haplogroups by APOE4 status.

Analyzing the three groups separately, we can see some haplogroups statistically associated with APOE4 status (**Table 9**). In particular, in DS subjects haplogroups K is statistically associated with the absence of ε4 allele (OR = 0.0, 95% C.I.= 0.0-0.67), also in DSS subjects, haplogroups K is statistically associated with the APOE4- (OR = 0.0, 95%C.I. = 0.0-0.83). In DSM groups the haplogroups statistically significant are HV*, associated with APOE4-, and T, associated with APOE4+, (OR = 0.45, 95%C.I. = 0.24-0.81 and OR = 4.89, 95%C.I. = 2.03-13.13). Finally, in DSS subjects also haplogroup T is statistically significant (OR = 8.01, 95%C.I. = 2.26-43.62) and associated with the presence of APOE ε4 allele.

	APOE4- *vs* APOE4+								
	DS			**DSM**			**DSS**		
mtDNA macro-haplogroups	**p-value**	**OR**	**95%C.I.**	**p-value**	**OR**	**95%C.I.**	**p-value**	**OR**	**95%C.I.**
HV*	0.5712	0.81	0.45-1.48	**0.00706**	**0.45**	**0.24-0.81**	0.5673	1.23	0.67-2.24
J	0.05938	0.0	0.0-1.07	0.2462	0.0	0.0-2.41	0.2462	0.0	0.0-2.41
T	0.1165	1.99	0.84-5.02	**0.0000356**	**4.89**	**2.03-13.13**	**0.000224**	**8.01**	**2.26-43.62**
K	**0.01402**	**0.0**	**0.0-0.67**	0.05938	0.0	0.0-1.07	**0.02893**	0.0	**0.0-0.83**
U	0.1941	1.61	0.81-3.25	0.1941	1.61	0.81-3.25	0.1881	0.61	0.30-1.23
Other	0.4975	0.0	0.0-5.32	0.2462	0.0	0.0-2.41	0.2462	0.0	0.0-2.41

Table 9: Fisher exact test (p-value), Odds ratio and 95% Confidence Intervals for macro-haplogroups associated with APOE4 status.

In order to evaluate if ε4 might correlate with the mental retardation, we analysed the APOE4 status with the cognitive scores of the DS subjects. In fact 47 DS subjects were carefully evaluated for their cognitive status and we got access to these data; 37 subjects (mean age 32, SD 13.5) have no ε4 allele and 10 subjects (mean age 25, SD 8.3) have at least one ε4 allele. All subjects had full IQ value < 65 points. The two DS subgroups were not statistically different in mean total IQ (TIQ), verbal IQ (VIQ) and performance IQ (PIQ) values (**Table 10**).

	APOE4- (N=37)	**APOE4+ (N=10)**
TIQ	44.4 ± 14.7	43.6 ± 17.0
VIQ	47.8 ± 15.7	46.6 ± 17.7
PIQ	46.8 ± 15.3	45.6 ± 17.7

Table 10: neuropsychological profile of DS subjects, subdivided by APOE4 status.

Chapter 7

DISCUSSION AND CONCLUSIONS

Down Syndrome (DS) is defined as a genetic disorder in which multiple major aspects of the senescent phenotype appear. In fact, it has been shown that most features of physiological aging such as skin and hair changes, visual and hearing impairments, thyroid dysfunction, diabetes, obesity, immune and central nervous systems changes [Esbensen, 2010] appear in DS earlier than in karyotypically normal age-matched subjects. In particular, adults with DS experience a decline of cognitive function which is reported to be in many cases an Alzheimer-like dementia, as well as an altered free radical metabolism and impaired mitochondrial function that lead to neuronal degeneration from 30-35 years of age onwards – thirty to forty years earlier than in the normal population. [Lott et al. 2001; Busciglio et al. 2002 and 2007; Haan et al. 2003],

It is thought that the oxidative stress, resulting from an imbalance in the metabolism of free radicals such as reactive oxygen species (ROS), have a direct role in the development of neuropathological changes of AD in DS [Kedziora and Bartosz, 1988; Busciglio et al. 1998]. Predisposing factors such as aging, DS, or gene mutations associated with familial AD contribute by either increasing oxidative stress [Carney J and Carney A, 1994] or Aβ production and deposition [Selkoe, 1997], which impairs mitochondrial function, resulting in energy depletion followed by further neuronal degeneration [Flint Beal, 1992].

Mitochondria are the most susceptible target of ROS formation and mutations in the mtDNA control region occur in demented individuals with DS [Coskun et al. 2010].

In the present study, we investigated an integrated set of molecular genetic parameters (sequencing of complete mtDNA, heteroplasmy of the mtDNA control region, genotypes of APOE gene) in order to identify a possible association with the early neurocognitive decline observed in DS.

1. mtDNA analysis

Previous reports have suggested that mtDNA mutations have a role in the pathogenesis and aetiology of DS [Arbuzova, 1995, 1996; 1998 and 2002]. In particular three new point mutations were found in the complete mtDNA sequence of three DS individuals in the following genes: ATP-ase (G8764A) and

ND5 (G13243A) with the same aminoacid substitutions Ala/Thr, and ND1 (G3337A) with aminoacid substitution Val/Met; Arbuzova hypothesized that these mtDNA mutations explain the association of DS with premature ageing and diabetes, which are more common in the mothers of DS individuals. Furthermore mtDNA is entirely of maternal origin, as is the extra chromosome 21 in the majority of DS cases, and examination of pedigrees from families with aneuploidy recurrence indicates cytoplasmic inheritance as a risk factor [Arbuzova, 2002]. In this regard, it was shown that in amniocytes of DS fetuses decreased ATPase6 gene expression during oocyte meiotic maturation reduces the capacity for oxidative phosphorylation and influences ATP generation, leading to chromosomal nondisjunction [Lee et al. 2003].

Coskun et al. (2010) support the hypothesis that mitochondrial dysfunction is a major factor in the age related dementia associated with advanced age DS patients. In fact, they observed a high frequency of mtDNA regulatory control region homoplasmic and heteroplasmic mutations in the brains of AD and DS and dementia patients. These mutations would be expected to affect mtDNA replication and L-strand transcription, following by reduction in the mtDNA copy number and an overall decline in OXPHOS. These reductions would result in reduced mitochondrial energy output, increased mitochondrial ROS production, reduced mitochondrial membrane potential and thus altered calcium regulation, and an increased probability for the activation of the mtPTP, predisposing neurons to loss of processes and ultimately death.

Our database is the first of this kind in the literature because it compares DS subjects with their mothers and siblings matched for age and gender. So we can be sure that any mutations found in the DS subjects would be typical of the syndrome or maternally inherited.

In our DS subjects we found 19 novel mutations in DS subjects (seven of them non-synonymous), but the same SNPs were also found in the complete mtDNA sequences of mothers and siblings. Only two novel mutations (het4569, 10365) are DS specific, of which one is non-synonymous. Comparing the number of mutations along mtDNA molecule by mtDNA regions, we found that the mutations accumulate equally in the three groups analyzed and even when we analyzed the sporadic mutations (showing up in one sample only), the DS, DSM and DSS groups under study resulted balanced. We hypothesize that oxidative stress, documented in DS, does not depend firstly by specific mtDNA mutations, but rather by a general dysfunction of mitochondria, involving the antioxidant systems.

Michikawa et al. (1999) reported that some mutations in the CR, such as T414G, T408A, A189G, accumulate with age in specific tissues, but most recently a C150T mutation has been identified in lymphocytes of centenarians and twins [Zhang et al. 2003]. This study is of great value because it indicates a possible beneficial effect on longevity by a mtDNA somatic mutation able to restore the mitochondrial replication machinery. In this regard, Rose et al. (2007 and 2010) argued that the patterns of mtDNA CR heteroplasmy do not differ between centenarians and their descendants, but differ between relatives of centenarians and age-matched controls, in which heteroplasmy is lower. Therefore it seems that mtDNA

CR heteroplasmy is genetically controlled and that it overcomes the age-related mitochondrial dysfunction with mitochondria complementation.

Rose et al. were the first to compare the levels of heteroplasmy between relatives, but there are many reports [Meierhofer et al. 2005; Irwin et al. 2009; Sondheimer et al. 2011] which, analyzing the heteroplasmy with other methods, as well as DHPLC, have demonstrated that different sites and portions of the control region exhibit greatly different levels of population variation and rates of phylogenetic sequence evolution. Moreover, Sondheimer et al. (2011), examining mother-child pairs, found that heteroplasmy was inherited for the 30% and for the 70%, occur *de novo* in offspring or, conversely, be present in mothers but eliminated in their children.

Most of our samples do not display heteroplasmy, (92.45% of DS, 87.23% of DSM and 92.68% of DSS, respectively). Approximately 4% of DS subjects and 2% of both DSM and DSS subjects have levels of heteroplasmy lower than 2.5%. Levels of heteroplasmy between 2.5%-5% are present only in both DSM and DSS (2.13% and 2.44% respectively), while 2% of both DS and DSS and 8% of DSM have an heteroplasmy between 5%-10%. Finally only one DS subject has an heteroplasmy at 33%. Our data are in the same direction of previous reports since we observe an upward trend in the levels of heteroplasmy with age in DS and DSS groups, but there is no correlation in the members of the same family, except for two families: in one there is correlation between DS and DSM, in the other the correlation is between DSM and DSS. So it seems that heteroplasmy accumulates because of stochastic events.

2. APOE genotypes analysis

We have already said that DS subjects are characterized by a dementia Alzheimer-like, with also neuropathological characteristics of AD. The recognized genetic risk factor for developing AD is represented by the presence of APOE ε4 allele; however, several studies [Lott, 1992; Wisniewski et al. 1995] have demonstrated that the development of pathological changes in DS does not always parallel that observed in AD patients and that the ε4 allele frequency is the same between the AD-DS cases and DS controls [Val Gool et al. 1995]; on the contrary, others studies found that demented adults with DS showed a significantly higher frequency of ε4 allele compared to non-demented adults [Deb et al. 2000].

Within our sample we have observed that DS patients do not differ in terms of APOE alleles frequency neither from the general population nor from DSM and DSS subjects. It can be hypothesized that the early neurocognitive decline of DS subjects depends on the overexpression of the other proteins encoded by Chr 21, such as APP (amyloid precurson protein), that is the precursor of Aβ and consequently determines the formation of senile plaques.

Furthermore, there is large amount of evidence about the role of mitochondria in the pathogenesis of AD even if several studies reported lack of association between mtDNA haplogroups and AD [van der Walt et al. 2005; Mancuso et al. 2007], while others found positive associations [van der Walt et al. 2004; Maruszak et al. 2009; Santoro et al. 2010]. In our samples we found that haplogroup T appears to be associated with APOE ε4 allele in the three groups analyzed; nevertheless the amount of our samples is too low for considering reliable this result.

Summarizing, with the present work we aimed at identifying genetic determinants of Down Syndrome. Specifically, the major objectives were the following:

1. to analyze the mtDNA from a quantitative and qualitative perspective, in order to argue if it can be considered risk factor for the DS decline;
2. to genotype APOE gene, in order to evaluate ε4 allele and its association with the DS cognitive status.

In conclusion, our study highlighted that mtDNA somatic mutations (either in heteroplasmy or homoplasmy) does not play a role in the development of AD-like dementia in our sample of DS subjects, that was bona fide present in 9 subjects (scoring in the stage of profound cognitive impairment according to IQ assessment – for instance indicated as score 19). We could exclude also that these subjects were bringing the APOE4 allele more frequently than those who scored higher.

Probably, the analysis of mitochondria is the correct way to understand the complex traits of Down Syndrome; nevertheless studies should focus on the mitochondrial functionality and/or on another tissue other than whole blood.

APPENDIX

TBE 10X (TRIS BORATO EDTA) 1L

108 gr TRIS BASE

55 gr boric acid

40 ml EDTA 0.5X pH=8

TBE 1X 1L

100 ml TBE 10X

900 ml distilled H_2O

AGAROSE GEL 0.8 %, 1.5%, 2% IN TBE 1X 250 ML (it changes only the amount of agarose)

250 ml TBE 1X

2.0 gr, 3.75 gr, 5.0 gr agarose

12.5 ml Gel Red staining (5 µl in 100 ml)

MS AGAROSE GEL 4.5% IN TBE 1X 250 ML

250 ml TBE 1X

11.25 gr MS agarose

12.5 ml Gel Red staining (5 µl in 100 ml)

NaAc

P.M.=82.03 g/moli

A final concetration of 3M pH=4.6.

Moles= 3M*0.2 l=0.6 moles

Grams= 0.6 moli*82.03 g/moles=49.218g

PRIMERS FOR COMPLETE mtDNA AMPLIFICATION

Name	Start (5')	End (5')	Length	Primer Fw	Primer Rv
38	16187	275	657	5' TGT AAA ACG ACG GCC AGT CCT CCC CAT GCT TAC A 3'	5' CAG GAA ACA GCT ATG ACC CTG TGT GGA AAG CGG 3'
22	6	503	497	5' TGT AAA ACG ACG GCC AGT CAG GTC TAT CAC CCT ATT 3'	5' CAG GAA ACA GCT ATG ACC GGG TTG TAT TGA TGA GAT TA 3'
39	318	927	609	5' TGT AAA ACG ACG GCC AGT TGG CCA CAG CAC TTA AAC 3'	5' CAG GAA ACA GCT ATG ACC CTA TTG ACT TGG GTT AAT CG 3'
21	720	1074	354	5' TGT AAA ACG ACG GCC AGT CCC GTT CCA GTG AGT T 3'	5' CAG GAA ACA GCT ATG ACC CCC AGT TTG GGT CTT AG 3'
31	873	1431	558	5' TGT AAA ACG ACG GCC AGT GGT TGG TCA ATT TCG TGC 3'	5' CAG GAA ACA GCT ATG ACC CTG CTA AAT CCA CCT TCG 3'
18	1174	1770	596	5' TGT AAA ACG ACG GCC AGT TGG CGG TGC TTC ATA TC 3'	5' CAG GAA ACA GCT ATG ACC CGC CAG GTT TCA ATT TCT 3'
1	1485	2078	593	5' TGT AAA ACG ACG GCC AGT GCC CGT CAC CCT C 3'	5' CAG GAA ACA GCT ATG ACC GGG ATT TAG AGG GTT CT 3'
2	1873	2441	568	5' TGT AAA ACG ACG GCC AGT AAC TTT GCA AGG AGA GCC 3'	5' CAG GAA ACA GCT ATG ACC GCA TGC CTG TGT TGG 3'
3	2201	2797	596	5' TGT AAA ACG ACG GCC AGT GCG TTC AAG CTC AAC AC 3'	5' CAG GAA ACA GCT ATG ACC GCA GGT TTG GTA GTT TAG 3'
4	2571	3170	599	5' TGT AAA ACG ACG GCC AGT GCG GTA CCC TAA CCG 3'	5' CAG GAA ACA GCT ATG ACC GGG AAG GCG CTT TGT 3'
23	2927	3527	600	5' TGT AAA ACG ACG GCC AGT CCC TAG GGA TAA CAG 3'	5' CAG GAA ACA GCT ATG ACC GCG GTG ATG TAG AGG 3'
5	3306	3890	584	5' TGT AAA ACG ACG GCC AGT CAT ACC CAT GGC CAA C 3'	5' CAG GAA ACA GCT ATG ACC CGG TTG GTC TCT GC 3'
24	3640	4167	527	5' TGT AAA ACG ACG GCC AGT CCT CTA GCC TAG CCG 3'	5' CAG GAA ACA GCT ATG ACC GTG TAT GAG TTG GTC GTA 3'
40	3897	4533	636	5' TGT AAA ACG ACG GCC AGT CTT CGA CCT TGC CGA A 3'	5' CAG GAA ACA GCT ATG ACC GCG CTG TGA TGA GTG T 3'
25	4389	4987	598	5' TGT AAA ACG ACG GCC AGT CAC CCC ATC CTA AAG TAA 3'	5' CAG GAA ACA GCT ATG ACC GTT TGG TTT AAT CCA CCT CAA 3'
41b	4851	5484	633	5' TGT AAA ACG ACG GCC AGT CTG CTT CTT CTC ACA TGA 3'	5' CAG GAA ACA GCT ATG ACC TAG GTA GGA GTA GCG TG 3'
26	5261	5930	669	5' TGT AAA ACG ACG GCC AGT GGC CAT TAT CGA AGA ATT C 3'	5' CAG GAA ACA GCT ATG ACC AGA GAA TAG TCA ACG GTC 3'
27	5696	6263	567	5' TGT AAA ACG ACG GCC AGT CAG CTA AGC ACC CTA ATC 3'	5' CAG GAA ACA GCT ATG ACC GGC CTC CAC TAT AGC A 3'
28	5995	6537	542	5' TGT AAA ACG ACG GCC AGT CAG CTC TAA GCC TCC T 3'	5' CAG GAA ACA GCT ATG ACC CTG TTA GTA GTA TAG TGA TG 3'
42	6425	6892	467	5' TGT AAA ACG ACG GCC AGT TGC CAT AAC CCA ATA CCA 3'	5' CAG GAA ACA GCT ATG ACC CTT CCG TGG AGT GTG 3'
43b	6791	7398	607	5' TGT AAA ACG ACG GCC AGT ATC AAT TGG CTT CCT AGG 3'	5' CAG GAA ACA GCT ATG ACC GGC ATC CAT ATA GTC ACT 3'
29	7233	7805	572	5' TGT AAA ACG ACG GCC AGT CCC GAT GCA TAC ACC 3'	5' CAG GAA ACA GCT ATG ACC CTA GGA TGA TGG CGG G 3'
44b	7681	8274	593	5' TGT AAA ACG ACG GCC AGT CCT TAT CTG CTT CCT AGT 3'	5' CAG GAA ACA GCT ATG ACC GGG TGC TAT AGG GTA AAT 3'
19	8161	8671	510	5' TGT AAA ACG ACG GCC AGT CTA CGG TCA ATG CTC TG 3'	5' CAG GAA ACA GCT ATG ACC GTC ATT GTT GGG TGG TG 3'
30	8533	9060	527	5' TGT AAA ACG ACG GCC AGT GAA AAT CTG TTC GCT TCA TT 3'	5' CAG GAA ACA GCT ATG ACC GGT GGC GCT TCC AAT T 3'
6	8775	9336	561	5' TGT AAA ACG ACG GCC AGT CCT CCT CGG ACT CC 3'	5' CAG GAA ACA GCT ATG ACC TGA GGA GCG TTA TGG AG 3'
16	9046	9642	596	5' TGT AAA ACG ACG GCC AGT ATT GGA AGC GCC ACC C 3'	5' CAG GAA ACA GCT ATG ACC AGG TGA TTG ATA CTC CTG 3'
32	9443	10005	562	5' TGT AAA ACG ACG GCC AGT CGA TAC GGG ATA ATC CT 3'	5' CAG GAA ACA GCT ATG ACC TTA TAC TAA AAG AGT AAG ACC C 3'
33	9751	10280	529	5' TGT AAA ACG ACG GCC AGT CGA GTC TCC CTT CAC 3'	5' CAG GAA ACA GCT ATG ACC GGG TAA AAG GAG GGC A 3'
45b	10121	10724	603	5' TGT AAA ACG ACG GCC AGT ATT TTG ACT ACC ACA ACT CA 3'	5' CAG GAA ACA GCT ATG ACC ATA TGT GTT GGA GAT TGA GA 3'
7	10657	11237	580	5' TGT AAA ACG ACG GCC AGT TAC TAG TCT TTG CCG CC 3'	5' CAG GAA ACA GCT ATG ACC GGG GAA GGG AGC CTA 3'
8	10999	11595	596	5' TGT AAA ACG ACG GCC AGT CCA ACG CCA CTT ATC C 3'	5' CAG GAA ACA GCT ATG ACC TGT CGT AGG CAG ATG G 3'
34	11368	11934	566	5' TGT AAA ACG ACG GCC AGT CTT ATG ACT CCC TAA AGC 3'	5' CAG GAA ACA GCT ATG ACC GTG ATA TTT GAT CAG GAG AA 3'
35	11754	12194	440	5' TGT AAA ACG ACG GCC AGT CAA ACT ACG AAC GCA CTC 3'	5' CAG GAA ACA GCT ATG ACC GTC GTA AGC CTC TGT TG 3'

17	12007	12560	553	5' TGT AAA ACG ACG GCC AGT GGG CTC ACT CAC CCA 3'	5' CAG GAA ACA GCT ATG ACC TGG GTT GTT TGG GTT G 3'
9	12395	12994	599	5' TGT AAA ACG ACG GCC AGT TTA CCA CCC TCG TTA ACC 3'	5' CAG GAA ACA GCT ATG ACC TGC TAG GAG GAG GC 3'
36	12787	13346	559	5' TGT AAA ACG ACG GCC AGT CCT TCT TGC TCA TCA GTT 3'	5' CAG GAA ACA GCT ATG ACC GCT TTG AAG AAG GCG TG 3'
10	13198	13712	514	5' TGT AAA ACG ACG GCC AGT GCA GCA GTC TGC GC 3'	5' CAG GAA ACA GCT ATG ACC GCT GCC AGG CGT TTA A 3'
46	13515	14124	609	5' TGT AAA ACG ACG GCC AGT CCA CAT CAT CGA AAC CG 3'	5' CAG GAA ACA GCT ATG ACC GAT GAG TGG GAA GAA GAA 3'
11	13802	14373	571	5' TGT AAA ACG ACG GCC AGT CAG CCC TCG CTG TC 3'	5' CAG GAA ACA GCT ATG ACC GGA TTG GTG CTG TGG G 3'
20	14224	14811	587	5' TGT AAA ACG ACG GCC AGT ACG CCC ATA ATC ATA CAA AG 3'	5' CAG GAA ACA GCT ATG ACC GGG AGG TCG ATG AAT G 3'
12	14453	15051	598	5' TGT AAA ACG ACG GCC AGT GCC ATC GCT GTA GTA TAT 3'	5' CAG GAA ACA GCT ATG ACC AGG CCT CGC CCG AT 3'
13	14860	15455	595	5' TGT AAA ACG ACG GCC AGT CGC CTG CCT GAT CC 3'	5' CAG GAA ACA GCT ATG ACC GAA GGA AGA GAA GTA AGC 3'
14	15257	15812	555	5' TGT AAA ACG ACG GCC AGT GAC AGT CCC ACC CTC 3'	5' CAG GAA ACA GCT ATG ACC GGA TGC TAC TTG TCC 3'
15	15608	16185	577	5' TGT AAA ACG ACG GCC AGT CTA GGA GGC GTC CTT G 3'	5' CAG GAA ACA GCT ATG ACC GGT TTT GAT GTG GAT TGG 3'
37	15960	16548	588	5' TGT AAA ACG ACG GCC AGT AGA AAA AGT CTT TAA CTC CAC 3'	5' CAG GAA ACA GCT ATG ACC GGG AAC GTG TGG GC 3'

REDESIGNED PRIMERS FOR LONG PCR

Name	Start (5')	End (5')	Length	Primer Fw	primer lenght	Tm	%GC
7b	10610	11334	724	AAC CCT CAA CAC CCA C	16	46	56,3
20b	14222	14897	675	CAA CGC CCA TAA TCA TAC	18	46	44,4
26c	5311	5990	679	TCA TAG CCA CCA TCA CC	17	47	51
27b	5690	6360	670	AGT TAA CAG CTA AGC ACC C	19	49	48
45c	10128	10830	702	CTA CCA CAA CTC AAC GG	17	47	52
47	245	965	720	TTG AAT GTC TGC ACA GCC	18	48	50
482F	482	1180	698	TCA TCA ATA CAA CCC CCG	18	48	50
3113F	3113	3798	685	ATT CCT CCC TGT ACG AA	17	45	47,1
3316F	3316	4020	704	GCC AAC CTC CTA CTC CT	17	49	58,8
4143F	4143	4840	697	ATT CCG CTA CGA CCA ACT	18	48	50
4897F	4897	5573	676	ACC AAA TCT CTC CCT CA	17	45	47,1
5807F	5807	6450	643	ATG AAA ATC ACC TCG GAG C	19	49	47,4
7706F	7706	8372	666	GCC CTT TTC CTA ACA CTC	18	48	50
14985F	14985	15637	652	GCT ACC TTC ACG CCA AT	17	47	52

SUB-HAPLOGROUP OF EACH FAMILY RECRUITED

Family (N=37)		Family (N=10)		Family (N=4)		Family (N=2)	
DS-DSM-DSS	**Sub-Haplogroup**	**DS-DSM**	**Sub-Haplogroup**	**DS-DSS**	**Sub-Haplogroup**	**DS**	**Sub-Haplogroup**
1	V	17	H2a2	27	J1b1a	1047	K2a
2	H2a2a	24	H2a2a	41	H5a1	1053	T2b
3	K1a	25	H6a1	45	U5a1a1		
4	U5b2a	31	T2b	49	H5b		
5	HV0	32	H2a2a				
6	H1	33	J2b1				
7	U4b1a1	34	H3				
8	H1	35	T2e1				
9	U5a1a1	48	H6a1b				
10	H7a1	50	U5b1c				
11	H3						
12	U4a						
13	H						
14	H1						
15	L2a1						
16	U5a2a						
18	U3b2						
19	H4a						
20	T1						
21	U5a2b						
22	HV2						
23	U5a1b						
26	T2b3a						
28	K2a						
29	U4c1a						
30	H3						
36	T2c1b						
37	H1c						
38	HV0						
39	H14a						
40	H1e1						
42	H1e2						
43	H5a1						
44	U5b2a1a						
46	HV10						
51	H13a1a						
52	HV0c						

REFERENCES

Adayev, T., Chen-Hwang, M.C., Murakami, N., Wang, R. and Hwang, Y.W. (2006) MNB/DYRK1A phosphorylation regulates the interactions of synaptojanin 1 with endocytic accessory proteins. Biochem. Biophys. Res. Commun., 351, 1060–1065.

Ahn, K.J., Jeong, H.K., Choi, H.S., Ryoo, S.R., Kim, Y.J., Goo, J.S., Choi, S.Y., Han, J.S., Ha, I. and Song, W.J. (2006) DYRK1A BAC transgenic mice show altered synaptic plasticity with learning and memory defects. Neurobiol. Dis., 22, 463–472.

Aitchinson C, Easty DL, Jancar J. Eye abnormalities in the mentally handicapped. Journal of Mental Deficiency Research 1990;34:41–48

Alexeyev M. F., LeDoux S. P., Wilson G. L.: Mitochondrial and aging, Clin Sci (Lond) 2004;107(4): 355-64

Allen EG, (2009) EG, Freeman SB, Druschel C, Hobbs CA, O'Leary LA, Romitti PA, Royle MH, Torfs CP, Sherman SL.: "Maternal age and risk for trisomy 21 assessed by the origin of chromosome nondisjunction: a report from the Atlanta and National Down Syndrome Project."- Hum Genet. Feb;125(1):41-52

Altafaj, X., Dierssen, M., Baamonde, C., Marti, E., Visa, J., Guimera, J., Oset, M., Gonzalez, J.R., Florez, J., Fillat, C. et al. (2001) Neurodevelopmental delay, motor abnormalities and cognitive deficits in transgenic mice overexpressing Dyrk1A (minibrain), a murine model of Down's syndrome. Hum. Mol. Genet., 10, 1915–1923.

Amano, K. et al. (2004) Dosage-dependent over-expression of genes in the trisomic region of Ts1Cje mouse model for Down syndrome. Hum. Mol. Genet. 13, 1333–1340

Anderson, S., Bankier, A. T., Barrell, B. G. et al. (1981) Sequence and organization of the human mitochondrial genome. Nature (London) 290, 457–465

Andreu AL, Bruno C, Dunne TC, Tanji K, Shanske S, et al. 1999. A nonsense mutation (G15059A) in the cytochrome b gene in a patient with exercise intolerance and myoglobinuria. Ann. Neurol. 45:127–30

Anneren KG, Epstein CJ. Lipid peroxidation and superoxide dismutase-1 and glutathione peroxidase activities in trisomy 16 fetal mice and human trisomy 21 fibroblasts. Pediatr Res 1987;21:88–92

Antonarakis S.E. and Epstein C.J. (2006) The challenge of Down syndrome. Trends in Mol. Med. 12, 473-479

Appel E, Eisenberg S, Roitelman J. 1995. Improved PCR amplification/Hhal restriction for unambiguous determination of apolipoprotein E alleles. Clin Chem.; 41(2):187-90

Aranda, S., Alvarez, M., Turro, S., Laguna, A. and de la, L.S. (2008) Sprouty2-mediated inhibition of fibroblast growth factor signaling is modulated by the protein kinase DYRK1A. Mol. Cell. Biol., 28, 5899–5911.

Arbuzova S. About the role of the mitochondrial DNA in the etiology of the regular trisomy 21. Cytology and Genetics 1995; 29: 77–80 (in Russian)

Arbuzova S. Free radicals in aetiology and pathogenesis of Down's syndrome. Cytology and Genetics 1996;30:25–34 (in Russian)

Arbuzova S. Why it is necessary to study the role of the mitochondrial genome in trisomy 21 pathogenesis? Down's Syndrome Research and Practice 1998;5:26–29

Arbuzova S, Cuckle H, Mueller R, Sehmi I. Familial Down syndrome: evidence supporting cytoplasmic inheritance. Clin Genet 2001; 60: 456–462

Arbuzova S, Hutchin T, and Cuckle H. 2002. Mitochondrial dysfunction and Down's syndrome. BioEssays 24: 681-684

Armstrong RA, Smith CUM. 1994. b-Amyloid (b/A4) deposition in the medical temporal lobe in Down syndrome: Effects of brain region and patient age. Neurobiol Dis 1:139–144

Arron, J.R. et al. (2006) NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. Nature 441, 595–600

Atzmon G, Rincon M, Rabizadeh P, Barzilai N: Biological evidence for inheritance of exceptional longevity. Mech Ageing Dev 2005, 126:341-345.

Aylward, E.H. et al. (1997) Cerebellar volume in adults with Down syndrome. Arch. Neurol. 54, 209–212

Azizeh BY, Head E, Ibrahim MA, et al. 2000. Molecular dating of senile plaques in aged Down syndrome and canine brains. Exp Neurol 163:111–122

Ballinger SW, Shoffner JM, Hedaya EV, Trounce I, Polak MA, et al. 1992. Maternally transmitted diabetes and deafness associated with a 10.4 kb mitochondrial DNA deletion. Nat. Genet. 1:11–15

Ballinger SW, Shoffner JM, Gebhart S, Koontz DA,Wallace DC. 1994. Mitochondrial diabetes revisited. Nat. Genet. 7:458–59

Bambrick L, Fiskum G. 2008. Mitochondrial dysfunction in mouse trisomy 16 brain. Brain Res 1188:9–16

Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116, 281–297.

Battaglia, D. E., Goodwin, P., Klein, N. A., and Soules, M. R. (1996) Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. Hum. Reprod. 11, 2217−2222.

Becker, L.E., Mite, T., Takashima, S., Onodera, K., Friend, W.C. (1993) Association of phenotypic abnormalities of Down syndrome with an imbafance of genes on chromosome 21. APMIS, Suppl. 40, 101: 57-70

Beerman, F. and Hansmann, I. (1986) Follicular maturation, luteinization and first meiotic division in oocytes after inhibiting mitochondrial function in mice with chloramphenicol. Mut. Res. 160, 47−54.

Beerman, F., Hummler, E., Franke, U., and Hansmann, I. (1988) Maternal modulation of the inheritable meiosis I error Dipl in mouse oocytes is associated with the type of mitochondrial DNA. Hum. Genet. 79, 338−340.

Behl C, Davis JB, Lesley R & Shubert D. 1994. Hydrogen peroxide mediates amyloid β protein toxicity. Cell 77: 817–827.

Belichenko, P.V. et al. (2004) Synaptic structural abnormalities in the Ts65Dn mouse model of Down Syndrome. J. Comp. Neurol. 480, 281– 298

Bellance N, Lestienne P, Rossignol R: Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis, 2009; Front Biosci.1;14: 4015-34

Bertrand I, Koffas D. 1946. Cas d'idiotie mongolienne adulte avec nombrueses plaues seniles et concretions calcarires pallidales. Rev Neurol 78:338–43

Bialowas-McGoey L, Lesicka A, Whitaker-Azmitia P. 2008. Vitamin E increases S100B-mediated microglial activation in an S100B overexpressing mouse model of pathological aging. Glia 56:1780–1790

Bittles AH, Glasson EJ. Clinical, social, and ethical implications of changing life expectancy in Down syndrome. Developmental Medicine and Child Neurology 2004;46:282–286

Bras A, Monteiro C, Rueff J. Oxidative stress in trisomy 21. A possible role in cataractogenesis. Ophthalmic Paediatr Genet 1989;10:271–277

Brooksbank BW, Balazs R. Superoxide-dismutase, glutathione peroxidase and lipoperoxidation in Down's syndrome fetal brain. Brain Res 1984;318:37–44

Brown MD, Sun F, Wallace DC. 1997. Clustering of Caucasian Leber hereditary optic neuropathy patients containing the 11778 or 14484 mutations on an mtDNA lineage. Am. J. Hum. Genet. 60:381–87

Brown MD, Trounce IA, Jun AS, Allen JC,Wallace DC. 2000. Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's Hereditary Optic Neuropathy mtDNA mutation. J. Biol. Chem. 275:39831–36

Brown MD, Zhadanov S, Allen JC, Hosseini S, Newman NJ, et al. 2001. Novel mtDNA mutations and oxidative phosphorylation dysfunction in Russion LHON families. Hum. Genet. 109:33–39

Brugge KL, Grove GL, Clopton P, Grove MJ, Piacquadio DJ. Evidence for accelerated skin wrinkling among developmentally delayed individuals with Down's syndrome. Mechanics of Ageing and Development 1993;70:213–225

Bruno C, Martinuzzi A,Tang Y, Andreu AL, Pallotti F, et al. 1999. A stop-codon mutation in the human mtDNA cytochrome c oxidase I gene disrupts the functional structure of complex IV. Am. J. Hum. Genet. 65:611–20

Buchanan LH. Early onset of presbyacusis in Down syndrome. Scandinavian Audiology 1990;19:103–110

Burt DB, Loveland KA, Primeaux-Hart, et al. 1998. Dementia in adults with Down syndrome: diagnostic challenges. AmJ Ment Retard103(2):130–45

Busciglio J, Yankner BA. Apoptosis and increased generation of reactive oxygen species Down's syndrome neurons in vitro. Nature 1995;378: 776–779

Busciglio J, Andersen J, Schipper H, Gilad G, McCarty R, Marzatico F, Toussaint O. 1998. Stress, aging, and neurodegenerative disorders. Molecular mechanisms. Ann NY Acad Sci 851:429–443

Busciglio, J., Pelsman, A., Wong, C., Pigino, G., Yuan, M., Mori, H., Yankner, B.A., 2002. Altered metabolism of the amyloid _ precursor protein is associated with mitochondrial dysfunction in Down's syndrome. Neuron 33, 677–688.

Busciglio, J., Pelsman, A., Helguera, P., Ashur-Fabian, O., Pinhasov, A., Brenneman, D.E., Gozes, I., 2007. NAP and ADNF-9 protect normal and Down's syndrome cortical neurons from oxidative damage and apoptosis. Curr. Pharm. Des. 13, 1091–1098.

Canfield MA, Honein MA, Yuskiv N, Xing J, Mai CT, Collins JS, Devine O, Petrini J, Ramadhani TA, Hobbs CA: "National estimates and race/ethnic-specific variation of selected birth defects in the United States, 1999–2001."- Birth Defects Res. A Clin. Mol. Teratol., 76, 747–756 (2006).

Cann RL, Stoneking M, Wilson AC. 1987. Mitochondrial DNA and human evolution. Nature 325: 31–36

Canzonetta C, Mulligan C, Deutsch S, Ruf S, O'Doherty A, Lyle R, Borel C, Lin-Marq N, Delom F, Groet J: "DYRK1A-dosage imbalance perturbs NRSF/REST levels, deregulating pluripotency and embryonic stem cell fate in Down syndrome."- Am. J. Hum. Genet., 83, 388–400 (2008).

Capone GT. Down syndrome: advances in molecular biology and the neurosciences. J Dev Behav Pediatr 2001;22(1):40–59

Carney J. & Carney A. 1994. Role of protein oxidation in aging and in age-associated neurodegenerative diseases. Life Sci. 55: 2097–2103.

Carter DM, Jegasothy BV. Alopecia areata and Down syndrome. Archives of Dermatology 1976;112:1397–1399

Cavelier, L., Johannisson, A. and Gyllensten, U. (2000) Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. Exp. Cell Res. 259, 79–85

Chen XJ, Butow RA. 2005. The organization and inheritance of the mitochondrial genome. Nat Rev Genet 6: 815-825

Cheon MS, Shim KS, Kim SH, Hara A, Lubec G. Protein levels of genes encoded on chromosome 21 in fetal Down syndrome brain: challenging the gene dosage effect hypothesis (Part IV). Amino Acids 2003;25(1):41–7

Cleves MA, Hobbs CA, Cleves PA, Tilford JM, Bird TM, Robbins JM. 2007. Congenital defects among liveborn infants with Down syndrome. Birth Defects Res A Clin Mol Teratol.; 79(9): 657-63.

Coleman M. Thyroid dysfunction in Down's syndrome: A review. Down Syndrome Research and Practice 1994;2:112–115

Comi GP, Bordoni A, Salani S, Franceschina L, Sciacco M, et al. 1998. Cytochrome c oxidase subunit I microdeletion in a patient with motor neuron disease. Ann. Neurol. 43:110–16

Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, PC., Small, G.W., Roses, A.D., Haines, J.L., Pericak-Vance, M.A. (1993) Gene dose of apolipoprotein E type 4 aflele and the risk of Alzheimer's disease in late onset families. Science, 261: 921–923

Coskun PE, Pesini ER, Wallace DC (2003) Control region mtDNA variants: longevity, climatic adaptation, and a forensic conundrum PNAS 100, 2174-2176

Coskun P, Wyrembak J, Derbereva O, Melkonian G, Doran E, Lott I, Head E, Cotman C, Wallace D. 2010. Systemic mitochondrial dysfunction and the etiology of Alzheimer's disease and Down syndrome dementia. J Alzheimers Dis 20:S293–S310

Cossarizza A, Monti D, Montagnani G, Ortolani C, Masi M, Zannotti M, Franceschi C. 1990. "Precocious aging of the immune system in Down syndrome: alteration of B lymphocytes, T-lymphocyte subsets, and cells with natural killer markers." - Am J Med Genet Suppl, 7: 213-8

Cossarizza A, Ortolani C, Forti E, Montagnani G, Paganelli R, Zannotti M, Marini M, Monti D, Franceschi C. 1991. "Age-related expansion of functionally inefficient cells with markers of natural killer activity in Down's syndrome." - Blood, Mar 15;77(6): 1263-70

Cournil A, Kirkwood TB: If you would live long, choose your parents well. Trends Genet 2001, 17:233-235.

Coyle JT, Oster-Granite ML, Gearhart JD. 1986. The neurobiologic consequences of Down syndrome. Brain Res Bull 16:773–787

Dauphinot, L. et al. (2005) The cerebellar transcriptome during postnatal development of the Ts1Cje mouse, a segmental trisomy model for Down syndrome. Hum. Mol. Genet. 14, 373–384

Deb S, Braganza J, Norton N, et al. 2000. APOE epsilon 4 influences the manifestation of Alzheimer's disease in adults with Down syndrome. Br J Psychiatry 176:468–472

De Benedictis G, Rose G, Carrieri G, De Luca M, Falcone E, Passarino G, Bonafè M, Monti D, Baggio G, Bertolini S, Mari D, Mattace R, Franceschi C: Mitochondrial DNA inherited variants are associated with successful aging and longevity in human, FASEB J., 1999; 13: 1532-1536

de Graaf, K., Hekerman, P., Spelten, O., Herrmann, A., Packman, L.C., Bussow, K., Muller-Newen, G. and Becker, W. (2004) Characterization of cyclin L2, a novel cyclin with an arginine/serine-rich domain: phosphorylation by DYRK1A and colocalization with splicing factors. J. Biol. Chem., 279, 4612–4624.

de Graaf, K., Czajkowska, H., Rottmann, S., Packman, L.C., Lilischkis, R., Luscher, B. and Becker, W. (2006) The protein kinase DYRK1A phosphorylates the splicing factor SF3b1/SAP155 at Thr434, a novel in vivo phosphorylation site. BMC Biochem., 7, 7.

Del Bo R., Comi G.P., Bresolin N., Castelli E., Conti E., Degiuli A., Ausenda C.D., Scarlato G. (1997) The apolipoprotein E epsilon4 allele causes a faster decline of cognitive performances in Down's syndrome subjects. J Neurol Sci.; 145(1): 87-91

Dermitzakis, E.T. et al. (2005) Conserved non-genic sequences – an unexpected feature of mammalian genomes. Nat. Rev. Genet. 6, 151– 157

Dickinson, M.J., Singh, I. (1993) Down's syndrome, dementia, and superoxide dismutase. British J. Psychiatry, 162: 811–817

DiMauro S, Schon EA. 2003. Mitochondrial respiratory-chain diseases. N. Engl. J. Med. 348:2656–68

DiMauro S, Davidzon G: Mitochondrial DNA and disease. Ann Med 2005, 37:222-232.

Dinani S, Carpenter S. Down's syndrome and thyroid disorder. Journal of Mental Deficiency Research 1990;34:187–193

Dowjat, W.K., Adayev, T., Kuchna, I., Nowicki, K., Palminiello, S., Hwang, Y.W. and Wegiel, J. (2007) Trisomy-driven overexpression of DYRK1A kinase in the brain of subjects with Down syndrome. Neurosci. Lett., 413, 77–81.

Dumoulin R, Sagnol I, Ferlin T, Bozon D, Stepien G, Mousson B. 1996. A novel gly290asp mitochondrial cytochrome b mutation linked to a complex III deficiency in progressive exercise intolerance. Mol. Cell. Probes 10:389–91

du Vivier A, Munro DD. Alopecia areata, autoimmunity, and Down's syndrome. British Medical Journal 1975;1:191–192

Dyrks TE, Dyrks T, Hartmann C, Masters K, Beyreuther. 1992. Amyloidogenicity of β/A4 and β/A4-bearing amyloid protein precursor fragments by metal-catalyzed oxidation. J. Biol. Chem. 267: 18210–18217.

Edwards JSH. Skin age in Down's syndrome: A note on the findings of Murdoch and Evans. Journal of Mental Deficiency Research 1978;22:223

Emi M, Wu LL, Robertson MA, Myers RL, Hegele RA, Williams RR, White R, Lalouel JM. 1988. Genotyping and sequence analysis of apolipoprotein E isoforms. Genomics. 3(4):373-9

Epstein, C.J. (2001) Down Syndrome (trisomy 21). In The metabolic and molecular bases of inherited diseases (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., eds), pp. 1223–1256, New York, McGraw-Hill

Esbensen AJ, Health conditions associated with aging and end of life of adults with Down syndrome Int Rev Res Ment Retard. 2010 ; 39(C): 107–126

Fabia J, Drolette M. Malformations and leukemia in children with Down's syndrome. Pediatrics 1970: 45: 60–70

Fish J, Raule N, and Attardi G: Discovery of a major D-loop replication origin reveals two modes of human mtDNA synthesis, Science, 2004: 306: 2098-101.

Flint Beal M. 1992. Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? Ann. Neurol. 31: 119–130.

Fonseca MI, Head E, Velasquez P, et al. 1999. The presence of isoaspartic acid in b-amyloid plaques indicates plaque age. Exp Neurol 157: 277–288

Franceschi C., Licastro F., Chiricolo M., Zannotti M., MASI M. 1986. Premature senility in Down's syndrome: A model for and an approach to the molecular genetics of the ageing process. In

Immunoregulation in Ageing. A Facchini, J. J. Haaijman & G. Labo, eds.: 77-83. Eurage Pub]., Rijswijk, The Netherlands.

Franceschi C: Cell proliferation and cell death in the aging process, Aging Clin. Exp. Res., 1989; 1: 3-13

Franceschi, C., Cossarizza, A., Monti, D., Comaschi, V., Farruggia, G., Sartor, S., Masotti, L., (1990) Cellular defense mechanisms, cell death and ageing: An integrated view. In Modern Aging Research, Volume 9, Protein Metabolism in Aging. H.L. Segal, M. Rothstein & E. Bergamini, eds.: 381-386. Wiley-Liss Inc., New York

Franceschi, C.. F. Licastro, P. Paolucci, M. Masi. S. Cavicchi, and M. Zannotti. 1978. T and B lymphocyte subpopulations in Down's syndrome. A study on non-institutionalised subjects. J. Ment. Defic. Res. 22: 179-91

Franceschi C, Licastro F, Chiricolo M, Bonetti F, Zannotti M, Fabris N, Mocchegiani E, Fantini MP, Paolucci P, Masi M. 1981. Deficiency of autologous mixed lymphocyte reactions and serum thymic factor level in Down's syndrome. J Immunol.;126(6): 2161-4

Franceschi C., Monti D., Barbieri D., Grassilli E., Troiano L. et al., Immunosenescence in humans: deterioration or remodelling? (1995) Intern. Rev. Immunol., 12: 57-74

Franceschi C and Cossarizza A: The reshaping of the immune system with age, Int. Rev. Immunol, 1995; 12: 1-4

Franceschi C, Valensin S, Bonafe M, Paolisso G, Yashin AI, Monti D, and De Benedictis G. The network and the remodeling theories of aging: historical background and new perspectives. Exp Gerontol 35: 879–896, 2000a.

Franceschi C, Bonafè M, Valesin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G: Inflamm-aging. An evolutionary perspective on immunosenescence, Ann. N. Y. Acad. Sci., 2000b; 908: 244-254

Franceschi C and Bonafè M: Centenarians as a model of healthy aging, Biochemical Society, 2003; 31: 457-461

Freeman SB, Taft LF, Dooley KJ, Allran K, Sherman SL, Hassold TJ, Khoury MJ, Saker DM. 1998. Population-based study of congenital heart defects in Down syndrome. Am J Med Genet 80:213–217

Fuentes, J.J., Genesca, L., Kingsbury, T.J., Cunningham, K.W., Perez-Riba, M., Estivill, X. and de la, L.S. (2000) DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. Hum. Mol. Genet., 9, 1681–1690.

Gabuzda D, Busciglio J, Chen LB, Matsudaira P & Yankner BA. 1994. Inhibition of energy metabolism alters the processing of amyloid precursor protein and induces a potentially amyloidogenic derivative. J. Biol. Chem. 269: 13623–13628

Gadaleta MN et al: Mitochondrial-DNA copy number and mitochondrial-DNA deletion in adult and senescent rats, Mutat. Res., 1992; 275, 181-193

Gamis, A.S. (2005) Acute myeloid leukemia and Down syndrome evolution of modern therapy – state of the art review. Pediatr. Blood Cancer 44, 13–20

Gardiner PA. Visual defects in cases of Down's syndrome and in other mentally handicapped children. British Journal of Ophthalmology 1967;51:469–474

Gardiner K, Costa AC: "The proteins of human chromosome 21."- Am. J. Med. Genet. C Semin. Med. Genet., 142C, 196–205 (2006).

Gardiner K. 2010. Molecular basis of pharmacotherapies for cognition in Down syndrome. Trends Pharmacol Sci 31:66–73

Ghosh S, Feingold E, Dey SK: "Etiology of Down syndrome: Evidence for consistent association among altered meiotic recombination, nondisjunction, and maternal age across populations."- Am J Med Genet A. Jul;149A(7):1415-20 (2009).

Glasson EJ, Sullivan SG, Hussain R, Petterson BA, Montgomery PD, Bittles AH: "The changing survival profile of people with Down's syndrome: implications for genetic counseling."- Clin.Genet., 62, 390–393 (2002).

Goto Y, Nonaka I, Horai S. 1990. A mutation in the tRNALeu(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348:651–53

Granholm, A.C. et al. (2000) Loss of cholinergic phenotype in basal forebrain coincides with cognitive decline in a mouse model of Down's syndrome. Exp. Neurol. 161, 647–663

Griffin WS, Stanley LC, Ling C, et al. 1989. Brain interleukin I and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer's disease. Proc Natl Acad Sci USA. 86:7611

Gudmundsson H, Gudbjartsson DF, Frigge M, Gulcher JR, Stefansson K: Inheritance of human longevity in Iceland. Eur J Hum Genet 2000, 8:743-749.

Gwack, Y., Sharma, S., Nardone, J., Tanasa, B., Iuga, A., Srikanth, S., Okamura, H., Bolton, D., Feske, S., Hogan, P.G. et al. (2006) A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. Nature, 441, 646–650.

Haan, J.B., Susil, B., Pritchard, M., Kola, I., 2003. An altered antioxidant balance occurs in Down syndrome fetal organs: implications for the 'gene dosage effect' hypoth-esis. J. Neural Transm. 67, 67–83.

Haldane JBS. New Paths in Genetics. London: Allen & Unwin, 1941.

Harman D. Aging: A theory based on free radical and radiation chemistry. J. Gerontol. 11, 298–300, 1956.

Harman D. The biologic clock: The mitochondria? J. Am. Geriatr. Soc. 20, 145–147, 1972.

Hattori, M. et al. (2000) The DNA sequence of human chromosome 21. Nature 405, 311–319

Hayn M, Kremser K, Singewald N, et al. 1996. Evidence against the involvement of reactive oxygen species in the pathogenesis of neuronal death in Down syndrome and Alzheimer's disease. Life Sci 59(7):537– 44

Head E, Azizeh BY, Lott IT, et al. 2000. Complement association with Neurons and b-amyloid deposition in the brains of aged individuals with Down syndrome. Neurobiol Dis. 8(2):252-65.

Hill, D.A. et al. (2003) Mortality and cancer incidence among individuals with Down syndrome. Arch. Intern. Med. 163, 705–711

Hitzler, J.K. et al. (2003) GATA1 mutations in transient leukemia and acutemegakaryoblastic leukemia of Down syndrome. Blood 101, 4301– 4304

Hixson JE, Vernier DT. 1990. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. J Lipid Res. 31(3):545-8

Hjelmborg JV, Iachine I, Skytthe A, Vaupel JW, McGue M, Koskenvuo M, Kaprio J, Pedersen NL, Christensen K: Genetic influence on human lifespan and longevity. Hum Genet 2006, 119:312-321.

Hof PR, Bouras C, Perl DP. 1995. Age-related distribution of neuropathologic changes in the cerebral cortex of patients with Down syndrome. Arch Neurol 52:379 –391

Holt IJ, Harding AE, Morgan-Hughes JA. 1988. Deletions of muscle mitochondrialDNA in patients with mitochondrial myopathies. Nature 331:717–19

Holt IJ, Harding AE, Petty RK, Morgan-Hughes JA. 1990. A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. Am. J. Hum. Genet. 46:428–33

Howells G. Down's syndrome and the general practitioner. Journal of the Royal College of General Practitioners 1989;39:470–475

Hsieh, R., Tsai, N., Au, H., Chang, S., Cheng, Y., and Tzeng, C. (2001) Multiple rearrangements of mitochondrial DNA and defective oxidative phosphorylation gene expression in unfertilized human oocytes. Fertil. Steril. 76 (Suppl. 1), S8−S9

Huang, Y., Chen-Hwang, M.C., Dolios, G., Murakami, N., Padovan, J.C., Wang, R. and Hwang, Y.W. (2004) Mnb/Dyrk1A phosphorylation regulates the interaction of dynamin 1 with SH3 domain-containing proteins. Biochemistry, 43, 10173–10185.

Ishihara K, Amano K, Takaki E, Shimohata A, Sago H, Epstein C, Yamakawa K. 2010. Enlarged brain ventricles and impaired neurogenesis in the Ts1Cje and Ts2Cje mouse models of Down syndrome. Cereb Cortex 20:1131–1143

Jacobson L. Ophthalmology in mentally retarded adults. A clinical survey. Acta Ophthalmologica 1988;66:457–462

Jervis G. 1948. Early senile dementia in mongoloid idiocy. Am J Psychiat 105:102–106

Johnson MJ,Wallace DC, Ferris SD, Rattazzi MC, Cavalli-Sforza LL. 1983. Radiation of human mitochondria DNA types analyzed by restriction endonuclease cleavage patterns. J. Mol. Evol. 19: 255–71

Johnson BA, Aswad DW. 1993. Kinetic properties of bovine brain protein L-isoaspartyl methyltransferase determined using a synthetic isoaspartyl peptide substrate. Neurochem Res 18: 87–94

Jorde, L. B., Carey, J. C., Bamshad, M. J., and White, R. L. (1999) Medical genetics 2nd rev. ed. (Mosby inc: St. Louis)

Jovanovic SV, Clements D, MacLeod K. Biomarkers of oxidative stress are significantly elevated in Down syndrome. Free Radic Biol Med 1998; 25:1044–1048

Jun AS, Brown MD,Wallace DC. 1994. A mitochondrial DNA mutation at np 14459 of the ND6 gene associated with maternally inherited Leber's hereditary optic neuropathy and dystonia. Proc. Natl. Acad. Sci. USA 91:6206–10

Kallen B, Mastroiacovo P, Robert E. 1996. Major congenital malformations in Down syndrome. Am J Med Genet. 65: 160–166

Kang J, Lemaire HG, Unterbeck A, et al. 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature. 325:733–736

Kasamatsu, H. and Vinograd, J. (1974) Replication of circular DNA in eukaryotic cells. Annu. Rev. Biochem. 43, 695–719

Kedziora J, Bartosz G. 1988. Down's syndrome: A pathology involving the lack of balance of reactive oxygen species. Free Radic BiolMed4:317–330.

Keefe, D. L., Niven-Fairchild, T., Powell, S., and Buradagunta, S. (1995) Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. Fertil. Steril. 64, 577-583.

Kerins G, Petrovic K, Bruder MB, Gruman C. Medical conditions and medication use in adults with Down syndrome: A descriptive analysis. Down Syndrome Research and Practice 2008;12:141– 147

Khachaturian ZS. 1985. Diagnosis of Alzheimer's disease. Arch Neurol 42:1097–1106

Kim, E.J., Sung, J.Y., Lee, H.J., Rhim, H., Hasegawa, M., Iwatsubo, T., Min, d.S., Kim, J., Paik, S.R. and Chung, K.C. (2006) Dyrk1A phosphorylates alpha-synuclein and enhances intracellular inclusion formation. J. Biol. Chem., 281, 33250–33257.

Kirkwood, T.B., 1977. Evolution of ageing. Nature 270, 301-304.

Kirkwood, T.B., Holliday, R., 1979. The evolution of ageing and longevity. Proc. R. Soc. Lond. B: Biol. Sci. 205, 531-546.

Kivisild T, Shen P, Wall DP, Do B, Sung R, Davis K, Passarino G, Underhill PA, Scherfe C, Torroni A, Scozzari R, Modiano D, Coppa A, de Knijff P, Feldman M, Cavalli-Sforza LL, Oefner PJ (2005) The role of selection in the evolution of human mitochondrial genomes Genetics 172: 373-387

Kleschevnikov, A.M. et al. (2004) Hippocampal long-term potentiation suppressed by increased inhibition in the Ts65Dn mouse, a genetic model of Down syndrome. J. Neurosci. 24, 8153–8160

Korenberg JR, Kawashima H, Pulst SM, Ikeuchi T, Ogasawara N, Yamamoto K, et al. Molecular definition of a region of chromosome 21 that causes features of the Down syndrome phenotype. Am J Hum Genet 1990;47(2):236–46

Korsager S, Chatham EM, Ostergaard-Kristensen HP. Thyroid function tests in adults with Down's syndrome. Acta Endocrinology 1978;88:48–54

Kowald A and Kirkwood TB. A network theory of ageing: the interactions of defective mitochondria, aberrant proteins, free radicals and scavengers in the ageing process. Mutat Res 316: 209–236, 1996.

Kuhn DE, Nuovo GJ, Martin MM, Malana GE, Pleister AP, Jiang J, Schmittgen TD, Terry AV Jr, Gardiner K, Head E, Feldman DS, Elton TS: "Human chromosome 21-derived miRNAs are overexpressed in down syndrome brains and hearts."- Biochem Biophys Res Commun. 2008 Jun 6;370(3):473-7. Epub 2008 Apr 1

Kusters MA, Verstegen RH, Gemen EF, de Vries E. 2009. "Intrinsic defect of the immune system in children with Down syndrome: a review." - Clin Exp Immunol, May; 156(2):189-93

Lai F, Williams MD. 1989. A prospective study of Alzheimer disease in Down syndrome. Arch Neurol 46:849

Lee CM et al: Age-associated alterations of the mitochondrial genome, Free Radic. Biol. Med., 1997; 22: 1259-1269

Lee SH, Lee S, Jun HS, Jeong HJ, Cha WT, Cho YS, Kim JH, Ku SY, Cha KY. 2003. Expression of the mitochondrial ATPase6 gene and Tfam in Down syndrome Mol Cells.; 15(2): 181-5.

Lemere CA, Blusztajn JK, Yamaguchi H, et al. 1996. Sequence of deposition of eterogeneous amyloid beta-peptides and APOE in Down Syndrome: Implications for initial events in amyloid plaque formation. Neurobiol Dis 3:16 –32

Leonard, S. et al. (2000) Survival of infants born with Down's syndrome: 1980-96. Paediatr. Perinat. Epidemiol. 14, 163–171

Leverenz JB, Raskind MA. 1998. Early amyloid deposition in the medial temporal lobe of young Down syndrome patients: A regional quantitative analysis. Exp Neurol 150:296–304

Lim, S.E., Longley, M.J. and Copeland,W.C. (1999) The mitochondrial p55 accessory subunit of human DNA polymerase γ enhances DNA binding, promotes processive DNA synthesis, and confers N-ethylmaleimide resistance. J. Biol. Chem. 274, 38197–38203

Lim KS, Naviaux RK, Haas RH. 2007. Quantitative mitochondrial DNA mutation analysis by denaturing HPLC. Clin Chem.; 53 (6):1046-52

Linnane, A. W., Marzuki, S., Ozawa, T., and Tanaka, M. (1989) Mitochondrial DNA mutations as an important contributor to aging and degenerative diseases. Lancet 1, 642−645.

Liu, F., Liang, Z., Wegiel, J., Hwang, Y.W., Iqbal, K., Grundke-Iqbal, I., Ramakrishna, N. and Gong, C.X. (2008) Overexpression of Dyrk1A contributes to neurofibrillary degeneration in Down syndrome. FASEB J., 22, 3224–3233.

Lockrow J, Prakasam A, Huang P, Bimonte-Nelson H, Sambamurti K, Granholm A. 2009. Cholinergic degeneration and memory loss delayed by vitamin E in a Down syndrome mouse model. Exp Neurol 216:278–289

Loison A, Festa-Bianchet M, Gaillard JM, Jorgenson JT, and Jullien JM. Age-specific survival in five populations of ungulates: evidence of senescence. Ecology 80: 2539–2554, 1999

Lott IT, Lai F. Dementia in Down's syndrome: Observations from a neurology clinic. Applied Research in Mental Retardation 1982;3:233–239

Lott IT. 1982. Down syndrome, aging and Alzheimer's disease: A clinical review. In: Sinex FM, Merril CR, editors. Alzheimer's disease Down Syndrome and Aging. NY Acad Sci 396:15–28

Lott IT. 1986. The neurology of Down syndrome. In: Epstein CJH, editor. The Neurobiology of Down Syndrome. New York: Raven Press p 17–29

Lott IT.1992. The neurology of Alzheimer disease. In: Nadel L, Epstein C, editors. Down Syndrome and Alzheimer Disease. New York: Wiley-Liss 379:1–14

Lott, I.T., Head, E., 2001. Down syndrome and Alzheimer's disease: a link between development and aging. Ment. Retard. Dev. Disabil. Res. Rev. 7, 172–178.

Lott IT, Doran E, Nguyen V, Tournay A, Head E, Gillen DL. 2011.Down syndrome and dementia: A randomized, controlled trial of antioxidant supplementation. Am J Med Genet Part A 155:1939–1948

Mahle WT, Visconti KJ, Freier MC, Kanne SM, Hamilton WG, Sharkey AM, Chinnock RE, Jenkins KJ, Isquith PK, Burns TG, Jenkins PC. 2006. Relationship of surgical approach to neurodevelopmental outcomes in hypoplastic left heart syndrome. Pediatrics 117:e90–e97.

Mahley RW, Rall SC Jr. 2000. Apolipoprotein E: far more than a lipid transport protein. Annu Rev Genomics Hum Genet. 1:507-37.

Malamud NH. Neuropathology of organic brain syndromes associated with aging. In: Gaitz CM, editor. 1972. Aging and the Brain: Advances in Behavioral Biology, vol 3. New York: Plenum Press. p 63–87

Malec E, Mroczek T, Pajak J, Januszewska K, Zdebska E. 1999. Results of surgical treatment of congenital heart defects in children with Down's syndrome. Pediatr Cardiol 20:351–354

Malyarchuk BA, Rogozin IB, Mutagenesis by transient misalignment in the human mitochondrial DNA control region Ann Hum Genet. 2004; 68(Pt 4): 324-39.

Mann DMA. 1988. The pathological association between Down syndrome and Alzheimer disease. Mech Aging Dev 43:99 –136

Mann DMA, Esiri MM. 1989. The pattern of acquisition of plaques and tangles in the brains of patients under 50 years of age with Down syndrome. J Neurol Sci 89:169 –179

Mann DM. 1992. Down syndrome and Alzheimer's disease: Towards an understanding of pathogenesis. Neurodegeneration. Hunter AJ, Clarke S, editors. San Diego: Academic Press p 21–31

Martinez, D.L., Altafaj, X., Gallego, X., Marti, E., Estivill, X., Sahun, I., Fillat, C. and Dierssen, M. (2004) Motor phenotypic alterations in TgDyrk1a transgenic mice implicate DYRK1A in Down syndrome motor dysfunction. Neurobiol. Dis., 15, 132–142.

Mattiazzi M, Vijayvergiya C, Gajewski CD, DeVivo DC, Lenaz G, et al. 2004. The mtDNA T8993G (NARP) mutation results in an impairment of oxidative phosphorylation that can be improved by antioxidants. Hum. Mol. Genet. 13:869–79

McCarron M, Gill M, McCallion P, Begley C. Health co-morbidities in ageing persons with Down syndrome and Alzheimer's dementia. Journal of Intellectual Disability Research 2005;49:560–566

Medawar PB. An Unsolved Problem in Biology. London: Lewis, 1952.

Meierhofer D, Mayr JA, Ebner S, Sperl W, Kofler B. 2005. Rapid screening of the entire mitochondrial DNA for low-level heteroplasmic mutations. Mitochondrion; 5 (4):282-96

Merriwether DA, Clark AG, Ballinger SW, Schurr TG, Soodyall H, et al. 1991. The structure of human mitochondrial DNA variation. J. Mol. Evol. 33: 543–55

Michikawa Y, Mazzucchelli F, Bresolin N, Scarlato G, Attardi G: Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication, Science, 1999; 286: 774-779

Miller J. 1988. The developmental asynchrony of language development in children with Down syndrome. In: Nadel L, editor. The Psychobiology of Down Syndrome. Cambridge, MA: MIT Press

Mirra SS, Heyman A, McKeel D, et al. 1991. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41:479–486

Miquel J: An update of the oxygen stress-mitochondrial mutation theory of aging: genetic and evolutionary implications, Exp Gerontol., 1998 Jan-Mar; 33 (1-2): 113-26.

MITOMAP. 2005. A Human Mitochondrial Genome Database. http://www.mitomap.org

Mootha, V. K., Bunkenborg, J., Olsen, J. V. et al. (2003) Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. Cell (Cambridge, Mass.) 115, 629–640

Moraes CT, DiMauro S, Zeviani M, Lombes A, Shanske S, et al. 1989. Mitochondrial DNA deletions in progressive external ophthalmoplegia and Kearns-Sayre syndrome. N. Engl. J. Med. 320:1293–99

Morris JK, Wald NJ, Watt HC: "Fetal loss in Down syndrome pregnancies."- Prenat. Diagn., 19, 142–145 (1999).

Murphy, M. et al. (1995) A hypothesis to explain the immune defects in Down syndrome. Prog. Clin. Biol. Res. 393, 147–167

Nadel, L. (2003) Down's syndrome: a genetic disorder in biobehavioral perspective. Genes Brain Behav. 2, 156–166

National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's disease. 1997. Consensus recommendations for the post mortem diagnosis of Alzheimer's disease. Neurobiol Aging 18(S4):1–2

Nelson LD, Lott IT, Touchette PE, et al. 1995. Detection of Alzheimer's Disease in individuals with Down syndrome: Possible relevance of depression. Amer J Ment Ret 99:616 –22

Nicolaidis, P. and Petersen, M. B. (1998) Origin and mechanisms of non-disjunction in human autosomal trisomies. Hum. Reprod. 13, 313-319.

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

Odetti P, Angelini G, Dapino D, Zaccheo D, Garibaldi S, Dagna-Bricarelli F, Piombo G, Perry G, Smith M, Traverso N, et al. 1998. Early glycoxidation damage in brains from Down's syndrome. Biochem Biophys Res Commun 243:849–851

Ojala, D., Montoya, J. and Attardi, G. (1981) tRNA punctuation model of RNA processing in human mitochondria. Nature (London) 290, 470–474

Olson, L.E., Richtsmeier, J.T., Leszl, J. and Reeves, R.H. (2004) A chromosome 21 critical region does not cause specific down syndrome phenotypes. Science, 306, 687–690.

Ono T, Isobe K, Nakada K, Hayashi JI: Human cells are protected from mitochondrial dysfunction by complementation of DNA products in fused mitochondria. Nat Genet 2001, 28:272-275.

Park J, Oh Y, Chung KC : “Two key genes closely implicated with the neuropathological characteristics in Down syndrome: DYRK1A and RCAN1.”- BMB Rep. Jan 31;42(1):6-15 (2009).

Patcher J. 1997. Inflammatory mechanisms in Alzheimer disease: The role of betaamyloid/glial interactions. Mol. Psychiatry 2: 91–95.

Penrose LS. The incidence of Mongolism in the general population. Journal of Mental Science, 1949;95:685–688.

Perls T, Kunkel LM, Puca AA: The genetics of exceptional human longevity. J Am Geriatr Soc 2002, 50:359-368.

Philip, R. et al. (1986) Abnormalities of the in vitro cellular and humoral responses to tetanus and influenza antigens with concomitant numerical alterations in lymphocyte subsets in Down syndrome (trisomy 21). J. Immunol. 136, 1661–1667

Prasher VP. Prevalence of thyroid dysfunction and autoimmunity in adults with Down syndrome. Down Syndrome Research and Practice 1994a;2:67–70

Prasher VP. Screening of medical problems in adults with Down syndrome. Down Syndrome Research and Practice 1994b;2:59–66

Pueschel SM, Louis S, McKnight P. Seizure disorders in Down syndrome. Archives of Neurology 1991;48:318–320

Raule N, Sevini F, Santoro A, Altilia S, Franceschi C: Association studies on human mitochondrial DNA: methodological aspects and result in the most common age-related diseases, Mitochondrion, 2007; 7: 29-38

Reeves RH, Baxter LL, Richtsmeier JT. Too much of a good thing: mechanisms of gene action in Down syndrome. Trends Genet 2001;17(2):83–8

Richter, C. (1995) "Oxidative damage to mitochondrial DNA and its relationship to ageing" Int J Biochem Cell Biol 27: 647-53.

Richtsmeier, J.T. (2002) Craniofacial phenotypes in segmentally trisomic mouse models for Down syndrome. Am. J. Med. Genet. 107, 317–324

Ritz S, Turzynski A, Schultz HW. 1994. Estimation of age at death based on aspartic acid racemization in noncollagenous bone proteins. Forensic Sci Int 69:149 –159.

Ritz S, Turzynski A, Schultz HW, et al. 1996. Identification of osteocalcin as a permanent aging constituent of the bone matrix: Basis for an accurate age at death determination. Forensic Sci Int 77:13–26

Roat E, Prada N, Ferraresi R, Giovenzana C, Nasi M, Troiano L, Pinti M, Nemes E, Lugli E, Biagioni O, Mariotti M, Ciacci L, Consolo U, Balli F, Cossarizza A. 2007. Mitochondrial alterations and tendency to apoptosis in peripheral blood cells from children with Down syndrome. FEBS Lett 581:521–525

Roizen NJ, Patterson D. Down's syndrome. Lancet 2003;361:1281–1289

Rose G, Passarino G, Scornaienchi V, Romeo G, Dato S, Bellizzi D, Mari V, Feraco E, Maletta R, Bruni A, Franceschi C, De Benedictis G. 2007. The mitochondrial DNA control region shows genetically correlated levels of heteroplasmy in leukocytes of centenarians and their offspring. BMC Genomics; 29; 8:293.

Rose G, Romeo G, Dato S, Crocco P, Bruni AC, Hervonen A, Majamaa K, Sevini F, Franceschi C, Passarino G; GEnetics of Healthy Ageing Project Consortium. Somatic point mutations in mtDNA control region are influenced by genetic background and associated with healthy aging: a GEHA study. 2010. PLoS One; 5 (10): e13395

Rovelet-Lecrux, A. et al. (2006) APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. Nat. Genet. 38, 24–26

Royston, MC., Mann, D,, Pickering-Brown, S., Owen, F., Perry R., Raghavan, R., Khin-Nu, C., Tyrer, S., Day, K., Crook, R. (1994) Apolipoprotein E epsilon 2 aflele promotes longevity and protects patients with Down's syndrome from dementia. Neuroreport, 5: 2583-2585

Rueda N., Flórez J., Martínez-Cué C., 2011. The Ts65Dn mouse model of Down syndrome shows reduced expression of the Bcl-XL antiapoptotic protein in the hippocampus not accompanied by changes in molecular or cellular markers of cell death. Int. J. Devl Neurosci.

Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC. 2004. Effects of purifying and adaptive selection on regional variation in human mtDNA. Science 303:223–26

Rumble, B. et al. (1989) Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer's disease. N. Engl. J. Med. 320, 1446– 1452

Ryoo, S.R., Jeong, H.K., Radnaabazar, C., Yoo, J.J., Cho, H.J., Lee, H.W., Kim, I.S., Cheon, Y.H., Ahn, Y.S., Chung, S.H. et al. (2007) DYRK1A-mediated hyperphosphorylation of Tau. A functional link between Down syndrome and Alzheimer disease. J. Biol. Chem., 282, 34850–34857.

Ryoo, S.R., Cho, H.J., Lee, H.W., Jeong, H.K., Radnaabazar, C., Kim, Y.S., Kim, M.J., Son, M.Y., Seo, H., Chung, S.H. et al. (2008) Dual-specificity tyrosine(Y)-phosphorylation regulated kinase 1A-mediated phosphorylation of amyloid precursor protein: evidence for a functional link between Down syndrome and Alzheimer's disease. J. Neurochem., 104, 1333–1344.

Salehi, A. et al. (2006) Increased App expression in a mouse model of Down's syndrome disrupts NGF transport and causes cholinergic neuron degeneration. Neuron 51, 29–42

Santoro A, Balbi V, Balducci E, Pirazzini C, Rosini F, Tavano F, Achilli A, Siviero P, Minicuci N, Bellavista E, Mishto M, Salvioli S, Marchegiani F, Cardelli M, Olivieri F, Nacmias B, Chiamenti AM, Benussi L, Ghidoni R, Rose G, Gabelli C, Binetti G, Sorbi S, Crepaldi G, Passarino G, Torroni A, Franceschi C. Evidence for sub-haplogroup h5 of mitochondrial DNA as a risk factor for late onset Alzheimer's disease. 2010. PLoS One.; 5 (8): e12037

Sato A, Nakada K, Hayashi JI: Mitochondrial dynamics and aging: mitochondrial interaction preventing individuals from expression of respiratory deficiency caused by mutant mtDNA. Biochim Biophys Acta 2006, 1763:473-481.

Saunders, A.M., Strittmatter, W.J., Schmechel, D., George-Hyslop, St,, Pericak-Vance, M.A., Joo, S.H., Rosi, B.L., Gusella, J.F., Crapper- MacLachlrm, D.R., Abcrts, M.J., Hrdette, C., Crain, B., Goldgaber, D.,

Roses, A.D. (1993) Association of apolipoprotein E allele e4 with late-onset familial and sporadic Alzheimer's disease. Neurology, 43: 1467-1472

Savva GM, Morris JK, Multon DE, Alberman E: "Maternal age-specific fetal loss rates in Down syndrome pregnancies."- Prenat Diagn Jun;26(6):499-504 (2006).

Schar P, Herrman G, Daly G, Lindahl T. A newly identified DNA ligase of Saccharomyces cerevisiae involved in RAD52-independent repair of DNA double-strand breaks. Genes Dev 1997; 11: 1912–1924

Schmechel, D.E., Saunders, A.M., Strittmatter, W.J., Cmin, B.J., Hulette, C.M., Joo, S.H., Pericak-Vance, M.A., Goldgaber, D., Roses, A.D. (1993) I amyloid /3-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. Proc. Natl. Acad. Sci. USA, 90: 9649–9653

Schuchmann S, Heinemann U. Increased mitochondrial superoxide generation in neurons from trisomy 16 mice: a model of Down's syndrome. Free Radic Biol Med 2000;28(2):235–50

Seidl R, Greber S, Schuller E, et al. 1997. Evidence against increased oxidative DNA-damage in Down syndrome. Neurosci Lett 235(3):137–140

Selkoe D. 1997. Amyloid β-protein and the genetics of Alzheimer's disease. J. Biol. Chem. 271: 18295–18298.

Selwood SP, Chrzanowska-Lightowlers ZM, Lightowlers RN. 2000. Does the mitochondrial transcription-termination complex play an essential role in controlling differential transcription of the mitochondrial DNA? Biochem Soc Trans., 28 (2): 154-9

Sethupathy, P., Borel, C., Gagnebin, M., Grant, G.R., Deutsch, S., Elton, T.S., Hatzigeorgiou, A.G. and Antonarakis, S.E. (2007) Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3' untranslated region: a mechanism for functional single-nucleotide polymorphisms related to phenotypes. Am. J. Hum. Genet., 81, 405–413.

Shadel GS, Clayton DA, Mitochondrial DNA maintenance in vertebrates Annu Rev Biochem. 1997; 66: 409-35.

Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW,Wallace DC. 1990. Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNALys mutation. Cell 61:931–37

Slonim D, Koide K, Johnson K, Tantravahi U,Cowan J, Jarrah Z, Bianchi D. 2009. Functional genomic analysis of amniotic fluid cell-free mRNA suggests that oxidative stress is significant in Down syndrome fetuses. Proc Natl Acad Sci USA 106:9425–9429

Solano A, Roig M, Vives-Bauza C, Hernandez-Pena J, Garcia-Arumi E, et al. 2003. Bilateral striatal necrosis associated with a novel mutation in the mitochondrial ND6 gene. Ann. Neurol. 54:527–30

Strick TR, Croquette V, Bensimon D. Single-molecule analysis of DNA uncoiling by a type II topoisomerase. Nature 2000;404:901–904

Struwe F. 1929. Histopathology Untersuchungen uber Entstenhung und Wesen der senile Plaques Z. ges. Neurol Psychiat 122:291–307

Strydom A, Dickinson M, Shende S, Pratico D, Walker Z. 2009. Oxidative stress and cognitive ability in adults with Down syndrome. Prog Neuropsychopharmacol Biol Psychiatry 33:76–80

Taanman, J.W. (1999) The mitochondrial genome: structure, transcription, translation and replication. Biochim. Biophys. Acta 1410, 103–123

Takamatsu, C., Umeda, S., Ohsato, T. et al. (2002) Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. EMBO Rep. 3, 451–456

Tanzi RE, Gusella JF, Watkins PC, et al. 1987. Amyloid b protein genes: cDNA, mRNA distribution and genetic linkage near the Alzheimer locus. Science 235:880–884

Tatuch Y, Christodoulou J, Feigenbaum A, Clarke JTR, Wherret J, et al. 1992. Heteroplasmic mtDNA mutation (T-G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high. Am. J. Hum. Genet. 50:852–58

Taylor, S. W., Fahy, E. and Ghosh, S. S. (2003) Global organellar proteomics. Trends Biotechnol. 21, 82–88

Theuns, J. et al. (2006) Promoter mutations that increase amyloid precursor-protein expression are associated with Alzheimer disease. Am. J. Hum. Genet. 78, 936–946

Torroni A, Achilli A, Macaulay V, Richards M, Bandelt HJ: Harvesting the fruit of the human mtDNA tree, Trends in Genetics, 2006; 22: 339-345

Trounce I, Neill S,Wallace DC. 1994. Cytoplasmic transfer of the mtDNA nt 8993 TG (ATP6) point mutation associated with Leigh syndrome into mtDNA-less cells demonstrates cosegregation with a decrease in state III respiration and ADP/O ratio. Proc. Natl Acad. Sci. USA 91:8334–38

Tsai, M.-S,, Tangalos, E.G., Petersen, R.C., Smith, G.E., Schaid, D.J., Kokmen, E., Ivnik, R.J., Thibodeau, S.N. (1994) Apolipoprotein E: risk factor for Alzheimer's disease. Am. J. Hum. Genet., 54: 643-649

Van Allen MI, Fung J, Jurenka SB. Health care concerns and guidelines for adults with Down syndrome. American Journal of Medical Genetics 1999;89:100–110

Van Blerkom, J. and Runner, M. N. (1984) Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocytes. Am. J. Anat. 171, 335−355.

Van Blerkom, J., Davis, P., and Lee, J. (1995) ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. Hum. Reprod. 10, 415−454.

Van Blerkom, J. (1997) Can the developmental competence of early human embryos is predicted effectively in the clinical IVF laboratory. Hum. Reprod. 12, 1610−1614.

Van Blerkom, J., Sinclair, J., and Davis, P. (1998) Mitochondrial transfer between oocytes: potential applications of mitochondrial donation and the issue of heteroplasmy. Hum. Reprod. 3, 2857-2868

van den Ouweland JM, Lemkes HH, Trembath RC, Ross R, Velho G, et al. 1994. Maternally inherited diabetes and deafness is a distinct subtype of diabetes and associates with a single point mutation in the mitochondrial tRNALeu(UUR) gene. Diabetes 43:746–51

Van Gool, W.A., Evenhuis, H.M., Van Duijn, C.M. (1995) A case-control study of apolipoprotein E genotypes in Alzheimer's disease associated with Down's syndrome. Ann. Neurol., 38: 225–230

van Oven M, Kayser M. 2009. Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Hum Mutat 30 (2): E386-E394. http://www.phylotree.org/

Varnhagen CK, Das JP, Varnhagen S. 1987. Auditory and visual memory span: cognitive processing by TMR individuals with Down syndrome and other etiologies. Am J Ment Defic 91:398–405

Veall RM. The prevalence of epilepsy among Mongols related to age. Journal of Mental Deficiency Research 1974;18:43

Visootsak J, Mahle WT, Kirshbom PM, Huddleston L, Caron-Besch M, Ransom A, Sherman SL. 2011. Neurodevelopmental outcomes in children with Down syndrome and congenital heart defects. Am J Med Genet A.; 155A(11): 2688-91

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, et al. 1988a. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242:1427–30

Wallace DC, Zheng X, Lott MT, Shoffner JM, Hodge JA, et al. 1988b. Familial mitochondrial encephalomyopathy (MERRF): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. Cell 55:601–10

Wallace DC, Brown MD, Lott MT. 1999. Mitochondrial DNA variation in human evolution and disease. Gene 238: 211–30

Wallace DC. 1999. Mitochondrial diseases in man and mouse. Science 283:1482–88

Wallace, DC. 1992. Mitochondrial genetics: a paradigm for aging and degenerative diseases? Science (Washington, D.C.) 256, 628–632

Wallace DC. 1999. Mitochondrial Diseases in Man and Mouse. Science; 283: 1482–1488

Wallace DC, Lott MT, Brown MD, Kerstann K. 2001. Mitochondria and neuroophthalmological diseases. In The Metabolic and Molecular Basis of Inherited Disease, ed. CR Scriver, AL Beaudet, WS Sly, D Valle, pp. 2425–512. New York: McGraw-Hill

Wallace DC, Lott MT. 2002. Mitochondrial genes in degenerative diseases, cancer and aging. In Emery and Rimoin's Principles and Practice of Medical Genetics, ed. DL Rimoin, JM Connor, RE Pyeritz, BR Korf, pp. 299–409. London: Churchill Livingstone

Wallace DC, A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine, Annu. Rev. Genet., 2005; 39: 359-407

Wechsler, J. et al. (2002) Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. Nat. Genet. 32, 148– 152

Weinert BT, Timiras PS. Invited review: Theories of aging. J Appl Physiol. 2003 Oct;95(4):1706-16

Weisgraber, K.H., Roses, A.D,, Strittmatter, W,J. (1994) The role of apolipoprotein E in the nervous system. Curr. Opin. Lipidol., 5: 110-116

Wiseman F.K., Alford K. A., Tybulewicz V. L.J., and Fisher E. M.C. Down syndrome—recent progress and future prospects Hum. Mol. Genet. (2009) 18(R1): R75-R83

Wisniewski, T., Morelli, L., Wegiel, J., Levy, E., Wisniewski, H.M., Frangione, B. (1995) The influence of apolipoprotein E isotypcs on Alzheimer's disease pathology in 40 cases of Down's syndrome. Ann. Neurol., 37: 136-138

Woods, Y.L., Cohen, P., Becker, W., Jakes, R., Goedert, M., Wang, X. and Proud, C.G. (2001) The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bepsilon at Ser539 and the microtubule-associated protein tau at Thr212: potential role for DYRK as a glycogen synthase kinase 3-priming kinase. Biochem. J., 355, 609–615.

Yang, Q. et al. (2002) Mortality associated with Down's syndrome in the U. S. A. from 1983 to 1997: a population-based study. Lancet 359, 1019–1025

Zana M, Janka Z, Kàlmàn J. 2007. Oxidative stress: A bridge between Down's syndrome and Alzheimer's disease. Neurobiol. Aging. 28: 648-676

Zeviani, M. and Antozzi, C. (1997) Mitochondrial disorder. Mol. Hum. Reprod. 3, 133-148.

Zhang J, Asin-Cayuela J, Fish J, Michikawa Y, Bonafè M, Olivieri F, Passarino G, De Benedictis G, Franceschi C, Attardi G, Strikingly higher frequency in centenarians and twins of mtDNA mutation causing remodeling of replication origin in leukocytes, Proc. Natl. Acad. Sci., 2003; 100: 1116-1121

Zhu X, Raina AK, Perry G, Smith MA. Alzheimer's disease: the two hit hypothesis. Lancet Neurol 2004;3(4):219–26