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MITOCHONDRIAL DNA HAPLOGROUP-DEPENDENCE OF DRUGS AND XENOBIOTICS TOXICITY

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ABSTRACT

Pharmacogenomics is the study of how genes affect a individual response to drugs to develop medications tailored to a person's genetic makeup because the efficacy/safety profile have not be the same way for everyone.

Mitochondria are characterized by a unique milieu, with an alkaline and negatively charged interior (pH value 8) due to the proton pumping associated with OXPHOS and a series of specific channels and carrier proteins. As a consequence, mitochondria can easily accumulate lipophilic compounds of cationic character and weak acids in their anionic form, particularly amphiphilic xenobiotics including ethidium bromide, 1-methyl-4 phenylpyridinium (MPP⁺), paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; PQ) and others that can penetrate the inner mitochondrial membrane (IMM) freely since in their undissociated forms. Indeed, it is well understood that many drugs and chemicals can cause mitochondrial dysfunction (mitotoxicity) by interacting with mitochondrial DNA (mtDNA), protein synthesis, respiratory chain, other metabolic processes, channels and transporters. Moreover, due to its peculiar uniparental maternal inheritance and high mutation rate, mtDNA presents different clusters of population-specific-polymorphism (SNPs) that characterize different maternal lineages (mitochondrial haplogroups). It has been demonstrated that many non-synonymous SNPs, cause amino acid variations in the mitochondrial-encoded proteins, potentially modifying OXPHOS activity and ROS production. Some of these haplotypes may confer vulnerability to, or protection from, various common diseases. Well-documented examples are the role of mtDNA haplotype in Parkinson's disease (PD) and Leber's hereditary optic neuropathy (LHON). It has been proposed that European haplogroups J and K are protective for PD. On the other hand the haplogroups J and T may influence mitochondrial dysfunction, resulting in an increased risk of PD. In addition the 11778/ND4, 14484/ND6 and 3460/ND1 LHON mutations are associated respectively with mitochondrial subhaplogroup J2b, J1c and K, as these mtDNA backgrounds may increase penetrance of LHON mutations.

Several reports suggest that environmental factors such as pesticides (e.g. rotenone), herbicides (e.g. paraquat) and MPTP or 1-methyl 4-phenyl 1,2,3,6-tetrahydro-pyridine (contaminant in the illicit synthesis of opiates) increase the risk of PD due to a

reduction in ATP synthesis and increase of reactive oxygen species (ROS). Furthermore, tobacco smoking has been proposed as an environmental trigger of visual loss in LHON, due to the presence of substances, contained in the tobacco that can directly act inhibiting CI.

Researchers have also associated non synonymous variants in mtDNA with the development of side effects of drugs. Effectively the analysis of mtDNA haplogroup in patients with cancer treated with chemioterapic agent cisplatin (cisPt) revealed an increased incidence of hearing loss in haplogroup J, due to inhibition of mtDNA replication. It has also been shown that patients treated with the antibiotic Linezolid may develop LHON-like optic neuropathy, myelosuppression and lactic acidosis. This is possibly due to the inhibition of mitochondrial protein synthesis, which is modulated by the SNPs at positions 2706 and 3010, in the 16S gene of mtDNA. This sequence region is predicted to be very close to the Peptidyl Transferase Center (PTC) that is the binding site of several antibiotics.

To demonstrate that mitochondrial genetic variability may influence individual susceptibility to drugs toxicity (Linezolid and CisPt) or to toxic environmental factors (rotenone, MPP⁺, paraquat and cigarette smoking) we assessed *in vitro* cell viability, mitochondrial functions including ATP synthesis, activity of OXPHOS complexes and ROS generation, and biogenesis (mtDNA copy number) in a collection of transmitochondrial cytoplasmic hybrids (cybrids) carrying divergent human mtDNA haplogroups (N1b, H, J, T, U, and K) or LHON mutations, that have been defined by sequencing of D-loop region and then of the entire mtDNA. Cybrids were constructed from fibroblasts obtained, after informed consent, from skin biopsies of unrelated healthy subjects and LHON patients.

The results of this study demonstrated that mitochondrial genetic variability may influence individual susceptibility to drugs or environmental factors toxicity, highlighting interesting associations between specific haplogroups, mitochondrial functional alterations, and toxic agent. More in details: 1) haplogroup K1 was found to play a protective role against rotenone toxicity, whereas haplogroup J1 seem to be more susceptible to the action of both rotenone and MPP⁺; 2) haplogroup T seems to be more susceptible to the action of paraquat; 3) haplogroups H12 and T1 in association with the LHON mutation 3460/ND1, and haplogroups J1c and J2A all

increase the susceptibility to mitochondrial damage after smoke exposure. Moreover haplogroup H1, characterized by SNPs 2706A/3010A in 16SrRNA is the most sensitive to Linezolid toxicity and haplogroup J appears to act as risk factor in CisPt toxicity.

Even though future studies will be necessary to better understand the mechanism of action of some of these molecules, studying the association between mitochondrial haplogroup and toxicity of drugs and chemicals is extremely useful to prevent toxicity in predisposed subjects. This may avoid the occurrence of adverse reactions leading to the withdrawal of drugs from the market or Black Box warnings by FDA. For these reasons, pharmaceutical companies have introduced early in the drug-development process stringent *in vitro* studies to evaluate mitochondrial function (respiratory chain, ROS, membrane potential and mtDNA).

RIASSUNTO

La farmacogenomica si occupa di indagare gli effetti di un determinato farmaco o sostanza chimica in base al genotipo dell'individuo con lo scopo di personalizzare le cure e fornire le terapie adeguate.

I mitocondri presentano un potenziale di membrana ($\Delta\psi$) di 180mV, una matrice alcalina (pH 8) con carica negativa e possono accumulare al loro interno sia sostanze cariche positivamente sia acidi deboli in forma anionica, rendendosi bersaglio primario o secondario dell'azione di farmaci e agenti tossici. I mitocondri sono dotati di un proprio corredo genomico (mtDNA) che si caratterizza per un'ereditarietà uniparentale materna un elevato tasso di mutazione e numerose varianti genetiche, distinte in aplogruppi. È noto che variazioni non sinonime nel mtDNA causano alterazioni funzionali di proteine implicate nel processo OXPHOS, tali da supportare studi per comprendere se la variabilità mitocondriale possa svolgere un'azione protettiva o rappresentare un fattore di rischio per l'insorgenza di patologie. In particolare è stato dimostrato che soggetti appartenenti agli aplogruppi J e K e con polimorfismo 10398G nel gene ND3 (CI) si correlano a minore rischio di sviluppare il Morbo di Parkinson (PD). Al contrario la variante 4216C nel gene ND1 (CI), comune al sottogruppo JT, parrebbe correlata a un aumentato rischio di malattia. Inoltre è stato dimostrato un maggiore rischio di Neuropatia Ottica di Leber (LHON) se le mutazioni 11778/ND4 (CI),

14484/ND6 (ND1) e 3460/ND1 (CI) si associano agli aplogruppi J2b, J1c e K rispettivamente.

Ulteriormente studi ipotizzano che agenti inquinanti ambientali quali pesticidi (es. rotenone), erbicidi (es. paraquat) e MPTP o 1-metil 4-fenil 1,2,3,6-tetraidro-piridina (composto secondario contaminante nella sintesi illecita di oppiacei) siano causa di un maggiore rischio di PD per un'alterata funzionalità mitocondriale con riduzione della sintesi di ATP e aumento di specie reattive dell'ossigeno (ROS). In altri studi s'ipotizza che il rischio di perdita della vista in carrier di mutazioni LHON, aumenta se il soggetto è fumatore, per azione sul CI della catena respiratoria.

Infine ancora dati di letteratura associano numerose variazioni mitocondriali non sinonime allo sviluppo di patologie collaterali a trattamenti farmacologici. E' stato dimostrato che soggetti appartenenti all'aplogruppo J, presentano un aumento del rischio di sviluppare ototossicità dopo trattamento con l'antitumorale Cisplatino (CisPt) per inibizione della replicazione del mtDNA mentre pazienti con SNPs nella regione 16SrRNA in posizione 2706A e 3010A trattati con l'antibiotico Linezolid, sviluppano mielosoppressione, neuropatia e acidosi lattica in seguito a inibizione della sintesi proteica mitocondriale.

L'attività di ricerca ha avuto come oggetto di studio il ruolo della variabilità del genoma mitocondriale (mtDNA) nella suscettibilità individuale alla tossicità da farmaci (Linezolid e Cisplatino) o da agenti tossici inquinanti ambientali (rotenone, MPP⁺, paraquat ed estratto di fumo) in un modello in vitro costituito, da ibridi transcitoplasmatici (cibridi) creati mediante fusione di fibroblasti da donatore, dopo enucleazione, con cellule di osteosarcoma private di mtDNA (rho0). Sono così stati originati cloni di cibridi appartenenti ai principali aplogruppi mitocondriali europei (N1b, H, J, T, U, K) o portatori di diverse mutazioni LHON, e caratterizzati mediante analisi di sequenza della regione non codificante di 1122 bp o "Displacement loop" (Dloop) e dell'intero genoma mitocondriale. Gli studi sono stati eseguiti valutando la vitalità cellulare, la funzionalità (sintesi di ATP, produzione di ROS) e la biogenesi mitocondriale (numero di copie di mtDNA).

I risultati ottenuti da queste analisi hanno dimostrato che una variabilità genetica mitocondriale può influenzare la suscettibilità individuale alla tossicità da agenti tossici inquinanti ambientali e farmaci. In particolare sono state evidenziate alcune

interessanti associazioni tra specifici aplogruppi mitocondriali, alterazioni mitocondriali e agente tossico: 1) l'aplogruppo K1 sembra svolgere un'azione protettiva rispetto al rotenone mentre l'aplogruppo J1 sembra più sensibile all'azione del pesticida e del MPP⁺ anche se quest'ultimo è meno specifico e affine al Cl₂; 2) l'aplogruppo T sembra più suscettibile all'azione del paraquat.; 3) gli aplogruppi H12 e T1 quando associati a mutazione LHON 3460/ND1 e gli aplogruppi J1c e J2a aumentano la suscettibilità al danno mitocondriale da fumo. Successive evidenze hanno dimostrato che l'aplogroup H1, caratterizzato dai polimorfismi 2706A e 3010A nel gene 16SrRNA, sembra essere il più sensibile all'azione tossica del Linezolid così come l'aplogruppo J è risultato più sensibile alla citotossicità da Cisplatino.

Questi dati suggeriscono che procedere nello studio di associazioni tra aplogruppo mitocondriale e tossicità da farmaci e agenti chimici potrebbe consentire di individuare precocemente il rischio tossicologico individuale. Queste conoscenze potrebbero essere di particolare impatto per prevenire reazioni avverse a farmaci in alcuni casi causa di ritiro dal commercio o black box. Con questo scopo di recente le aziende farmaceutiche hanno introdotto nelle fasi iniziali dello sviluppo di un farmaco studi *in vitro* per valutare eventuali effetti sulla funzionalità mitocondriale (catena respiratoria, ROS, potenziale di membrana e mtDNA).

Introduction

INTRODUCTION

1. PHARMACOGENOMICS

Pharmacogenomics is the study of how genes affect a person's response to drugs. This relatively new field combines pharmacology and genomics to develop effective and safe medications tailored to a person's genetic make up. Many drugs that are currently available are "one size fits all," but they don't work the same way for everyone. It can be difficult to predict which person will benefit from a medication, which person will not respond at all, and which person will experience negative side effects (adverse drug reactions). With the knowledge gained from the Human Genome Project, researchers are learning how inherited differences in genes affect the individual response to medications. Many studies describe the role of nuclear genome (nDNA) variability in toxicity/pharmacological efficacy using single nucleotide polymorphisms (SNPs) [<http://qhr.nlm.nih.gov/handbook/genomicresearch/pharmacogenomics>]. Interestingly the mitochondrial genome (mtDNA), present in multiple copies within the cell, may also play a role in the toxicity/pharmacological efficacy. Medically applied substances that interact with mitochondria can be specifically designed to affect mitochondrial structures and functions as primary or secondary targets facilitating a better understanding of their mechanism of action and opening new perspectives to their application [[Szewczyk A. et al., 2002](#); [Don A.S et al., 2004](#); [Duchen M.R, 2004](#)]. Mitochondria are not merely the center for energy metabolism, but are also the headquarters for various catabolic and anabolic processes, calcium fluxes, and various signaling pathways. In this context, the interaction of pharmacological agents with mitochondria is an aspect of molecular pharmacology that is recently considered of interest not only in terms of toxicology but also from a therapeutic point of view [[Scatena .R, 2012](#); [Scatena R. et al., 2007](#)].

2. MITOCHONDRIA

Mitochondria firstly discovered in 1890 were defined "bioblast" and were described as "elementary organism" living inside the cells with "vital functions" [[Altmann R., 1890](#)]. Mitochondria [from the Greek mitos (thread-like) and khondros (grain or granule)] are bacterium-sized organelles found in all nucleated cells [[Schon E.A. et al., 2012](#)]. Their

origin is explained by the endo-symbiont theory, which proposes that more than a billion years ago these subcellular organelles originate from aerobic bacteria, incorporated into an oxidative proto-eukaryote host cell and maintained during evolution [[Sagal L., 1967](#); [Margulis L., 1981](#)]. Mitochondria are the ‘powerhouses of the cell’ because they are responsible for producing the most of cellular ATP through the process of oxidative phosphorylation (OXPHOS) [[Schon E.A. et al., 2012](#)]. Moreover, they host numerous biochemical pathways, including pyruvate oxidation and tricarboxylic acid cycle (TCA), fatty acid oxidation and metabolism of amino acids (urea cycle) [[Greaves L.C. et al., 2012](#)]. Mitochondria are also important regulators of several other physiological processes that include buffering of cytoplasmic calcium [[Pozzan T. et al., 2000](#)], controlling of cellular redox status, generating and releasing of reactive oxygen species (ROS) [[Murphy M.P., 2009](#); [Galluzzi L. et al., 2012](#)], and are central to iron–sulphur cluster biogenesis [[van der Giezen M. et al., 2005](#)]. Overall, mitochondria maintained over the evolution their own multicopy circular bacter-like DNA, reduced in size, which is packaged with proteins in structures called nucleoids [[Miyakawa I et al., 1987](#); [Bogenhagen D.F., 2012](#)].

2.1 Mitochondrial Architecture

Mitochondria are very small organelles about 0.5–1µm in diameter and up to 7µm long. Their shape and number (about 100-1000/cell) depends on the energy requirements. They have two membranes, each composed of a phospholipid bilayer, distinct in appearance and in physic-chemical properties, thus determining the biochemical function of each one [[Krauss S. et al., 2001](#)] (Figure 1).

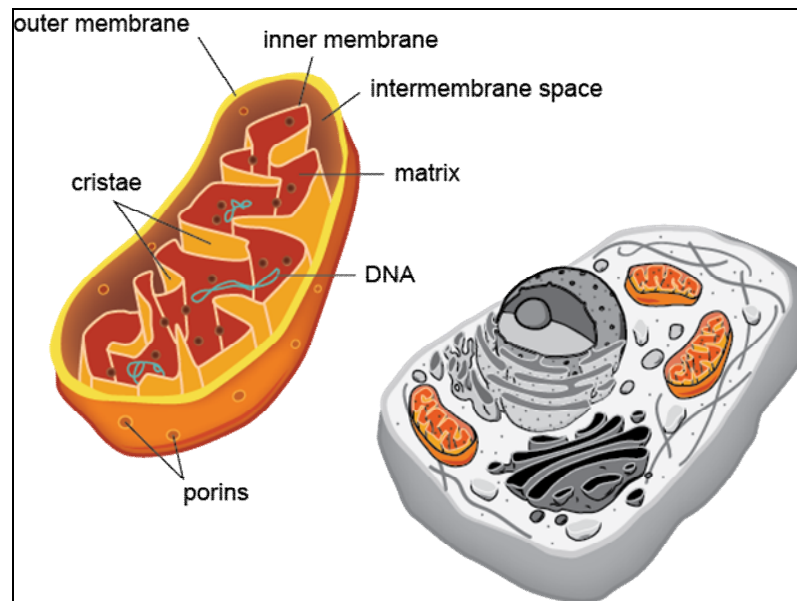


Figure 1. Mitochondrial structure

The outer mitochondrial membrane (OMM), delimitating and containing the organelle, is a relatively simple phospholipid bilayer identical to plasma membrane containing many copies of integral protein called porins. Porins are aqueous channels that render OMM free permeable to molecules of about 5000 Dalton or less such as ions, nutrient molecules, ATP, ADP, etc. [Bruce A. et al., 1994; Subhashini B. et al., 2013]. The OMM contains also a large multisubunit complex called translocase of the outer membrane (TOM), which actively translocates proteins with a specific signalling sequence into the mitochondrion [Kulawiak B. et al., 2013]. OMM interacts physically with endoplasmic reticulum (ER) membrane forming inter-organellar membrane micro domains MAMs (mitochondria-associated ER-membrane). Recently, it has been demonstrated that MAMs are crucial for highly efficient transmission of Ca^{2+} from the ER to mitochondria, enhancing the importance of the relationships between the two cellular compartments in controlling fundamental processes involved in energy production and also determining cell fate by triggering or preventing apoptosis [Hayashi T. et al, 2009]. The inner mitochondrial membrane (IMM) is folded to form invaginations called cristae, which protrude into the mitochondria defining two spaces: the intermembrane space between the OMM and IMM and the matrix, the space limited by the internal membrane. The IMM has a unique structure, which invaginations that form narrow tubular structures that connect the cristae to the periphery of the IMM. The cristae form an intracristal and intermembrane space that might produce a

microcompartmentation within the IMM space with implication for the formation of the membrane potential [[Mannella C.A. et al., 2013](#)]. Also the composition of proteins and phospholipids of IMM is very peculiar and crucial for mitochondrial function. In particular this membrane is highly enriched in proteins specific for OXPHOS and transport and contains an unusual lipid cardiolipin in addition to the major classes of phospholipids found in all cell membranes. Cardiolipin is located predominantly but not exclusively in the mitochondria membrane, it contains four fatty acids and plays an important role in maintaining the mitochondrial membrane potential ($\Delta\psi$) contributing to make the IMM strongly impermeable to polar molecules, in particular ions [[Bruce A. et al., 1994](#); [Herrmann J.M. et al., 2000](#); [McMillin J.B. et al., 2002](#)]. Nevertheless the IMM is freely permeable to oxygen (O_2), carbon dioxide (CO_2), and water (H_2O).

The internal compartment enclosed by IMM is called matrix and contains the enzymes for the TCA cycle and the other metabolic pathways, mitochondrial ribosomes transfer RNAs and several copies of the mtDNA [[Bruce A. et al., 1994](#)].

Mitochondria are highly dynamic organelles that response to cellular stress through changes in overall mass, interconnectedness, and sub-cellular localization. Changes in overall mitochondrial mass reflects and altered balance between mitochondrial biogenesis (increased mitochondrial genome duplication combined with increased protein mass added to mitochondria) and rate mitophagy (degradation of mitochondria at the autophagosome) [[Boland M.L et al., 2013](#)]. In addition mitochondria continually undergo a process of fusion and fission depending on the functional state of the cell and the balance between these opposing events determines the morphology of the organelle [[Bereiter-Hahn J. et al., 1994](#); [Westermann B., 2010](#); [Kanamuru Y et al., 2012](#)]. Mitochondria fusion occurs when mitofusin proteins (MFN) link the OMM of two separated mitochondria, and the protein OPA1, which resides in the IMM, facilitate the lengthening and tethering of adjacent mitochondria to form a connected tubular network [[Martinez T.N et al., 2012](#)]. It is thought to allow the exchange of contents between intact and dysfunctional mitochondria consenting the replacement of damage material. Therefore fusion contributes to the suppression of further damage and mitochondrial homogeneity [[Kanamuru Y. et al., 2012](#)]. On the contrary, mitochondria fission involves division of mitochondria in punctuate structures and it is mediated by fission-1 (FIS1) and dynamin-related protein 1 (Drp-1)

[[Subhashini B. et al., 2013](#)]. Fission is thought to allow the segregation of severely damaged mitochondria from healthy mitochondrial networks. These segregated damaged mitochondria are delivered to autophagosomes and ultimately degraded (mitophagy) [[Kanamaru Y. et al., 2012](#)] (Figure 2).

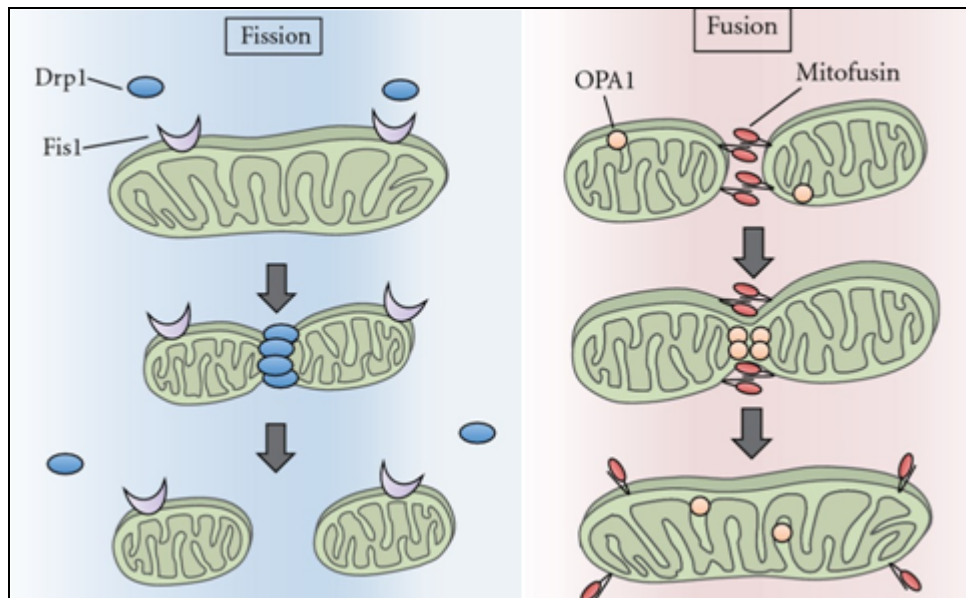


Figure 2. Pool of viable mitochondria: Fusion and Fission [[Kanamaru Y et al., 2012](#)]

2.2 Mitochondrial Functions

➤ Oxidative phosphorylation system

Mitochondrial reaction chain (MRC) is classically defined as an electron-transfer chain driven by a sequence of prosthetic groups (flavins and cytochromes) localized in the IMM and that catalyzes redox reaction from nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH₂) originating in different metabolic pathways (glycolysis, fatty acid oxidation or the TCA cycle) to oxygen (O₂) [[Chance B. et al., 1955](#)]. Subsequently, the MRC has been depicted as the functional sequence of four multi-subunit complexes (CI-IV), randomly dispersed in the IMM where reducing equivalents enter the MRC through CI (NADH-coenzyme Q reductase) or several FADH₂ dehydrogenases (e.g. succinate-coenzyme Q reductase or CII, glycerol-3-phosphate dehydrogenase, electron-transfer flavoprotein (ETF)-ubiquinone oxidoreductase, dihydroorotate dehydrogenase, etc) to reduce coenzyme Q (CoQ₁₀). CoQ₁₀ is a lipophilic mobile redox-active molecule that transports electrons to ubiquinone: cytochrome c reductase (CIII) for reducing cytochrome c (cyt c), that in

turn is oxidized by cytochrome c oxidase (CIV) to reduce O_2 as the final acceptor. The oxidation of NADH and FADH₂ is coupled to the pumping of protons ($\Delta p H^+$) into the IMM space, and the resulting proton gradient ($\Delta \mu H$) is used by the ATPase to generate ATP [Lenaz G. et al., 2010] (Figure 3).

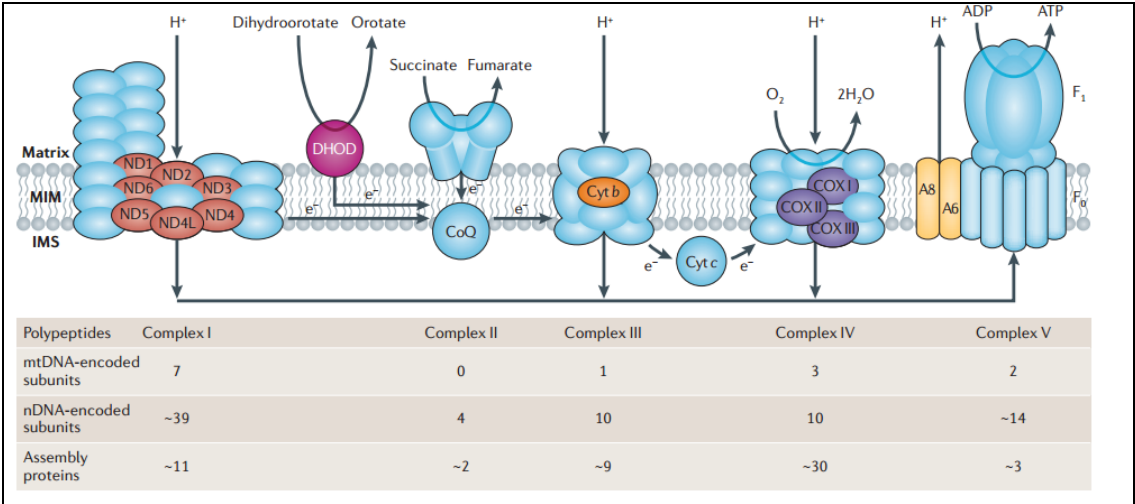


Figure3. OXPHOS complexes. CI, III, IV and ATPase contain both mtDNA and nuclear DNA (nDNA)-encoded subunits, whereas CII, which is also part of the TCA cycle, has only nDNA-encoded. Polypeptides encoded by nDNA are in blue (except for Dihydroorotate dehydrogenase (DHOD), which is in pink); those encoded by mtDNA are in colours [Schon E.A. et al., 2012]

- NADH-coenzyme Q reductase or Complex I

Although it has not been possible to crystallize the human CI due to the difficult to preserve the enzyme, excellent progression has been made in revealing the structure [Gabaldón T. et al., 2005]. CI has an L-shaped structure consisting of 2 arms: a hydrophobic membrane region which is embedded in the IMM and a hydrophilic peripheral or matrix region which protrudes into the matrix [Leonard K. et al., 1987; Efremov R.G et al., 2011]. It is composed of 45 subunits, seven of which are mitochondrially encoded [Carroll J. et al., 2002] and constitute the hydrophobic arm of the complex. The remaining 38, encoded by nDNA, are present in both the hydrophilic and hydrophobic arms of the complex [Iommarini L et al. 2013]. It also includes a flavine mononucleotide (FMN)-containing flavoprotein and six iron-sulfur (Fe-S) center. The latter are one of the three types of electron-carrying molecules function in the MRC in which the iron (Fe ion) is surrounded by sulfur (S) atoms of four Cys residues. Nevertheless the function and the molecular mechanism involved in CI assembly are still poorly understood. Three functional modules can be distinguished for bovine CI i)

the NADH dehydrogenase part, responsible for the oxidation of NADH, consisting of at least the NDUFV1, NDUFV2, NDUFV3 and NDUFS1 subunits; ii) hydrogenase module, which guides the released electrons to CoQ₁₀, consisting of at least the NDUFS2, NDUFS3, NDUFS7 and NDUFS8 subunits and iii) proton traslocating unit or membrane arm, which consist of at least the ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 subunits [[Galkin A. et al., 2006](#); [Raqaan C.I. et al., 1986](#)] (Figure 4).

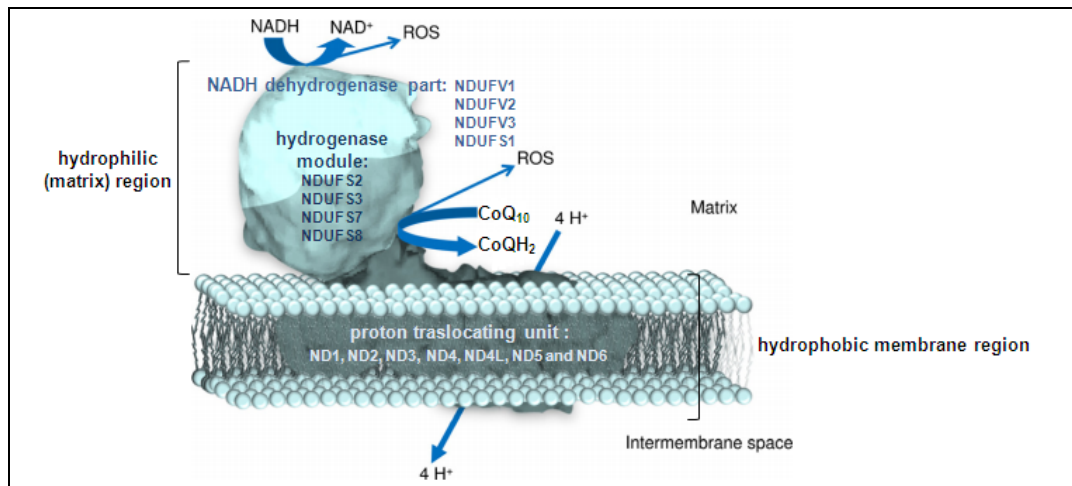


Figura 4. CI structure and function [modified from [Iommarini L. et al., 2013](#)]

CI is the first and crucial component of MRC. Electrons derived from NADH, generated by Krebs Cycles, are transferred to FMN bound to NDUFV1 subunit (51 kDa) that then transferred them to a series of Fe-S clusters. Thus electrons would flow to N3 in the same subunit and to N4 and N5 in the NDUFS1 subunit (75 kDa), and then to N6a and N6b in the NDUFS8 subunit (TYKY) and to N2 in the NDUFS7 subunit (PSST). Finally, the electrons are transferred from N2 Fe-S cluster, that is likely located between the hydrophobic membrane and hydrophilic arm to CoQ₁₀, in the Q-pocket (ND1) where it initiates a cascade of conformational changes (Figure 5) [[Lenaz G. et al., 2010](#)].

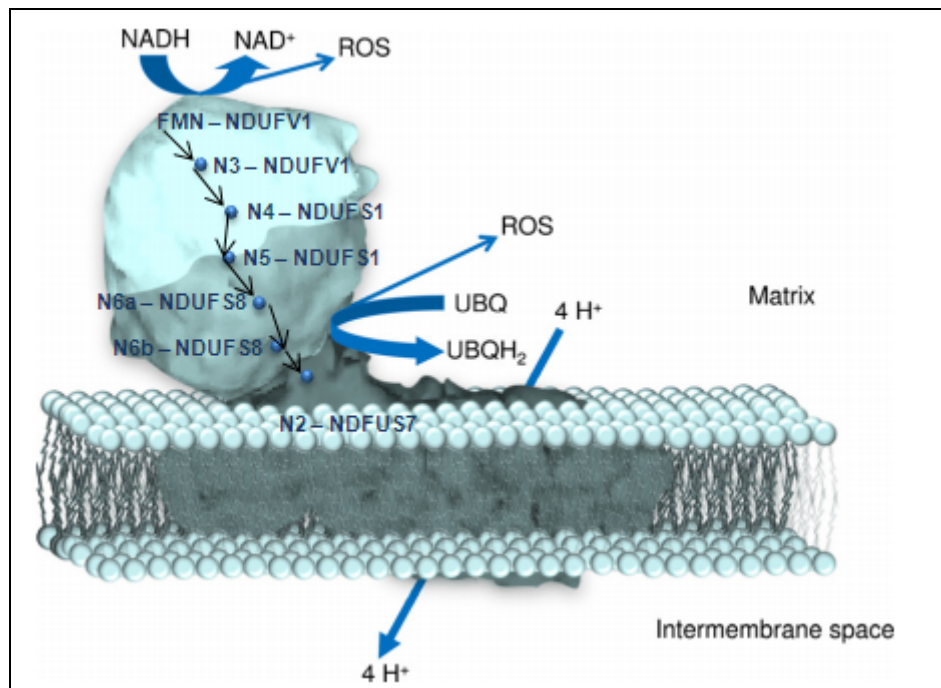


Figure 5. A schematic representation of the electron pathway from NADH to physiologic acceptor CoQ₁₀ through FMN and the Fe-S clusters [modified from [Iommarini L. et al., 2013](#)]

Effectively a suitable conformational change occurs after the first electron delivery to CoQ₁₀ to provide a gating mechanism for the second electron to semiquinone to produce ubiquinol (CoQH₂). Reduction of CoQ₁₀ to CoQH₂ also contributes to the generation of ΔpH at ND2, ND4 and ND5 subunits across the IMM from the matrix side to the intermembrane space [[Lenaz G. et al., 2007](#)]. The ND2, ND4 and ND5 subunits known as Nqo14, Nqo13 and Nqo12 respectively in *T. thermophilus* are homologous to Na⁺/H⁺ antiporter complex subunits and contain a putative proton-translocation channel [[Baradaran R. et al., 2013](#)]. Three mechanisms of ΔpH have been hypothesized: 1) a revised direct model for coupling the transport of H⁺ to electron transfer [[Ohnishi, T. et al., 2005; 2012](#)]; 2) an indirect coupling, which involves a conformational change of the enzyme [[Belogrudov G. et al., 1994; Euro L. et al., 2008](#)], and 3) a chimera models of having both direct and indirect mechanism [[Friedrich T. et al., 2004; Sazanov et al., 2000; Baranova, E.A., 2007](#)]. The revised direct model simultaneously involves 2H⁺/2e⁻ stoichiometry via conformation-coupled indirect proton (H⁺)-pumping plus (2H⁺/2e⁻) stoichiometry by CoQ₁₀ redox-coupled direct H⁺-pump [[Ohnishi, T. et al., 2012](#)]. As regards the chimera model, it has been discovered that ND5 subunit extends a long amphipathic α -helix (called HL) aligned with the membranes close to the end of the electron transfer chain. HL links most subunits

together (ND 3, 4L, 6), separating antiporter homologs from the putative Q site in ND1 subunit. Therefore a “piston-like rod” method has been proposed in which HL will simultaneously open and close three antiporter pumps (ND2, ND4 and ND5) subunits for the total (3H⁺/2e⁻) stoichiometry. And by the reduction of the CoQ₁₀ likely in ND1 subunit, conducts the remaining one H⁺ pumping with the (1 H⁺/2e⁻) stoichiometry, by an unknown mechanism [[Mathiesen C. et al., 2002](#)]

CI is also considered one of the main site of ROS production having two sites, NADH-binding and the CoQ₁₀-binding sites, accessible to O₂ where formation of superoxide anion (O₂^{•-}) may occur [[Murphy M.P., 2009](#); [Iommarini L et al. 2013](#)]. Effectively the Fe-S cluster (N2-N6) in the hydrophilic arm is reasonably well shielded from O₂ thus O₂ is more likely to access electron carriers at FMN and CoQ₁₀ sites. Nonetheless the involvement of the N2 Fe-S cluster is not excluded. The other mechanism by which CI produced O₂^{•-} is during reverse electron transport (RET) even though the site of ROS production is not known. RET occurs when electron supply reduces the CoQ pool, which in the presence of a significant ΔpH, forces electrons back from CoQH₂ into CI reducing NAD⁺ to NADH at the FMN site [[Murphy M.P., 2009](#)].

In conclusion, CI is inhibited by more than 60 different families of natural and commercial compounds from rotenone to a number of synthetic neurotoxins including 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) and its active metabolite 1-methyl-4 phenylpyridinium (MPP⁺). They have been grouped into three classes: 1a) antagonists of CoQ₁₀ substrate including roliniastatin-2, pieridin A and idebenone, 1b) antagonists of semiquinone intermediate including pesticide rotenone, pieridin A and B, aureothin, amytal, phenoxan and MPP⁺ analogue and 1c) antagonists of CoQH₂ including stigmatellin, reduced Q-2, myxothiazol and meperidine [[Lenaz G. et al., 2010](#)]. Both rotenone and MPP⁺ displace the ubiquinone intermediate and they might act at the hydrophobic pocket where CoQ₁₀ access to the catalytic site of the enzyme [[Degli Esposti M. et al., 1994a](#); [Vinogradov A.D., 1993](#)]. Their specific interactions with CI were demonstrated by replacing the endogenous subunit in human neuroblastoma cells with the single-subunit NADH dehydrogenase from *Saccharomyces Cerevisiae*. The substitution of this neurotoxic-insensitive CI attenuated rotenone and MPP⁺ toxic effect [[Shere T.B. et al., 2003](#); [Richardson J.R. et al., 2007](#)].

- Succinate-coenzyme Q reductase or Complex II

Besides its functional role in the Krebs cycle, CII is involved in the respiratory chain because it can couple the two-electron oxidation of succinate to fumarate with the electron transfer from FADH₂ to CoQ₁₀. It is the only respiratory enzyme completely encoded by nDNA. [Rustin P. *et al.*, 2002]. Mammalian CII contains a single *b* heme, a binding site for CoQ₁₀ and it has anchored to the IMM by two hydrophobic subunits, SdhC (14.2 kDa) and SdhD (12.8 kDa). Moreover the primary sequence of the soluble domain consists of a flavoprotein subunit (SdhA, 79 kDa) containing covalently linked FAD and a Fe-S protein subunit (SdhB, 31 kDa) both located on the matrix side of the membrane. Electrons derived from oxidation of succinate to fumarate are transferred to Fe-S redox co-transport chain that extends from FAD to the CoQ₁₀ site. Heme *b* does not seem to be directly involved in the transfer of electrons within the enzyme but it may serve to reduce the frequency with which electrons “leak” out the system, moving from succinate to O₂ to produce ROS (Figure 6).

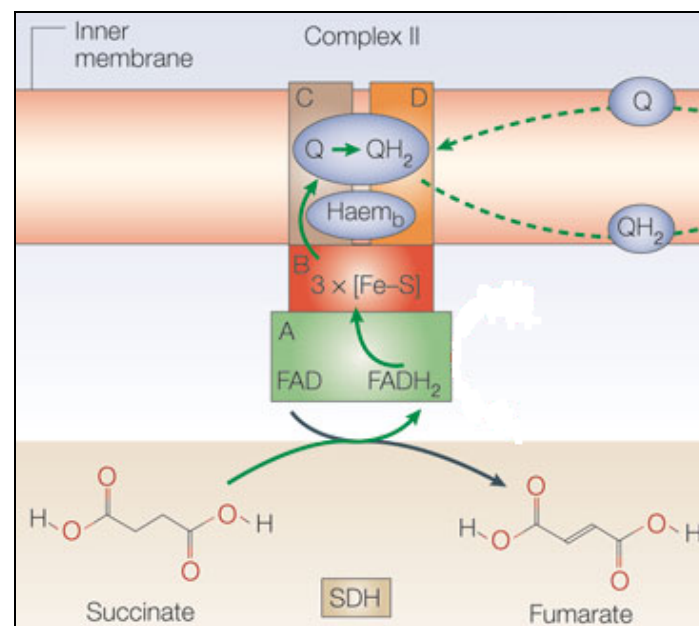


Figure 6. CII structure [Gottlieb E. *et al.*, 2005]

CII is the only enzyme that does not pump protons from the matrix to the intermembrane space and it has inhibited by malonate [Lenaz G. *et al.*, 2010].

- Coenzyme Q

In addition to NAD and flavoproteins, CoQ₁₀ is another type of electron-carrying molecule in the MRC. It is a lipid-soluble benzoquinone with a long isopropenyl side chain formed by ten units. The quinone chemical group allows the CoQ₁₀ to function as electron transporter. Effectively this molecule exists in three different oxidation states: the completely oxidized CoQ₁₀ can accept one electron to become the semiquinone radical or two electrons to form the completely reduced CoQH₂ (Figure 7).

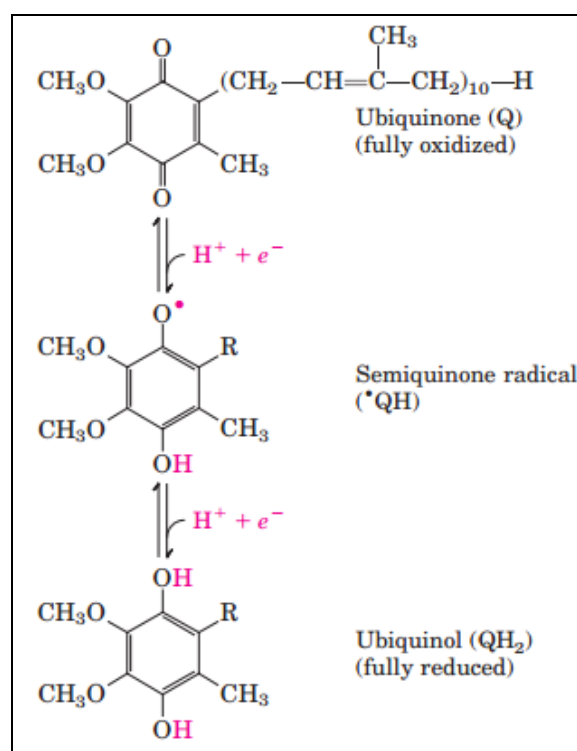


Figure 7. Three different oxidation states of CoQ₁₀

The isoprenoid hydrophobic tail allows to freely diffuse within the lipid bilayer of the IMM where CoQ₁₀ transfers electrons from CI and CII to CIII. It receives electrons both from MRC, and glycerol 3-phosphate and ETF dehydrogenase, etc.

- Ubiquinone:cytochrome c reductase or Complex III

CIII catalyze the transfer of electrons from CoQH₂ to cyt c and it can, concomitantly, link this redox reaction to translocation of H⁺ across the membrane. [[Lenaz G. et al., 2010](#)]. It is a symmetrical, oligomeric dimer of identical monomers, each with 11 subunits. Only one is mtDNA-encoded, cytochrome b, whereas the other 10 are nDNA-

encoded, and at least one of the nDNA-encoded subunits has been reported to be essential for the enzyme assembly [[Berry E.A. et al., 2000](#); [Zeviani M. et al., 2003](#)]. Each monomer presents three protein subunits with redox prosthetic groups: i) a di-heme cytochrome b containing two hemes b_H (or b_{566}) and b_L (or b_{562}), ii) cytochrome c1 and iii) a Fe-S protein (Rieske protein) with a 2Fe-2S cluster (figure 8). The other seven non-redox subunits are also present but not required for electron-transfer and proton translocation activities of the enzyme so their possible functions include structural stability and regulation of coordinated activity of the dimeric enzyme [[Lenaz G. et al., 2010](#)]. These structural details provide a confirmatory evidence of the the protonmotive Q-cycle mechanism of the enzyme proposed by Mitchell (Q cycle), with protons being carried across the IMM, whereas electrons from CoQH_2 are transferred through the bc1 complex. According to Mitchell, CoQH_2 delivers the first electron at the outer positive site called site Q_o of the IMM to the Rieske Fe-S protein and hence to cytochrome c1 that then reduces cyt c. The result is the release of two protons in the intermembrane space and the formation of semiquinone anion at the Q_o site, which is immediately oxidized to CoQ_{10} by cytochrome b_L . The electron is then delivered to the cytochrome b_H at the internal negative site (site Q_i), and then b_H is reoxidized by CoQ_{10} at the Q_i site, forming another semiquinone. The cycle is completed by oxidation of a second molecule of CoQH_2 . The Q-cycle cycle determines the oxidation of the two CoQH_2 molecules resulting in the release of four H^+ in the intermembrane space, the reduction of two molecules of cyt c and the traslocation of two H^+ from the matrix to the intermembrane space (Figure 8) [[Mitchell P.,1975](#); [Lenaz G. et al., 2010](#)]

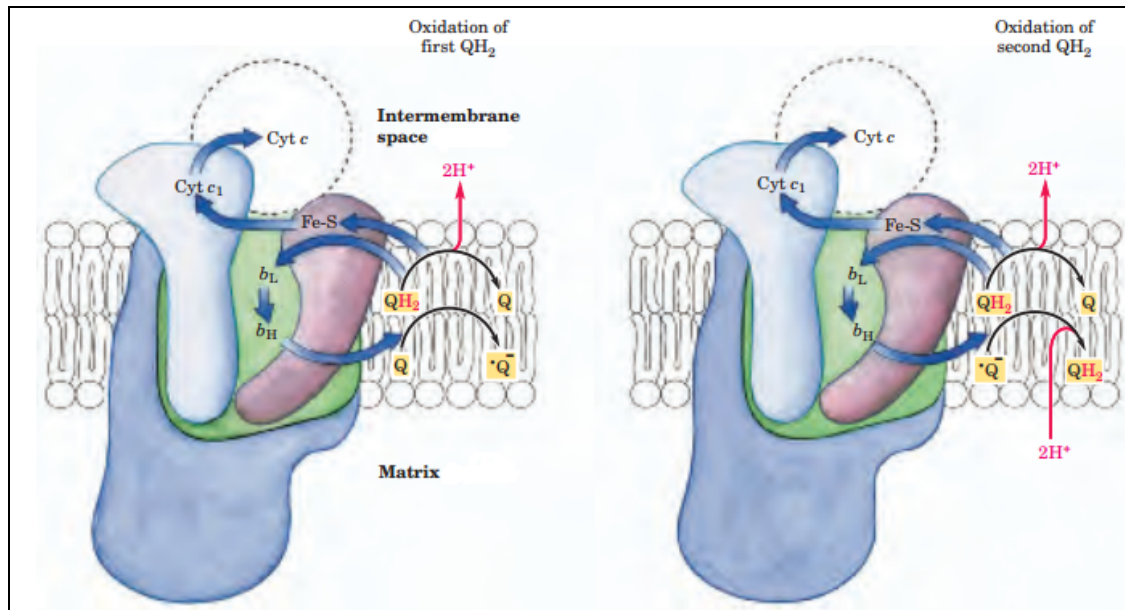


Figure 8 The Q cycle.

The formation of $O_2^{\bullet-}$ in CIII depends on this peculiar mechanism of electron transfer. It is possible that both center Qo and semiquinone are the main responsible for ROS production. Effectively Muller et al. suggested that oxidation of CoQH₂ at center Qo is characterized by the delivery of the first electron to the Rieske Fe-S cluster, producing a semiquinone that, in the absence of further oxidation by cytochrome b_L, would interact with oxygen, forming $O_2^{\bullet-}$. It is directed toward intermembrane space due to the position of the Qo site [Muller F.L. et al, 2003]. Antimycin A (AA) is known to block CoQ₁₀ reduction by cytochrome b_H at center Qi enhancing the production of $O_2^{\bullet-}$ [Lenaz G. et al., 2010]

CIII inhibitors have been grouped into two classes: i) class I inhibitors that target the Qo center and were further divided into three subclasses (Ia, Ib and Ic) and ii) class II inhibitors that act on center Qi.

- **Cytochrome c**

The third type of electron-carrying molecules function in MRC is an iron-containing protein as cytochromes. Mitochondria contains three classes of cytochromes, designed a, b and c. The heme factors of a and b cytochromes are not covalently bound to their associated proteins, whereas the hemes of c-type cytochromes are covalently attached through Cys residues. The latter is a small soluble protein of the intermembrane space. It is responsible for the transfer of electron from CIII to CIV. After its single heme

accepts an electron from CIII, cyt c move to CIV to donate electron to a binuclear copper center

- **Cytochrome c oxidase or Complex IV**

In the final step of the respiratory chain, CIV carries electrons from cyt c to O_2 , reducing it to H_2O . CIV is a large enzyme composed of 13 subunits that belongs to the heme-copper oxygen reductase superfamily. Tree (subunit I,II and III) of the 13 subunits are encoded by mtDNA and represent the catalytic center of the enzyme, whereas the remaining ten subunits are encoded by nDNA and they have been identified as essential to the enzyme assembly [Zeviani M et al., 2003; Shoubridge E.A., 2001b]. Mitochondrial subunit I contains two heme group, designed a and a_3 , and an other copper ion (Cu_B). Heme a_3 and Cu_B form a second binuclear center that accept electrons from heme a and transfer them to O_2 bound to heme a_3 . Subunit II contains two Cu ions complexed with the $-SH$ group of two Cys residues in a binuclear center (Cu_A) that resembles the 2Fe-S center of Fe-S proteins (Figure 9).

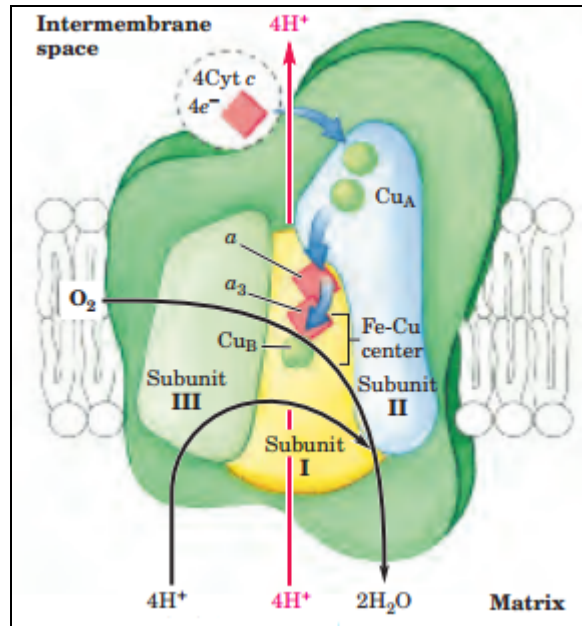


Figure 9. Path of electrons through CIV

Electron transfer through CIV occurs from cyt c to the Cu_A center which acts as a single-electron receptor, then to heme a_3 - Cu_B center, and finally to O_2 bound to heme a_3 . For every four electrons passing through this complex, the enzyme consumes four

“substrate” H^+ from the matrix in converting O_2 to $2H_2O$. It also uses the energy of this redox reaction to pump one H^+ outward into the intermembrane space for each electron that passes through, adding to $\Delta\mu H$ produced by redox-driven proton transport through CI and CIII [[Lenaz G. et al., 2010](#)]. This four-electron reduction of O_2 must occur without release of incompletely reduced intermediates such as hydrogen peroxide (H_2O_2) or hydroxyl free radicals ($OH^\bullet$) that remains bound to the complex until completely converted to H_2O .

CIV is potently inhibited by potassium cyanide (KCN), azide (N_3), nitric oxide (NO) and carbon monoxide (CO), which bind at the O_2 binding site (heme a_3).

In other words CI, III and IV are considered the core proton translocating complexes because are involved in the ΔpH coupled to the ATP synthesis. The organization of respiratory complexes in the IMM is an object of intense debate. For many years, the most accepted model for the MRC organization was the fluid or random collision model [[Hackenbrock C.R. et al., 1986](#)] opposite to the original model that proposed the respiratory components closely packed to guarantee high efficiency in electron transport [[Chance B. et al., 1955](#)]. At the beginning of 2000 years, some evidences for a supermolecular organization of respiratory complexes were obtained by introducing a sensitive analytical approach as the BN-PAGE in digitonin-solubilized mitochondria [[SchäggerH. et al., 2000](#)]. With this technique, it was demonstrated in different organisms that respiratory CI, III and IV are involved in supramolecular association to form the “respirasome” leading to a reformulation of the solid model proposed by Chance [[SchäggerH. Et al., 2000](#)]. However, none of the two models satisfactory explain the functional studies on mitochondrial respiratory chain. In a very recent work, Lapeunte-Brun and colleagues [[Lapiente-Brun E. et al., 2013](#)] showed that in vivo, the MRC should be able to work both when supercomplexes are present and when the formation of supercomplexes is prevented. This work confirm the previously proposed model, “the plasticity model” in which the respiratory chain is a very dynamic organization that can move from respirasome to dispersed respiratory complexes allowing the cell to adapt to different carbon sources and varying physiological conditions [[Acin Perez R. et al., 2008](#); [Lapiente-Brun E. et al., 2013](#); [Acin-Perez R. et al., 2013](#)].

- **F1Fo ATPase or Comple V**

According to the chemiosmotic model proposed by Mitchell, the electron flow through the MRC is coupled with a proton transfer across the membrane, producing both a chemical gradient (ΔpH) and an electrical gradient ($\Delta\psi$). The energy associated to electrochemical gradient $\Delta\mu\text{H}$ drives the synthesis of ATP by the molecular motor ATP synthase (F1-FoATPase or CV) [[Mitchell P., 1961](#); [Sgarbi G. et al., 2006](#)]. CV is a ubiquitous enzyme that catalyses the terminal step in OXPHOS. The enzyme consists of two structurally and functionally distinct sectors termed F1, the proper catalytic domain, where ATP synthesis or hydrolysis takes place, and Fo (oligomycin-sensible), the membrane bound-portion that sustains H^+ transport. ATPase has two subunits encoded by mtDNA (ATPase6 and ATPase8), that take part to the Fo portion, and about 13 other subunits encoded by nDNA [[Abrahams J.P et al., 1994](#)]. F1 has nine subunits of five different types with the composition $\alpha_3\beta_3\gamma\delta\epsilon$. Each of the three β subunits has a nucleotide-binding site critical to the catalytic activity and together with three α subunits are arranged like the segment of an orange with alternating α and β subunits around a central shaft, the γ subunit. The γ subunit is associated with one of the three $\alpha\beta$ pairs forcing each β subunit into slightly different conformations, with different nucleotide-binding sites: one subunit has ADP (β -ADP) in its binding site, the next has ATP (β -ATP), and the third has no bound nucleotide (β -empty). The Fo complex is composed of three subunits a, b and c in the proportion $\text{ab}2\text{c}_{10-12}$. Subunit c is a small, very hydrophobic peptide consisting of two transmembrane helices with a loop extending from the matrix side of the membrane. It is attached to the shaft (subunits γ). The two b subunits of Fo associate firmly with α and β subunits of F1, holding them fixed relative to the membrane (Figure 10). [[Baker L.A. et al., 2012](#)].

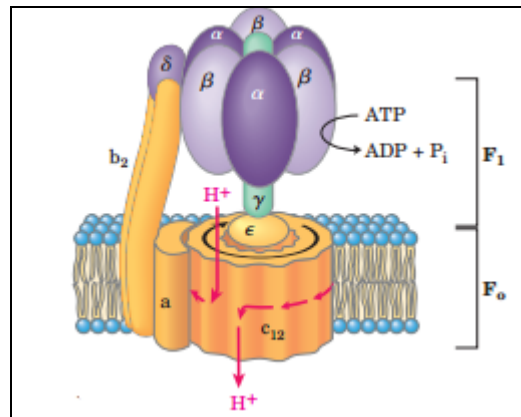


Figure 10. Structure of F1Fo ATPsynthase

As protons flow through Fo, the cylinder of c subunits and shaft (γ subunit) rotate forcing each β subunit into slightly different conformations in which the β -ATP site is converted to the β -empty conformation and dissociate ATP; the β -ADP site is converted to the β -ATP conformation, which promotes condensation of bound ADP+Pi to form ATP; and the β -empty site becomes a β -ADP site. ATP can not be released from one site unless and until ADP and Pi are bound at the other. Therefore one complete rotation of the γ subunit causes each β subunit to cycle through all three of its possible conformations, and for each rotation, three ATP are synthesized and released from the enzyme surface [[Gresser M.J. et al., 1982](#)].

Oligomycin and Dicyclohexylcarbodiimide (DCDD) are ATPase inhibitors that block the transfer of electrons through the Fo portion and then ATP synthesis.

Chemiosmotic theory explains the dependence of electron transfer on ATP synthesis. Nonetheless certain chemical compounds including 2,4-dinitrophenol (DNP) and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) cause uncoupling without disrupting mitochondrial structure. They are weak acids with hydrophobic properties that permit them to diffuse across the IMM. After entering the matrix in the protonated form they can release H^+ thus dissipating the ΔpH allowing respiration to continue without ATP synthesis. On the other hand ionophores such as valinomycin allow inorganic ions (K^+) to pass through the membrane dissipating the $\Delta\psi$ across the IMM.

The ATP synthesized in the matrix is transported across the IMM with an exchange mechanism, importing cytosolic ADP and phosphate (Pi) by the adenine nucleotide and phosphate translocase system. Adenine nucleotide translocase (ANT) is an antiporter

thus the same protein moves ADP into the matrix and ATP out. The effect is the net flux of one negative charge which is favored by $\Delta\psi$. The phosphate translocase is specific for H_2PO_4^- . There is not a net flux of charge during symport of H_2PO_4^- and H^+ . Therefore $\Delta\mu\text{H}$ is also responsible for transporting substrates (ADP and Pi) in and product (ATP) out of the matrix.

➤ Reactive Oxygen Species and Antioxidant Agent

During NADH oxidation or CoQ_{10} reduction by CI and CoQ_{10} oxidation by CIII, electrons may escape leading to Reactive Oxygen Species (ROS) generation. ROS are dangerous for the cell since they can damage mtDNA, proteins and lipids [[Adam-Vizi V. et al., 2006](#); [Murphy M.P., 2009](#)] but also function as molecular signaling molecules [[D'Autreaux B. et al., 2007](#)].

Free radicals were described as “any species capable of independent existence that contains one or more unpaired electrons” [[Halliwell B. et al., 1984](#)]. The term ROS refers to a variety of reactive molecules that are derived from oxygen (O_2) and can be free radicals such as superoxide ($\text{O}_2^{\bullet-}$), hydroxyl radical ($\text{OH}^\bullet$) and non-radicals (hydrogen peroxide (H_2O_2)). It was demonstrated that ROS are generated by loose electron spilling from CI and CIII, and reacting with O_2 to form $\text{O}_2^{\bullet-}$ in IMS through the transfer of electrons from NADH to CoQ_{10} , which is accompanied by translocation of protons from the matrix to the IMM. Similarly, CII is responsible for the reduction of CoQ_{10} generating low levels of $\text{O}_2^{\bullet-}$ [[McLennan H.R. et al., 2000](#); [Fato R. et al., 2008](#); [Murphy M.P., 2009](#); [Yankovskaya V. et al., 2003](#)]

Given that low levels of $\text{O}_2^{\bullet-}$ are constantly generated, evolution has selected antioxidant system enable to detoxify this anion. Basically manganese superoxide dismutase (MnSOD, SOD2), that is a mitochondrial matrix enzyme, rapidly converts $\text{O}_2^{\bullet-}$ to H_2O_2 which is subsequently converted to H_2O by glutathione peroxidase (GPx) and catalase in the mitochondria or follows diffusion into the cytosol. GPx oxidizes reduced glutathione (GSH) to oxidized glutathione (GSSG) that is reproduced by glutathione reductase starting from GSH (Figure 11). $\text{O}_2^{\bullet-}$ can be also converted to H_2O_2 by copper-zinc superoxide dismutase (CuZnSOD) in the IMS. Nevertheless, in the presence of iron, H_2O_2 is rapidly converted to the highly reactive $\text{OH}^\bullet$ via the Fenton reaction. $\text{OH}^\bullet$ may

further react with bicarbonate to yield the very reactive carbonate radical anion ($\text{CO}_3^{\bullet-}$) [Szeto H.H., 2006] (Figure 11).

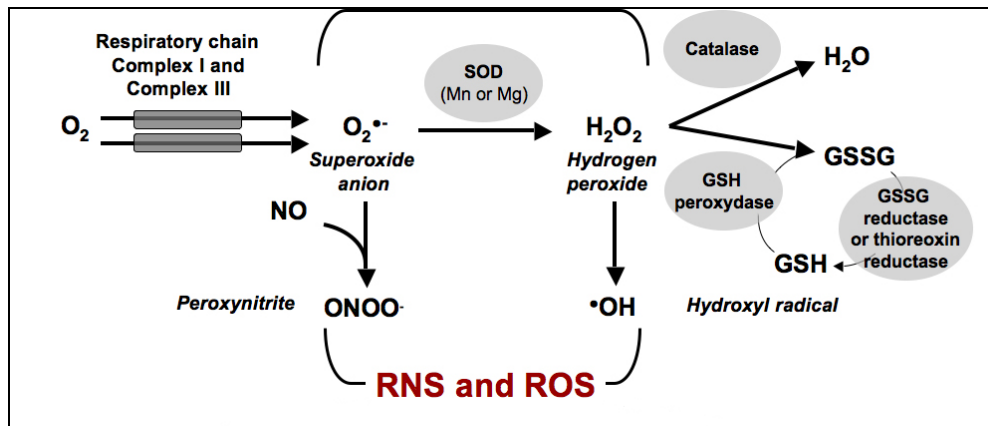


Figure 11. Generation of mitochondrial reactive species [Bellance N. et al.2009]

On the other hand, reactive nitrogen species (RNS) refers to reactive species derived from nitrogen (NO) and can be broadly classified as ions (peroxynitrite (ONOO^-)) or non-ions (Nitric Oxide ($\text{NO}^\bullet$)) [Subhashini B. et al., 2013]. ROS are formed at low levels during normal respiration by healthy mitochondria. In fact, $\text{O}_2^{\bullet-}$, generated during the electron transport chain through partial reduction of O_2 , can react with $\text{NO}^\bullet$ to form ONOO^- (Figure 11).

In conclusion, ROS are formed at different rates in a cell and differ in their activity. In terms of activity, $\text{OH}^\bullet$ is the most reactive species known and is by in large responsible for the cytotoxic effects of ROS. In contrast, reactive species such as $\text{NO}^\bullet$ and H_2O_2 are less reactive and have shown to play an important role in several cellular activities. [Subhashini B. et al., 2013]. However, damaged and dysregulated mitochondria generate excessive amounts of $\text{O}_2^{\bullet-}$, which can damage several mitochondrial components and functions and ultimately lead to cell death via apoptosis and necrosis [Subhashini B. et al., 2013; Szeto H.H., 2006].

➤ Apoptosis

Apoptosis is a major pathway of programmed cell death and is extremely important both in several physiological conditions and pathological events, including neurodegenerative, cardiovascular and immunological disorders [Zimmermann K.C. et al., 2001]. It is characterized by a cascade of controlled events that leads to specific

morphological changes in the cell: loss of adhesion, cell shrinkage, plasmatic membrane blebbing, chromatin condensation, and DNA fragmentation, proteolytic cut of specific substrates and exposure of phosphatidylserine on the external surface of the cell [[Kerr J.F. et al., 1972](#)]. The final event of this cascade is the phagocytosis of the apoptotic cell, without any release of cytoplasmic content into the extracellular matrix or inflammatory response induction. Apart the granzyme B pathway, there are two other apoptotic cascades: the “extrinsic” or death receptor pathway, and the “intrinsic” or mitochondrial pathway. The mitochondrial pathway is a complex signaling cascade, regulated by the Bcl-2 family proteins, that needs the release of apoptogenic factors (cyt c, AIF, Apoptotic protease activating factor-1 (Apaf 1), endonuclease G, Smac/DIABLO and Omi/HtrA2) from mitochondria for the caspase activation. It can be divided in three well-defined phases: induction, mitochondrial and execution phases. During the induction phase external and internal stimuli activate different signals which are transduced to mitochondria by the Bcl2-family proteins [[Adams J.M. et al., 1998](#)]. The second apoptotic step is the mitochondrial phase characterized by an alteration of the IMM/OMM permeability and the release of apoptogenic factors to the cytosol. Two mechanisms are hypothesized to explain this phenomenon, involving two distinct channels, which are the permeability transition pore (PTP) in the IMM and the mitochondrial apoptosis-induced channel (MAC) in OMM. The last step in apoptosis is the executive phase. Cyt c, released from the mitochondria into the cytosol, binds to APAF-1 and to pro-caspase-9 to create a protein complex called apoptosome. Caspases (cysteine aspartyl-specific protease) are specific protease that can be activated by proteolytic cleavage at conserved Aspartic Acid (Asp) residues. Effectively procaspase-9 binds Apaf-1 at a conserved amino acid sequence called the caspase recruitment domain or CARD, leading to the activation of procaspase-9. Caspases collaborate in a proteolytic cascades, where caspases activate themselves and each other, and finally cleave their substrate at conserved Asp residues [[Thornberry N. et al, 1998](#); [Cryns V. et al. 1999](#)] (Figure 12).

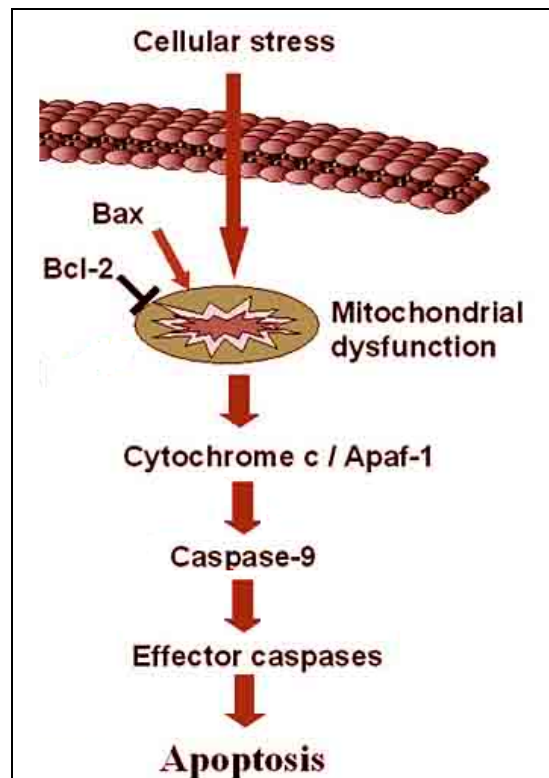


Figure 12. Mitochondrial pathway of apoptosis. It is regulated by the Bcl-2 family proteins with pro-apoptotic functions: the Bax type proteins (Bax, Bak and Bok) that cause the release of apoptogenic factors (cytochrome c, AIF, Apaf 1, endonuclease G, Smac/DIABLO and Omi/HtrA2) to the cytosol for the caspase activation [Feldstein A.E. et al., 2005]

3. MITOCHONDRIAL DNA

The human mtDNA is a double-stranded, circular molecule of 16,569 bp, now completely decoded [Anderson S. et al., 1981], containing 37 genes. Of these 24 genes - consisting of 2 ribosomal RNAs (rRNAs) and 22 tRNAs — are used for translation of 13 polypeptides [Wallace D.C., 1995] that encode subunits of the OXPHOS multimeric enzymes located in the IMM: 7 subunits of CI (ND1, 2, 3, 4, 4L, 5, and 6), 1 subunit of CIII (cyt *b*), 3 subunits of CIV (COX I, II, and III), and 2 subunits of ATP synthase (A6 and A8) [DiMauro S. et al., 2003]. More than 99% of mitochondrial proteins are encoded by nDNA, translated on cytoplasmic ribosomes, and selectively imported into the appropriate mitochondrial compartment [Johns D. R., 1995]. The mtDNA is composed of 2 strains: the guanosine (G)-rich heavy (H)-strand (O_H) and the cytosine (C)-rich light (L)-strand (O_L) [Schon E.A. et al., 2012]. All of the mtDNA mRNAs, except for ND6, are encoded by O_H and are derived from one long polycistronic transcript. ND6, by contrast, is transcribed from O_L using an independent L-strand promoter (Figure13)

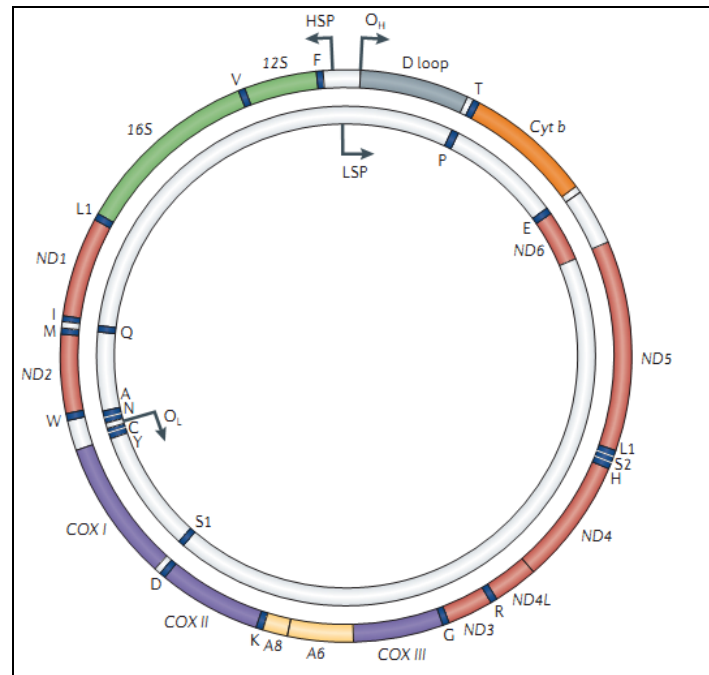


Figure 13. The mtDNA [[Schon E.A. et al., 2012](#)]

Almost the entire genome sequence is coding because there are no introns or intergenic regions. Some respiratory protein genes overlap and protein coding and rRNA genes are interspersed with tRNA genes that represent the signal for cleavage sites of RNA processing. However, there are two non-coding regions. One of 1122bp called Displacement Loop (Dloop), characterized by the presence of a triple strand structure due to the association of the new H-strand in this region. It contains the origin of H-strand DNA replication and is also the site of transcription from opposing heavy and light strand promoters [[Clayton D.A., 2000](#); [Scarpulla R.C., 2008](#)]. The other of 30bp represents the replication origin for the L-strand (Figure 13).

3.1 Mitochondrial replication, transcription and translation

Mitochondrial DNA replication is independent from the cell cycle (relaxed replication) [[Bogenhagen D.F. et al., 2003](#); [Clayton D.A., 2003](#)]. Originally it has been described as a strand-asymmetric and asynchronous replication, in which the primers for the H-strand replication are provided by the transcription mechanism. When H-strand synthesis has reached 3/4 of the DNA molecule, it exposes the origin of L-strand DNA replication (OL) and lagging-strand DNA synthesis initiates in the opposite direction. New complete mtDNA molecules are finally ligated [[Clayton D.A., 1991](#); [Falkenberg M. et al., 2007](#)] (Figure 14).

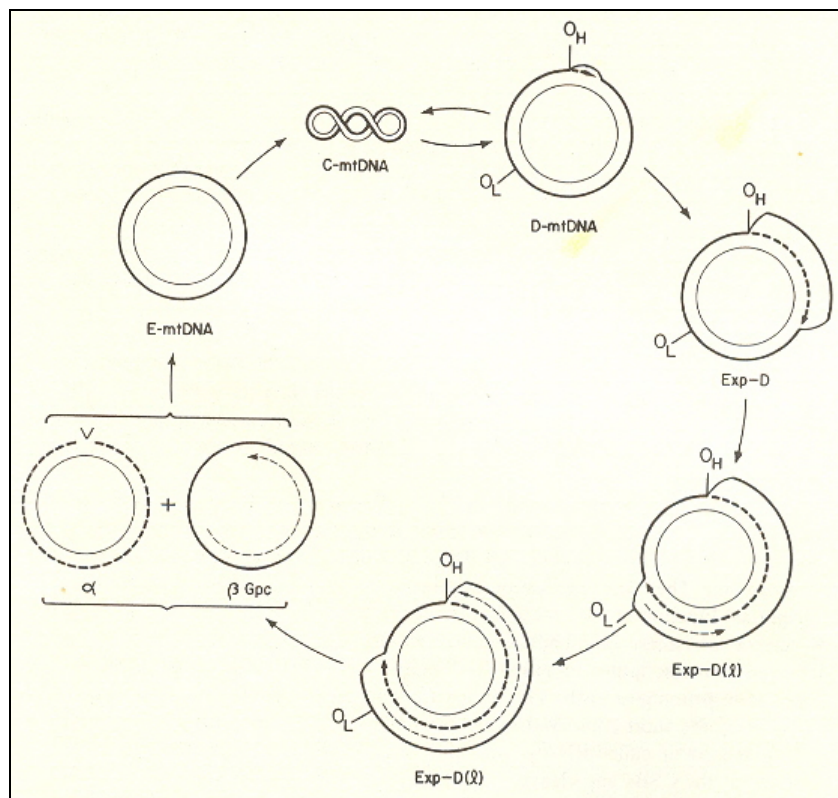


Figure 14. Strand-asymmetric and asynchronous replication of mtDNA [Clayton D.A et al., 1991]

More recently another model has been more proposed in which mtDNA replicates symmetrically, with leading and lagging strands synthesis progressing from multiple, bidirectional replication forks [Holt I.J. et al., 2000] (Figure 15).

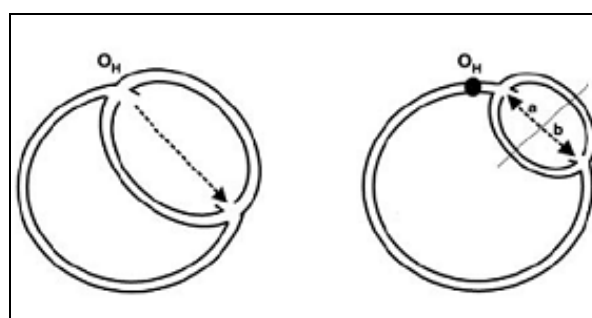


Figure 15. Symmetrically and synchronous replication of mtDNA [Holt I.J. et al., 2000]

The enzyme responsible for mtDNA replication is DNA polymerase γ (POL γ), an RNA dependent DNA polymerase, discovered in human HeLa cells [Fridlender B. et al., 1972], composed by a catalytic subunit (POL γ A, 140kDa), with polymerase 3'-5' exonuclease, and 5'-deoxyribose phosphate lyase activities, and 2 smaller accessory subunits (POL γ B, 55kDa), able to increase the catalytic activity of POL γ A [Gray H. et al.,

[1992](#); [Kaguni L.S., 2004](#); [Pinz K.G. et al., 2000](#)]. Other 2 proteins are necessary for mtDNA replication: the helicase TWINKLE and the mitochondrial single-stranded DNA-binding protein (mtSSB). Together with POLy, they form a processive replisome, able to replicate the entire mtDNA [[Falkenberg M. et al., 2007](#)]. Moreover, mtDNA replication requires a RNA primer synthesized by the action of the mitochondrial transcription factor A (TFAM) and the mitochondrial RNA polymerase (mtRPOL). TFAM is able to wrap, bend and unwind DNA by HMG-boxes (High Mobility Group) inducing a structural change in the promoter region of mtDNA that allows the mtRPOL to initiate transcription of the RNA primer necessary for the replication of mtDNA. The synthesized RNA fragment is stably hybridized to parental L-strand of mtDNA to form a triple helix causing the displacement of the parental H-strand. The hybrid RNA-DNA starts the H-strand replication following the binding of the POLy [[Shadel G.S. et al., 1997](#)].

Mitochondrial transcription starts from 3 different transcription origins, one for the L-strand (O_L) and 2 for the H-strand (H₁ and H₂), producing 3 polycistronic molecules [[Montoya J. et al., 2006](#)]. The machinery required for mtDNA transcription includes the mtRPOL, the initiations factors TFAM, TFB1M and TFB2M and the termination factor mTERF. ORI H₁ (nucleotide 561) is responsible for the synthesis of the 2 rRNAs (12S and 16S), tRNA^{Phe} and tRNA^{Val}. This short transcript is terminated thanks to the binding of mTERF protein to a specific sequence within the tRNA^{Leu} gene, and is needed to produce the appropriate amount of ribosome for translation. ORI H₂ (position 646) produces a polycistronic molecule that is subsequently processed in 12 mRNAs and 14 tRNA [[Clayton D.A. 1991](#)]. ORI_L generates a single polycistron starting at position 407, from which 8 tRNAs and the ND6 mRNA are derived [[Montoya J. et al., 2006](#)]. The primary transcripts are processed, according to the “tRNA punctuation” model, to generate the mature RNAs after an endonucleolytic cleavage, triggered by the maturation of tRNAs secondary structure [[Montoya J. et al., 1983](#); [Ojala D. et al., 1981](#)] (Figure 16).

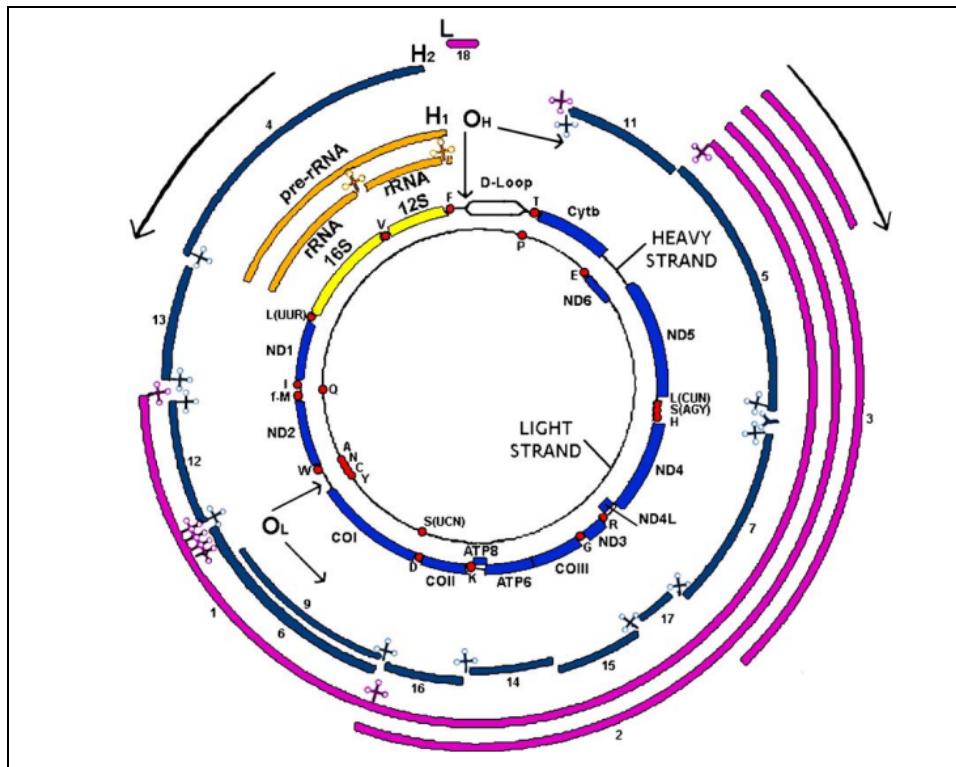


Figure 16. Mitochondrial transcription. The 2 internal circles represent both mtDNA strands with the encoded genes in yellow (rRNAs), red dots (tRNAs) and blue (protein coding genes). External circles represent the RNAs transcribed from the H- strand (in orange or in blue for the RNAs derived from the H1 or H2 transcription units) and L- strand (in pink). Arrows at the OH and OL, and in the outside part of the figure, indicate the direction of replication and transcription of both strands. [[Montoya J. et al., 2006](#)].

Mitochondrial encoded mRNAs are translated in the matrix with specific translational machinery represented by the mitoribosomes composed by 2 mitochondrial rRNAs (12s and 16s) and nuclear encoded proteins.

The genetic code of mtDNA is also slightly different. Thus, UGA in mitochondrial translation does not specify for a tryptophan amino acid, but a stop codon; moreover AUA represent an isoleucine and not a methionine and AGA/AGG are not stop codons but specify for arginine [Attardi G. et al., 1988].

3.2 Mitochondrial genetics

Mitochondrial genetics follows its specific rules and encompasses the rules of both Mendelian and non-Mendelian genes [[Coskun P. et al., 2012](#)]. The mtDNA is maternally inherited [[Wallace D.C. 2005](#)] so every mitochondrion and every mtDNA, in the zygote derives from the oocyte, because after the fecundation process all mitochondria from the spermatocytes are degraded in a ubiquitin-dependent fashion [[Sutovsky P. et al.,](#)

[1999](#); [2000](#)]. Thus, mtDNA molecules and, if present, mtDNA mutations are transmitted in the progeny, along the maternal lineage. However it has been reported a case of paternal transmission of a pathogenic microdeletion of 3bp in the context of a mitochondrial diseases affecting the skeletal muscle [[Schwartz M., 2002](#)]. Each mitochondrion contains several hundred to several thousand copies of mtDNA molecules packaged in nucleoids that are anchored to the IMM [[Scheffler I.E., 2001](#); [Meyer J.N. et al., 2013](#)]; the number varies according to the bioenergetic needs of each particular tissue [[Schon E.A. et al., 2012](#)]. Whether all mtDNA molecules are identical (*wild type* (*wt*) or mutant), this condition is called homoplasmy. When a mutation occurs, *wt* and mutant mtDNA can coexist within the same cell, a condition known as heteroplasmy [[Wallace D.C., 1999](#)]. The proportions of mutant and *wt* mtDNAs are distributed to the daughter cells stochastically during both mitosis and meiosis according to the distribution of mutant mtDNAs at cytokinesis. Therefore, the percentage of mutant mtDNAs can drift during cell division toward either more or less mutant. As the percentage of mutant mtDNAs increases, the energy output of the cell declines until it crosses the minimum energy threshold for that tissue to function, the bioenergetic threshold, at which point symptoms appear [[Coskun P., 2012](#)]. A heteroplasmic mutation can be transmitted with different mutation load because during the oogenesis there is a preferential amplification of only few mtDNA molecules (bottleneck) (Figure 17) [[Marchington D.R. et al., 1998](#)].

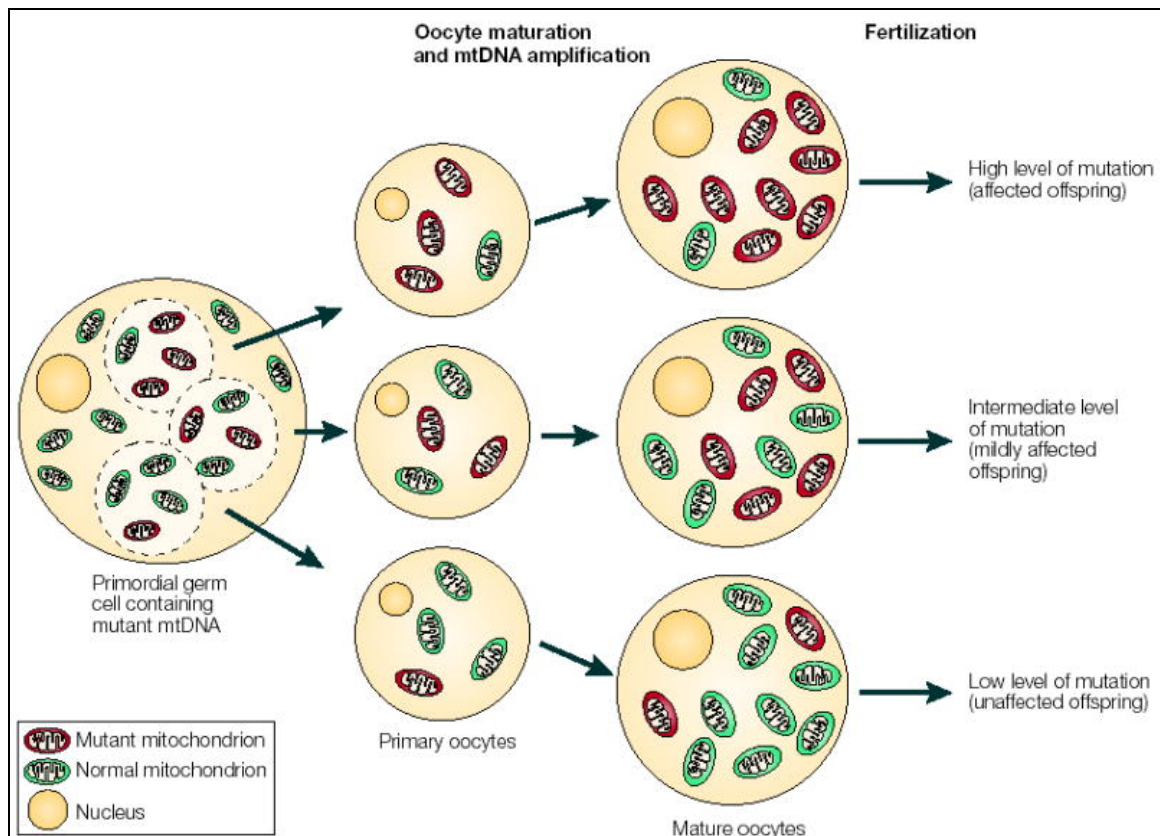


Figure 17. The mitochondrial genetic bottleneck. During fertilization, a selected number of mtDNA molecules are transferred into each oocyte which maturation is associated with the rapid replication of this mtDNA population. This restriction-amplification event can lead to a random shift of mtDNA mutational load between generations and is responsible for the variable levels of mutated mtDNA observed in offspring (heteroplasmy) [Taylor R.W. et al., 2005]

The bottleneck phenomenon explains the rapid shift of some heteroplasmic mutation to homoplasmy, in a few generations. In the majority of cases, mutations do not cause a biochemical phenotype until they reach a threshold level, which has been shown to vary for different types of mutation, in the range of 50–60% [Shoubridge E.A., 1994; Moraes C.T. et al., 1992; Mita S. et al., 1990; Hayashi J. et al., 1991], for deleted mtDNA molecules, and up to >90% for some tRNA point mutations [Boulet L. et al., 1992; Chomyn A. et al., 1992]. However, there has been one recent report of a dominant mutation, m.5545C → T, in the *MT-TW* gene, which showed tissues to be clinically affected with a mutation level of <25% [Sacconi S. et al., 2008; Greaves L.C. et al., 2012]

The mtDNA has a very high mutation rate, presumably due to its chronic exposure to mitochondrial ROS [DiMauro S. et al., 2008] because it is hypomethylated, employs a relatively inefficient repair mechanism, and is proximal to the ETC in the IMM

[[Martinez N.T. et al., 2012](#)]. The absence of protective histones, the lack of effective repair mechanism, and the high mtDNA replication rate are all factors increasing the likelihood of errors [[Yu-Wai-Man P. et al., 2011](#)].

3.3 Mitochondrial-nucleus communications: mitochondrial biogenesis

Mitochondrial biogenesis is a complex and regulated process that involves the coordinated expression of mitochondrial and nuclear genes. As a matter of fact mitochondria only have limited autonomy and they rely heavily on the nDNA for the majority of their structural and functional subunits [[Yu-Wai-Man P. et al., 2011](#)]. Mitochondrial biogenesis is finely tuned by different signaling cascades that involve transcription factors and co-activators that regulate the expression of genes coding for mitochondrial components, which include nuclear encoded mitochondrial proteins participating in OXPHOS, heme biosynthesis, mitochondrial protein import, and mtDNA transcription and replication. The most important transcription factors activating promoters of mitochondrial genes are transcription factor A mitochondrial (TFAM), NRF (nuclear respiratory factor)-1, NRF-2, PPARs (peroxisome proliferator associated receptors) and ERR α (estrogen related receptor), together with transcriptional co-activators belonging to the peroxisome proliferator-activated receptor γ -coactivator-1 (PGC-1) family [[Diaz F. et al., 2008](#)]. Many other nuclear factors have been implicated in the expression of respiratory genes and in the control of mitochondrial biogenesis. For example, the cyt c promoter contains *cis*-elements that recognize transcription factors ATF/CREB, c-Myc and Sp1, while muscle-specific CIV subunits are regulated by MEF-2 and/or YY1 (YingYang1) [[Alaynick W.A. 2008](#)]

3.4 Mitochondrial DNA variability

Due to its peculiar uniparental maternal inheritance and high mutation rate, mtDNA has been extensively used to study population genetics by phylogenetic analysis since a great number of mtDNA variants have been fixed and accumulated sequentially characterizing different maternal lineages. These mtDNA lineages have diverged from the first “Eve” and colonized different geographic regions. Based on different clusters of population-specific polymorphism, present both in coding and control regions, it is possible to define the mitochondrial haplogroups clusters of mitochondrial genomes,

which are continent-specific and defined by the presence of ancestral polymorphisms in the maternal line [[Torroni A. et al., 1993](#); [Graven L. et al., 1995](#); [Torroni A. et al., 1994](#); [1994b](#)]. The origin of modern humans dates back to about 70,000 years ago when some *sapiens* (haplogroup L3) left the Horn of Africa (“out of Africa” model) to direct toward the coasts of Arabia, Iran, India, arriving in East Asia (haplogroups N, M). Macrohaplogroup N moved along north into the Middle East and radiated to create submacrohaplogroup R. Both R and N lineage spread into Europe to generate the European-specific haplogroups (H, I, J, K, T, U, V, W, X) It also moved along Southeast Asia to Australia and from southern Asia north into central Asia to form haplogroups A and Y, whereas the N-derived R lineage generated haplogroup B and F. Macrohaplogroup M moved along tropical Southeast Asia, ultimately reaching Australia, and later moved north out of Southeast Asia to form Asian-specific mtDNA haplogroup (C,D,G and M1-M40). Ultimately, haplogroups A, B, C, and D migrated into the American to found the Native American populations. [[Maca-Meyer et al., 2001](#); [Olivieri A. et al., 2006](#); [Wallace D.C., 2013a](#)]. Thus haplogroups, designated by a capital letter followed by a number that represents the subcluster of haplogroups [[Torroni A. et al., 1996](#)], tend to be limited to specific geographic areas and population groups (Figure 18).

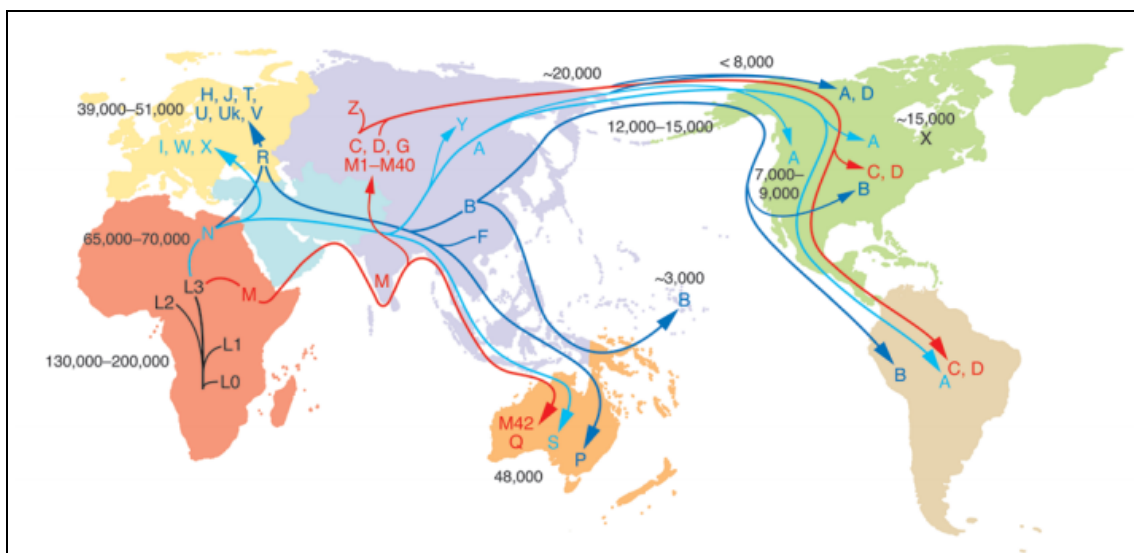


Figure 18. The migration history of human mtDNA haplogroups [[Wallace D.C. 2013a](#)]

The mtDNA polymorphisms might produce important mtDNA evolutionary changes that coincide with the major human geographical transitions facilitating the human

adaptation to different regional environments. Indeed the macrohaplogroup N migration from Sub-Saharan Africans into Eurasia have not been facilitated by the cold of the northern latitudes. Thus to survive, early humans would have needed to produce more core body heat to increase resistance to cold. It has been achieved by the mtDNA mutations that decreased the “coupling efficiency” of OXPHOS. Macrohaplogroup M, in contrast, stayed in the semi-tropic Southeast Asia, so cold-adaptative variants were not fixed in this lineage [[Wallace D.C. 2013a](#); [2013b](#)].

It has also been hypothesized that many non-synonymous polymorphisms in mtDNA (SNPs), causing aminoacid variations in the mitochondrial-encoded proteins, have the potential to modify OXPHOS activity and ROS production [[Wallace D.C., 2005](#)]. Therefore some of these haplogroups may confer vulnerability to, or protection from, a wide range of metabolic and degenerative disease, cancer and longevity [[Schon E.A., 2012](#); [Wallace D.C. 2013b](#)].

➤ Mitochondrial Medicine

The term “mitochondrial medicine” was introduced for the first time by Rolf Luft in 1994 and now is commonly used to indicate the branch of medicine dedicated to study mitochondrial dysfunctions [[Luft R., 1994](#)]. Mitochondrial disorders can be divided in two classes: the first comprise Mendelian diseases associated with nDNA mutations or rearrangements in genes encoding mitochondrial proteins, whereas the second is due to mtDNA mutations or rearrangements [[DiMauro S. et al., 2008](#)].

Major rearrangements of mtDNA usually come as deletions that are caused by defects in nuclear genes encoding for enzymes involved in mtDNA maintenance and nucleotide metabolism, whereas single deletions are usually sporadic [[DiMauro S. et al., 2008](#)]. The main syndromes associated with single sporadic deletions are Kearns-Sayre Syndrome (KSS), Pearson marrow-pancreas Syndrome (PS) and some forms of Chronic Progressive External Ophthalmoplegia (CPEO). CPEO is clinically defined by palpebral ptosis, generalized weakness and progressive limitation of ocular movements [[Spinazzola A. et al., 2005](#); [2007](#)]. Sporadic forms are reported but there are also inherited CPEO, that can be autosomal dominant (adCPEO), autosomal recessive (arCPEO) and maternally inherited [[DiMauro S. et al., 2008](#)]. The latter is caused by point mutations in mitochondrial tRNA genes specifying for leucine,

isoleucine and alanine, whereas autosomal forms are caused by mutations in POLG1, POLG2, Twinkle and ANT-1 and others. The large majority of these genes is involved in mtDNA replication or nucleotide metabolism and lead to the accumulation of multiple deletions in postmitotic tissue, mainly skeletal muscle and CNS [[Kaukonen J. et al., 2000](#); [Spelbrink J.N. et al., 2001](#); [Longley M.J. et al., 2006](#)].

Moreover it has been recently described a syndrome, characterized by dominant optic atrophy, sensorineural deafness, ataxia, axonal sensory-motor polyneuropathy, CPEO and mitochondrial myopathy with cytochrome c oxidase negative and ragged-red fibres (RRFs), in which patients harbour missense mutations in the OPA1 GTPase domain and accumulated multiple deletions of mtDNA in skeletal muscle [[Ferraris S. et al., 2008](#); [Amati-Bonneau P. et al., 2008](#); [Hudson G. et al., 2008](#)].

A more complex syndrome is MNGIE (Mitochondrial NeuroGastroIntestinal Encephalomyopathy), an autosomal recessive disorder affecting young adults. It is characterized by CPEO, peripheral neuropathy, leukoencephalopathy and gastrointestinal dysmotility [[Hirano M. et al., 2004](#)]. MNGIE is caused by different mutations in the gene TP, encoding the enzyme thymidine phosphorylase [[Nishino I. et al., 1999](#)] that is necessary in the thymine metabolism, and acting in the thymine recycle pathway.

Other recessive disorders leading to mtDNA depletion and multiple deletions are Sensory-Ataxia Neuropathy, Dysarthria and Ophthalmoplegia (SANDO), spinocerebellar ataxia epilepsy syndrome and Alpers' syndrome, all associated with recessive mutations in the POLG1 gene [[Spinazzola A. et al., 2007](#)].

Mitochondrial DNA depletion syndromes are also recessive traits with various phenotypical expressions, which are caused by mutations in several genes. The two major syndromes are (1) hepatocerebral syndrome, caused by mutations in POLG1 (Alpers' syndrome), DGUOK (deoxyguanosine kinase, involved in nucleotide metabolism) and MPV17 (an IMM protein with unknown function) [[DiMauro S. et al., 2008](#); [Spinazzola A. et al., 2006](#); [2007](#)] and (2) pure myopathic syndromes, due to mutations in TK2, SUCLA2 (encoding the β -subunit of succinylCoA synthetase) and RRM2B (p53 inducible ribonucleotide reductase small subunit) [[DiMauro S. et al., 2008](#); [Elpeleg O. et al., 2005](#); [Bourdon A. et al., 2007](#)].

The mtDNA is also a hot spot for pathogenic point mutations accumulation, causing MELAS (Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like episodes) a multisystem disorder characterized by stroke-like episodes in young age, encephalopathy with seizures and/or dementia and mitochondrial myopathy with lactic acidosis and RRFs [[Di Mauro S. et al., 2008](#); [Zeviani M. et al., 2007](#); [Hirano M. et al., 2006](#)]. More than 80% of the patients harbor the common mutation A3243G in the tRNA^{Leu} gene resulting in biochemical dysfunction affecting CI, III, IV and ATP synthase [[Yasukawa T et al., 2000](#)]. Mutations in structural genes, such as COIII, ND1, ND5 and ND6, have been also reported in association with the MELAS phenotype [<http://www.mitomap.org/MITOMAP>].

Furthermore, Leigh syndrome is a complex, early-onset, disease with a heterogeneous clinical manifestation, due to different mutations in several genes (nuclear and mitochondrial) [[Hirano M et al., 2006](#)]. Mutations in structural genes ND1-ND6, with the only exception of ND4L have been reported [[Santorelli F.M. et al., 1993](#); [Ugalde C. et al., 2004](#)].

Another syndrome characterized by the presence of mitochondrial tRNAs point mutations is MERRF (Myoclonus, Epilepsy and Ragged-Red Fibers). In literature are described at least 6 different mutations, especially in tRNA^{Lys} (A8344G, T8356C, G8361A) [[Mancuso M. et al., 2004](#)]. Mutations can also occur in structural mitochondrial genes and the major disorders associated with these mutations are NARP (Neuropathy, ataxia, retinitis pigmentosa), MILS (Maternally Inherited Leigh Syndrome) and Leber's hereditary optic neuropathy (LHON). NARP is a multisystem disorder affecting young adults characterized by a single heteroplasmic point mutation in the ATPase6 gene (T8993G/C) [[Hirano M. et al., 2006](#); [Hirano M. et al., 1994](#); [Holt I.J. et al. 1990](#)]. The same mutations, at high levels of mutational load may lead to MILS [[Santorelli F.M. et al., 1993](#)].

Disorders caused by CI deficiency such as MELAS, Leigh Syndrome and LHON, are usually progressive, multi-systemic, have a poor prognosis and lead to early death or permanent disabilities of the patients [[Shoubridge E.A. et al., 2001](#); [Janssen R.J. et al., 2006](#); [Koene S. et al., 2012](#)]. They result in a broad spectrum of clinical signs and symptoms involving energy demanding tissue, like brain and muscle, but also other organ system can be affected.

There is growing evidence that mitochondrial dysfunctions play a key pathogenic mechanism in many neurodegenerative disorders including LHON and Dominant Optic Atrophy (DOA), even in those without a primary mitochondrial etiology such as Parkinson's disease (PD) and Alzheimer's disease (AD). Both LHON and DOA are associated with a CI defect [[Carelli V. et al., 1999](#); [2007](#); [2009](#); [Zanna C. et al., 2008](#)] which is also recognized as a key feature in the pathogenesis of PD, both in the sporadic cases as well as in the genetic forms [[Schapira A.H., 2008](#)].

- **Leber's Hereditary Optic Neuropathy**

LHON is a maternally inherited blinding disease characterized by the degeneration of retinal ganglion cells and their axons causing an acute or sub-acute loss of central vision and affecting predominantly young males [[Wallace D.C. et al., 1988](#); [Carelli V. et al., 2004](#); [Sadun A.A. et al., 2011](#); [Leber T. 1871](#)]. This usually monosymptomatic disease was clinically defined by Leber and is now recognized as the most frequent mitochondrial disease [[Man P.Y. et al., 2003](#)]. Clinically it is characterized by degeneration of retinal ganglion cells (RGCs) leading to optic nerve atrophy and bilateral loss of central vision explained by selective damage to the papillomacular bundle (PMB) associated with dyschromatopsia. Fundus examination during the acute/subacute stage reveals circumpapillary telangiectatic microangiopathy, swelling of the nerve fiber layer around the disc (pseudoedema) and, absence of leakage on fluorescein angiography [[Nikoskelainen E. et al., 1983](#); [1984](#)].

About 95% of LHON cases carry mtDNA point mutations that affect subunits of the respiratory chain CI subunits genes: m.3460G>A in ND1 (3460/ND1) [[Howell N. et al., 1991](#); [Huoponen K. et al., 1991](#)], m.11778G>A in ND4 (11778/ND4) [[Wallace D.C. et al., 1988](#)], and m.14484T>C in ND6 (14484/ND6) [[Johns D.R et al., 1992](#); [Mackey D. et al., 1992](#); [Rizzo G. et al., 2012](#); [Pello R. et al., 2008](#)]. Several studies showed that CI dysfunction leads at least to two main pathogenic mechanisms: a bioenergetic defect of oxidative phosphorylation and a cronical production of ROS [[Carelli V. et al 2007](#)]. The mutation G3460A results in the substitution of threonine for alanine at position 52 of the ND1 protein. The threonine substitution is adjacent to an invariant aspartic acid residue and the additional electronegativity of the threonine hydroxyl group -and/or

its greater side chain volume might distort or weaken the electrostatic interactions among the charged residues, thereby altering the tertiary structure of this loop and/or its dielectric environment [[Howell N. et al., 1991](#)].

The mutation G11778A induces changes an evolutionarily conserved arginine with a histidine at position 340 in subunit ND4 of CI involved in CoQ₁₀ binding site. It alters the affinity of CI for CoQ₁₀ reflecting a substantial loss in the energy conserving function of CI and thus explain the pathological effect of the mutation [[Degli Esposti M. et al., 1994b](#)].

The mutation T14484C, on ND6 subunit gene, converts the amino acid methionine in position 64, with a valine in the C strand that damage the conformation of CI [[Carelli V. et al., 1999](#)].

However, two unresolved questions in the understanding of LHON remain unexplained: the variability of intra and interfamilial penetrance (percent affect of total number of mutation carriers), especially when the pathogenic mutation is homoplasmic in all maternally related individuals and the male prevalence, with only 50% of male and 10% of female carriers eventually losing vision in their life time [[Pello R. et al., 2008](#); [Howell N. 1998](#); [Chalmers R.M. et al., 1999](#)]. Factors proposed as possible contributors to LHON penetrance, may include heteroplasmy, environmental factors, like tobacco smoking [[Howell N. 1998b](#); [Chalmers R.M. et al., 1999](#); [Tsao K et al., 1999](#); [Sadun A.A. et al., 2003](#); [Newman N.J. 2009](#)] and mtDNA background as well as nuclear modifying gene [[Carelli, V. et al., 2006](#); [Hudson G. et al., 2007](#); [Sadun A.A. et al., 2011](#)]. Haplogroup analyses of LHON mutations have revealed an increased risk of vision loss with suhaplogroup J1, J2 and K, increasing the expressivity and penetrance of different primary LHON mutations. Basically 11778/ND4 and 14484/ND6 mutations are associated respectively with mitochondrial subhaplogroup J2b and J1c, and that the 3460/ND1 mutation is associated with haplogroup K. Probably, accumulation of non-synonymous polymorphisms in ND subunits can increase the penetrance of LHON [[Carelli, V. et al., 2006](#); [Hudson G. et al., 2007](#)]. To further complicate the scenario for LHON mutations environmental factors such as tobacco smoking have been proposed as further contributors for developing visual loss in LHON due to the presence of substances, contained in the tobacco, that can directly act on CI [[Yu-Wai-Man P. et al., 2011](#)]. Moreover the rare exposure to toxic vapors from solvents such as n-hexane and

toluene has also been associated with LHON as triggering factors for the disease. Interestingly, haplogroup J was shown to be more sensitive to these chemicals, thus highlighting a potentially very complex interplay among a pathogenic mtDNA mutation and the interactive role played by both mtDNA haplogroup and environmental factors [[Ghelli A. et al., 2009](#)]. Other studies provide new insights for LHON unexplained male prevalence. These observations suggest that oestrogens may influence mitochondrial functions by acting on the mitochondrial respiratory chain, oxidative stress and mitochondrial biogenesis and they might be responsible for female longevity. Thus the different exposure to oestrogens between males and females is sufficient to modify the severity of mitochondrial dysfunction induced by mitochondrial DNA mutations affecting complex I in LHON [[Giordano C. et al., 2011](#)]

- **Parkinson's disease**

PD is the second most common neurodegenerative disease after Alzheimer disease, affecting over 4 million people with pronounced degeneration of the dopaminergic neurons of the substantia nigra and formation of fibrillar cytoplasmic inclusions, known as Lewy bodies, which contain ubiquitin and α -synuclein [[Ghezzi D. et al., 2005](#) ; [Martin I., 2011](#); [Giordano S., 2012](#)]. It is characterized by bradykinesia, tremor, rigidity and impaired postural reflexes. In addition mental disorders like depression of psychosis and autonomic and gastrointestinal dysfunction may occur [[Lopez-Gallardo E. et al., 2011](#)]. Roughly 10% of total cases are thought to stem from mutations in several nuclear genes including α -synuclein, Parkin, PINK1, DJ-1, LRRK2 and Omi/HTRA2, which play a prominent role in mitochondrial dysfunction in familial PD [[Cannon J.R. et al., 2011](#); [Lopez-Gallardo E. et al., 2011](#); [Winklhofer K.F et al., 2010](#); [Banerjee R. et al., 2009](#); [Schapira A.H. et al., 2008](#); [Bueler H. 2009](#)]. However, the most frequent form arises from unknown causes (idiopathic PD) [[Cannon J.R. et al., 2011](#)]. In these regards, a number of toxicants present in the environment are capable of selectively damaging dopaminergic neurons, contributing to PD by inducing mitochondrial dysfunction due to their major role in the production of cellular ROS [[Drechsel D.A. et al., 2008](#)]. CI and CIV activities has been reported to be reduced in mitochondria isolated from central nervous system (CNS), skeletal muscle, and platelets of PD patients [[Parker W.D. Jr, 1989](#); [Bindoff L.A., 1989](#); [Parker W.D. Jr., 1998](#);

[Trimmer P.A., 2000](#); [Smigrodzki R., 2004](#); [Trimmer P.A., 2004](#); [Parker W.D. Jr., 2008](#); [Lopez-Gallardo E. et al., 2011](#)]. Furthermore cybrid cell lines with mitochondria from sporadic PD patients also exhibit decreased CI activity, increased levels of ROS, increased expression of antioxidative proteins (bcl-2 and bcl-XL), the accumulation of protein inclusion bodies and enhanced susceptibility to cell death [[Trimmer P.A., 2004](#); [Esteves A.R., 2008](#); [2010](#); [Swerdlow R.H., 2012](#)].

Although parkinsonian features have occasionally been reported in mtDNA related encephalomyopathies, neither maternal transmission nor specific mtDNA mutations have been associated with idiopathic PD. Nevertheless, the analysis of mtDNA haplogroups in idiopathic PD suggested that European mtDNA haplogroups J and K, and their common SNP 10398G in ND3 gene, exert a protective effect from the risk of PD [[Ghezzi D. et al., 2005](#)]. On the contrary the root for haplogroups J and T, defined by 4216C in the ND1 gene, seems to be more frequent among PD patients [[Ross O.A. et al., 2003](#)].

To further complicate this scenario, genetic susceptibility factors may interact with environmental toxins such as the pesticide rotenone, MPP⁺, and the herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; PQ) increasing the risk of PD due to a reduction in ATP synthesis and increase of reactive oxygen species (ROS) [[Swerdlow R.H. et al., 2012](#)].

4. DRUG INDUCED MITOCHONDRIAL DYSFUNCTION

Why mitochondria are so special for pharmacogenomics? Because of the special properties of these organelles which are characterized by a unique milieu, with an alkaline and negatively charged interior (pH value 8) due to the proton pumping associated with OXPHOS and a series of specific channels and carrier proteins. As a consequence, mitochondria can easily accumulate lipophilic compounds of cationic character and weak acids in their anionic form, particularly amphiphilic xenobiotics including ethidium bromide, MPP⁺, paraquat and others that can penetrate the IMM freely since in their undissociated form [[Meyer J.N et al., 2013](#)]. When inside the alkaline matrix, their protons dissociate rendering the molecules much less permeable and trapping them inside the organelle [[Blaikie F.H. et al., 2006](#); [Olszewska A. et al., 2013](#)]. The high lipid content of mitochondrial membrane facilitates accumulation of

lipophilic compounds such as polycyclic aromatic hydrocarbons (PAHs) and some alkylating agents. Cationic metals, such as lead, cadmium, mercury and manganese, have also been shown to accumulate in mitochondria due to both calcium transporters and mitochondrial pH and charge. Cytochrome P450 in mitochondrial can activate chemicals that are relatively nonreactive prior to metabolism, such as PAHs and mycotoxins [[Meyer J.N. et al., 2013](#)] (Figure 19).

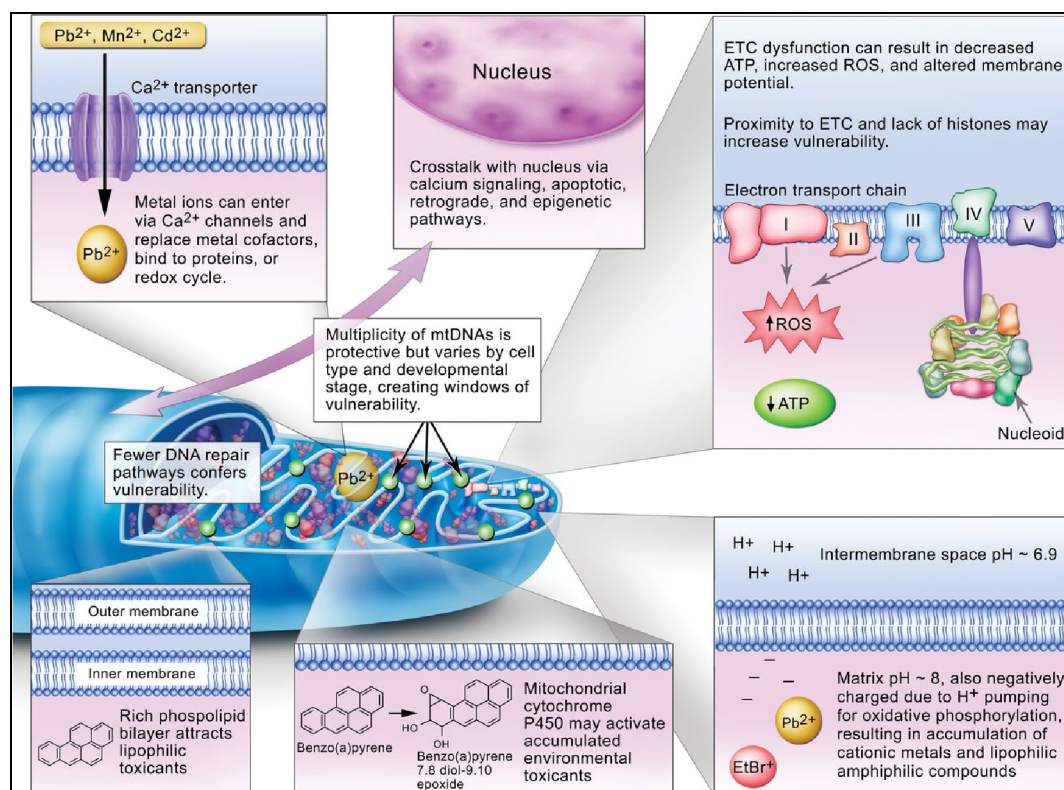


Figure 19. Drug-induced mitotoxicity [[Meyer J.N et al., 2013](#)]

Thus the progressive accumulation of these compounds inside mitochondria damage their function and permeability properties. Depending on the level and number of mitochondria, the cell could go toward a severe denegerization state that in turn can lead to necrosis or more localized damage of a few mitochondria with a collapse of their $\Delta\psi$. The resultant pH modification could reprotonate the substance, rendering it freely permeant in the cell and capable of entering other mitochondria [[Scatena R et al., 2003](#); [2004](#); [2007](#)].

Mitochondrial dysfunction (mitotoxicity) has been demonstrated in the etiology of drug-induced toxicities, causing withdrawn from the market, or Black Box warnings. Thus to increase the drugs safety, pharmaceutical companies have started to assay the

mitochondrial toxicity early in the drug-development process because severe *in vitro* mitochondrial impairment might be sufficient to abandon the development process of a new drug, whereas mild impairment may be acceptable. Early identification of mitochondrial liabilities during compound series selection and lead selection using *in vitro* screens allows for structure–activity relationship (SAR) studies to avoid it. General cytotoxicity is identified by cell-based screening. If a mitochondrial etiology is suspected, assessment of isolated mitochondrial function can be performed. If positive, additional *in vitro* mechanistic studies can examine effects on OXPHOS, permeability transition, ROS production, $\Delta\Psi_m$, and mtDNA status. Therefore mitochondrial assessment should be completed before a compound moves into further development and is elevated to drug candidate level (Figure 20) [Dyken J.A. et al., 2007].

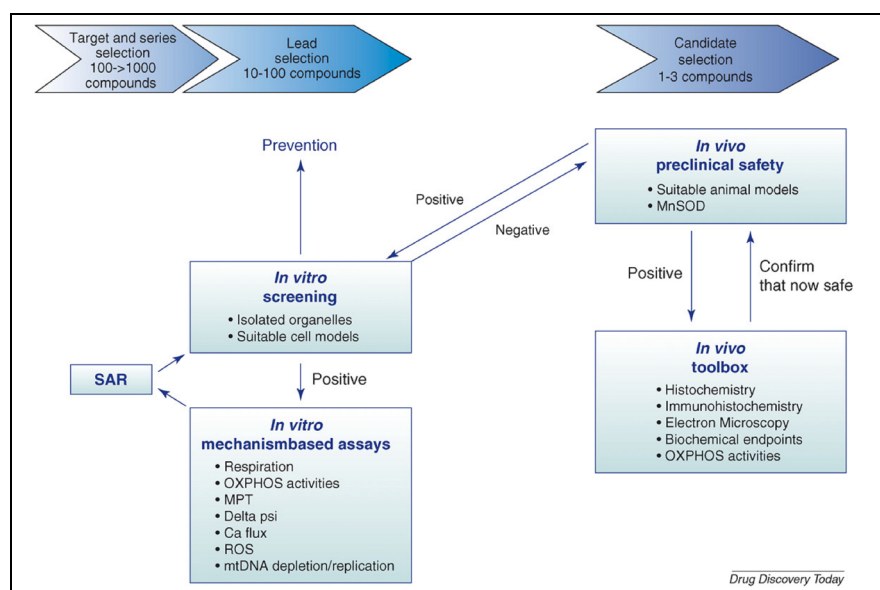


Figure 20. Mitochondrial toxicity screening [Dyken K.A. et al., 2007]

All in all, mitochondria seem to be a potential primary or secondary target of xenobiotics and drugs even though the interaction with mitochondria or mitochondrial components should not always be considered negative. These substances can act on mtDNA, mitochondrial protein synthesis, mitochondrial respiratory chain (MRC), mitochondrial metabolic processes and mitochondrial channels and transporters, leading to mitotoxicity [Figure 18] [Meyer J.N et al., 2013; Dyken K.A et al., 2007].

4.1 Drugs targeting mitochondrial transporters and channels.

The integrity of mitochondrial membrane is crucial for mitochondrial function. Not only IMM and OMM, but proteins, ion channels and transporters embedded within lipid membrane are also target by drugs [[Olszewska A. et al., 2013](#)] disrupting ion homeostasis or affecting the mPTP. [[Scatena R. 2012](#); [2007](#)].

IMM potassium channel openers (e.g. nicorandil, diazoxide, antidiabetic and antitumor sulfonylureas) modify the activity of different mitochondrial channels, which have a fundamental role in maintaining the electrolyte homeostasis.

Valproic acid, t-butyl-hydroperoxide, diclofenac and other NSAIDs are responsible for the opening of mPTP provoking cell death. It is a high-conductance; nonspecific pore in the IMM composed of proteins that link the IMM and OMM. When it is opened, low molecular weight substrates can freely penetrate the MM, carrying along with them H₂O resulting in mitochondrial swelling and the release of cyt c into the cytosol that triggers a cascade of events that will lead to either apoptosis or necrosis [[Scatena R. et al., 2007](#)]. The mPTP represents the best targets for many drugs producing mitotoxicity [[Bouchier-Hayes L. et al., 2005](#); [Renner K. et al., 2003](#)].

4.2 Drugs targeting mitochondrial metabolism

Xenobiotics and drugs can interfere with catabolic and anabolic pathways in mitochondria, including pyruvate oxidation, TCA, and fatty acid β -oxidation [[Di Mauro S. et al., 2008](#); [Greaves L.C. et al., 2012](#)]. Isoniazid inhibits the conversion of pyruvate to lactate and interferences with NADH synthesis in the TCA contributing to the lactic acidosis [[Scatena R et al., 2007](#)]. Cyclosporin reduces the concentration of TCA intermediates and inhibites OXPHOS at the level of ATP synthesis (CV) [[Christians U. et al., 2004](#)]. The inhibition of fatty acid β -oxidation can result in the accumulation of free fatty acids and triglycerides in the cytoplasm that can have toxic effects on mitochondrial respiration, ATP synthesis, ketone body production and gluconeogenesis [[Rial E. et al., 2010](#)]. Valproate, tetracycline derivatives, NSAIDs such as ibuprofen and irprofen, glucocorticoids, antidepressants such as amineptine and tianeptine, statins, fibrates, estrogens, antiarrhythmics and antianginal drugs such as amiodarone and perhexiline, can directly and/or indirectly interference with mitochondrial fatty acid oxidation [[Scatena R et al., 2007](#)]. Valproate enters the mitochondrial where it

activated to valproyl-CoA that sequesters CoA preventing the oxidation of fatty acids and resulting in failure of energy generation. Valproyl-CoA also inhibits carnitine palmitoyltransferase I (CPTI), a critical enzyme for acylcarnitine transport into mitochondria [[Cohen B.H. et al., 2010](#)].

4.3 Drugs targeting mitochondrial DNA

Several drugs interfere with mtDNA and mtDNA processes, such as replication and transcription through interaction with mitochondrial topoisomerase (e.g. amsacrine, etoposide, teniposide and tamoxifen), mitochondrial rRNA (e.g. chloramphenicol and thiamphenicol) and mitochondrial POLy (e.g. menadione and antiviral drugs) that is the unique enzyme responsible for mtDNA replication, required for normal mitochondrial maintenance and duplication [[Neuzil J. et al., 2013](#); [Olszewska A. et al., 2013](#)]. Although POLy is a nuclear protein, it has no known function other than mtDNA replication, and thus any mutation or inhibition of this enzyme will be manifested only in mtDNA. Drugs may damage POLy through different mechanisms. Antiviral drugs used in the treatment of human immunodeficiency virus or hepatitis B infections have shown mitochondrial toxicity through the inhibition of POLy [[Scatena R. 2012](#); [2007](#); [Cohen B.H., 2010](#); [Apostolova N. et al., 2011](#); [Olszewska A. et al., 2013](#)].

The nucleotide reverse transcriptase inhibitors (NRTIs) (e.g. zidovudine, didanosine, zalcitabine, lamivudine, stavudine and abacavir) are nucleoside analogs that are taken up by cells and sequentially phosphorylated to the active triphosphate form [[Collins M.L. et al., 2004](#); [Lewis W. et al., 2006](#); [Apostolova N. et al., 2011](#); [Meyer J.N. et al., 2013](#)]. After phosphorylation they can be used as substrates by retroviral reverse transcriptase competing with natural nucleosides and they can be incorporate into the nascent DNA chain resulting in chain termination [[Collins M.L. et al., 2004](#); [Cohen B.H., 2010](#); [Olszewska A. et al., 2013](#)]. Because the analog does not have a second hydroxyl group to support the lengthening of the chain, the nascent strand can no longer grow, resulting in a DNA pol- γ dysfunction and reduction of mtDNA copy number. This interferes with the synthesis of essential proteins of MRC [[McComsey G. et al., 2005](#)] resulting in reduced ATP synthesis, electron leakage and increased ROS production [[Scatena R et al., 2007](#); [Cohen B.H., 2010](#)].

Protonated tacrine, that is a reversible cholinesterase inhibitor approved for the treatment of Alzheimer's disease, intercalates between mtDNA bases inhibiting mtDNA replication both directly and by inhibition of topoisomerases [[Olszewska A. et al., 2013](#)].

Finally recent studies revealed that a variety of chemotherapeutic agents, such as Cis-diaminedichloroplatinum (II) (cisplatin), induce cell death generating a significant amount of ROS and impairing MRC after interaction with mtDNA [[Qian W. et al., 2005](#)]. Cisplatin (cisPt) is used to treat solid tumor including ovarian, testicular, cervical, lung, head, and neck and bladder cancer in adult patient [[Podratz J.L et al., 2011](#); [Mukherjea D. et al., 2011](#)]. However side effects such as ototoxicity, nephrotoxicity and neurotoxicity are limiting factors in clinic [[Pasetto L.M. et al., 2006](#); [Cepeda V et al., 2007](#)]. Although nephrotoxicity may be prevented by saline hydration or mannitol diuresis, there are no known preventive treatments available for ototoxicity and neurotoxicity [[Mukherjea D. et al., 2011](#)]. CysPt, that is hydrolyzed to a positive charged metabolite, becomes activated intracellularly through the aquation to mono-aqua species (in which one of the two chlorine groups is replaced by H₂O) [[Wang D. et al., 2005](#)] and accumulates inside mitochondrion because the membrane potential is negative inside (~180mV), being its uptake increasing proportionally to both the membrane potential and mitochondrial density. Consequently cells with higher density of mitochondria seem to accumulate a larger amount of cisPt and exhibit mitochondrial dysfunction due to enhanced mtDNA injury [[Qian W. et al., 2005](#)]. As a result mtDNA appears to serve as one of the primary targets for cisPt because of: i) it is more susceptible to oxidative stress than nDNA since it does not contain histone-like proteins and it has a less efficient repair mechanism for injured DNA [[Sawyer D.E et al., 1999](#)], ii) preferential formation of cisPt-mtDNA adduct, and iii) a low activity of decomposing them [[Reedii JK J et al., 1985](#); [Olivero O.A. et al., 1997](#)]. cisPt forms a preferential covalent binding to the nitrogen on position 7 of guanine. The major covalent bis-adduct that is formed involves adjacent guanines on the same strand of DNA (the intrastrand crosslink); a minor adduct involves binding to guanines on opposite DNA strands (the interstrand crosslink) [[Wang D. et al., 2005](#)] (Figure 21).

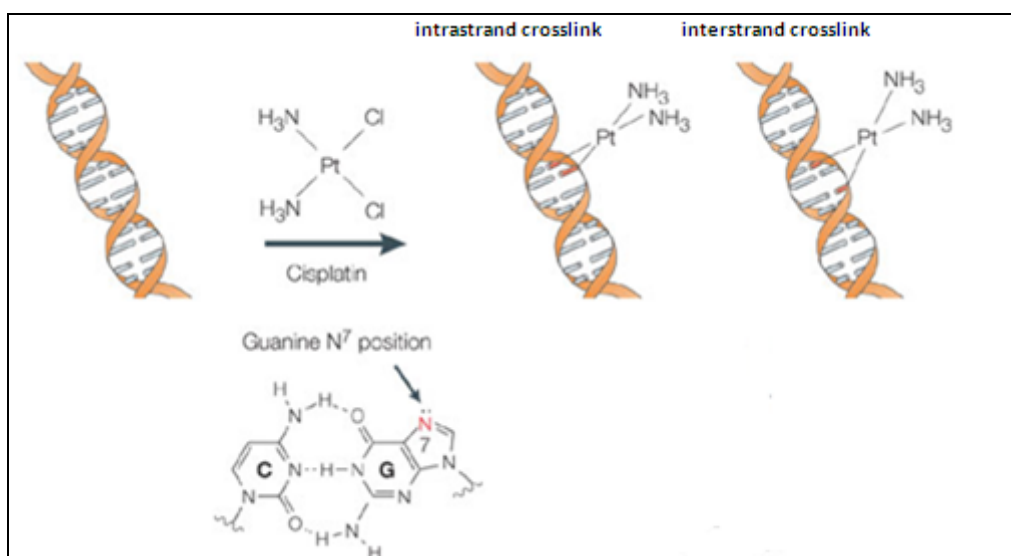


Figure 21. Formation and effects of cisplatin - DNA adducts [Wang D. et al, 2005]

The mtDNA-cisPt adduct inhibits mtDNA replication, disrupt mtDNA transcription, generate ROS production, impair mitochondrial respiratory chain causing morphological changes within mitochondria that induce apoptosis. These findings are important because they provide a theoretical target for interventions to help prevent cisPt-induced neuropathy [Qian W. et al., 2005; Podratz J.L et al., 2011]. Furthermore analysis of mtDNA haplogroup revealed an increased incidence of cisPt-induced hearing loss in mitochondrial haplogroup J, which, however, did not reach statistical significance [Peter U. et al., 2003].

4.4 Drug targeting mitochondrial transduction

During evolution, the mtDNA from the endosymbiont decrease its size because redundant genes were lost, many other genes of the primitive bacteria were transferred to the nDNA and the remaining ones were reduced in size [Schneider A. et al., 2004]. Therefore the mtDNA presents 2 genes of rRNAs – 12S and 16S – contained in the 55S mitochondrial ribosome (mitoribosome) that consists of 2 subunits of 28S and 39S. The 28S comprises 12S and 28 proteins, and the large subunit contains the 16S rRNA and 48 proteins [Pacheu-Grau D et al., 2010]. Because of the bacterial origin of mitochondria, mitochondrial rRNAs (rRNAs 12S and 16S) resemble eubacterial rRNAs (16S and 23S) thus several bacterial antibiotics such as chloramphenicol, aminoglycosides, tetracycline, erythromycin and the oxolidozones also act on mitochondrial protein synthesis and, therefore, this mechanism is involved in side

effects for humans [[Scatena R et al., 2007](#); [Olszewska A. et al., 2013](#); [Cohen B.H, 2010](#); [Pacheu-Grau D. et al., 2010](#)]. Aminoglycosides may induce hearing loss, especially when in the 12S rRNA gene, sequence is present the A>G transition at nucleotide 1555, where the codon-anticodon interaction occurs and aminoglycosides bind the bacterial ribosome [[Cohen B.H, 2010](#)]. This SNP has arisen in different mtDNA haplogroups, suggesting that there is no common haplogroup susceptibility [[Tang H-Y et al., 2002](#)]. Moreover chloramphenicol bind the bacterial 23S rRNA, which is the homolog of human mitochondrial 12S rRNA [[Burk A. et al., 2002](#)].

Oxazolidinones antibiotics (e.g. linezolid (Zyvox)) inhibit bacterial protein synthesis binding the domain V of 23S rRNA, which constitutes the A site of ribosomal peptidyl transferase center (PTC), thus stopping the growth of bacteria. The PTC is in the middle of the 23S rRNA subunit in the bottom of the cleft where the 3' ends of aminoacyl-tRNA (A-site) and peptidyl-tRNA (P-site) are positioned for peptide transfer [[Long S. K. et al., 2012](#); [Leach K.L et al., 2007](#)]. Linezolid comprises four chemical moieties, namely three aromatic rings (ring A, B and C) and an acetamidomethyl tail (C₅ tail) (Figure 22).

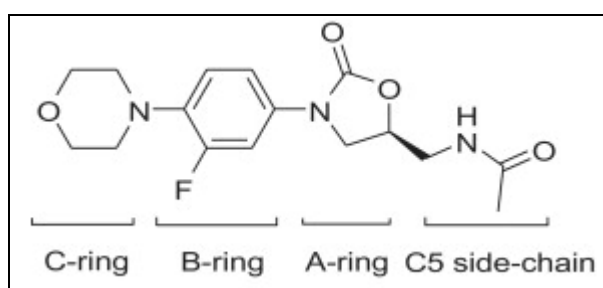


Figure 22. Structure of Linezolid [[Palumbo Piccionello A. et al., 2012](#)]

Linezolid binds at the PTC within a pocket composed of universally conserved residues and is located such that ring C orients toward the intersubunit interface, whereas ring A as well as the C₅ tails head in the general direction of the ribosomal tunnel. Within the pocket, the oxazolidinone ring stacks on U2504, and the C₅-tail extends toward A2503. Ring-B is sandwiched between A2451 and U2506 [[Wilson D.N. et al., 2008](#)] (Figure 23).

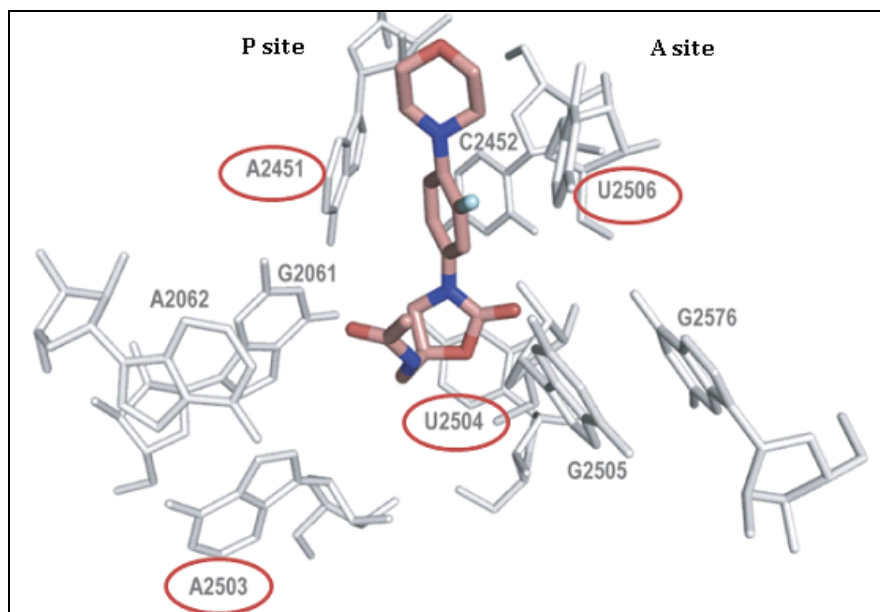


Figure 23. The binding site of oxazolidinones [[Leach K.L et al., 2007](#)]

Not surprisingly, genetic polymorphism in domain V of bacterial 23S rRNA have been associated with Linezolid resistance even if they are not located in the binding site of Linezolid [[Leach K.L. et al., 2007](#); [Palenzuela L. et al., 2005](#)].

Given the similarities between the conserved domains of rRNAs in bacterial and human mitoribosomes, Linezolid can bind to similar position in mitoribosomes causing mitochondrial toxicity by inhibiting protein synthesis. Furthermore, human mtDNA variation in the mitochondrial 16S rRNA gene may confer genetic susceptibility to Linezolid toxicity. Indeed some individuals treated with Lin developed myelosuppression, lactic acidosis and optic and peripheral neuropathy as a result of inhibition of mitochondrial protein synthesis. Sequencing the mitochondrial 12S and 16S rRNA gene in all individual, it was revealed a homoplasmic A2706G and G3010A polymorphism in the mitochondrial 16S rRNA gene [[Palenzuela L. et al., 2005](#)]. Replacement of guanine by adenine, and vice versa, at base pairs 2706 and 3010 in 16S rRNA gene, have been suggested to increase the susceptibility of certain individuals to the toxic effect of Linezolid through inhibition of mitochondrial protein synthesis and activity of the mitochondrial respiratory complexes , especially CIV [[Velez J.C. Q et al., 2010](#); [Pacheu-Grau D. et al., 2013](#)].

4.5 Drugs targeting mitochondrial respiratory chain

Substances may impair MRC via direct inhibition of any of the 4 protein complexes or via acceptance of electrons instead of natural acceptors like CoQ₁₀ or cyt c [[Scatena R et al., 2007](#); [Olszewska A. et al., 2013](#)]. Specific inhibitors are rotenone (ROT), antimycin A, cyanide and carbone monoxide (CO). Some of these compounds are used routinely in laboratories in general mitochondrial pharmacology [[Olszewska A. et al., 2013](#)] such as ROT, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and its active metabolite MPP⁺ that have been shown to inhibit mitochondrial CI directly in assays involving isolated mitochondria [[Bové J., 2012](#); [Martinez T.N et al., 2012](#); [Parker W.D. Jr, 1989](#)]. They have been used in a variety of cell-based and animal models to investigate their role in the pathogenic mechanism of parkinsonianism [[Cannon J.R et al., 2011](#); [Gorell J. M. et al., 1998](#); [Dranka B.P et al., 2012](#); [Rodriguez-Pallares J. et al., 2007](#); [Bové J., 2012](#); [Martinez T.N. et al., 2012](#)].

Among medicines, papaverine, meperidine, cinnarizine, amytal, haloperidol, ketoconazole, fibrates (e.g. clofibric acid, bezafibrate, gemfibrozil) and their derivatives thiazolidinediones (e.g. ciglitazone, troglitazone, pioglitazone) share a common structural motif: a cyclic head and a hydrophobic tail by which they inhibit CI. [[Filser M. et al., 1988](#); [Morikawa N. et al., 1996](#); [Subramanyam B. et al., 1990](#); [Veitch K. et al., 1994](#); [Degli Esposti M, 1998](#); [Duchen M.R, 2004](#); [Miyoshi H, 1998](#)].

Artificial electron acceptors, including methylene blu, phanazine methosulfate, 2-6-indophenol, tetramethyl-p-phenylenediamine, ferricyanide, doxorubicina, menadione and paraquat, are capable of interfering with MRC acting as alternative electron acceptors by extracting electron from intermediates in the chain [[Scatena R. et al., 2007](#); [Meyer J.N. et al., 2013](#)]. In recent years the investigation of the herbicide PQ has suggested that it might be another environmental factor contributing to human PD [[Dinis-Oliveira R.J. et al., 2006](#); [Rodriguez-Pallares J. et al., 2007](#)]. These compounds convert O₂ to O₂⁻ by auto-oxidation that can combine with two protons to form H₂O₂ or can form via the Fenton reaction the OH[•] in the presence of ferrous iron (Fe²⁺) damaging DNA, lipid membrane and proteins [[Cohen B.H, 2010](#)]. Hexachlorobutadiene, 4-thiaalkanoates and valproic acid are also capable of inducing ROS generation without directly deranging the MRC [[Scatena R et al., 2007](#)].

Apart from the common CII inhibitors (e.g. malonate, carboxin, 3-nitropropionic acid), cis-crotonalide fungicides, diazoxide, fluoroquinolones, chloramphenicol succinate and anthracycline are also studied [[Szewczyk A. et al., 2002](#); [Wallace K.B. et al., 2000](#)].

There are a large number of CIII inhibitors, including myxothiazol and antimycin A, acting at different levels [[Szewczyk A. et al., 2002](#); [Walker U.A. et al., 2003](#)] and resulting in the generation of ROS [[Scatena R. 2012](#); [2007](#)].

Effects on CIV are the most severe because this is the step where O₂ is reduced to H₂O. [[Scatena R. 2012](#); [2007](#)]. Cyanide azide and carbon monoxide (CO) are common CIV inhibitor. Also NO interact at physiological levels with CIV, leading to a competitive and reversible inhibition [[Brown G.C. et al., 2004](#)], promoting alterations in the electrochemical gradient that could affect Ca²⁺ uptake and regulating processes such as MTP opening and release of proapoptotic proteins [[Ho W.P. et al., 2005](#)].

Oligomycin and drugs such as propranolol and diethylstilbestrol may interfere with OXPHOS by direct inhibition of ATP synthesis (CV) [[Scatena R. et al., 2007](#)].

Finally non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, diclofenac and nimesulide, antitumor, antipsychotic, hypolipidemic and antimycotic compounds may lead to uncoupling of OXPHOS dissipating the proton gradient. It can result in the generation of heat and, in extreme condition, a malignant hyperthermia syndrome can occur [[Duchen M.R. et al., 2004](#); [Ernster L., 1984](#)]. These compounds can act as direct protonophores, shutting hydrogen ions into the matrix or as ionophores, exchanging hydrogen ions for other mono-or divalent cations or may increase the permeability of IMM. For all these molecules, the main structure-activity relationship is based on a lipophilic weak acid group [[Fleischer S., 1979](#); [Moreno-Sanchez R. et al., 1999](#); [Wallace K.B. et al., 2000](#); [Scatena R. et al., 2007](#)].

Amiodarone and perhexiline, protonated in the acidic IMS, enter the MM where they uncouple OXPHOS and inhibit CI and CII, resulting in a reduction of mtDNA level and an impairment of fatty acid β -oxidation [[Olszewska A. et al., 2013](#)].

On the whole, tissue that rely on OXPHOS, are also targets of mitochondrial toxins and drugs. For this reason there are numerous common syndrome associated with mitochondrial toxicity including lactic acidosis, cardiac and skeletal myopathy, peripheral, central and optic neuropathy, retinopathy, ototoxicity, enteropathy, pancreatitis, diabetes, hepatic steatosis and hematotoxicity [[Scatena R et al., 2007](#)].

➤ **Mitochondrial neurotoxicity due to CI inhibitors**

Rotenone MPP⁺ and paraquat are popular toxicant agents that have been shown to inhibit CI, either directly or indirectly, in assays involving isolated mitochondria from a variety of cell-based and animal models to investigate their role in the pathogenic mechanism of parkinsonianism [[Swerdlow R.H. et al., 2012](#); [Cannon J.R et al., 2011](#); [Bové J., 2012](#); [Martinez T.N et al., 2012](#); [Parker W.D. Jr, 1989](#), [Betarbet R. et al.,2000](#); [Richardson J.R. et al., 2005](#)]. The specific interactions of rotenone and MPP⁺ with CI were confirmed bypassing CI, in human neuroblastoma cells, by expressing the single-subunit NADH dehydrogenase from *Saccharomyces Cerevisiae*. The introduction substitution of this neurotoxic-insensitive CI attenuated the rotenone and MPP⁺ toxic effects [[Shere T.B. et al., 2003](#); [Richardson J.R. et al., 2007](#)]. Paraquat's neurotoxicity action is not the results of CI specific inhibition but it is essentially due to its redox cycle [[Dinis-Oliveira R.J. et al, 2006](#); [Richardson J.R. et al., 2005](#)]. Overall, these compounds are known as inhibitor of the ETC, and the ECT is both a target and a source of ROS so that the common mechanism proposed for toxicity in all these pharmacological models of PD centers around two major pathways: inhibition of mitochondrial function, and increased ROS generation [[Dranka B.P et al., 2012](#)] (Figure 24).

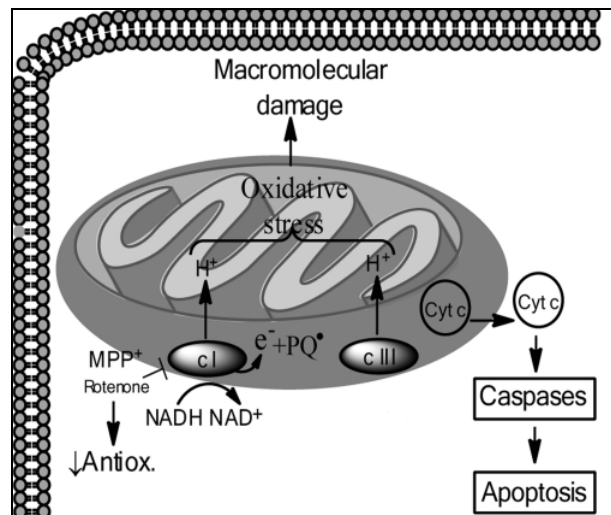


Figure 24. Mitochondrial dysfunction, ATP energy crisis and oxidative stress model of dopamine neuron cell death. Within DA neurons, MPP^+ and rotenone inhibit CI decreasing ATP levels and endogenous antioxidants and generating oxidative stress from CI and CIII which leads to oxidation of macromolecules. Additionally, cyt c is released from the IMS, activating caspase signaling and subsequent apoptotic cell death. PQ accepts electrons CI and functions as a redox cyler to generate oxidative stress and subsequent mitochondrial dysfunction leading to DA neuron death in a manner similar to MPP^+ and rotenone. [Martinez T.N et al., 2012].

As a result both mitochondrial dysfunction and oxidative stress are attractive targets for clinical intervention [Schapira A.H., 2012]. Nevertheless the lack of a direct connection between these events is also increasingly being recognized [Fukui H. et al., 2008; Surmeirer D.J. et al., 2010]: for example despite ROS increase in PD has been well documented, clinical trials using antioxidants have not been proven efficacious [Dranka B.P et al., 2012].

Furthermore tobacco smoking has been proposed as an environmental trigger of visual loss in LHON due to the impairment of OXPHOS through a direct effect on CI activity [Yu-Wai-Man P. et al., 2011]. It has been known that cigarette smoke contains many lipophilic compounds (phenols, aldehydes, and aromatic hydrocarbons, etc) that are able to accumulate in mitochondria and affect mitochondrial respiratory chain, by altering membrane potential ($\Delta\psi$), oxygen consumption and ATP production, thus leading to oxidative stress, mtDNA damage and cellular death [van der Toorn M. et al., 2007].

- **Rotenone**

The lipophilic pesticide rotenone (2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b]furo(2,3-h)chromen-6-one), a plant

derivate, has been considered an organic pesticide and was commonly used as a household insecticide in home gardening and agriculture and kills fish for the past 50–150 years. The ubiquitous use of rotenone in both work and home settings suggests that many people have been exposed [[Tanner C.M. et al., 2011](#)]. It is believed to have a relatively short environmental half-life and limited bioavailability so that a relationship to human disease has been questioned [[Hatcher J.M. et al., 2008](#)] (Figure 25).

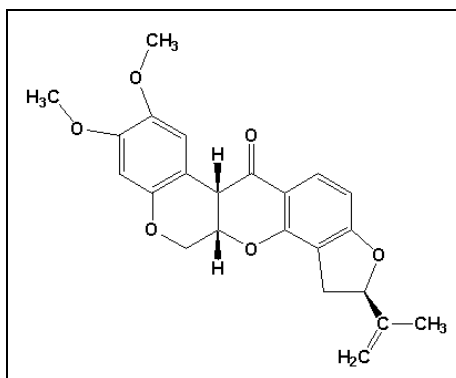


Figure 25. Structure of rotenone

Recent work in rodent models indicated that a temporally limited exposure to this pesticide later caused progressive functional and pathologic changes, mimicking changes found in human PD [[Drechsel A.D. et al., 2008](#)]. Chronic intravenous infusion of rotenone in rats caused a 75% reduction in CI activity and selective degeneration of nigrostriatal DA neurons that are associated behaviorally with hypokinesia and rigidity and for the first time fibrillar cytoplasmic α -synuclein and ubiquitin immunoreactive inclusions were accumulated in surviving neurons within the SN and striatum [[Betarbet R et al., 2000](#); [Lopez-Gallardo E. et al., 2011](#)]. Because it is extremely hydrophobic, rotenone crosses the blood-brain barrier (BBB) and biological membranes easily, independent of a receptor or transporter and accumulates in subcellular organelles including the mitochondria where therefore is well-suited to produce a systemic inhibition of electron flow through CI [[Drechsel A.D. et al., 2008](#); [Sherer T.B. et al. 2007](#)]. Particularly rotenone inhibits CI which shuts off the supply of electron to CIII [[Change B. et al., 1963](#); [Sherer T.B. et al. 2007](#)] blocking one of the eight iron/sulfur clusters in CI [[Dranka B.P et al., 2012](#)]. Consequently, electrons remains in the matrix site for a longer period of time than normal and O_2 can react via a one electron reduction to produce $O_2^{\bullet-}$ [[Drechsel A.D. et al., 2008](#)]. All in all the consequences of

inhibition of mitochondrial CI by rotenone are multifarious. It decreases ATP levels, which may lead to compromised function of the ubiquitin proteasome system (UPS) and mediates bioenergetics crisis within DA neurons, culminating in cells death. Moreover it can sensitize DA neurons to an N-methyl-D-aspartate receptor (NMDA) receptor and cav1.3 L-type Ca^{2+} channel increasing a glutamate and a cytoplasmic influx of Ca^{2+} that culminate in excitotoxic cell death [[Martinez T.N et al., 2012](#)]. Lastly rotenone has been shown to impinge on mitochondrial dynamics by inducing mitochondrial fission [[Arnold B. et al., 2011](#)].

- **1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine**

MPTP was first recognized as a neurotoxin when a group of intravenous heroin users developed an acute version of Parkinsonism that was symptomatically indistinguishable from idiopathic PD [[Langston J.W. et al., 1983](#)]. It was discovered to be an impurity in the illicit drug synthesis of a narcotic meperidine analogue. Basically after it was recognized to function as a DA neurotoxicant, animal models were quickly developed providing the first effective nonhuman primate model of Parkinsonism [[Burns R.S. et al., 1983](#)]. MPTP exhibits the major symptoms of human PD (with the exception of tremor) even if it is not progressive, does not affect other brain regions that are affected in human PD patients, and Lewy bodies are not observed [[Jenner P., 2009](#); [2008](#); [Langston J.W. et al., 1999](#); [Martinez T.N. et al., 2012](#)]. Following systemic injection, MPTP, that is highly lipophilic, readily crosses BBB [[Drechsel A.D. et al., 2008](#)]. It was shown that neurotoxicity depends on oxidation of MPTP by monoamino oxidase (MAO) in brain tissue to the dihydropyridinium form (MPDP⁺) which is further deprotonated (likely due to spontaneous oxidation) to the active neurotoxin N-methyl-4-pyridinium (MPP⁺) [[Ramsay R.R. et al 1986](#); [Martinez T.N. et al., 2012](#)]. Both the A and B types of MAO process MPTP relatively rapidly, particularly the B type, which is enriched in endothelial cells of the BBB microvasculature and in glial cells [[Martinez T.N. et al., 2012](#)]. The actively toxic species MPP⁺ diffuses from astrocytes into the extracellular milieu where it is selectively bound to the dopamine uptake system (DAT) in the synapses and transported to the stroma of dopaminergic neurons [[Ramsay R.R. et al., 1986](#); [Tolwani R. J. et al., 1999](#); [Martinez T.N. et al., 2012](#)] (Figure 26).

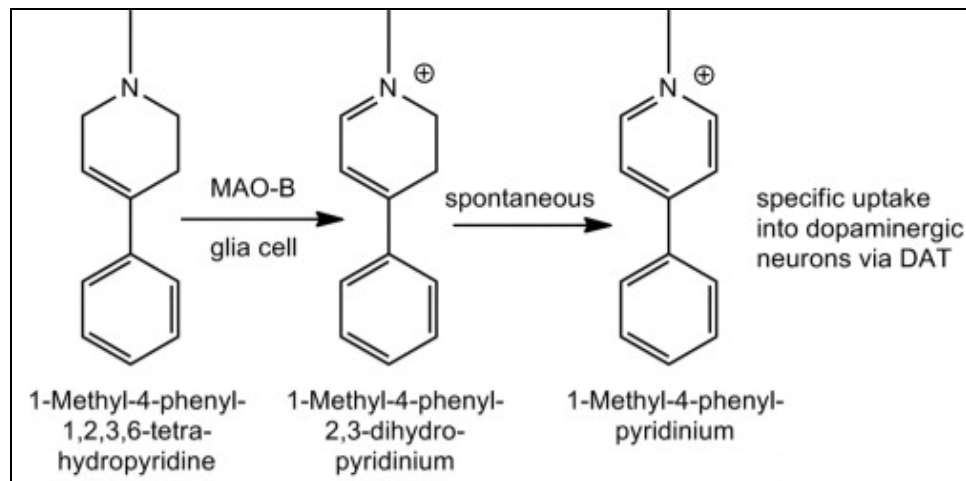


Figure 26. . Structure of the proto-toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) and its metabolic activation to MPP⁺ [Matusch A. et al., 2010]

Once inside the neuron and during the acute phase of MPTP-induced death, there are a few routes that MPP⁺ can take: it can enter the vesicular pathway, binds to vesicular monoamine transporters and translocates into synaptosomal vesicles [Liu Y. et al., 1992]. It can remain in the cytosol and interact with various cytosolic enzymes [Klaidman L.K. et al., 1993] or it can be concentrated within mitochondria via a mechanism dependent on mitochondrial transmembrane potential [Ramsay R.R. et al., 1986; Rossetti Z.L. et al., 1988]. Within the mitochondria, MPP⁺ binds to CI, uncoupling the oxidation of NADH-linked substrates and consequently disrupting the flow of electrons along the ETC, resulting in acute ATP deficiency and increased generation of ROS, particularly O₂^{•-} [Drechsel A.D. et al., 2008; Richardson J.R. et al., 2007; Nicklas W.J. et al., 1985]. Excessive oxidative stress resulting from MPP⁺ inhibition of CI is one proposed mechanism of DA neurotoxicity. Support for this hypothesis is provided by the observation that the reduction in ATP levels is not likely to be a severe enough to be the primary cause of DA neuron death [Chan P. et al., 1991; Davey G.P. et al., 1996]. Furthermore MPTP has been suggested to inhibit mitochondrial CIII and IV (but not II) in vitro [Desai V.G., et al, 1996].

- **Paraquat**

Paraquat was first recognized as a potential DAergic toxicant based on its structural similarity with MPP⁺. Paraquat treatment elicits a selective and dose-dependent loss of nigral DA neurons with associated modest decreases in striatal dopamine nerve

terminal density and mildly decreased motor behavior [Brooks A.I. et al., 1999; McCormack A.L. et al., 2002]. Long-term, systemic paraquat administration in rats produces slowly progressive degeneration of nigrostriatal neurons and reduced number of tyrosine hydroxylase-immunoreactive neurons in the substantia nigra, leading to delayed deficits in dopaminergic transmission that resemble early presymptomatic stage of PD [Ossowska K., et al., 2005]. Despite the striking resemblance, MPP⁺ and paraquat exert their deleterious cellular effect via different mechanism, although induction of oxidative stress is shared property [Richardson J.R. et al., 2005]. The mechanism by which the herbicide kills dopaminergic neurons is not clear because the neurotoxicity actions are not the results of transport by the DAT or Cl⁻ specific inhibition but it is essentially due to its redox cycle [Dinis-Oliveira R.J. et al., 2006; Richardson J.R. et al., 2005]. However paraquat is a potent redox cyclers. It is reduced, mainly by the mitochondrial CI [Fukushima T et al., 1993; Yamada K et al., 1993] and by the mitochondrial CIII [Castello P.R et al., 2007] to form a monocation free radical (PQ^{•+}). It is then rapidly reoxidized in the presence of O₂ generating the superoxide radical (O₂^{•-}) [Brush A.E. et al., 1998; Dicker E. et al., 1991; Jones G.M. et al., 2000; Yumino K. et al., 2002] (Figure 27).

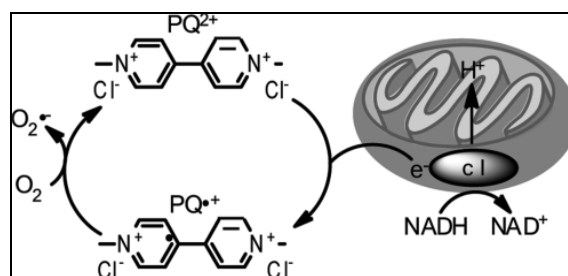


Figure 27. The redox cycling mechanism of paraquat [Martinez T.N et al., 2012].

This then set off the well-known cascade of reaction leading to the generation of other ROS that is a primary cause of paraquat-induced toxicity as evidenced by protection from adverse PQ effects by overexpression of SOD [Thiruchelvam M. et al., 2005] or via administration of SOD mimetics [Mollace V. et al., 2003]. Another important feature of paraquat toxicity is related to its ability to permeate the BBB into the CNS. This herbicide is a charged molecule, with a hydrophilic structure, and does not readily cross membranes [Dinis-Oliveira R.J. et al., 2006]. Paraquat might be specifically transported into the brain by a neutral aminoacid transporter, most likely the system L

Carrier (LAT-1) [[Shimizu K. et al., 2001](#); [Richardson J.R. et al., 2005](#); [Martinez T.N., 2012](#)]. However, in a new recent study, it was demonstrated that PQ, in high doses, employs the organic cationic transporter-3 (OCT-3) and DAT [[Rappold et al., 2011](#)].

- Tobacco smoking

Cigarettes, made from dried tobacco leaves, are a complex mixture of chemicals such as nicotine, cyanide, benzene, formaldehyde, methanol (wood alcohol), acetylene (the fuel used in welding torches), and ammonia - including over 60 carcinogens - produced by the burning of tobacco and its additives. Some of these substances cause heart and lung diseases, and all of them can be deadly. They also contain tar and the poison gases carbon monoxide and nitrogen oxide. The tobacco leaves used in making cigarettes also contain radioactive materials, whose amount depends on the soil the plants were grown in and fertilizers used (Figure 28).

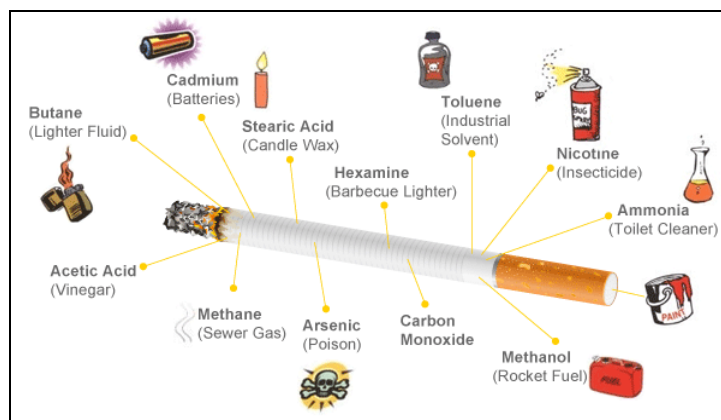


Figure 28. Chemicals inside a cigarette

Thus cigarettes contain small amounts of radioactive material which smokers take into their lungs as they inhale [www.cancer.org].

Aim

AIM

Adverse reactions associated with marketed drugs and xenobiotics are estimated to be between the fourth and sixth leading cause of death in the USA and an important reason for hospitalization, leading to increased health care costs [<http://ghr.nlm.nih.gov/>]. As a result many studies have just examined the role of nDNA variability in the toxicity or efficacy of xenobiotics and drugs among individuals. However, there is a lack of knowledge on the possible correlation between mtDNA variability and the different susceptibility to xenobiotics and drugs has been considered in research. Indeed mitochondria are not merely the center for energy metabolism, but are also the headquarters for various catabolic and anabolic processes, calcium fluxes, and various signaling pathways. In this context, the interaction of pharmacological agents with mitochondria make them a valuable target due to the special properties of these organelles which are characterized by a unique milieu, with an alkaline and negatively charged interior (pH value 8) and a series of specific channels and carrier proteins. In any case, identification of mitochondria as primary or secondary targets of drugs/xenobiotics may facilitate a better understanding of their mechanism of action and open new perspectives on their application.

The first aim of this research project was to understand whether the genetic polymorphisms within the mtDNA genome may act as susceptibility factors and/or influence the cytotoxicity of CI inhibitors such as rotenone, MPP⁺, paraquat and tobacco smoking, reflecting on the pathogenic mechanism leading to PD and LHON disease, both disorder being linked CI dysfunction and increased ROS production [Schapira A.H., 2008; Carelli V. et al., 2009]. Rotenone and MPP⁺ inhibit CI directly, leading to increased O₂^{•-}, whereas paraquat neurotoxicity is essentially due indirectly through its redox cycle by interfering with MRC and increasing ROS production.

Tobacco smoking has been proposed to impair OXPHOS and increase ROS, possibly through direct effect on CI [[Yu-Wai-Man P. et al., 2011](#)]

Moreover, it is accepted that mtDNA variation contributes to both PD and LHON pathogenic mechanism [[Carelli, V. et al., 2006](#); [Hudson G. et al., 2007](#); [Ghezzi D. et al., 2005](#); [Ross O.A. et al., 2003](#); [Schon E.A et al., 2012](#); [Wallace D.C., 2013b](#)]. This variation

may be influential also on sensitivity to rotenone, MPP⁺ and paraquat as well as to the deleterious effect of tobacco smoking, by this impinging on the complex relationship among genetic variation, its interaction with environmental factors and ultimately the pathogenic mechanism leading to both PD and LHON. This intricate relationship is the object of this aim.

The second aim is to understand whether the genetic variation of mtDNA may influence the neurotoxicity of Linezolid and CisPt, respectively. Linezolid is an antibacterial drug inhibiting bacterial protein synthesis which may interfere with mitochondrial function leading to severe side such as myelosuppression, lactic acidosis and optic and peripheral neuropathy - phenotypes frequently found in mitochondriopathies – [[Leach K.L. et al., 2007](#); [Palenzuela L. et al., 2005](#)]. CisPt, a drug used to treat solid tumor, inhibits mtDNA replication and may lead to ototoxicity, nephrotoxicity and neurotoxicity limiting the dose than can be administered to patient [[Qian W. et al., 2005](#); [Podratz J.L et al., 2011](#)].

Overall, we investigated the impact of population-specific mtDNA common variation on mitochondrial function, and its relevance to interaction with environmental toxic agents and drugs by using the cell model of transmitochondrial cytoplasmic hybrids (cybrids), in which common mtDNA haplogroups have been transferred.

Materials and Methods

MATERIALS AND METHODS

5.1 Cellular Lines and Culture Protocols

Transmitochondrial cytoplasmic hybrids (cybrids) were constructed from fibroblasts obtained, after informed consent, from skin biopsies of unrelated healthy subjects and LHON patients as previously reported [[King M.P. et al., 1996](#)]. Fibroblasts, grown in DMEM supplemented with 10% fetal calf serum, were then enucleated and fused with osteosarcoma (143.TK⁻)-derived 206 cell line as acceptor ρ^0 cell line. In the most common approach for making ρ^0 cells, a transformed cell line (143B) that is deficient in thymidine kinase activity (TK⁻) is exposed to ethidium bromide (EtBr), which is an inhibitor of mtDNA replication because mitochondria have a high membrane potential and take up EtBr, as it is ionizable. The ρ^0 line is auxotrophic for uridine and pyruvate (U⁻, P⁻). The ρ^0 cells are repopulated by forming hybrids with cytoplasts from unrelated controls that lack nuclei but still contain mitochondria. Fibroblasts were enucleated by exposing to cytochalasine B followed by centrifugation. After fusion with polyethylene glycol (PEG), cells are plated in media containing bromodeoxyuridine (+BrdU) and lacking either pyruvate or uridine (U⁻ or P⁻), which permits only the growth of ρ^0 cells that have fused with cytoplasts containing mitochondria by fibroblasts owing to TK⁺ fibroblasts are not able to grow in the presence of BrdU. After selection, several cybrid clones were isolated and expanded (Figure 29).

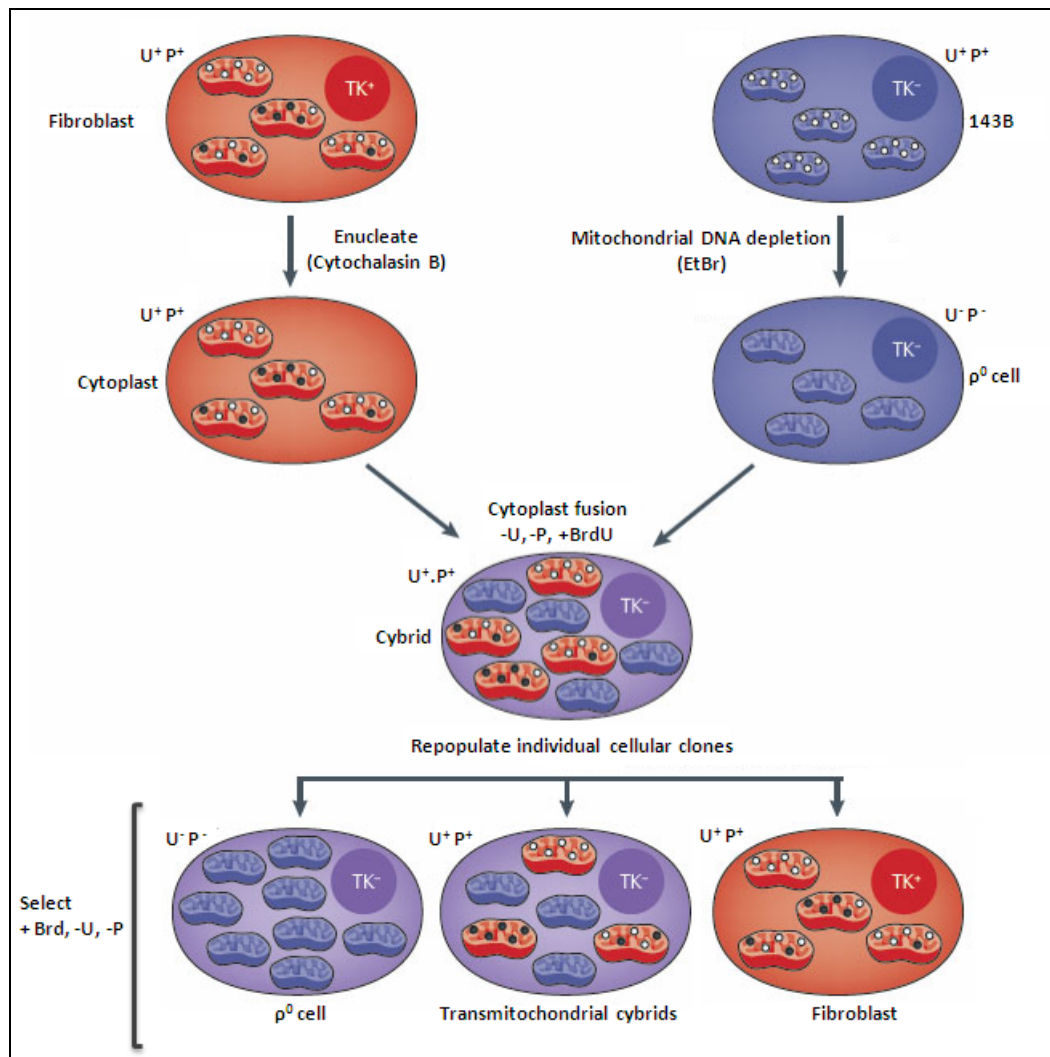


Figure 29. Generation of cybrids (modified by [Schon A.E. et al., 2012](#)).

Cybrid cell lines were grown in Dulbecco's modified Eagle's medium (DMEM-high glucose) supplemented with 10% foetal bovine serum (FBS), 25mM glucose, 5mM pyruvate, 2mM L-glutamina, 10mg/mL penicillin-streptomycin, in an incubator with a humidified atmosphere of 5% CO₂ at 37 C.

5.2 The mitochondrial DNA sequencing and haplogroup affiliation

The mtDNA sequence variation and haplogroup affiliation of cybrids except those already characterized [[Pello R. et al., 2008](#); [La Morgia C. et al., 2008](#); [Ghelli A. et al., 2009](#)] was determined by sequencing their entire mtDNA and their mtDNA non-coding region (D-loop). The complete mtDNA sequencing was carried out on DNA samples extracted from venous blood, in the Laboratory of Genetics, Dipartimento di Genetica e Microbiologia, Università di Pavia (Prof. Antonio Torroni), as previously described [[Achilli A. et al., 2004](#)]

D-loop region was determined by DNA extraction using the standard technique of extraction with phenol-chloroform [[Chomczynski P. et al, 1987](#)] and polymerase chain reaction (PCR) method by a GeneAmp PCR System 2700 (Applied Biosystems) thermal cycler. The PCR mixture contained 50-100ng of DNA, 1U Taq DNA Polymerase 5U/ul (Eppendorf), 1X Buffer Advanced 10X (Eppendorf), 100μM PCR nucleotide Mix (Roche), 100μM each Primer 2μM (Invitrogen), in a final volume of 50μL. Primers sequences and PCR conditions are reported in Table 1.

Table 1. Primers sequences and PCR conditions

mtDNA polymorphism D-loop		
Fw: CAAATGGGCCTCTCCTTGTA	Rv: GGAGTGGGAGGGGAAAATAA	
1 cycle	30 cycles	1 cycle
95°C x 5'	95°C x 30''	72°C x 7'
	55°C x 30''	
	72°C x 1'	

Sequence analysis requires the amplification of the fragment of interest using labeled nucleotides. The reaction mixture is composed of 0.5μl of DMSO (dimethylsulfoxide), 1.5 L of 5X Sequencing Buffer, 1μl of primer, 1μl of 1.1 ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), in a final volume of 10μl. The sequences of the primers and amplification condition used are shown in Table 2.

Table 2. Primers sequences and sequence analysis conditions

mtDNA D-loop Sequence Analysis	
Fw:	Rv:
CAAATGGGCCTGTCCTTGTA	AGGGTGGGTAGGTTTGTGG
CAGTCAAATCCCTCTCGTC	GGAACGTGTGGGCTATTTAGG
CTTGATTCTGCCTCATCC	ACAGATACTGCGACATAGGG
	TGGCTGTGACAGACATTCAAT
25 cycles	
96°C x 10''	
50°C x 5''	
60°C x 4'	

After sequence reaction, the mixture was purified with PERFORMA® DTR Gel Filtration Cartridges (EdgeBio) according to the manufacturer's instructions and a volume of Formamide was added. It was denatured for 5' at 95 °C, cooled to 4 °C in ice to avoid the re-annealing of the filaments, and loaded onto automated sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystem). The sequence analysis of D-loop was performed using Sequencing Analysis 5.2 software, while the search of nucleotide substitutions was made with Sequencher 4.8 Demo. All D-loop sequences are

compared to the revised Cambridge reference sequence ([rRCS](#)) to reveal the haplogroup.

5.3 Cellular Viability

Cell viability was determined using the colorimetric sulforhodamine B (SRB) assay as previously described [[Bonora E. et al., 2006](#)]. Cells were seeded 30.000 cells/well in DMEM-high glucose into 24-well plates and after 24h were washed twice with PBS and incubated in DMEM glucose-free medium or complete medium supplemented with 5nmoli/L galactose, 5nmoli/L Na-pyruvate and 5% FBS (DMEM galactose) in presence or absence of environmental factors, including rotenone, MPP⁺, paraquat and tobacco smoking, and drugs such as Linezolid and CisPt at different concentration. Briefly, at the end of incubation time, cells were fixed with 50% trichloroacetic acid (TCA) for 1 h at 4°C, washed five times with H₂O and dried for 1h at room temperature. Cell were then stained for 30 min with SRB dye 0.4% diluted in acetic acid 1%, washed four time with 1% acetic acid, and solubilized in Tris Buffer 10mM pH 9.8. Absorbance was measured at 540 nm by using a microplate reader [VICTOR³ Multilabel Plate Counter (PerkinElmer Life and Analytical Sciences, Zaventem Belgium)]. The SRB absorbance value in absence of rotenone corresponds to 100% viable cells.

5.4 ATP synthesis

The assay of mitochondrial ATP synthesis driven by CI or CII substrates was determined in digitonin-permeabilized cybrid cell lines, exactly as previously described in luciferin/luciferase assay [[Manfredi G. et al. 2002](#)], with minor modifications [[Giorgio V. et al. 2012](#)] by a Bio Orbit 1250 Luminometer. Briefly, after trypsinization, cells were resuspended (10×10^6 /mL) in buffer A [150mM KCl, 25mM Tris-HCl, 2mM EDTA, 10mM potassium phosphate (KH₂PO₄), 0.1mM MgCl₂ (pH 7.4)] and aliquot was taken to measure protein content [[Bradford M.M., 1976](#)]. Then they were incubated with 50µg/mL digitonin for 1' at room temperature in slight agitation. After centrifugation, the cells pellet was resuspended in buffer B [10mM KCl, 25mM Tris-HCl, 2mM EDTA, 0.1% bovine serum albumin (BSA), 10mM potassium phosphate, 0.1mM MgCl₂ (pH 7.4)] and aliquots were taken to measure ATP synthesis and citrate synthase (CS) activity [[Trounce I.A et al., 1996](#)]. Aliquots of cell were incubated in a tube assay

containing buffer B, 1mM malate plus 1mM piruvate (CI substrates) or with 5mM succinate (CII substrate) plus 5 μ M rotenone for CII to avoid reoxidation of CoH₂ from CI by RET

The reaction was started by addition of 0.1mM ADP in the presence of luciferine/luciferase kit (Sigma-Aldrich), as detailed by the manufacturer's instructions, and chemiluminescence was determined as a function of time with a luminometer. After addition of 1 μ M oligomycin, the chemiluminescence signal was calibrated with an internal ATP standard 10 μ M. The residual activity of ATP synthesis was normalized to protein content, and expressed as a ratio of CS activity and as percentage of the activity of untreated cells.

CS activity was measured at 412 nm (30°C) with UV-Vis spectrophotometer (V550 Jasco) in a buffer containing 125mM Tris-HCl (pH 8), 0,1% Triton X100, DTNB 100 μ M, Acetyl CoA 300 μ M. The reaction was started by addition of oxaloacetate 500 μ M. [[Trounce I.A et al., 1996](#)]

5.5 Respiratory chain complexes activity

Spectrophotometric assays for OXPHOS complexes were performed using a dual-wavelength UV-Vis spectrophotometer (Jasco V550 Europa, Italy), under stirring and controlled temperature as previously described [[Janssen A.J. et al., 2007](#)]. Respiratory chain complexes activity (CI+III, CIII and CIV) were evaluated in mitochondrial-enriched fraction isolated from cybrid cell lines grown in DMEM-high glucose in presence or absence of Lin. Cells, resuspended in Sucrose-Mannitol buffer (Sucrose 70mM, D-Mannitol 200mM, EGTA 1mM, HEPES 10mM, in MilliQ pH 7.6 with protease inhibitor cocktail), were mechanically homogenized each with 80 strokes using a glass-Teflon potter. Homogenates were low centrifuged at 3000 rpm for 10 min at 4 °C discarding the cellular debris pellet. Gently collected supernatants (containing the dirty mitochondrial fraction) were centrifuged at 13000 rpm for 20 min at 4°C. These pellets were then resuspended in Sucrose-Mannitol buffer and stored at - 80 °C.

CI+CIII activity was performed at double wavelength (540 nm-550 nm) at 37°C in agitation and follows the reduction of oxidized bovine heart cyt c exogenous. Kinetics was calculated using the cyt c molar extinction coefficient (19,1 mM⁻¹cm⁻¹). The activity

was performed in 50mM potassium phosphate buffer (KPi) (pH 7.6), 0,35% BSA, 0,3mM KCN, 20μM oxidized cyt c exogenous. The reaction was started by addition of 150μM NADH. In a separate assay, 1μM rotenone (CI inhibitor) and 1μM Antimycin (CIII inhibitor) were added at the beginning of the reaction to measure the non-specific activity.

Complex III activity was analyzed at 540 nm-550 nm wavelength at 37°C in agitation and follows the oxidation of reduced quinol:decylbenzoquinol (DBH₂) and the reduction of oxidized cyt c exogenous. Kinetics was calculated using the cyt c molar extinction coefficient (19,1 mM⁻¹cm⁻¹). The activity were performed in 50mM KPi (pH 7.6), 0,35% BSA, 0,3mM KCN, 20μM oxidized cyt c, 1μM rotenone (CI inhibitor), 5mM Malonate (CII inhibitor). The reaction was started by addition of 50μM reduced DBH₂. In a separate assay, 1μM Antimycin (CIII inhibitor) was added at the beginning of the reaction to measure the non-specific activity.

CIV activity follows the oxidation of reduced cyt c exogenous. It was performed at 540 nm-550 nm wavelength at 37°C in agitation and kinetics was calculated using cyt c molar extinction coefficient (19,1 mM⁻¹cm⁻¹). Buffer contained 50mM KPi (pH 7.6), 1mM EDTA, 1μM Antimycin (CIII inhibitor). The reaction was started by addition of 20μM reduced cyt c. The nonspecific activity measurement was performed in a separate assay, adding 300μM KCN at the beginning of the reaction.

The rates were normalized to CS activity and protein content.

5.6 Reactive Oxygen Species Production

ROS production was determined using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) as previously described [[Porcelli A.M. et al., 2010](#)]. Cells were seeded 40.000 cells/well and incubated in Hepes Ringer Solution Medium [NaCl 125mM, KCl 5mM, MgSO₄ 1mM, KH₂PO₄ 1mM, Hepes 20mM, CaCl₂ 1,3mM, Galactose 5mM (pH 7,4)] at 37°C in an incubator with a humidified atmosphere of 5% CO₂. The medium is supplemented with Calcein AM 100μM and H2DCFDA 2μM. After 30 min, cells were incubated with environmental factors including rotenone, MPP⁺, paraquat and CSE for 2h and 4h. Briefly upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Fluorescence was determined at 485 nm (excitation

wavelength) and 535 nm (emission wavelength) using a multilabel plate reader [VICTOR3 and Analytical Sciences, Zaventem Belgium]].

5.7 The mitochondrial DNA copy number/cell quantification

Absolute quantification of mtDNA relative to nuclear DNA (nDNA) was performed by real-time PCR using a LightCycler[®] 480 (Roche Applied Science, Penzberg, BY, DE) as previously described [[Mussini C et al., 2005](#)]. This method is a multiplex assay based on hydrolysis probe chemistry: 10-50ng of DNA extracted from cells was adequate. Briefly, a mitochondrial DNA fragment (*MTND2* gene) and a nuclear DNA fragment (*FASLG* gene) were extracted from cybrids and were co-amplified by multiplex PCR and concentrations were determined by absolute quantification.

In the first reaction, mtDNA was measured using a mix of 1x PCR buffer (Promega, Madison, Wisconsin, USA), 3mmol/L magnesium chloride, 400pmol primers for mtDNA, 0,2mmol/L dNTP and 2U *Taq* polymerase (Promega). The TaqMan probe used for mtDNA was included in the reaction mixture throughout PCR as a real-time detector for the amplified product. One cycle of denaturation (95°C for 6') was performed, followed by 45 cycles of amplification (94°C for 30', 60°C for 60'). The same approach was used to quantify nDNA, which was required to obtain the number of copies of mtDNA per cell. In this case, primers, designed on the gene sequence *FasL*, were used. The TaqMan probe Genprobe was included in the reaction mixture throughout PCR as a real-time detector for the amplified product.

An mtDNA fragment (*MTND2* gene) and an nDNA fragment (*FASLG* gene) were cloned tale to tale in a vector (pGEM-11Z; Promega) and serial dilutions were used to construct a standard curve, obtaining a ratio of 1:1 of the reference molecules. From the standard curve, the relative copy number of both mtDNA and nDNA was determined. The measured values for mtDNA and nuclear DNA were always within the range of the standard curve and the correlation coefficient was always > 0.995. Absolute values for the copies of mtDNA per cell were then simply obtained from the ratio between the relative values of mtDNA and nDNA (obtained versus the same vector), multiplied by 2 (as two copies of the nuclear gene are present in a cell). Primers, probes and conditions are shown in Table 3.

Table 3. Primers, probes and conditions for RT-PCR assay

RT-PCR assay	
mtDir: CACAGAAGCTGCCATCAAGTA	mtRev: CCGGAGAGTATATTGTTGAAGAG
GenDir: GGCTCTGTGAGGGATATAAGACA	GenRev: AAACCACCGAGCAACTAATCT
TaqMan probe(mtDNA): 5'-FAM-CCGGAGAGTATATTGTTGAAGAG-BlackHole Quencer 1-3'	
TaqMan probe (GenProbe): 5'-Texasred-CTGTTCCGTTTCTGCCGGTGC-BlackHole Quencer 2-3'	
1 cycle	45 cycles
95°C x 6'	94°C x 30''
	60°C x 60''

5.8 Cigarette smoking extract production

One Marlboro red cigarette was smoked via vacuum pump as previously described [[Facchinetti F. et al., 2007](#)]. Just before the experiments, the filter was cut from the cigarette and cigarette smoke was bubbled through 25 ml of cell growth medium without phenol to obtain the cigarette smoking extract (CSE). The absorbance (Abs) of this extract was measured at 320 nm.

5.9 Statistical analysis

All statistical analyses were performed using Sigma Stat software package ver 3.5, choosing the most appropriate test. Statistical significance was declared at $p \leq 0.05$. We performed the analysis using comparison between each haplogroups via Anova One Way followed by post-hoc Bonferroni Test or via Anova on Ranks followed by post-hoc Dunn's Test when the values from the sample were not normally distributed or don't have the same variance. For correlation between untreated and treated cells with Lin is used Student t. Data are presented as means $\pm$ SEM.

Results

RESULTS

6.1 The mitochondrial DNA sequencing of control cybrids

In this study we analyzed 11 control (wild type) cybrid cell lines constructed from healthy subjects belonging to European haplogroups N1b, H, J, T, U, and K, initially genotyped by sequence analysis of the D-loop region. To establish the full genotype/phenotype correlation of our functional studies we then sequenced the entire mtDNA molecules. The raw data of mtDNA sequencing are reported in [Appendix A](#) and the list of specific non-synonymous nucleotide changes (SNPs) is reported in table 4.

Table 4. Specific SNPs found in mtDNA of our cybrids with relative single amino acid substitutions (SAP) according to rCRS. SNPs and SAPs in ND subunits (CI) are shown in black bold

Wild-type cybrids	SNPs		SAPs	Haplogroup	Gene bank ID	References
	Position	Region				
H28705	C4960T	ND2	Ala164Val	N1b		
HAD	T9592C	COIII	Val129Ala	H1		
HF16W				H1		
HQB	G5460A T7407C G8557A T13879C	ND2 COI ATPase6 ND5	Ala331Thr Tyr502His Ala111Thr Ser515Pro	J1b1a2b	JQ797764.1	Pala M. et al. Am. J. Hum. Genet. (2012) 90(5):915-924
HGA	A13681G T14798C	ND5 Cytb	Thr449Ala Phe18Leu	J1c	GQ304740.1	Ghelli A. et al. PLoS ONE (2009) 4(11):e7922
HFG	G15257A	Cytb	Asp171Asn	J2a2c	JQ797930.1	Pala M. et al. Am. J. Hum. Genet. (2012) 90(5):915-924
H42	A5451G G7853A	ND2 COII	Thr328Ala Val90Ile	T1a	JQ798014.1	Pala M. et al. Am. J. Hum. Genet. (2012) 90(5):915-924
HPS	G6261A	COI	Ala120Thr	T2c1c1	JQ798099.1	Pala M. et al. Am. J. Hum. Genet. (2012) 90(5):915-924
HGDA				U1	JN872374.1	Perli E. et al. Hum Mol Genet (2012) 21(1): 85-100
HF01M/10	T14757C T15635C	Cytb Cytb	Met4Thr Ser297Pro	K1c	AY882394.1	Achilli A. et al. Am. J. Hum. Genet. (2005) 76(5): 883-886
HP27	A4024T T11025C	ND1 ND4	Thr240Ser Leu89Pro	K1a2	EU915473.1	Pello, R. Hum. Mol. Genet. (2008) 17(24): 4001-4011

Figure 30 shows the tree encompassing the data shown in table 5 of cell lines studied.

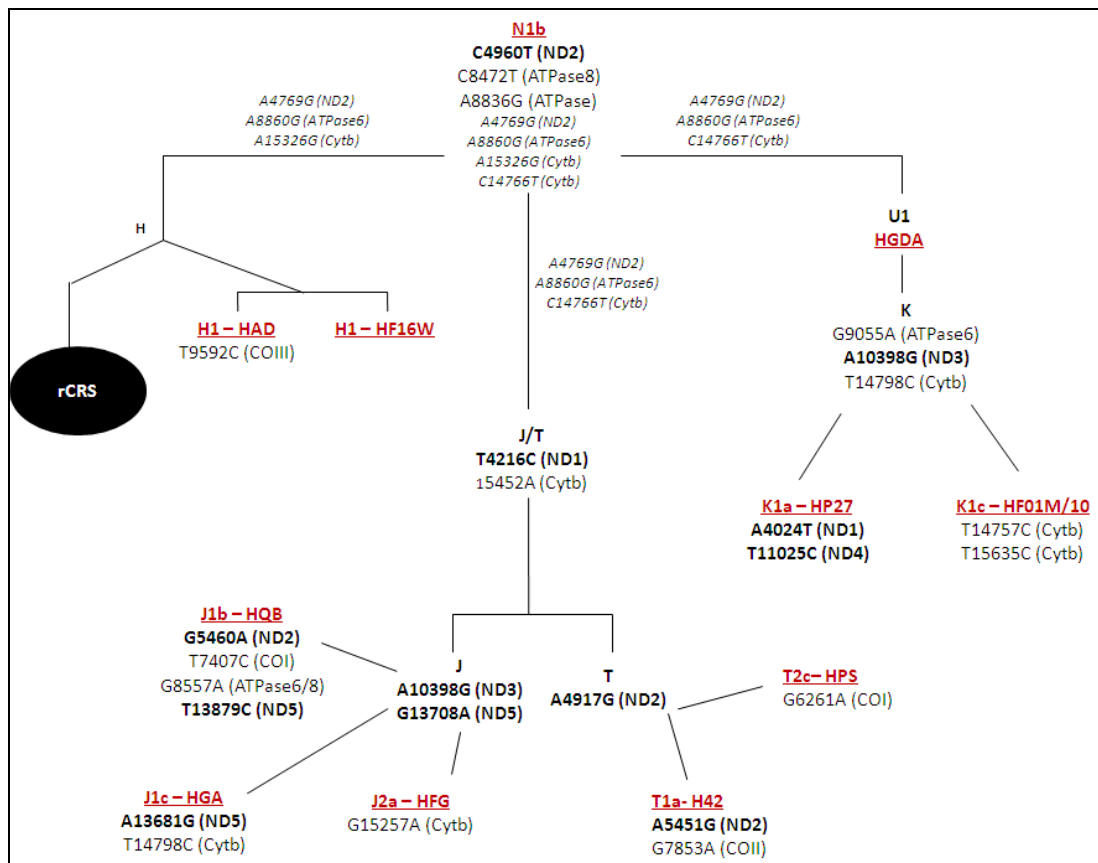


Figure 30. Tree encompassing the complete mtDNA sequences of wt cybrids compared to rCRS. SNPs in ND subunits (CI) are shown on the branches in black bold.

All mtDNA sequences were compared to the [rCRS](#), to assign haplogroup affiliation, because this is a commonly used reference human mtDNA sequence belonging to a European H2a2a1 haplogroup, published in 1981 [[Anderson S. et al., 1981](#)] and revised by [Andrews R. et al](#) in 1999 and [Bandelt H.J. et al](#) in 2013 [<http://www.mitomap.org/bin/view.pl/MITOMAP/MitoSeqs>]. Recently a “Copernican” reassessment of human mtDNA phylogeny has been proposed, switching to a Reconstructed Sapiens Reference Sequence (RSRS) as a valid reference derived from more than 8000 complete mtDNA sequences. This is assumed to be the root of the modern human mtDNA phylogeny [[Behar D.M. et al., 2012](#)] (Figure 31).

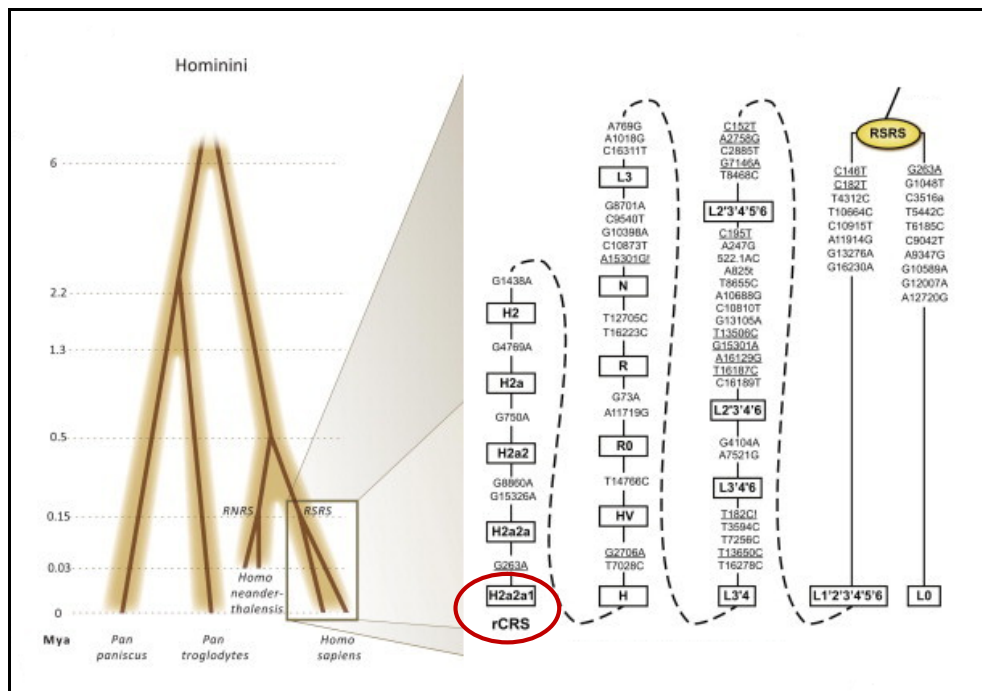


Figure 31. Schematic representation of modern human mtDNA phylogeny. The position of rCRS and RSRS are shown [modified by [Behar D.M. et al., 2012](#)]

A comparison of rCRS to RSRS and the corresponding nucleotide differences is reported in [Appendix B](#). We also compared mtDNA sequences of our hybrids to the RSRS. As expected, specific non-synonymous variants (shown in black bold) are essentially the same with the exception of the SNP 10398 in ND3 gene. This is shown as the substitution of adenine (A) to guanine (G) in rCRS, whereas in RSRS the nucleotide G is converted to A. (Figure 32).

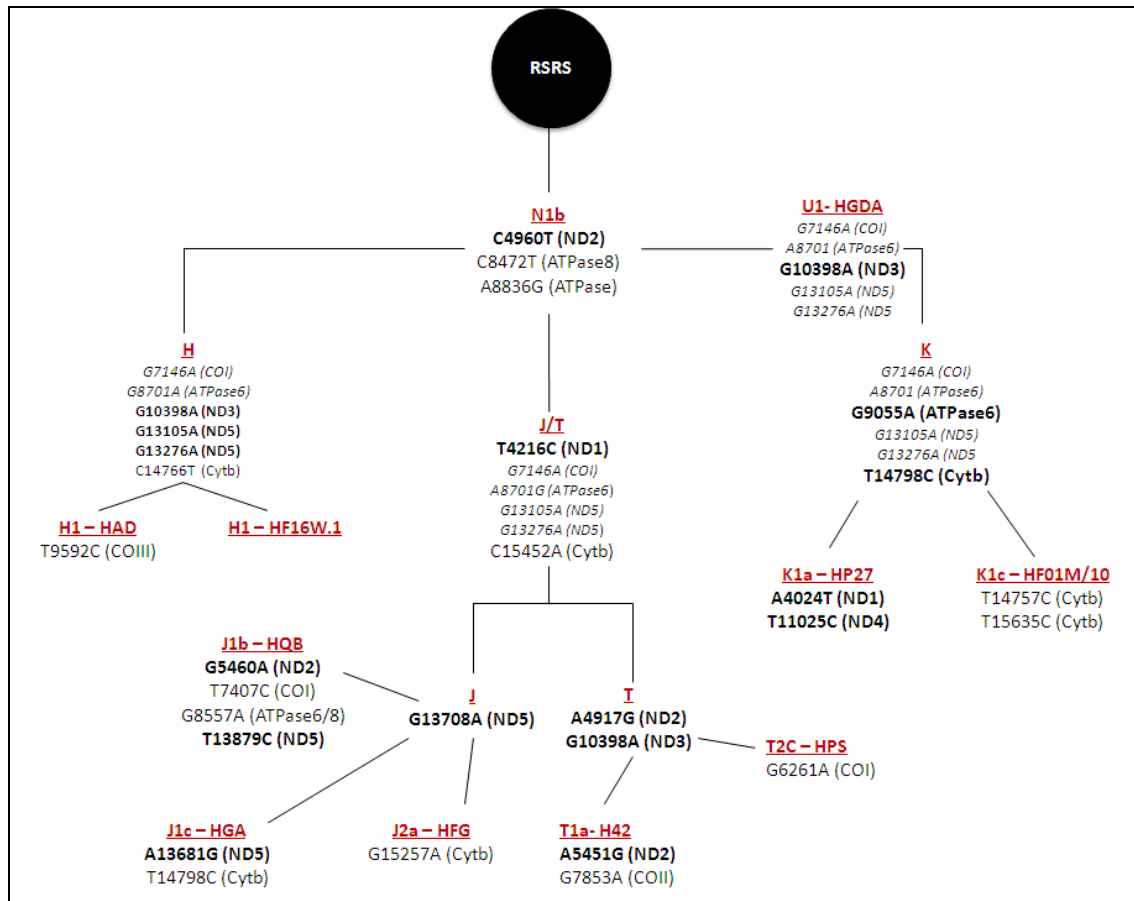


Figure32. Tree encompassing the complete mtDNA sequences of wt cybrids compared to RSRs. SNPs in ND subunits (CI) are shown on the branches in black bold.

The raw data of mtDNA sequencing compared to RSRs are reported in [Appendix C](#)

6.2 The mitochondrial DNA sequence alignment and conservation analysis

The complete mitochondrial sequences of 60 mammals species, according to mtSAP (single amino acid polymorphism) Evaluation, were aligned with UniProtKB. By this mean we compared the SNPs of cybrids with both reference sequences and identified the percentage of amino acid conservation for specif position in the query alignment (Table 5). The analysis reveals that only SNPs of haplogroup K1 - A4042T in ND1 and T11025C in ND4 corresponding to SAPs T240A and L89P respectively - are not conserved, whereas SAP Y304H in ND1 (SNP T4216C) of haplogroup J/T is the most conserved (63,3%) between mammals.

Table 5. SNPs, SAPs and the amino acid conservation analysis in mammals.
Specific SNPs and SAPs in ND1 subunits are shown in red bold and red italics

SNP					SAP				Haplogroup	Mammals (n=60)		
Position	Region	rCRS	wt cybrids	RSRS	Position	rCRS	wt cybrids	RSRS		Position	AA	% of AA conservation
4769	ND2	A	G	G	100	Met	Lys	Lys		100	Lys (n=0)	0
8860	ATPase6	A	G	G	112	Thr	Ala	Ala		112	Ala (n=19)	31,67
15326	Cytb	A	G	G	194	Thr	Ala	Ala		194	Ala (n=7)	11,67
14766	Cytb	C	T	T	7	Thr	Ile	Ile		7	Ile (n=1)	3,33
7146	COI	A	A	G	58	Thr	Thr	Ala		58	Ala (n=16)	26,67
8701	ATPase6	A	A	G	59	Thr	Thr	Ala		59	Thr (n=18)	30
13105	ND5	A	A	G	257	Ile	Ile	Val		257	Ile (n=27)	45
13276	ND5	A	A	G	314	Met	Val	Met		314	Met (n=48)	80
4960	ND2	C	T	C	164	Ala	Val	Ala	N1b	164	Val (n=21)	35
8472	ATPase8	C	T	C	36	Pro	Leu	Pro		36	Leu (n=2)	3,33
8836	ATPase6	A	G	A	104	Met	Val	Met		104	Val (n=0)	0
9592	COIII	T	C	T	129	Val	Ala	Val	H1	129	Ala (n=0)	0
4216	ND1	T	C	T	304	Tyr	His	Tyr	J/T	304	His (n=38)	63,33
15454	Cytb	C	A	C	236	Leu	Ile	Leu		236	Ile (n=26)	43,33
10398	ND3	A	G	G	114	Thr	Ala	Ala	J/K	114	Ala (n=17)	28,33
13708	ND5	G	A	G	458	Ala	Thr	Ala	J	458	Thr(n=7)	11,67
5460	ND2	G	A	G	331	Ala	Thr	Ala	J1b	331	Thr (n=11)	18,64
13879	ND5	T	C	T	515	Ser	Pro	Ser		515	Pro (n=5)	8,47
7407	COI	T	C	T	502	Tyr	His	Tyr		502	His (n=3)	5
8557	ATPase6	G	A	G	11	Ala	Thr	Ala		11	Thr (n=48)	80
13681	ND5	A	G	A	449	Thr	Ala	Thr	J1c	449	Ala (n=3)	5
14798	Cytb	T	C	T	18	Phe	Leu	Phe		18	Leu (n=9)	13,56
15257	Cytb	G	A	G	171	Asp	Asn	Asp	J2a	171	Asn (n=3)	5
4917	ND2	A	G	A	150	Asn	Asp	Asn	T	150	Asp (n=5)	8,33
5451	ND2	A	G	A	328	Thr	Ala	Thr	T1a	328	Ala (n=2)	1,67
7853	COII	G	A	G	90	Val	Ile	Val		90	Ile (n=53)	88,33
6261	COI	G	A	G	120	Ala	Thr	Ala	T2c	120	Thr (n=0)	0
9055	ATPase6	G	A	G	177	Ala	Thr	Ala	K	177	Thr (n=4)	6,67
14798	Cytb	T	C	T	18	Phe	Leu	Phe		18	Leu (n=9)	14,75
4024	ND1	A	T	A	240	Thr	Ala	Thr	K1a	240	Ala (n=0)	0

11025	ND4	T	C	T	89	Leu	Pro	Leu		89	Pro (n=12)	0
14757	Cytb	T	C	T	4	Met	Thr	Met	K1c	4	Thr(n=4)	20
15635	Cytb	T	C	T	29	Ser	Pro	Ser		29	Pro (n=0)	6,67

6.3 The mitochondrial DNA haplogroups sensitivity to the pesticide rotenone

In order to establish the cytotoxicity of rotenone we carried out a cell viability assay in a medium devoid of glucose and supplemented with galactose (stress medium). This assay detects the mitochondrial energetic efficiency, since galactose reduces the glycolytic rate and cells are forced to rely on the ATP produced almost exclusively through OXPHOS. We first measured the concentration-dependent effect of rotenone (10 to 40nM) in our cybrid cell lines collection (N1b, H1, J, T, U1 and K1 haplogroups). Thus after 24h incubation, viability of haplogroup J incubated with rotenone 10 and 20nM was markedly reduced compared to haplogroup K1 (Figure 33). Haplogroup J cybrids group included three different subhaplogroups carrying SNPs defining the J1b, J1c and J2a (Table 4). In particular, the viability of J1 was significantly reduced in the presence of rotenone 10 and 20nM compared to K1, which in turn was the average of K1a and K1c (not shown).

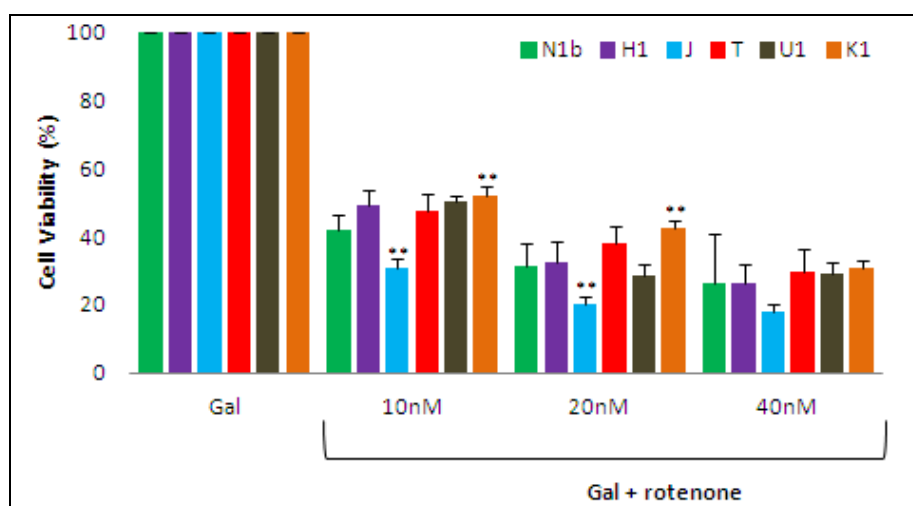


Figure33. Effect of rotenone on cybrids viability of different mtDNA haplogroups (N1b, H1, J, T, U1 and K1). Cells were incubated in galactose with rotenone for 24h. Data are reported as mean $\pm$ SEM (**p $\leq$ 0,001)

To better define the bioenergetic set up of our cybrids, the rate of ATP synthesis driven by CI (malate and pyruvate) and CII substrates (succinate) was measured in cells incubated with rotenone 10nM in galactose medium for 24h, and digitonin-

permeabilized. As reported in figure 34, the rate of ATP synthesis driven by CI was consistently more reduced by rotenone in cybrids with haplogroup J1 (74%) compared to K1 (52%). Conversely, we did not observe significant differences in reduction by rotenone of CII-driven ATP synthesis rate among mtDNA haplogroups J1, J2, H1 and K1.

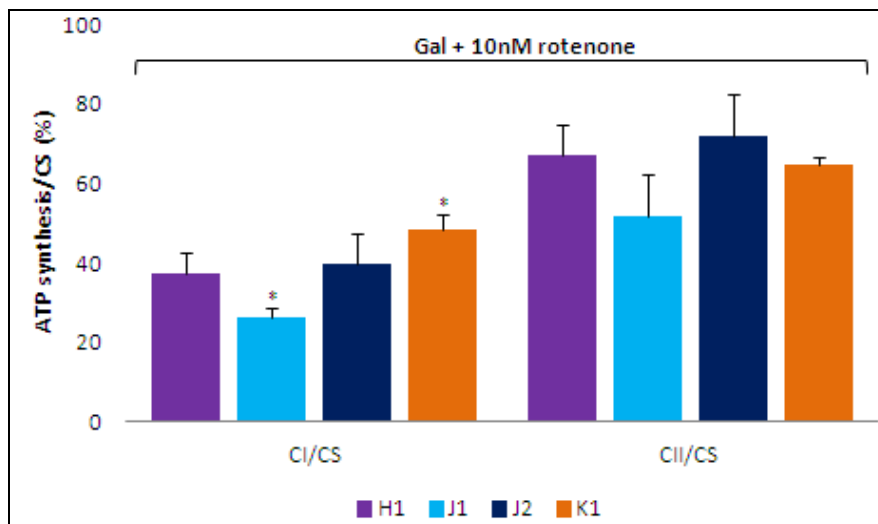


Figure 34. Effect of rotenone on ATP synthesis driven by CI and CII substrates in cybrids of different mtDNA haplogroups (H1, J1, J2 and K1). Cells were incubated with rotenone 10nM and galactose for 24h and then were digitonin-permeabilized. ATP synthesis rate was normalized for CS activity. Data are reported as mean $\pm$ SEM ($p \leq 0,05$)

The assays of mitochondrial ATP synthesis and viability in galactose medium represent a combination of outcome measures that evaluate the energetic state of cybrids incubated with or without rotenone. These results clearly indicated a selective impairment in CI of cybrids carrying variants defining mitochondrial haplogroup J1 in the presence of this pesticide. In addition, we also measured the direct effect of rotenone on ATP synthesis driven by CI substrates adding rotenone in the assay tube. Under this experimental condition, CI-driven ATP synthesis was reduced in a dose-dependent manner in all cybrid cell lines (H1, J1, J2 and K1 haplogroups), yet in presence of rotenone 20nM mitochondria belonging to haplogroup J1 had a significantly more reduced ATP synthesis compared to K1 (Figure 35).

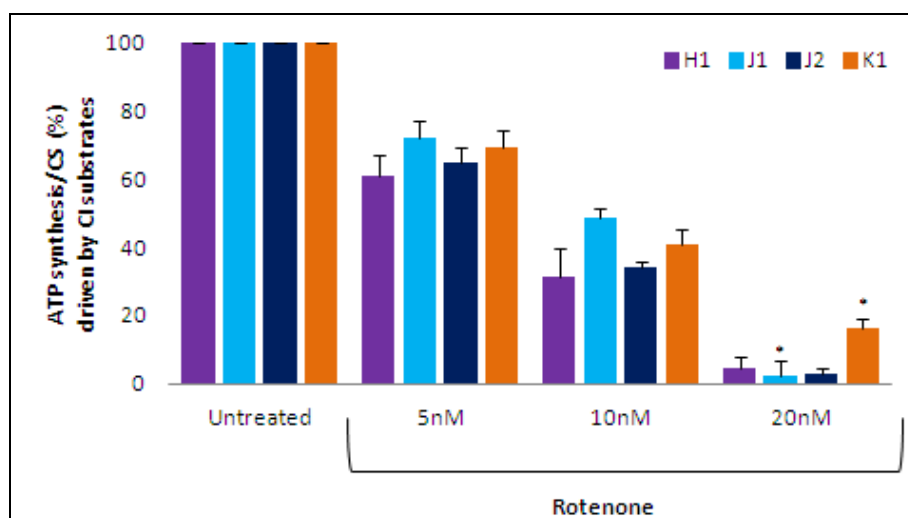


Figure 35. Effect of rotenone on ATP synthesis driven by CI substrates in cybrids of different mtDNA haplogroups (H1, J1, J2 and K1). Cells were incubated in glucose. Rotenone was added to digitonin-permeabilized cells directly in the assay tube. The rate of ATP synthesis was normalized for CS activity. Data are reported as mean $\pm$ SEM ($p < 0.05$)

These results are in agreement with previous studies suggesting a CI-dependent impairment of OXPHOS efficiency in haplogroup J [[Ghezzi D. et al., 2005](#); [Ross O.A. et al., 2003](#)] that our experiments highlight to enhance the sensitivity to rotenone exposure.

Moreover, it has been reported that after rotenone exposure there is a CI-induced increase in ROS production [[Dranka B.P. et al., 2012](#); [Drechsel A.D. et al., 2008](#)]. Hence, we measured the production of H_2O_2 in our cybrids incubated in galactose medium at incremental concentration of rotenone. After exposure for 2-4h, no differences in H_2O_2 levels between haplogroups H1, J1, J2 and K1 were observed (not shown). Thereafter, the effect of rotenone on H_2O_2 levels was investigated, limiting our analysis to the mitochondrial macro-haplogroups J and K, as the more divergent to rotenone toxicity as shown by the previous experiments. The shift from glucose to galactose medium increased H_2O_2 production as expected [[Floreani M. et al., 2006](#)], and haplogroup J cybrids had a slight but significant overproduction of ROS compared to K1. The addition of rotenone failed to further enhance ROS production, even if haplogroup J presented again a limited but significant higher production of ROS compared to K1, as shown in figure 36. Thus, we conclude that the poorly performing CI displayed by haplogroup J cybrids, as shown by the ATP synthesis assay, is also reflected in a slightly higher ROS production.

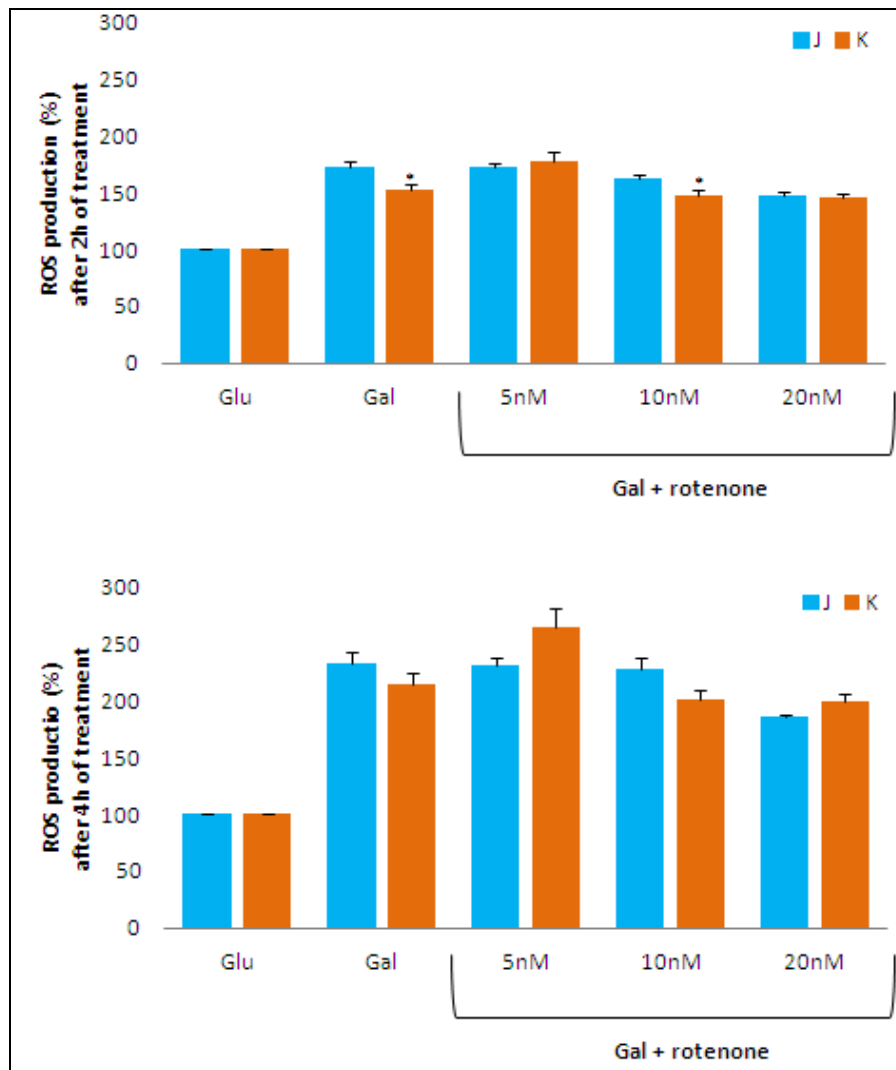


Figure 36. Effect of rotenone on H_2O_2 levels in cybrids of macro-haplogroup J and K. Cells are incubated in galactose (Hepes Ringer Solution Medium) with rotenone for 2 and 4 hours. Data are reported as mean $\pm$ SEM ($p < 0.05$)

As final experiment we also tested the effect of rotenone pesticide on mitochondrial biogenesis. The mtDNA copy number/cell was evaluated in cybrids (H1, J1, J2 and K1 haplogroups) treated with rotenone 10nM in galactose for 24h. As shown in figure 37, there was no significant difference in mtDNA content among different haplogroups induced by galactose only, but the mtDNA copy number in cells with haplogroup J1 was markedly reduced by rotenone 10nM compared to K1.

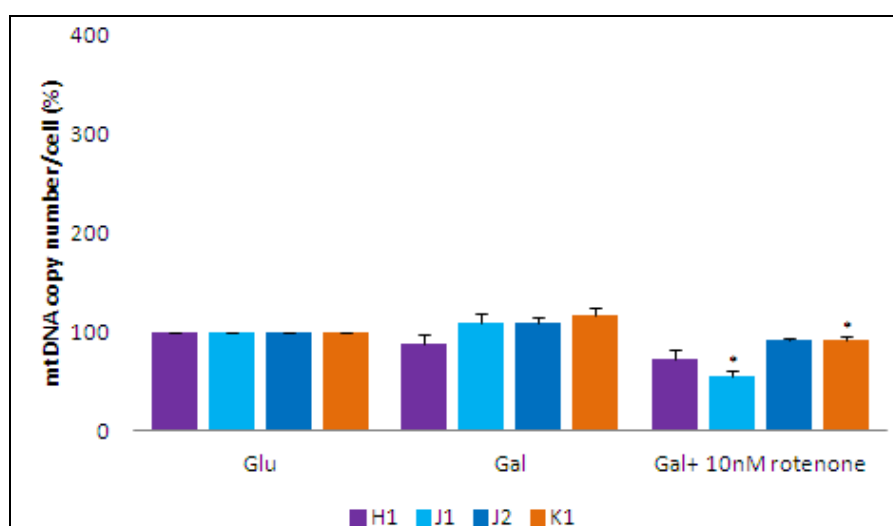


Figure 37. Effect of rotenone on the mtDNA quantification in cybrids of different mtDNA haplogroups (H1, J1, J2 and K1). The mtDNA copy number/cell was evaluated in wt cells treated with rotenone 10nM in galactose for 24h. Data are reported as mean $\pm$ SEM ($p \leq 0,05$)

6.4 The mitochondrial DNA haplogroups sensitivity to the MPP⁺ drug

Initially, we determined the concentration-dependent effect on cell viability of MPP⁺ in our cybrids (N1b, H1, J1, J2, T1, T2 and K1 haplogroups). Figure 38 shows the cell viability in a galactose medium supplemented with MPP⁺ at concentration of 0,1 and 0,2mM. After 24h incubation, cell viability of cybrids with haplogroup J1 was markedly reduced compared to both haplogroup T (T1 and T2) and K1.

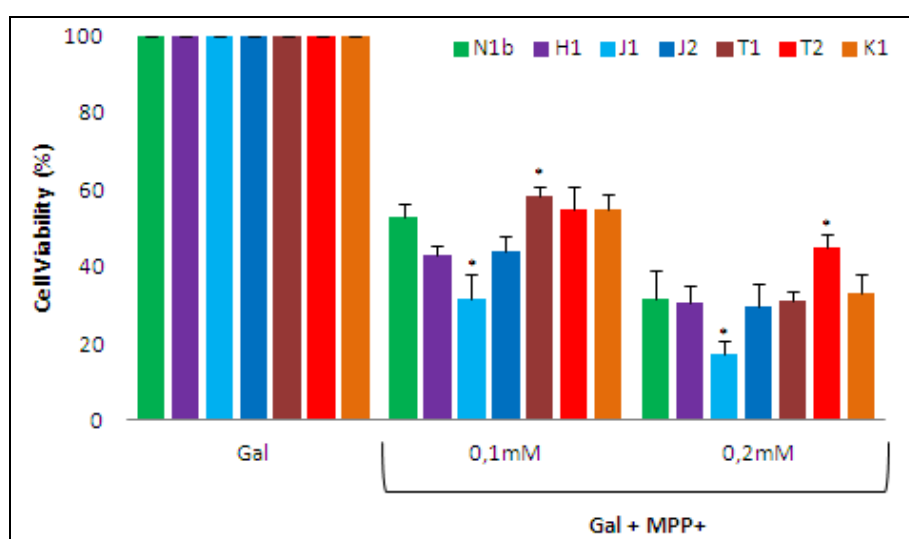


Figure 38. Effect of MPP⁺ on cybrids viability of different mtDNA haplogroups N1b, H1, J1, J2, T1, T2 and K1. Cells were incubated in galactose with MPP⁺ for 24h. Data are reported as mean $\pm$ SEM (* $p \leq 0,05$)

In figure 39, we show the rate of ATP synthesis driven by CI substrates titrating increasing concentration of MPP⁺ directly in the assay tube going up to 0,35mM and

0,5mM MPP^+ concentrations. Under these conditions, CI-driven ATP synthesis was incrementally reduced in a dose-dependent manner, but no significant difference was apparent between mtDNA haplogroups J1 and K1.

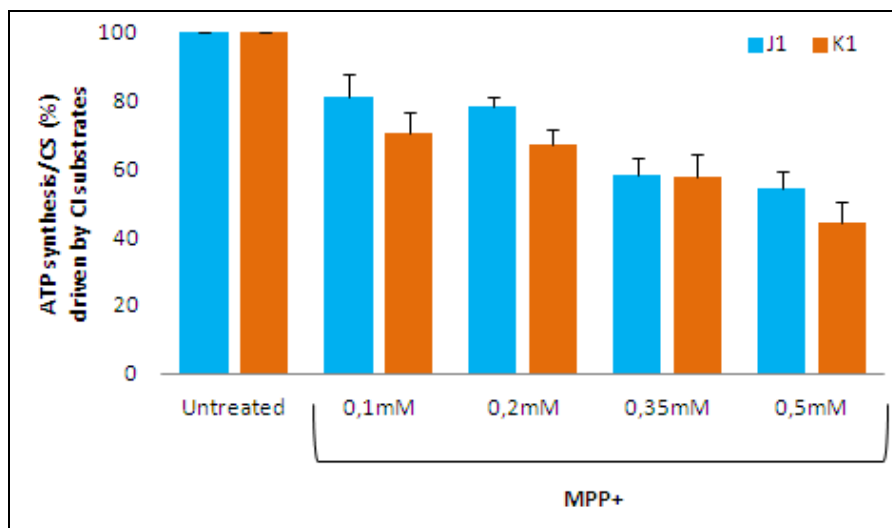


Figure 39. Effect of MPP^+ on ATP synthesis driven by CI substrates in cybrids of mtDNA haplogroups J1 and K1 Cells were incubated in glucose. MPP^+ was added to digitonin-permeabilized cells directly in the assaytube. The rate of ATP synthesis was normalized for CS activity. Data are reported as mean $\pm$ SEM

Based on the previous results, the CI inhibition occurs at high concentrations of MPP^+ . Thus, we also tested the effect of MPP^+ on H_2O_2 production in our cybrids (J1 and K1 haplogroups) incubated in galactose 0.2-0.35-0.5 mM MPP^+ for 2-4h. At high concentration of MPP^+ (0,35mM and 0,5mM), haplogroup J1 diverged displaying reduced ROS production as compared with haplogroup K1 (figure 40).

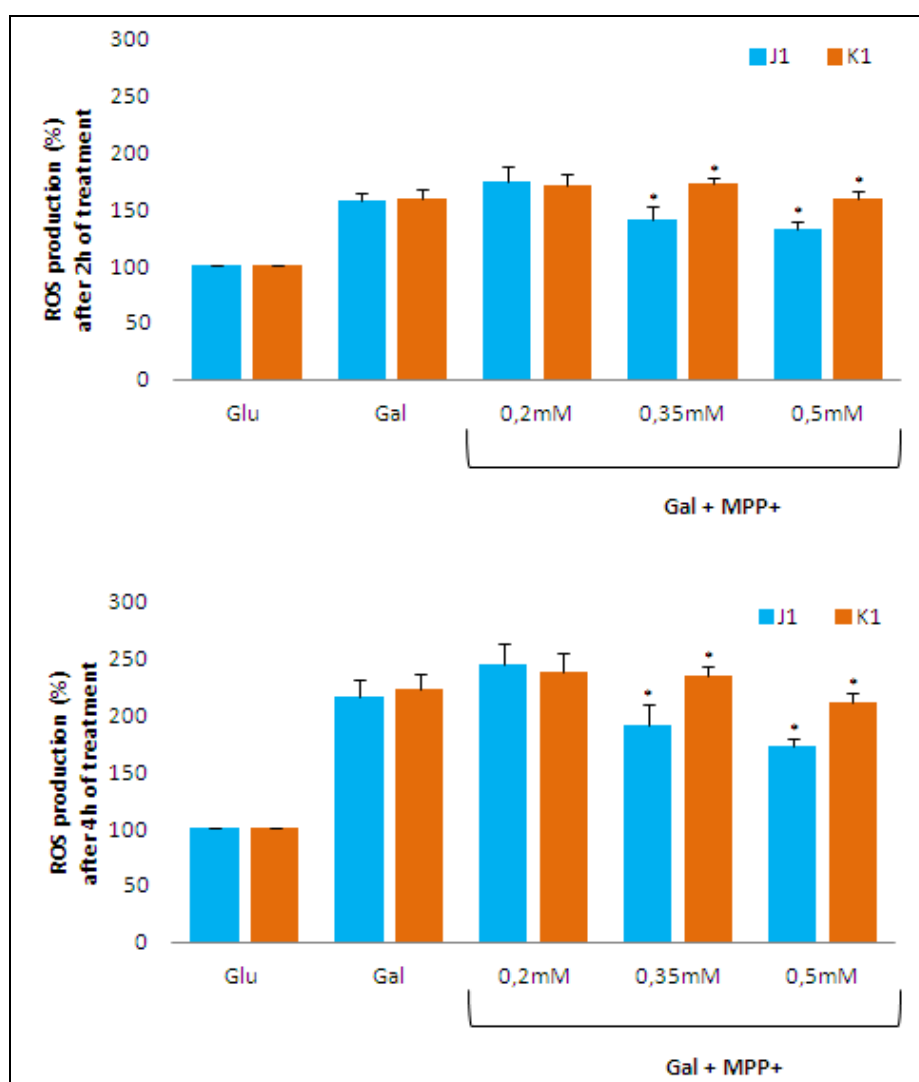


Figure 40. Effect of MPP⁺ on H₂O₂ levels in cybrids of mtDNA haplogroups J1 and K1. Cells are incubated in galactose (Hepes Ringer Solution Medium) with MPP⁺ for 2 and 4 hours. Data are reported as mean±SEM (*p<0,05)

6.5 The mitochondrial DNA haplogroups sensitivity to the herbicide paraquat

Figure 41 shows the cell viability in galactose medium with paraquat supplemented at different concentrations (0,5 mM and 1mM). T2 subhaplogroup was the most sensitive to this herbicide together with H1, whose viability also declined rapidly at incremental concentration of paraquat. Strikingly, a significant resistance to paraquat was shown by the T1 haplogroup.

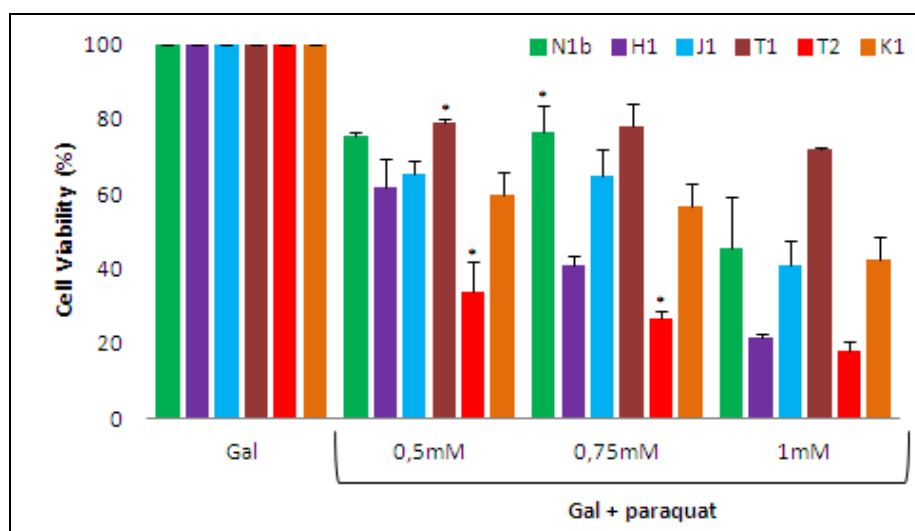


Figure 41 Effect of paraquat on *wt* cybrids viability of different mtDNA haplogroups (N1b, H1, T1, T2 and K1). Cells were incubated in galactose with paraquat for 24h. Data are reported as mean $\pm$ SEM (* $p \leq 0,05$)

The rate of ATP synthesis driven by CI substrates adding paraquat directly in the assay tube was not reduced by incremental concentrations of this herbicide, and no differences among mtDNA haplogroups J1, T2 and K1 were observed (figure 42). We also measured the rate of the rate of ATP synthesis driven by CI and CII substrates in cells incubated in galactose and paraquat 0,75mM, and digitonin-permeabilized. Preliminary results showed that T2 cybrids were most sensitive to inhibition of paraquat on ATP synthesis driven by CII, and most consistently by CI (not shown).

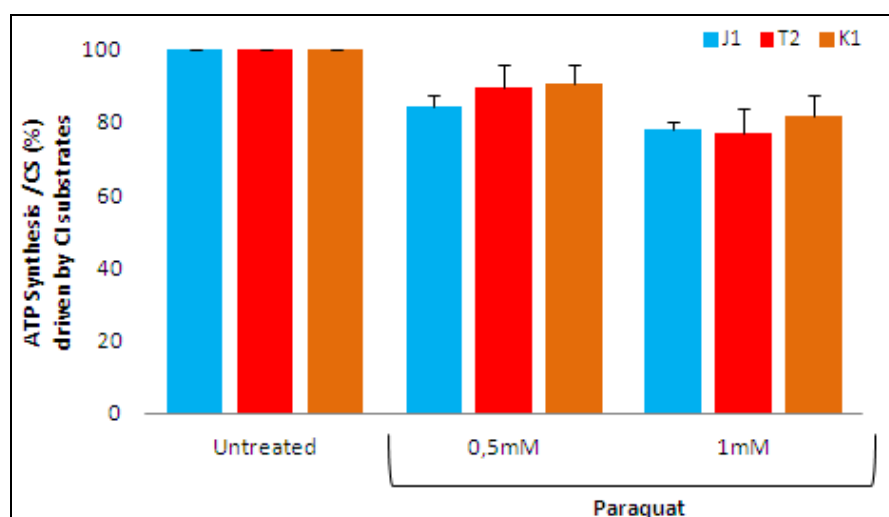


Figure 42. Effect of paraquat on ATP synthesis driven by CI substrates in cybrids of different mtDNA haplogroups (J1,T2 and K1). Cells were incubated in glucose. Paraquat was added to digitonin-permeabilized cells. The rate of ATP synthesis was normalized for CS activity. Data are are reported as mean $\pm$ SEM

According to literature, the mechanism by which paraquat kills dopaminergic neurons is essentially due to its redox cycle that interferes with MRC and increases ROS production [[Dinis-Oliveira R.J. et al, 2006](#); [Richardson J.R. et al., 2005](#)]. Hence, we measured the production of H_2O_2 in our cybrids (J1, T1, T2 and K1 haplogroups) incubated in galactose and paraquat 1mM. After exposure of 2-4h no differences in H_2O_2 levels were detected (figure 43) in contrast with previous reports [[Dinis-Oliveira R.J. et al, 2006](#); [Richardson J.R. et al., 2005](#)].

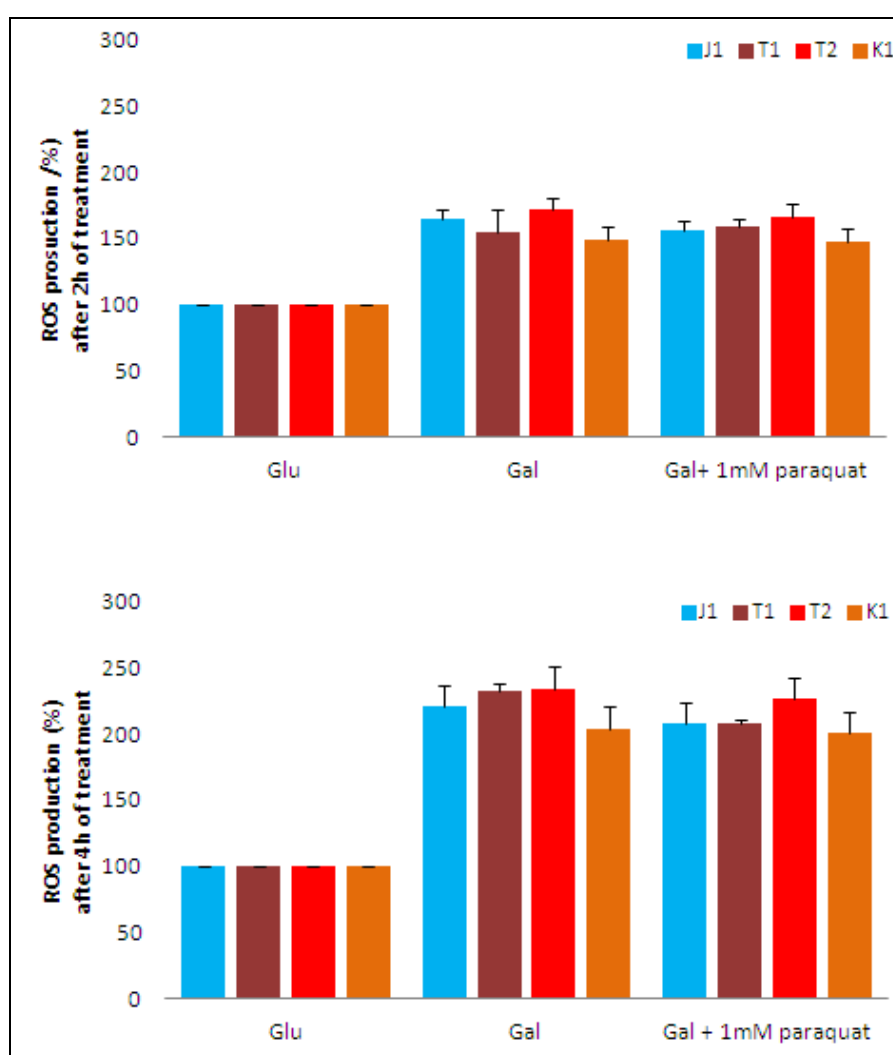


Figure 43. Effect of paraquat on H_2O_2 levels in cybrids of different mtDNA haplogroups (J1, T1, T2 and K1). Cells are incubated in galactose (Hepes Ringer Solution Medium) with paraquat 1mM for 2 and 4 hours. Data are reported as mean $\pm$ SEM

Finally we evaluated the mtDNA copy number/cell in cybrids (J1, T1, T2 and K1 haplogroups) in galactose for 24h and after addition of paraquat 0,75mM. As shown in figure 44, no significant difference was detected on mtDNA content in galactose, but

both haplogroup J1 and K1 displayed an increased mtDNA copy number after addition of paraquat.

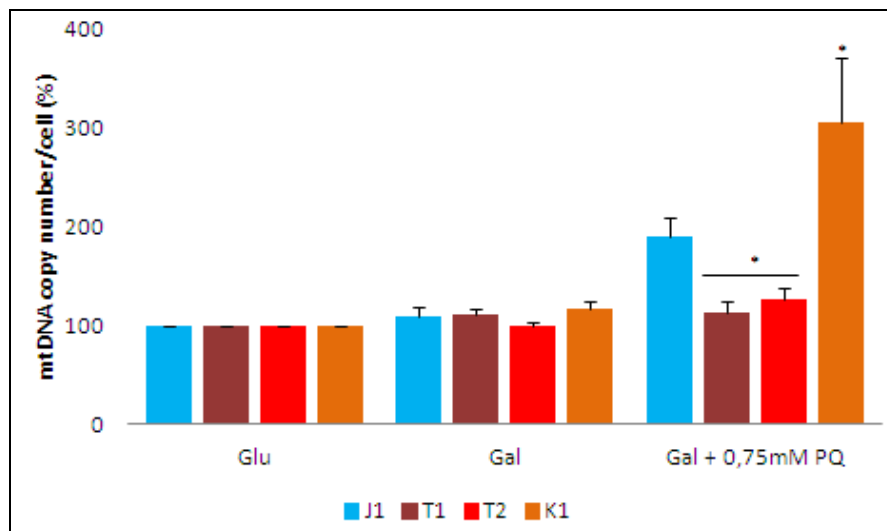


Figure 44 Effect of paraquat on the mtDNA quantification in cybrids of different mtDNA haplogroups (J1, T1, T2 and K1). The mtDNA copy number/cell was evaluated in wt cells treated with paraquat 0,75mM in galactose for 24h. Data are reported as mean $\pm$ SEM (* $p \leq 0,05$)

6.6 The mitochondrial DNA haplogroups and mtDNA LHON mutations sensitivity to cigarette smoking extract

We studied the effect of cigarette smoking extract (CSE) on cell viability of our cybrids generated from control subject. After 24h of incubation, we did not observe differences in cell growth among the macrohaplogroup investigated (N1b, H1, J1, J2, T1, T2, U1 and K1) (data not shown). Nevertheless, when we considered the subhaplogroup affiliation of all eleven cybrids, the cell viability of J1c and J2a subhaplogroups was markedly reduced (Figure 45)

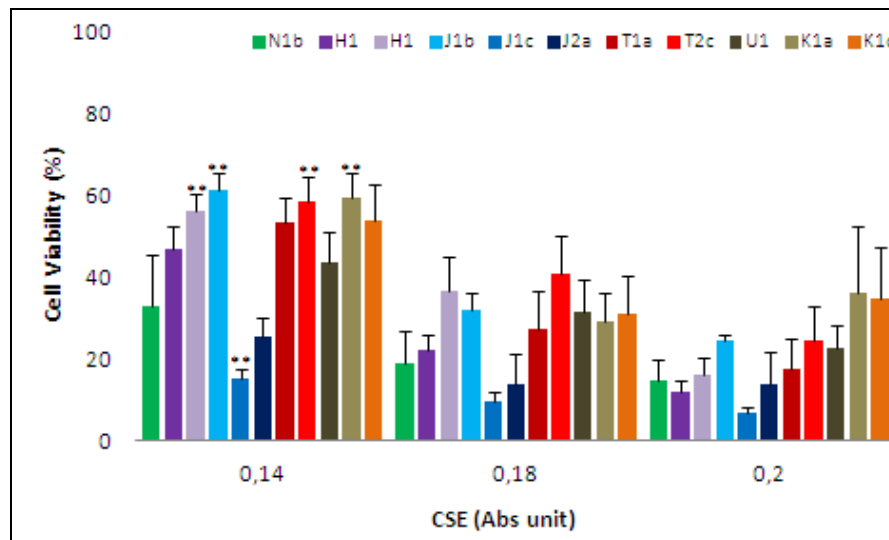


Figure 45. Effect of CSE on cybrids viability of different mtDNA haplogroups (N1b, H1, J1b, J1c, J2a, T1, T2, U1, K1a and K1c). Cells were incubated in glucose with CSE for 24h. Data are reported as mean $\pm$ SEM (** $p \leq 0,001$)

Tobacco smoking has been proposed to impair OXPHOS and increase ROS, possibly through a direct effect on CI [Yu-Wai-Man P. et al., 2011]. Nevertheless exposure of cybrids to CSE did not reduce ATP synthesis driven by CI in a dose-dependent manner and we did not observe differences between haplogroups in H_2O_2 levels (data not shown).

It has been previously described that haplogroups J1c and J2b increase penetrance of the 11778/ND4 and 14484/ND6 mutations associated with LHON [Carelli, V. et al., 2006; Hudson G. et al., 2007]. Furthermore, besides haplogroups it has been shown that also environmental factors such as tobacco smoking may trigger optic neuropathy in LHON [Yu-Wai-Man P. et al., 2011]. Therefore, given our results of a specific sensitivity of J subhaplogroup to CSE, we also investigated CSE effect on LHON cybrids. We first classified the haplogroup affiliation of LHON cybrids by sequencing the D-loop region and we further refined the haplotype by sequencing the entire mtDNA. The raw data are reported in Appendix D and the list of specific non-synonymous nucleotide changes is reported in table 6.

Table 8. SNPs found in mtDNA from LHON patients with relative SAPs compared to rCRS. Pathogenic point mutation are reported in red bold

LHON cybrids	SNP		SAP	Haplogroup	Gene bank ID	References
	Position	Region				
HPE	G11778A T12083G	ND4 ND4)	Arg340His Ser442Ala	J1c	EU915476.1	Pello R. et al. Hum. Mol. Genet. (2008) 17(24):4001-11
HL180	T7042C T14484C T14798C	COI ND6 Cytb)	Val380Ala Met64Val Phe18Leu	J1c	EU915479.1	Pello R. et al. Hum. Mol. Genet. (2008) 17(24):4001-11
HFF	G9477A A9667G G11778A A14793G	COIII COIII ND4 Cytb	Val91Ile Asn154Ser Arg340His His16Arg	U5a1	EU915477.1	Pello R. et al. Hum. Mol. Genet. (2008) 17(24):4001-11
RJ206	G3460A	ND1	Ala52Thr	T1a	GQ304742.1	Ghelli A. et al. PLoS ONE (2009) 4(11):e7922
HMM12	G3460A	ND1	Ala52Thr	H12	EU915475.1	Pello R. et al. Hum. Mol. Genet. (2008) 17(24):4001-11

Figure 42 shows the cell viability of LHON cybrids response to different CSE preparation (0,14-0,18-0,2 Abs unit). After 24h incubation, the viability of LHON G3460A/ND1 cybrids (H12 and T1a haplogroups) was strikingly reduced compared to LHON G1778A/ND4 cybrids (haplogroup U). The 14484/ND6 cybrids displayed an intermediate behaviour (Figure 46).

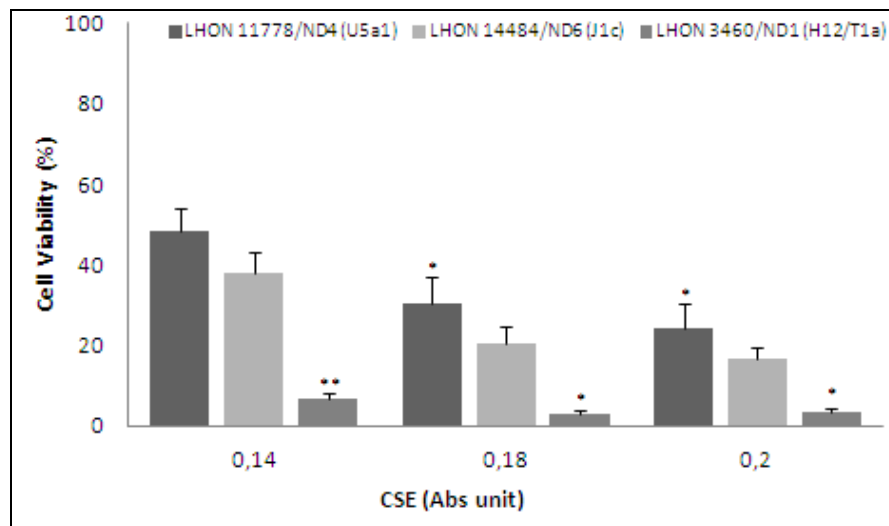


Figure 46. Effect of CSE on LHON cybrids viability of different mtDNA haplogroups. Cells were incubated in glucose with CSE for 24h. Data are reported as mean $\pm$ SEM (* $p \leq 0,05$; ** $p \leq 0,001$)

Moreover, we matched LHON cybrids with control cybrids according to their mtDNA haplogroups affiliation. As a result, cell viability of LHON G3460A/ND1 cells with haplogroup H12 and T1a showed a marked reduction also compared to their haplogroup-matched control cybrids (figure 47).

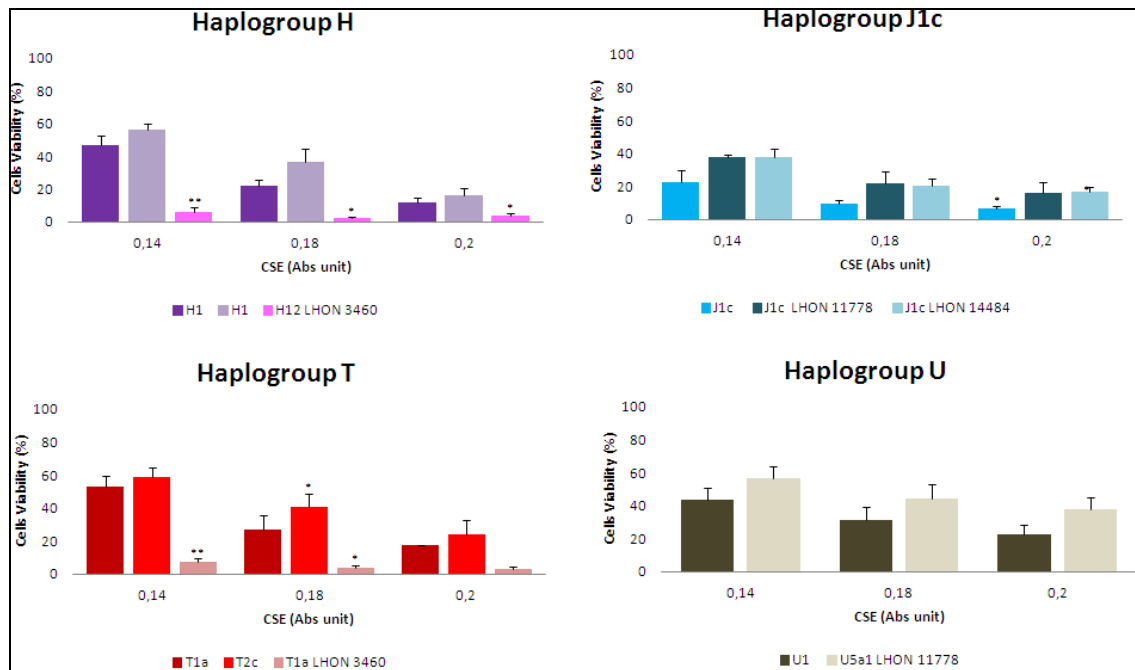


Figure 47. Effect of CSE on LHON and control cybrids variability marched for mtDNA haplogroups. Cells were incubated in glucose with CSE for 24h. Data are reported as mean $\pm$ SEM (* $p \leq 0,05$; ** $p \leq 0,001$)

6.7 The mitochondrial DNA polymorphisms sensitivity to antibiotic Linezolid

To evaluate the effect of the antibiotic Linezolid on mitochondrial function, we studied nine cybrids belonging to European mtDNA haplogroups N1b, H, J and T. As reported in table 9, cybrids were classified according to the different combinations of the two frequent SNPs at position 2706 and 3010 affecting the 16S rRNA in the sequence region predicted to be very close to the PTC. The list of specific non-synonymous nucleotide changes characterizing European mtDNA haplogroups is reported in table 5. We grouped the different combinations of the two variants in 2706G/3010G (haplogroups N1b, T1, T2 and J2 considered as control), 2706G/3010A (haplogroup J1 considered as single mutant), 2706A/3010G (haplogroup H5 considered as alternative single mutant) and 2706A/3010A (haplogroup H1 considered as “double mutant”).

Table 9. SNP variants at position 2706 and 3010 in 16SrRNA in cybrids

Cybrids	Haplogroup
2704G/3010G in rRNA 16S	
H28705	N1b
HFG5	J2a
H42	T1a
HPS11	T2c
2706G/3010A in rRNA 16S	
HQB17	J1b
HGA	J1c
2706A/3010G in rRNA 16S	
HPC7	H5
2706A/3010A in rRNA 16S	
HAD	H1
HF16W.1	H1

The first tested the viability of all cybrid clones after five days of exposure to increasing concentration of Linezolid (5-20 μ g/mL). Figure 48 shows that, despite a lack of statistical significance, the “double mutant” 2706A/3010A was the more susceptible to Linezolid. In a further analysis of the same assay we considered separately the subhaplogroup affiliation of our control group cybrids i.e. haplogroup N1b (at the root of all European haplogroups), haplogroup T and haplogroup J2. Haplogroup N1b was less susceptible to Linezolid compared to the other 2706G/3010G J2/T controls, whereas the double mutant 2706A/3010A H1 was constantly the worst (not shown).

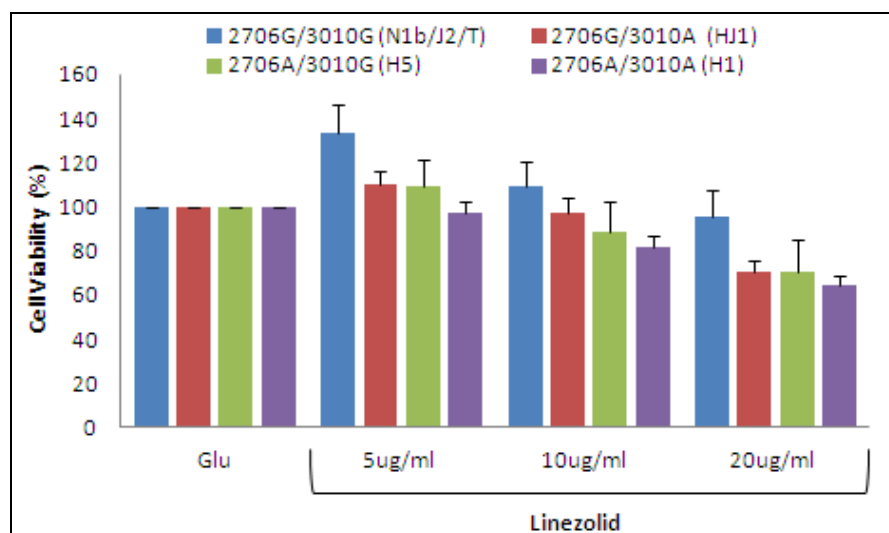


Figure 48. Effect of Linezolid on cybrids viability. Cells were incubated in glucose with CSE for five days. Data are reported as mean $\pm$ SEM

Next, the rate of ATP synthesis, after 24h exposure to 20 μ g/mL Linezolid in glucose medium, was evaluated on the different genetic combinations for 2706 and 3010

variants. As reported in figure 49 the differential ATP synthesis with and without Linezolid exposure was greater for the double mutant 2706A/3010A H1 cybrid clone.

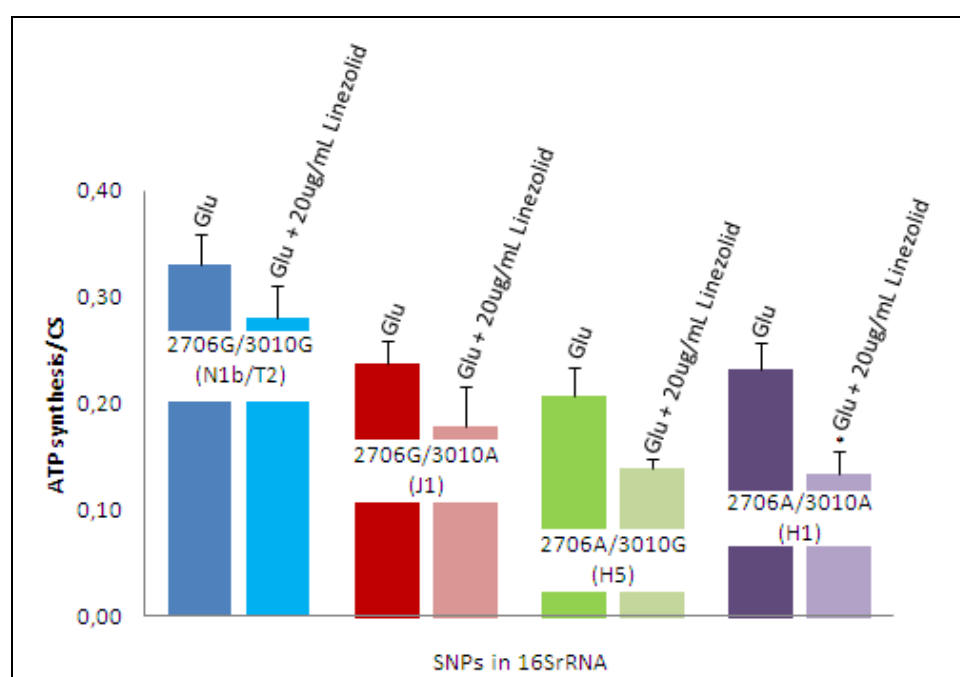


Figure 49. Effect of Linezolid on ATP synthesis driven by CI substrates in cybrids. Cells were incubated in glucose and Linezolid 20µg/mL for 24h and then were digitonin-permeabilized. ATP synthesis rate was normalized for CS activity. Data are reported as mean $\pm$ SEM ($p \leq 0,05$)

Finally, the specific activity of OXPHOS complexes was measured in presence or absence of Linezolid 20 µg/mL in mitochondrial-enriched fraction isolated from cybrid cell lines. Figure 50 shows that the “double mutant” 2706A/3010A H1 cybrid clone displayed a significant reduction in CI+CIII and CIV specific activity compared to untreated cells, whereas no difference were detected in CIII activity. CI+III activity is function of CI activity that is known to be limiting compared to CIII activity, and solves the problems of reproducibility of the CI isolated assay.

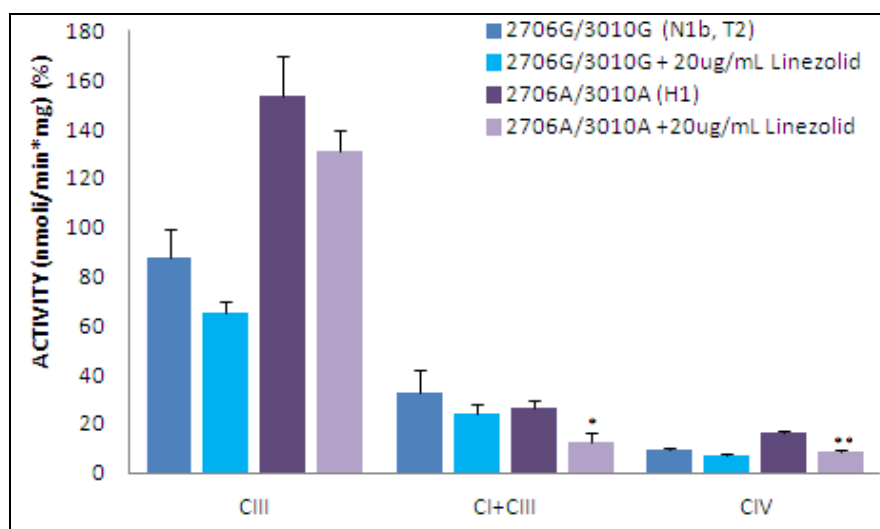


Figure 50. Effect of Linezolid on OXPHOS activities in cybrids. Cells were incubated with Linezolid 20 μ g/mL and glucose for 24h and then mitochondrial-enriched fraction was separated. OXPHOS activity was normalized for CS activity. Data are reported as mean $\pm$ SEM (* $p \leq 0,05$)

Overall these results indicate that the increased susceptibility to Linezolid of the 2706A/3010A combination of polymorphisms, already observed for cell viability and mitochondrial ATP synthesis experiments, is also reflected in functional activity of OXPHOS complexes (CI and CIV).

6.8 The mitochondrial DNA haplogroups sensitivity to chemioterapic cisplatin

Finally, we started to examine the effects of CisPt on mitochondrial function by testing the viability of our cybrids (N1b, H1, J, T2 and K1 haplogroups) after exposure to CisPt 5 and 10 μ M. It has been previously demonstrated that this chemioterapic agent induces hearing loss in patients with haplogroup J. [[Peter U. et al., 2003](#)]. Our preliminary results shows that despite cell viability was only slightly reduced, haplogroup J was more susceptible compared to K1a (Figure 51).

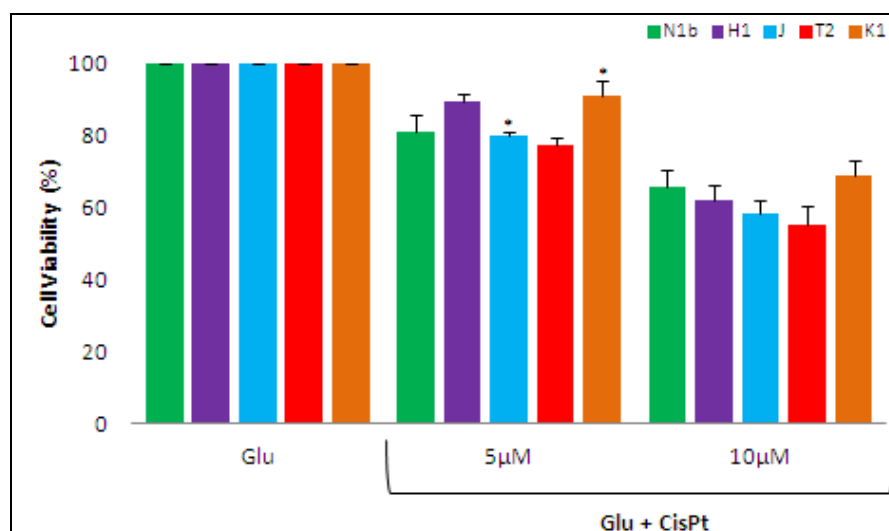


Figure 51. Effect of CisPt on cybrids viability of different mtDNA haplogroups (N1b, H1, J, T2 and K1). Cells were incubated in glucose with CisPt for 24h. Data are reported as mean $\pm$ SEM (** $p \leq 0,001$ all pairwise

Discussion

DISCUSSION

It is well known that medicines and other chemicals can interfere at various levels with mitochondrial function and frequently they cause mitochondrial dysfunction (mitotoxicity), affecting mtDNA maintenance and stability, protein synthesis of the 13 subunits encoded by mtDNA, MRC function, and other mitochondrial metabolic processes, channels and transporters. In this study we investigated the impact of mtDNA genetic variations on susceptibility to environmental agents and drugs previously implicated in predisposition to neurodegenerative disorders, or other kinds of neurotoxicity. To this end we selected compounds on a collection of transmitochondrial cytoplasmic hybrids (cybrids) constructed to carry mtDNAs from different human haplogroups and mtDNA mutations (G3460A/ND1, G11778A/ND4 and G14484A/ND6) affecting CI, pathogenic for LHON, a neurodegenerative disorder for which a modifying effect of mtDNA haplogroups has already been documented, as well as the influence of environmental factors (tobacco smoking) [[Carelli, V. et al., 2006](#); [Hudson G. et al., 2007](#); [Yu-Wai-Man P. et al., 2011](#)].

The cybrids cell lines generated for this study carried the most relevant mtDNA haplogroups of European populations (N1b, H, J, T, U, and K). This standard haplogroup affiliation of mtDNA sequence is normally obtained by comparison to the reference sequence known as rCRS that belongs to H2a2a1 haplogroup [[Andrews R.M. et al., 1999](#); [Bandelt H.J. et al., 2013](#)]. However recently Behar et al. proposed a new reference sequence denominated RSRS based on the assumption that all modern mtDNA sequences should be referred to an ancestral common root as best reference [[Behar D.M. et al., 2012](#)]. Therefore, we phylogenetically related all our mtDNA sequence with both rCRS and RSRS. As expected specific non-synonymous variants that define mtDNA haplogroups are essentially the same in both reference sequences with the exception of the SNP 10398 in ND3 gene. This is shown as the substitution of A to G in rCRS, whereas in RSRS the nucleotide G is converted to A. The SNP A10398G, obtained by comparison of our mtDNA sequences to rCRS, is a common variant for European macrohaplogroups J and K. The SNP G10398A, obtained by comparison to the RSRS, is a common variant for all European macrohaplogroups (H, T and U) except for N1b, J and K. Furthermore, to establish the conservation pattern of the amino acid

positions affected by haplogroup-specific SNPs (most conserved amino acid changes or SAPs) we analyzed 60 mammalian species, according to mtSAP Evaluation. Our analysis revealed that SAPs T240A in ND1 (SNP A4024T) and L89P in ND4 (SNP T11025C) of haplogroup K1a are not conserved, whereas SAP Y304H in ND1 (SNP T4216C) of haplogroups J/T is the most conserved (63,3%). Thus, different combinations of SNPs in different haplogroup backgrounds may exert both protective and/or predisposing roles, by interacting with environmental exposure to toxicants or drugs.

We first investigated a set of environmental agents, chosen because commonly implicated in PD such as rotenone, MPP⁺ and paraquat. These are all toxic agent that have been shown to inhibit CI, either directly or indirectly. CI is also a target and a source of ROS, thus the common mechanism proposed for toxicity in all these pharmacological models of PD centres around two major pathways: inhibition of mitochondrial function (CI), and increased ROS generation [[Dranka B.P et al., 2012](#)]. Although neither maternal transmission nor specific mtDNA mutations have been associated with idiopathic PD, Ghezzi D. et al suggested that the European mtDNA haplogroups J and K exert a protective role, lowering the risk of PD, possibly through the SNP A10398G in the ND3 gene [[Ghezzi D. et al, 2005](#)]. On the contrary, the root lineage originating both haplogroups J and T, defined by the T4216C SNP in the ND1 gene, seems to be more frequent among PD patients, thus exerting a predisposing role [[Ross O.A. et al., 2003](#)].

Our set of experiments with the pesticide rotenone revealed that cybrids carrying SNPs belonging to haplogroup J1 were most sensitive to the reduction of cell viability. This matched the impairment of OXPHOS through CI inhibition, as documented by the ATP synthesis assay with CI-dependent substrates. Furthermore, a slight chronic increase of ROS production was also evident, as predicted. However haplogroup J1 cybrids did not activate mitochondrial biogenesis efficiently, as a compensatory effect to the low OXPHOS efficiency. These results strikingly contrasted with in the performance of haplogroup K1 cybrids challenge with rotenone toxicity, supporting the indication that the genetic variation of this mtDNA haplogroup exerts a protective effect on PD, as indicated by population genetic studies [[Ghezzi D. et al, 2005](#); [Ross O.A. et al.,](#)

[2003](#)]. Hence it is possible that SAPs T240A of haplogroup K1a and Y304H in ND1 of haplogroups J/T can act as protective and risk factor in PD disease respectively.

As second well-known toxin inducing Parkinsonism in humans we tested MPP⁺. Again haplogroup J1 was the most sensitive to MPP⁺ exposure in term of cell viability, and incremental amounts of MPP⁺ progressively inhibited CI-driven ATP synthesis in both J and K haplogroup cybrids. These results are in agreement with previous reports [[Richardson J.R. et al., 2007](#); [Nicklas W.J. et al., 1985](#)] even though haplogroup J displayed reduced ROS production at high concentrations of MPP⁺ as compared to K haplogroup cells. These results confirm that MPP⁺ is toxic to mitochondrial OXPHOS through CI inhibition, however even if haplogroup J seemed the most sensitive, there was no clear cut difference with haplogroup K cybrids. A first comment is that the inhibitory mechanism of MPP⁺ is presumably different from that of rotenone, for which is known an interaction with the ND1 subunit that enters in the composition of the CoQ pocket/binding site of CI. Indeed the polymorphic variant in ND1 associated with haplogroups J/T may well play a modifying role in rotenone sensitivity/toxicity, as shown by our results. This does not apply to MPP⁺. Furthermore, rotenone is assumed to be a toxin widely present in the environment, thus reflected into the genetic epidemiology of PD patients for whom the protective role of haplogroup K was documented and here confirmed on a functional ground by our in vitro experiments. At difference, MPP⁺ is not present in the environment, being a contaminant of a recreational drug, thus limited to drug users with no epidemiological impact. In fact, our in vitro results do not suggest a relevant haplogroup-dependent protection from MPP⁺ of haplogroup K. However, haplogroup J remains the choke-point mtDNA background when environmental interaction with toxins comes into play.

The third toxin considered as risk factor for PD is the herbicide paraquat. Despite the drastic effect of paraquat on haplogroup T2c cell viability, its neurotoxicity does not result from a direct inhibition on CI, as shown by the CI-driven ATP synthesis experiments. Furthermore, in our hands, ROS production did not differ among haplogroups. Preliminary results on the rate of ATP synthesis driven by CI and CII substrates revealed that T2c cell lines, incubated in galactose and paraquat 0,75mM for 24h, were the most sensitive to its inhibition, corroborating the cell viability results. Moreover, haplogroup T also presented a lower mtDNA copy number, possibly

explaining the peculiar sensitivity of this haplogroup to 0.75mM paraquat, whereas J1 and most strikingly haplogroup K1 increased mtDNA copy number. This may be again relevant to the proposed protective effect of haplogroup K1. These experiments do not clearly indicate a major role for oxidative stress in paraquat toxicity. This may depend on the experimental cell model used, given that cybrids may be lacking paraquat transporters such as LAT-1 [[Shimizu K. et al., 2001](#); [Richardson J.R. et al., 2005](#); [Martinez T.N., 2012](#)], OCT-3 and DAT [[Rappold et al., 2011](#)], thus paraquat would need more time to diffuse within these cells to exert its toxicity.

In conclusion, this turn of experiments provides for the first time a functional basis for the proposed modifying role of mtDNA haplogroups in predisposition/protection to/from PD, and we show that this effect may be deeply dependent on a differential sensitivity of mtDNA haplogroups to the interaction with environmental factors relevant to PD pathogenesis, such as the pesticide/herbicide/toxins we here investigated.

In a second set of experiments, we have investigated the mitochondrial toxicity of tobacco smoking (CSE), the most common and relevant environmental toxic exposure for a large set of individuals, which has been proposed to impair OXPHOS and increase ROS, possibly through a direct effect on CI [[Yu-Wai-Man P. et al., 2011](#)]. Among many roles played by tobacco smoking in predisposing to cancer and other conditions, it is now well recognized its role in contributing to neurodegeneration in LHON patients, triggering RGCs cell death and visual loss. Moreover, the pathogenic potential of LHON mutations is modified by the mtDNA haplogroup, being haplogroups J1c and J2b well established as backgrounds that increase LHON penetrance

Thus we tested the effect of CSE on mitochondrial function, analyzing cells viability of control cybrids in presence of incremental CSE Abs unit in glucose medium. Haplogroups J1c and J2a viability was markedly reduced. However, despite the effect on cell viability of haplogroups J1c and J2b, there was nor haplogroup-dependent difference in CI-driven ATP synthesis, nor in ROS production. Therefore, we switched with the same experiment on five cybrids generated from LHON patient fibroblasts belonging to European mtDNA haplogroups J1c, H12, T1a and U. Cell viability was strikingly depressed by CSE in LHON 3460/ND1 cells (H12 and T1a haplogroups)

compared to LHON 11778/ND4 and 14484/ND6 cybrids (haplogroups J1c and U) and matched control cell lines (H and T haplogroup). Paradoxically, the 11778/ND4 and 14484/ND6 LHON cybrids exposed to CSE had better viability to their haplogroup-matched control cybrids. Thus, one conclusion is that smoking is particularly relevant as LHON trigger for patients with the 3460/ND1 mutations, whereas the relationship between LHON mutations and mtDNA background needs to be elucidated for the 11778/ND4 and 14484/ND6 mutations. Notably, these two mutations only are associated with haplogroup J, and do not affect CI activity. Thus, their pathogenic mechanism is recognized as different from the 3460/ND1 mutations that is not associated with haplogroup J and sharply decreases the CI activity.

The last set of experiments investigated two drugs for which there is documented mitochondrial toxicity and neurotoxic effect, namely the antibiotic Linezolid and the chemotherapeutic CisPt, respectively inhibiting mitochondrial protein translation and mtDNA replication.

Patients under prolonged Linezolid treatment develop myelosuppression, lactic acidosis and optic neuropathy indistinguishable from LHON, as a result of OXPHOS impairment. Sequencing the mitochondrial 16S rRNA gene in these patients, suggested that the combination of the A2706G and G3010A polymorphisms in the region that binds Linezolid may modulate the susceptibility to Linezolid [[Velez J.C. Q et al., 2010](#); [Pacheu-Grau D. et al., 2013](#)].

We studied nine cybrid cell lines harboring the European mtDNA haplogroups N1b, H, J and T. For this particular study we classified these cybrids according to the different combinations of the two mtDNA SNPs at position 2706 and 3010 in the 16S rRNA gene in the sequence region predicted to be very close to the PTC. For clarity we arbitrarily defined as “wild-type” the combination of 2706G/3010G (haplogroups N1b, J2a, T1a and T2c) and as “pathological” the following polymorphic combinations: single mutant 2706A/3010G (Haplogroup H5), alternative single mutant 2706G/3010A (haplogroup J1) and double mutant 2706G/3010G (haplogroup H1). In the human mtDNA, the haplogroup H is defined by the polymorphism G2706A (alternative single mutant as H5) and subhaplogroups H1 is characterized by SNP G3010A (“double mutant”). This SNP also defines subhaplogroup J1 (single mutant).

Our results demonstrate that the 2706A/3010A combination (haplogroup H1) enhances cell sensitivity to Linezolid toxicity as shown by inhibition of CI-driven ATP synthesis and specific activity of OXPHOS complexes (CI and CIV), in agreement with what recently reported by [Velez J.C. Q et al., 2010](#) and [Pacheu-Grau D. et al., 2013](#).

These results establish the possibility to test in advance the patients as eligible for Linezolid treatment or at risk for suffering the severe side effects, proposing a personalized medicine approach to circumvent or be aware of the intrinsic risk of using this antibiotic.

Concerning CisPt, this forms a preferential covalent binding to DNA (CisPt-DNA adduct) inhibiting mtDNA replication, disrupting mtDNA transcription, generating ROS production, impairing mitochondrial respiratory chain and inducing apoptosis [[Sawyer D.E et al., 1999](#); [Reedii JK J et al., 1985](#); [Olivero O.A. et al., 1997](#)]. We preliminarily examined the effect of CisPt on mitochondria, showing that haplogroup J seems to enhance cell sensitivity to CisPt toxicity, in agreement with what recently proposed by [Peter U. et al., 2003](#).

Overall, the studies carried out in this thesis highlight a proof of principle on how complex the interaction could be between mtDNA sequence variation, as exemplified by the haploypete definition through complete mtDNA sequencing, and sensitivity to environmental factors. We have chosen specific toxins or drugs previously involved in neurodegeneration and/or neurotoxicity and tested the haplogroup-sensitivity to their mitochondrial toxicity. The results for Parkinson-related toxins broadly agree with the predictions generated by the population-based genetic epidemiology of mtDNA haplogroups, validating on the functional ground these predictions. The results on CSE influence on LHON disclosed some unexpected differences due to the different LHON pathogenic mutations. Also testing the two drugs Linezolid and CisPt indicated the possibility to apply a personalized approach in administering them to patients.

One observation stands out from all these experiments. The haplogroup J and some specific subhaplogroups belonging to this affiliation display invariably a special sensitivity to compounds interfering with mitochondrial function, in particular with OXPHOS efficiency. This haplogroup is characterized for an accumulation by descent of multiple non-synonymous variants, which in particular generate variability in ND

subunits of CI and in the cyt b gene. Others and we have previously proposed that this genetic variability may affect partially the interaction between CI and CIII in the context of supercomplex assemblage. This peculiar feature of haplogroup J on the biochemical ground probably resulted from adaptative selection, as proposed by Wallace and colleagues [[Wallace D.C., 2013a](#); [2013b](#)]. The adaptative advantages derived from selecting this cluster of slightly functional variants reflects on the interaction with toxins and more in general with the predisposing/protective role that may occur under different conditions. The studies carried out here using cybrids may benefit of a higher throughput approach, as now provided by cutting edge techniques. The field of mitochondrial pharmacology is just at the beginning, but holds a lot of promises for personalized medicine and for new drugs development.

Conclusions

CONCLUSIONS

Pharmacogenomics is the study of how genes affect the individual response to drugs to develop medications tailored to a person's genetic makeup because the efficacy/safety profile is not the same for everyone.

It has been well demonstrated that medicines and other chemicals can cause mitochondrial dysfunction (mitotoxicity), causing alteration of mtDNA, protein synthesis, MRC, metabolic processes, channels and transporters.

The results here reported demonstrated that mitochondrial genetic variability may influence individual susceptibility to drugs or environmental factors toxicity highlighting interesting associations between specific haplogroups, mitochondrial functional alterations, and toxic agents. More in details: 1) haplogroups K1 was found to play a protective role against rotenone toxicity whereas haplogroup J1 was more susceptible to the action of both rotenone and MPP⁺ even if the latter affinity to CI is less marked; 2) haplogroup T seems to be more susceptible to the action of paraquat; 3) haplogroups H12 and T1 in association with the LHON mutation 3460/ND1 and haplogroups J1c J2A all increase the susceptibility to mitochondrial damage after CSE exposure. Moreover, haplogroup H1, characterized by SNPs 2706A/3010A in 16SrRNA is the most sensitive to Linezolid toxicity and haplogroup J appears to act as risk factor in CisPt toxicity.

Though future studies will be necessary to better understand the mechanism of action of some of these molecules, the study of associations between mitochondrial haplogroups and toxicity of drugs and chemicals is extremely useful to prevent toxicity in predisposed subjects.

Ultimately, to increase drugs safety, pharmaceutical companies have started to assay the mitochondrial toxicity early in the drug-development process because severe *in vitro* mitochondrial impairment might be sufficient to withdraw a development program.

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APPENDIX A – Raw data from complete mtDNA sequencing compared to the rRCS

H28705 – Haplogroup N1b				
Position	CRS	Mutation	Region	AA Change
73	A	G	D-loop	
152	T	C	D-loop	
263	A	G	D-loop	
309+C	:	C	D-loop	
315+C	:	C	D-loop	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
1598	G	A	12S_rRNA	
1703	C	T	16S_rRNA	
1719	G	A	16S_rRNA	
2639	C	T	16S_rRNA	
2706	A	G	16S_rRNA	
3921	C	A	ND1	Ser205Ser
4769	A	G	ND2	Met100Met/ Met100Lys
4960	C	T	ND2	Ala164Val
4967	T	C	ND2	
5471	G	A	ND2	
7028	C	T	COI	
8251	G	A	COII	
8472	C	T	ATPase8	Pro36Leu
8836	A	G	ATPase6	Met104Val
8860	A	G	ATPase6	Thr112Ala
9335	C	T	COIII	
10238	T	C	ND3	
11362	A	G	ND4	
11719	G	A	ND4	
12007	G	A	ND4	
12501	G	A	ND5	
12705	C	T	ND5	
12822	A	G	ND5	
14766	C	T	Cyt b	Ile7Thr
15326	A	G	Cyt b	Thr194Ala
16145	G	A	D-loop	
16176G	C	G	D-loop	
16209	T	C	D-loop	
16223	C	T	D-loop	
16390	G	A	D-loop	
16519	T	C	D-loop	

HAD – Haplogroup H1				
Position	rCRS	Mutation	Region	AA change
63	A	G	D-loop	
315+C	:	C	D-loop	
374	A	G	D-loop	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
3010	G	A	16S_rRNA	
4769	A	G	ND2	Met100Met/ Met100Lys
8860	A	G	ATPase6	Thr112Ala
9592	T	C	COIII	Val129Ala
15326	A	G	Cytb	Thr194Ala
16357	T	C	D-loop	
16519	T	C	D-loop	

HF16W – Haplogroup H1				
Position	rCRS	Mutation	Region	AA change
263	A	G	D-loop	
309.1	:	C	D-loop	
315.1	:	C	D-loop	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
3010	G	A	16S_rRNA	
4769	A	G	ND2	Met100Met/ Met100Lys
8860	A	G	ATPase6	Thr112Ala
9015	C	T	ATPase6	
15326	A	G	Cytb	Thr194Ala
16129	G	A	D-loop	
16240	A	G	D-loop	
16468	T	C	D-loop	
16519	T	C	D-loop	

HQB – Haplogroups J1b				
Position	rCRS	Mutation	Region	AA change
73	A	G	D-loop	
199	T	C	D-loop	
242	C	T	D-loop	
263	A	G	D-loop	
295	C	T	D-loop	
315.1	:	C	D-loop	
462	C	T	D-loop	
489	T	C	D-loop	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
2158	T	C	16S_rRNA	
2706	A	G	16S_rRNA	
3010	G	A	16S_rRNA	
4216	T	C	ND1	Tyr304His
4769	A	G	ND2	Met100Met/ Met100Lys
4772	T	C	ND2	
5460	G	A	ND2	Ala331Thr
5964	T	C	COI	
7028	C	T	COI	
7407	T	C	COI	Tyr502His
8269	G	A	COII	
8557	G	A	ATPase6	Ala11Thr
			ATPase8	Leu64Leu
8860	A	G	ATPase6	Thr112Ala
9968	C	T	COIII	
10398	A	G	ND3	Thr114Ala
11251	A	G	ND4	
11719	G	A	ND4	
12007	G	A	ND4	
12396	T	C	ND5	
12612	A	G	ND5	
13308	A	G	ND5	
13708	G	A	ND5	Ala458Thr
13879	T	C	ND5	Ser515Pro
14766	C	T	Cytb	Ile7Thr
15326	A	G	Cytb	Thr194Ala
15452A	C	A	Cytb	Leu236Ile
16069	C	T	D-loop	
16126	T	C	D-loop	
16145	G	A	D-loop	
16261	C	T	D-loop	
16468	T	C	D-loop	

HGA – Haplogroup J1c				
Position	rCRS	Mutation	Region	AA Change
73	A	G	D-loop	
146	T	C	D-loop	
185	G	A	D-loop	
228	G	A	D-loop	
263	A	G	D-loop	
295	C	T	D-loop	
315.1	:	C	D-loop	
462	C	T	D-loop	
489	T	C	D-loop	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
2706	A	G	16S_rRNA	
3010	G	A	16S_rRNA	
4216	T	C	ND1	Tyr304His
4769	A	G	ND2	Met100Met/ Met100Lys
6464A	C	A	COI	
6554	C	T	COI	
7028	C	T	COI	
8860	A	G	ATPase6	Thr112Ala
10398	A	G	ND3	Thr114Ala
11251	A	G	ND4	
11719	G	A	ND4	
12127	G	A	ND4	
12612	A	G	ND5	
13681	A	G	ND5	Thr449Ala
13708	G	A	ND5	Ala458Thr
14766	C	T	Cytb	Ile7Thr
14798	T	C	Cytb	Phe18Leu
15326	A	G	Cytb	Thr194Ala
15452A	C	A	Cytb	Leu236Ile
16069	C	T	D-loop	
16092	T	C	D-loop	
16126	T	C	D-loop	
16261	C	T	D-loop	

HFG – Haplogroup J2c				
Position	rCRS	Mutations	Region	AA Change
73	A	G	D-loop	
150	C	T	D-loop	
195	T	C	D-loop	
263	A	G	D-loop	
295	C	T	D-loop	
309.1	:	C	D-loop	
309.2	:	C	D-loop	
315.1	:	C	D-loop	
489	T	C	D-loop	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
2706	A	G	16S_rRNA	
4216	T	C	ND1	Tyr304His
4769	A	G	ND2	Met100Met/ Met100Lys
6671	T	C	COI	
7028	C	T	COI	
7476	C	T	tRNA_Ser(1)	
8860	A	G	ATPase6	Thr112Ala
10398	A	G	ND3	Thr114Ala
10499	A	G	ND4L	
11002	A	G	ND4	
11149	G	A	ND4	
11251	A	G	ND4	
11377	G	A	ND4	
11719	G	A	ND4	
12570	A	G	ND5	
12612	A	G	ND5	
13708	G	A	ND5	Ala458Thr
14766	C	T	Cytb	Ile7Thr
15257	G	A	Cytb	Asp171Asn
15326	A	G	Cytb	Thr194Ala
15452A	C	A	Cytb	Leu236Ile
15679	A	G	Cytb	
16069	C	T	D-loop	
16126	T	C	D-loop	
16231	T	C	D-loop	
16319	G	A	D-loop	

H42 – Gaplogroup T1a				
Position	rCRS	Mutation	Region	AA Change
73	A	G	D-loop	
152	T	C	D-loop	
263	A	G	D-loop	
315.1	:	C	D-loop	
471	T	C	D-loop	
709	G	A	12S_rRNA	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
1888	G	A	16S_rRNA	
2706	A	G	16S_rRNA	
4216	T	C	ND1	Tyr304His
4769	A	G	ND2	Met100Lys Met100Lys
4917	A	G	ND2	Asn150Asp
5451	A	G	ND2	Thr328Ala
7028	C	T	COI	
7853	G	A	COII	Val90Ile
8697	G	A	ATPase6	
8860	A	G	ATPase6	Thr112Ala
10463	T	C	tRNA_Arg	-
11251	A	G	ND4	-
11719	G	A	ND4	-
12633	C	T	ND5	-
12927	C	T	ND5	-
13368	G	A	ND5	-
14766	C	T	Cytb	Ile7Thr
14905	G	A	Cytb	-
15326	A	G	Cytb	Thr194Ala
15452A	C	A	Cytb	Leu236Ile
15607	A	G	Cytb	-
15721	T	C	Cytb	-
15928	G	A	tRNA_Thr	-
16126	T	C	D-loop	-
16163	A	G	D-loop	-
16186	C	T	D-loop	-
16189	T	C	D-loop	-
16294	C	T	D-loop	-
16311	T	C	D-loop	-
16519	T	C	D-loop	-

HPS – Haplogroup T2c				
Position	rCRS	Mutation	Region	AA Change
73	A	G	D-loop	
150	C	T	D-loop	
263	A	G	D-loop	
315+C	:	C Insertion	D-loop	
709	G	A	D-loop	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
1888	G	A	16S_rRNA	
2706	A	G	16S_rRNA	
3834	G	A	ND1	
3915	G	A	ND1	
4216	T	C	ND1	Tyr304His
4769	A	G	ND2	Met100Lys Met 100Met
4823	T	C	ND2	
4841	G	A	ND2	
4917	A	G	ND2	Asn150Asp
6261	G	A	COI	Ala120Thr
7028	C	T	COI	
7301	A	G	COI	
8697	G	A	ATPase6	
8860	A	G	ATPase6	Thr112Ala
10463	T	C	tRNA Arg	-
10822	C	T	ND4	-
11251	A	G	ND4	-
11719	G	A	ND4	-
11812	A	G	ND4	-
12378	C	T	ND5	-
13368	G	A	ND5	-
14233	A	G	ND6	-
14413	C	T	ND6	-
14766	C	T	Cytb	Thr7Ile
14905	G	A	Cytb	-
15326	A	G	Cytb	Thr194Ala
15452	C	A	Cytb	Leu236Ile
15454	T	C	Cytb	-
15601	T	C	Cytb	-
15607	A	G	Cytb	-
15928	G	A	tRNA Thr	-
16126	T	C	D-loop	-
16146	A	G	D-loop	-
16292	C	T	D-loop	-
16294	C	T	D-loop	-
16519	T	C	D-loop	-

HGDA – Haplogroup U1				
Position	rCRS	Mutations	Region	AA change
73	A	G	D-loop	
195	T	C	D-loop	
263	A	G	D-loop	
663	A	G	12S_rRNA	
750	A	G	12S_rRNA	
1438	A	G	12S_rRNA	
1861	T	C	16S_rRNA	
2218	C	T	16S_rRNA	
2706	A	G	16S_rRNA	
4769	A	G	ND2	Met100Met/ Met100Lys
4991	G	A	ND2	
6026	G	A	COI	
7028	C	T	COI	
7403	A	G	COI	
7581	T	C	COII	
8860	A	G	ATPase6	Thr 112 Ala
11116	T	C	ND4	
11467	A	G	ND4	
11719	G	A	ND4	
12308	A	G	tRNA-Leu	
12372	G	A	ND5	
13656	T	C	ND5	
14070	A	G	ND5	
14364	G	A	ND6	
14766	C	T	Cytb	Ile 7 Thr
15115	T	C	Cytb	
15148	G	A	Cytb	
15217	G	A	Cytb	
15326	A	G	Cytb	Thr 194 Ala
15954	A	C	Cytb	
16189	T	C		
16249	T	C	D-loop	

HP27 – Haplogroup K1a				
Position	rCRS	Mutations	Region	AA change
73	A	G	D-loop	
263	A	G	D-loop	
310	:	C	D-loop	
317	:	C	D-loop	
750	A	G	12s-rRNA	
1189	T	C	12s-rRNA	
1438	A	G	12s-rRNA	
1719	G	A	16s-rRNA	
1811	A	G	16s-rRNA	
2706	A	G	16s-rRNA	
3107d			16s-rRNA	
3480	A	G	ND1	
4024	A	T	ND1	Thr240Ser
4769	A	G	ND2	Met100Met/ Met100Lys
5773	G	A	tRNA-Cys	
7028	C	T	COI	
8468	T	C	ATPase8	
8860	A	G	ATPase6	Thr112Ala
9055	G	A	ATPase6	Ala177Thr
9698	T	C	COIII	
10398	A	G	ND3	Thr114Ala
10550	A	G	ND4L	
11025	T	C	ND4	Leu89Pro
11299	T	C	ND4	
11467	A	G	ND4	
11719	G	A	ND4	
12308	A	G	tRNA-Leu	
12372	G	A	ND5	
14167	C	T	ND6	
14766	C	T	Cytb	Thr7Ile
14798	T	C	Cytb	Phe18Leu
15326	A	G	Cytb	Thr194Ala
16145	G	A	D-loop	
16224	T	C	D-loop	
16244	T	C	D-loop	
16311	T	C	D-loop	
16497	A	G	D-loop	
16519	T	C	D-loop	

HF01M/10 – Haplogroup K1c				
Position	rCRS	Mutations	Region	AA change
73	A	G	D-loop	
146	T	C	D-loop	
152	T	C	D-loop	
263	A	G	D-loop	
316	:	C	D-loop	
498	C	:	D-loop	
750	A	G	12S rRNA	
1189	T	C	12S rRNA	
1438	A	G	12S rRNA	
1811	A	G	16S rRNA	
2706	A	G	16S rRNA	
3107			16S rRNA	
3480	A	G	ND1	
4769	A	G	ND2	Met100Met/ Met100Lys
7028	C	T	COI	
7082	C	T	COI	
8860	A	G	ATPase6	Thr112Ala
9055	G	A	ATPase6	Ala177Thr
9093	A	G	ATPase6	
9698	T	C	COIII	
10398	A	G	ND3	Thr114Ala
10550	A	G	ND4L	
11299	T	C	ND4	
11377	G	A	ND4	
11467	A	G	ND4	
11719	G	A	ND4	
12308	A	G	tRNA-Leu	
12372	G	A	ND5	
14167	C	T	ND6	
14757	T	C	Cytb	Met4Thr
14766	C	T	Cytb	Thr7Ile
14798	T	C	Cytb	Phe18Leu
15326	A	G	Cytb	Thr194Ala
15635	T	C	Cytb	Ser297Pro
16224	T	C	D-loop	
16301	C	T	D-loop	
16311	T	C	D-loop	
16519	T	C	D-loop	

APPENDIX B - Raw data from complete mtDNA sequencing of the rCRS compared to the RSRS and the corresponding nucleotide differences

Position	rCRS	RSRS	Region	AA Change
73	A	G	D-loop	
146	T	C	D-loop	
152	T	C	D-loop	
195	T	C	D-loop	
247	G	A	D-loop	
263	A	G	D-loop	
750	A	G	12S rRNA	
769	G	A	12S rRNA	
825	T	A	12S rRNA	
1018	G	A	12S rRNA	
1438	A	G	12S rRNA	
2706	A	G	16S rRNA	
2758	G	A	16S rRNA	
2885	C	T	16S rRNA	
3594	C	T	ND1	
4104	A	G	ND1	
4312	C	T	tRNA-Ile	
4769	A	G	ND2	Met100Lys
	G			Met100Met
7028	C	T	COI	
7146	A	G	COI	Thr 415Ala
7256	C	T	COI	
7521	G	A	tRNA-Asp	
8468	C	T	ATPase8	
8655	C	T	ATPase6	
8701	A	G	ATPase6	Thr 59Ala
8860	A	G	ATPase6	Thr112Ala
9540	T	C	COIII	
10398	A	G	ND3	Thr114Ala
10664	C	T	ND4L	
10688	G	A	ND4L	
10810	T	C	ND4	
10873	T	C	ND4	
10915	T	C	ND4	
11719	G	A	ND4	
11914	G	A	ND4	
12705	C	T	ND5	
13105	A	G	ND5	Ile257Val
13276	A	G	ND5	Met314Val
13506	C	T	ND5	
13650	C	T	ND5	
14766	C	T	Cytb	Tht7Ile
15326	A	G	Cytb	Thr194Ala
16129	G	A	D-loop	
16187	C	T	D-loop	
16189	T	C	D-loop	
16223	C	T	D-loop	
16230	A	G	D-loop	
16278	C	T	D-loop	
16311	T	C	D-loop	
16519	T	C	D-loop	

APPENDIX C - Raw data from complete mtDNA sequencing compared to the RSRS

H28705 – Haplogroup N1b				
Position	RSRS	Mutations	Region	AA change
146	C	T	D-loop	
195	C	T	D-loop	
247	A	G	D-loop	
309.1	:	C	D-loop	
315.1	:	C	D-loop	
769	A	G	12S rRNA	
825	A	T	12S rRNA	
1018	A	G	12S rRNA	
1598	G	A	12S_rRNA	
1703	C	T	16S_rRNA	
1719	G	A	16S_rRNA	
2639	C	T	16S_rRNA	
2758	A	G	16S_rRNA	
2885	T	C	16S_rRNA	
3594	T	C	ND1	
3921	C	A	ND1	
4104	G	A	ND1	
4312	T	C	tRNA-Ile	
4960	C	T	ND2	Ala164Val
4967	T	C	ND2	
5471	G	A	ND2	
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7521	G	A	tRNA-Asp	
8251	G	A	COII	
8468	T	C	ATPase8	
8472	C	T	ATPase8	Pro36Leu
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
8836	A	G	ATPase6	Met104Val
9335	C	T	COIII	
9540	C	T	COIII	
10238	T	C	ND3	
10398	G	A	ND3	Ala114Thr
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11362	A	G	ND4	
11914	A	G	ND4	
12007	G	A	ND4	
12501	G	A	ND5	
12822	A	G	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13506	T	C	ND5	
16129	A	G	D-loop	
16145	G	A	D-loop	
16176	C	GT	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16209	T	C	D-loop	
16230	G	A	D-loop	
16278	T	C	D-loop	
16311	C	T	D-loop	
16390	G	A	D-loop	

HAD – Haplogroup H1				
Position	RSRS	Mutations	Region	AA Change
73	G	A	D-loop	
146	C	T	D-loop	
152	C	T	D-loop	
195	C	T	D-loop	
247	A	G	D-loop	
315+C	:	C	D-loop	
374	A	G	D-loop	
769	A	G	12S_rRNA	
825	A	T	12S_rRNA	
1018	A	G	12S_rRNA	
2706	G	A	16S_rRNA	
2758	A	G	16S_rRNA	
2885	T	C	16S_rRNA	
3010	G	A	16S_rRNA	
3594	T	C	ND1	
4104	G	A	ND1	
4312	T	C	tRNA-Ile	
7028	T	C	COI	
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7521	A	G	tRNA-Asp	
8468	T	C	ATPase8	
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9540	C	T	COIII	
9592	T	C	COIII	Val129Ala
10398	G	A	ND3	Ala114Thr
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11719	A	G	ND4	
11914	A	G	ND4	
12705	T	C	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13506	T	C	ND5	
13650	T	C	ND5	
14766	T	C	Cytb	Ile7Thr
16129	A	G	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16223	T	C	D-loop	
16230	G	A	D-loop	
16278	T	C	D-loop	
16311	C	T	D-loop	
16357	T	C	D-loop	

HF16W – Haplogroup H1				
Position	RSRS	Mutations	Region	AA Change
73	G	A	D-loop	
146	C	T	D-loop	
152	C	T	D-loop	
195	C	T	D-loop	
247	A	G	D-loop	
309.1	:	C		
315+C	:	C	D-loop	
769	A	G	12S_rRNA	
825	A	T	12S_rRNA	
1018	A	G	12S_rRNA	
2706	G	A	16S_rRNA	
2758	A	G	16S_rRNA	
2885	T	C	16S_rRNA	
3010	G	A	16S_rRNA	
3594	T	C	ND1	
4104	G	A	ND1	
4312	T	C	tRNA-Ile	
7028	T	C	COI	
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7521	A	G	tRNA-Asp	
8468	T	C	ATPase8	
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9015	C	T	ATPase6	
9540	C	T	COIII	
10398	G	A	ND3	Ala114Thr
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11719	A	G	ND4	
11914	A	G	ND4	
12705	T	C	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13506	C	T	ND5	
13650	T	C	ND5	
14766	C	T	ND5	Ile7Thr
16129	A	G	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16223	T	C	D-loop	
16230	G	A	D-loop	
16240	A	G	D-loop	
16278	T	C	D-loop	
16311	C	T	D-loop	
16468	T	C	D-loop	

HQB – Haplogroup J1b				
Position	RSRS	Mutations	Region	AA Change
146	C	T	D-loop	
152	C	T	D-loop	
195	C	T	D-loop	
199	T	C	D-loop	
242	C	T	D-loop	
247	A	G	D-loop	
295	C	T	D-loop	
315.1	:	C	D-loop	
462	C	T	D-loop	
489	T	C	D-loop	
769	A	G	12S_Rrna	
825	A	T	12S_Rrna	
1018	A	G	12S_Rrna	
2158	T	C	16S_rRNA	
2758	A	G	16S_Rrna	
2885	C	T	16S_Rrna	
3010	G	A	16S_Rrna	
3594	T	C	ND1	
4104	G	A	ND1	
4216	T	C	ND1	Tyr304His
4312	T	C	Trna-Ile	
4772	T	C	ND2	
5460	G	A	ND2	Ala331Thr
5964	T	C	COI	
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7407	T	C	COI	Tyr502His
7521	A	G	tRNA-Asp	
8269	G	A	COII	term
8468	T	C	ATPase8	
8557	G	A	ATPase6 ATPase8	Ala11Thr Leu64Leu
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9540	C	T	COIII	
9968	C	T	COIII	
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11251	A	G	ND4	
11914	A	G	ND4	
12007	G	A	ND4	
12396	T	C	ND5	
12612	A	G	ND5	
12705	T	C	ND5	
13107	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13308	A	G	ND5	
13506	T	C	ND5	
13650	T	C	ND5	
13708	G	A	ND5	Ala458Thr
13879	T	C	ND5	Ser515Pro
15452A	C	A	Cytb	Leu236Ile
16069	C	T	D-loop	
16126	T	C	D-loop	
16129	A	G	D-loop	
16145	G	A	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16223	T	C	D-loop	
16230	G	A	D-loop	
16261	C	T	D-loop	
16278	T	C	D-loop	
16310	C	T	D-loop	
16468	T	C	D-loop	
16519	C	T	D-loop	

HGA – Haplogroup J1c				
Position	RSRS	Mutations	Region	AA Change
152	C	T	D-loop	
185	G	A	D-loop	
195	C	T	D-loop	
228	G	A	D-loop	
247	A	G	D-loop	
295	C	T	D-loop	
315.1	:	C	D-loop	
462	C	T	D-loop	
489	T	C	D-loop	
769	A	G	12S_rRNA	
825	A	T	12S_rRNA	
1018	A	G	12S_rRNA	
2758	A	G	16S_rRNA	
2885	C	T	16S_rRNA	
3010	G	A	16S_Rrna	
3594	T	C	ND1	
4104	G	A	ND1	
4216	T	C	ND1	Tyr304His
4312	T	C	tRNA-Ile	
6464	C	A	COI	
6554	C	T	COI	
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7521	A	G	Trna-Asp	
8468	T	C	ATPase6	
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9540	C	T	COIII	
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11251	A	G	ND4	
11914	A	G	ND4	
12127	G	A	ND4	
12612	A	G	ND5	
12705	T	C	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13506	T	C	ND5	
13650	T	C	ND5	
13681	A	G	ND5	Thr449Ala
13708	G	A	ND5	Ala->Thr
14798	T	C	Cytb	Phe18Leu
15452	C	A	Cytb	Leu->Ile
16069	C	T	D-loop	
16092	T	C	D-loop	
16126	T	C	D-loop	
16129	A	G	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16223	T	C	D-loop	
16230	G	A	D-loop	
16261	C	T	D-loop	
16278	T	C	D-loop	

HFG – Haplo J2a				
Position	RSRS	Mutations	Region	AA Change
146	C	T	D-loop	
150	C	T	D-loop	
152	C	T	D-loop	
247	A	G	D-loop	
295	C	T	D-loop	
309.1	:	C	D-loop	
309.2	:	C	D-loop	
315.1	:	C	D-loop	
489	T	C	D-loop	
769	A	G	12S_rRNA	
825	A	T	12S_rRNA	
1018	A	G	12S_rRNA	
2758	A	G	16S_Rrna	
2885	C	T	16S_Rrna	
3594	T	C	ND1	
4104	G	A	ND1	
4216	T	C	ND1	Tyr304His
4312	T	C	Trna-Ile	
6671	T	C	COI	
7146	C	A	COI	Ala415Thr
7256	T	C	COI	
7476	C	T	tRNA_Ser	
7521	A	G	tRNA_Asp	
8468	T	C	ATPase8	
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9540	C	T	COIII	
10499	A	G	ND4L	
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11002	A	G	ND4	
11149	G	A	ND4	
11251	A	G	ND4	
11377	G	A	ND4	
11719	A	A	ND4	
11914	A	G	ND4	
12570	A	G	ND5	
12612	A	G	ND5	
12705	T	C	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13506	T	A	ND5	
13650	T	C	ND5	
13708	G	A	ND5	Ala458Thr
15257	G	A	Cytb	Asp->Asn
15452A	C	A	Cytb	Leu236Ile
15679	A	G	Cytb	
16069	C	T	D-loop	
16126	T	C	D-loop	
16129	A	G	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16223	T	C	D-loop	
16230	G	A	D-loop	
16231	T	C	D-loop	
16278	T	C	D-loop	
16311	C	T	D-loop	
16319	G	A	D-loop	
16519	C	T	D-loop	

H42 – Haplogroup T1a				
Position	RSRS	Mutations	Region	AA Change
146	C	T	D-loop	
165	C	T	D-loop	
247	A	G	D-loop	
315.1		C insertion	D-loop	
471	T	C	D-loop	
709	G	A	12S_rRNA	
769	A	G	12S_Rrna	
825	A	T	12S_Rrna	
1018	A	G	12S_Rrna	
1888	G	A	16S_rRNA	
2758	A	G	16S_rRNA	
2885	T	C	16S_rRNA	
3594	T	C	ND1	
4104	G	A	ND1	
4216	T	C	ND1	Tyr340His
4312	T	C	tRNA-Ile	
4917	A	G	ND2	Asn150Asp
5451	A	G	ND2	Thr328Ala
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7521	A	G	tRNA-Asp	
7853	G	A	COII	Val90Ile
8468	T	C	ATPase8	
8655	T	C	ATPase6	
8697	G	A	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9540	C	T	COIII	
10398	G	A	ND3	Ala114Thr
10463	T	C	Trna_Arg	
10644	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11251	A	G	ND4	
11914	A	G	ND4	
12633	C	T	ND5	
12705	T	C	ND5	
12927	C	T	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13368	G	A	ND5	
13506	T	C	ND5	
14905	G	A	Cytb	
15452A	C	A transversion	Cytb	Leu236Ile
15607	A	G	Cytb	
15721	T	C	Cytb	
15928	G	A	tRNA_Thr	
16126	T	C	D-loop	
16129	T	C		
16163	A	G	D-loop	
16186	C	T	D-loop	
13187	T	C		
16223	T	C		
16230	G	A		
16278	T	C		
16294	C	T	D-loop	

HPS – Haplogroup T2c				
Map position	RSRS	Mutations	Region	AA Change
146	C	T	D-loop	
150	C	T	D-loop	
152	C	T	D-loop	
195	C	T	D-loop	
247	A	G	D-loop	
315+C	:	C	D-loop	
709	G	A	D-loop	
769	A	G	12S_rRNA	
825	A	T	12S_rRNA	
1018	A	G	12S_rRNA	
1888	G	A	16S_rRNA	
2758	A	G	16S_rRNA	
2885	C	T	16S_rRNA	
3564	T	C	ND1	
3834	G	A	ND1	
3915	G	A	ND1	
4104	G	A	ND1	
4216	T	C	ND1	Tyr340His
4312	T	C	tRNA-Ile	
4823	T	C	ND2	
4841	G	A	ND2	
4917	A	G	ND2	Asn150Asp
6261	G	A	COI	Ala120Thr
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7301	A	G	COI	
7521	A	G	tRNA-Asp	
8468	T	C	ATPase6	
8568	T	C	ATPase6	
8697	G	A	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9540	C	T	COIII	
10398	G	A	ND3	Ala114Thr
10463	T	C	tRNA Arg	
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10822	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11251	A	G	ND4	
11812	A	G	ND4	
11914	A	G	ND4	
12378	C	T	ND5	
12705	T	C	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13278	G	A	ND5	
13368	G	A	ND5	
13506	T	C	ND5	
13650	T	C	ND5	
14233	A	G	ND6	
14413	C	T	ND6	
14905	G	A	Cytb	
15452A	C	A	Cytb	Leu236Ile
15454	T	C	Cytb	
15601	T	C	Cytb	
15607	A	G	Cytb	
15928	G	A	tRNA Thr	
16126	T	C	D-loop	
16129	A	G	D-loop	
16146	A	G	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16223	T	C	D-loop	
16230	G	A	D-loop	
16278	T	C	D-loop	
16292	C	T	D-loop	
16294	C	T	D-loop	
16311	C	T	D-loop	

HGDA – Haplogroup U1				
Position	RSRS	Mutations	Region	AA change
146	C	T	D-loop	
152	C	T	D-loop	
247	A	G	D-loop	
663	A	G	12S_rRNA	
769	A	G	12S_Rrna	
825	A	T	12S_rRNA	
1018	A	G	12S_rRNA	
1861	T	C	16S_rRNA	
2218	C	T	16S_rRNA	
2758	A	G	16S_rRNA	
2885	C	T	16S_rRNA	
3594	T	C	ND1	
4104	G	A	ND1	
4312	T	C	tRNA-Ile	
4991	G	A	ND2	
6026	G	A	COI	
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7403	A	G	COI	
7521	A	G	tRNA-Asp	
7581	T	C	COII	
8468	T	C	ATPase8	
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9540	C	T	COIII	
10398	G	A	ND3	Ala114Thr
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11116	T	C	ND4	
11467	A	G	ND4	
11914	A	G	ND4	
12308	A	G	tRNA-Leu	
12372	G	A	ND5	
12705	T	C	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13506	T	C	ND5	
13650	T	C	ND5	
13656	T	C	ND5	
14070	A	G	ND5	
14364	G	A	ND6	
15115	T	C	Cytb	
15148	G	A	Cytb	
15217	G	A	Cytb	
15954	A	C	Cytb	
16129	A	G	D-loop	
16145	G	A	D-loop	
16187	T	C	D-loop	
16223	T	C	D-loop	
16230	G	A	D-loop	
16249	T	C	D-loop	
16278	T	C	D-loop	
16311	C	T	D-loop	
16519	C	T	D-loop	

HP27 – Haplogroup K1a				
Position	RSRS	Mutations	Region	AA change
146	C	T	D-loop	
150	C	T	D-loop	
195	C	T	D-loop	
247	A	G	D-loop	
310	:	C	D-loop	
317	:	C	D-loop	
497	C	T	D-loop	
769	A	G	12s rRNA	
825	A	T	12s rRNA	
1018	A	G	12s rRNA	
1189	T	C	12s-rRNA	
1719	G	A	16s-rRNA	
1811	A	G	16s-rRNA	
2758	A	G	16s-rRNA	
2885	C	T	16s-rRNA	
3107d	N	:	16s-rRNA	
3480	A	G	ND1	
3594	T	C	ND1	
4024	A	T	ND1	Thr240Ser
4104	G	A	ND1	
4312	T	C	tRNA-Ile	
5773	G	A	tRNA-Cys	
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7521	A	G	trNA-Asp	
8468	T	C	ATPase8	
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9055	G	A	ATPase6	Ala177Thr
9540	C	T	COIII	
9698	T	C	COIII	
10550	A	G	ND4L	
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11025	T	C	ND4	Leu89Pro
11299	T	C	ND4	
11467	A	G	ND4	
11914	A	G	ND4	
12308	A	G	tRNA-Leu	
12372	G	A	ND5	
12705	T	C	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13506	T	C	ND5	
13650	T	C	ND5	
14167	C	T	ND6	
14798	T	C	Cytb	Phe18Leu
16129	A	G	D-loop	
16145	G	A	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16223	T	C	D-loop	
16224	T	C	D-loop	
16230	G	A		
16278	T	C		
16497	A	G	D-loop	

HF01M/10 – Haplogroup K1c				
Position	RSRS	Mutations	Region	AA change
195	C	T	D-loop	
247	A	G	D-loop	
316	:	C	D-loop	
498	C	:	D-loop	
769	A	G	12S rRNA	
825	A	T	12S rRNA	
1018	A	G	12S rRNA	
1189	T	C	12S rRNA	
1811	A	G	16S rRNA	
2758	A	G	16S rRNA	
2885	C	T	16S rRNA	
3480	A	G	ND1	
3595	T	C	ND1	
4104	G	A	ND1	
4312	T	C	tRNA-Ile	
7082	C	T	COI	
7146	G	A	COI	Ala415Thr
7256	T	C	COI	
7521	A	G	tRNA-Asp	
8468	T	C	ATPase8	
8655	T	C	ATPase6	
8701	G	A	ATPase6	Ala59Thr
9055	G	A	ATPase6	Ala177Thr
9093	A	G	ATPase6	
9540	C	T	COIII	
9698	T	C	COIII	
10550	A	G	ND4L	
10664	T	C	ND4L	
10688	A	G	ND4L	
10810	C	T	ND4	
10873	C	T	ND4	
10915	C	T	ND4	
11299	T	C	ND4	
11377	G	A	ND4	
11467	A	G	ND4	
11914	A	G	ND4	
12308	A	G	tRNA-Leu	
12372	G	A	ND5	
12705	T	C	ND5	
13105	G	A	ND5	Val 257Ile
13276	G	A	ND5	Val 314Met
13506	T	C	ND5	
13650	T	C	ND5	
14167	C	T	ND6	
14757	T	C	Cytb	Met4Thr
14798	T	C	Cytb	Phe18Leu
15635	T	C	Cytb	Ser297Pro
16129	A	G	D-loop	
16187	T	C	D-loop	
16189	C	T	D-loop	
16223	T	C	D-loop	
16224	T	C	D-loop D5	
16230	G	A	D-loop	
16278	T	C	D-loop	
16301	C	T	D-loop	

APPENDIX D - Raw data from complete mtDNA sequencing of LHON patients compared to the RSRS and rCRS

HMM12 - LHON 3460/ND1 – Haplogrpu H12					
Positions	RSRS	Mutations	rCRS	Region	AA Change
73	G	A	A	D-loop	
146	C	T	T	D-loop	
152	C	T	T	D-loop	
195	C	C	T	D-loop	
247	A	G	G	D-loop	
263	G	G	A	D-loop	
750	G	G	A	12S_rRNA	
769	A	G	G	12S_rRNA	
825	A	T	T	12S_rRNA	
1018	A	G	G	12S_rRNA	
1438	G	G	A	12S_rRNA	
2706	G	A	A	16S_rRNA	
2758	A	G	G	16S_rRNA	
2885	C	T	T	16S_rRNA	
3460	G	A	G	ND1	Ala52Thr
3594	T	C	C	ND1	
3936	C	T	C	ND1	
4104	G	A	A	ND1	
4312	T	C	C	tRNA-Ile	
4769	G	G	A	ND2	
7028	T	C	C	COI	
7146	G	A	A	COI	Ala415Thr
7256	T	C	C	COI	
7521	A	G	G	tRNA-Asp	
8468	T	C	C	ATPase8	
8655	T	C	C	ATPase6	
8701	G	A	A	ATPase6	
8860	G	G	A	ATPase6	Thr112Ala
9540	C	T	T	COIII	
10398	G	A	A	ND3	Ala114Thr
10664	T	C	C	ND4L	
10688	A	G	G	ND4L	
10810	C	T	T	ND4	
10873	C	T	T	ND4	
10915	C	T	T	ND4	
11719	A	G	G	ND4	
11914	A	G	G	ND4	
12705	T	C	C	ND5	
13105	G	A	A	ND5	Val 257Ile
13276	G	A	A	ND5	Val 314Met
13506	T	C	C	ND5	
13650	T	C	C	ND5	
14552	A	G	A	ND6	Val41Ala
14766	T	C	C	ND5	Ile7Thr
15326	G	G	A	Cytb	Thr->Ala
16129	A	G	G	D-loop	
16187	T	C	C	D-loop	
16189	C	T	T	D-loop	
16223	T	C	C	D-loop	
16230	G	A	A	D-loop	
16274	G	A	G	D-loop	
16278	T	C	C	D-loop	
16311	C	T	T	D-loop	
16519	C	C	T	D-loop	

HL180 – LHON 1448/ND6 – Haplogroup J1c					
Position	RSRS	Mutations	rCRS	Region	AA change
73	G	G	A	D-loop	
146	C	T	T	D-loop	
152	C	T	T	D-loop	
185	G	A	G	D-loop	
188	A	G	A	D-loop	
195	C	T	T	D-loop	
247	A	G	G	D-loop	
263	G	G	A	D-loop	
295	C	T	C	D-loop	
310+C	:	C	:	D-loop	
317 +C	:	C	:	D-loop	
462	C	T	C	D-loop	
489	T	C	T	D-loop	
750	G	G	A	12S_rRNA	
769	A	G	G	12S_rRNA	
825	A	T	T	12S_rRNA	
1018	A	G	G	12S_rRNA	
1438	G	G	A	12S_rRNA	
2706	G	G	A	16S_rRNA	
2758	A	G	G	16S_rRNA	
2885	C	T	T	16S_rRNA	
3010	G	A	G	16S_rRNA	
3594	T	C	C	ND1	
4104	G	A	A	ND1	
4216	T	C	T	ND1	Tyr 304 His
4312	T	C	C	tRNA-Ile	
4769	G	G	A	ND2	
7028	T	T	C	COI	
7042	T	C	T	COI	Val 380 Ala
7146	G	A	A	COI	Ala415Thr
7256	T	C	C	COI	
7521	A	G	G	tRNA-Asp	
8468	T	C	C	ATPase8	
8655	T	C	C	ATPase6	
8701	G	A	A	ATPase6	
8727	C	T	C	ATPase6	
8860	G	G	A	ATPase6	Thr 112 Ala
9540	C	T	T	COIII	
10398	G	G	A	ND3	Thr 114 Ala
10664	T	C	C	ND4L	
10688	A	G	G	ND4L	
10810	C	T	T	ND4	
10873	C	T	T	ND4	
10915	C	T	T	ND4	
11251	A	G	A	ND4	
11719	A	A	G	ND4	
11914	A	G	G	ND4	
12612	A	G	A	ND5	
12705	T	C	C	ND5	
13105	G	A	A	ND5	Val 257Ile
13276	G	A	A	ND5	Val 314Met
13506	T	C	C	ND5	
13650	T	C	C	ND5	
13708	G	A	G	ND5	Ala458Thr
14279	G	A	G	ND6	Ser 132 Leu
14484	T	C	T	ND6	Met 64 Val
14766	T	T	C	Cytb	Ile 7 Thr
14798	T	C	T	Cytb	Phe 18 Leu
15326	G	G	A	Cytb	Thr 194 Ala
15452A	C	A	C	Cytb	Leu 236 Ile
16069	C	T	C	D-loop	
16126	T	C	T	D-loop	
16129	A	G	G	D-loop	
16187	T	C	C	D-loop	
16189	C	T	T	D-loop	
16223	T	C	C	D-loop	
16230	G	A	A	D-loop	
16278	T	C	C	D-loop	
16311	C	T	T	D-loop	
16362	T	C	T	D-loop	
16390	G	A	G	D-loop	
16519	C	T	T	D-loop	

HPE9 – LHON 11778/ND4 – Haplogroup J1c					
Position	RSRS	Mutations	rCRS	Region	AA change
73	G	G	A	D-loop	
146	C	T	T	D-loop	
152	C	T	T	D-loop	
185	G	A	G	D-loop	
195	C	T	T	D-loop	
228	G	A	G	D-loop	
247	A	G	G	D-loop	
263	G	G	A	D-loop	
295	C	T	C	D-loop	
310+C	:	C	:	D-loop	
317+C	:	C	:	D-loop	
462	C	T	C	D-loop	
489	T	C	T	D-loop	
750	G	G	A	12S_rRNA	
769	A	G	G	12S_rRNA	
825	A	T	T	12S_rRNA	
1018	A	G	G	12S_rRNA	
1438	G	G	A	12S_rRNA	
2706	G	G	A	16S_rRNA	
2758	A	G	G	16S_rRNA	
2885	C	T	T	16S_rRNA	
3010	G	A	G	16S_rRNA	
3594	T	C	C	ND1	
4104	G	A	A	ND1	
4216	T	C	T	ND1	Tyr 304 His
4312	T	C	C	tRNA-Ile	
4769	G	G	A	ND2	
7028	T	T	C	COI	
7146	G	A	A	COI	Ala415Thr
7256	T	C	C	COI	
7521	A	G	G	tRNA-Asp	
8468	T	C	C	ATPase8	
8655	T	C	C	ATPase6	
8701	G	A	A	ATPase6	
8860	G	G	A	ATPase6	Thr 112 Ala
9540	C	T	T	COIII	
9632	A	G	A	COIII	
10398	G	G	A	ND3	Ala 114 Thr
10664	T	C	C	ND4L	
10688	A	G	G	ND4L	
10810	C	T	T	ND4	
10873	C	T	T	ND4	
10915	C	T	T	ND4	
11251	A	G	A	ND4	
11719	A	A	G	ND4	
11778	G	A	G	ND4	Arg340His
11914	A	A	G	ND4	
12083	T	G	T	ND4	Ser442Ala
12612	A	G	A	ND5	
12705	T	C	C	ND5	
13105	G	A	A	ND5	Val257Ile
13276	G	A	A	ND5	Val314Met
13506	T	C	C	ND5	
13650	T	C	C	ND5	
13708	G	A	G	ND5	Ala 458 Thr
14766	T	T	C	Cytb	Ile 7 Thr
14798	T	C	T	Cytb	Phe 18 Leu
15326	G	G	A	Cytb	Thr 194 Ala
15452A	C	A	C	Cytb	Leu 236 Ile
16069	C	T	C	D-loop	
16126	T	C	T	D-loop	
16129	A	G	G	D-loop	
16187	T	C	C	D-loop	
16189	C	T	T	D-loop	
16223	T	C	C	D-loop	
16230	G	A	A	D-loop	
16278	T	C	C	D-loop	
16311	C	T	T	D-loop	
16519	C	T	T	D-loop	

HFF3 – LHON 11778/ND4 – Haplogroup U5a1					
Position	RSRS	Mutations	rCRS	Region	AA change
73	G	G	A	D-loop	
146	C	T	T	D-loop	
152	C	T	T	D-loop	
195	C	T	T	D-loop	
247	A	G	G	D-loop	
263	G	G	A	D-loop	
316 +C	:	C	:	D-loop	
358 +C	:	C	:	D-loop	
750	G	G	A	12S_rRNA	
769	A	G	G	12S_rRNA	
825	A	T	T	12S_rRNA	
1018	A	G	G	12S_rRNA	
1438	G	G	A	12S_rRNA	
2706	G	G	A	16S_rRNA	
2758	A	G	G	16S_rRNA	
2885	C	T	T	16S_rRNA	
2906	C	T	C	16S_rRNA	
3197	T	C	T	16S_rRNA	
3594	T	C	C	ND1	
4014	G	A	A	ND1	
4312	T	C	C	tRNA-Ile	
4553	T	C	T	ND2	
4655	G	A	G	ND2	
4769	G	G	A	ND2	
4914	C	T	C	ND2	
7028	T	T	C	COI	
7146	G	A	A	COI	Ala415Thr
7256	T	C	C	COI	
7521	A	G	G	tRNA-Asp	
8468	T	C	C	ATPase8	
8655	T	C	C	ATPase6	
8701	G	A	A	ATPase6	
8860	G	G	A	ATPase6	Thr 112 Ala
9477	G	A	G	COIII	Val91Ile
9540	C	T	T	COIII	
9667	A	G	A	COIII	Asn154Ser
10398	G	A	A	ND3	Ala 114 Thr
10664	T	C	C	ND4L	
10688	A	G	G	ND4L	
10810	C	T	T	ND4	
10873	C	T	T	ND4	
10915	C	T	T	ND4	
11467	A	G	A	ND4	
11719	A	A	G	ND4	
11778	G	A	G	ND4	Arg340His
11914	A	G	G	ND4	
12308	A	G	A	tRNA-Leu	
12372	G	A	G	ND5	
12705	T	C	C	ND5	
13105	G	A	A	ND5	
13276	G	A	A	ND5	
13506	T	C	C	ND5	
13617	T	C	T	ND5	
13650	T	C	C	ND5	
14766	T	T	C	Cytb	Ile 7 Thr
14793	A	G	A	Cytb	His16Arg
15326	G	G	A	Cytb	Thr 194 Ala
16129	A	G	G	D-loop	
16186	T	C	C	D-loop	
16189	C	T	T	D-loop	
16192	C	T	C	D-loop	
16223	T	C	C	D-loop	
16230	G	A	A	D-loop	
16256	C	T	C	D-loop	
163270	C	T	C	D-loop	
16278	T	C	C	D-loop	
16291	C	T	C	D-loop	
16311	G	T	T	D-loop	
16399	A	G	A	D-loop	
16519	C	T	T	D-loop	

RI206 – LHON 3460/ND1 – Haplogroup T1a					
Position	RSRS	Mutations	rCRS	Region	AA Change
73	G	G	A	D-loop	
146	C	T	T	D-loop	
152	C	C	T	D-loop	
195	C	T	T	D-loop	
247	A	G	G	D-loop	
263	G	G	A	D-loop	
310+C	:	C	:	D-loop	
317+C	:	C	:	D-loop	
709	G	A	G	12S_rRNA	
750	G	G	A	12S_rRNA	
769	A	G	G	12S_rRNA	
825	A	T	T	12S_rRNA	
1018	A	G	G	12S_rRNA	
1438	G	G	A	12S_rRNA	
1888	G	A	G	16S_rRNA	
2706	G	G	A	16S_rRNA	
2758	A	G	G	16S_rRNA	
2885	c	t	t	16S_rRNA	
3460		A	G	ND1	Ala52Thr
3594	T	C	C	ND1	
4104	G	A	A	ND1	
4216	T	C	T	ND1	Tyr304His
4312	T	C	C	tRNA-Ile	
4769	G	G	A	ND2	
4917	A	G	A	ND2	Asn150Asp
7028	T	T	C	COI	
7146	G	A	A	COI	Ala415Thr
7256	T	C	C	COI	
7521	A	G	G	tRNA-Asp	
8468	T	C	C	ATPase8	
8655	T	C	C	ATPase6	
8697	G	A	G	ATPase6	
8701	G	A	A	ATPase6	
8860	G	G	A	ATPase6	Thr112Ala
9540	C	T	C	COIII	
9899	T	C	T	COIII	
10398	G	A	A	ND3	Ala114Thr
10463	T	C	T	tRNA-Arg	
10664	T	C	C	ND4L	
10688	A	G	G	ND4L	
10180	C	T	T	ND4	
10873	C	T	T	ND4	
10915	C	T	T	ND4	
11251	A	G	A	ND4	
11719	A	A	G	ND4	
11914	A	G	G	ND4	
12633		T	C	ND5	
12705				ND5	
13105	G	A	A	ND5	Val257Ile
13276	G	A	A	ND5	Val314Met
13368	G	A	G	ND5	
13506	T	C	C	ND5	
13650	T	C	C	ND5	
14766	T	T	C	Cytb	Ile7Thr
14905	G	A	G	Cytb	
15326	G	G	A	Cytb	Thr194Ala
15452A	C	A	C	Cytb	Leu236Ile
15607	A	G	A	Cytb	
15928	G	A	G	tRNA_Thr	
16126	T	C	T	D-loop	
16129	A	G	G	D-loop	
16163	A	G	A	D-loop	
16186		T	C	D-loop	
16187	T	C	C	D-loop	
16189	C	C	T	D-loop	
16223	T	C	C		
16230	G	A	A		
16278	T	C	C		
16294	C	T	C	D-loop	
16311	C	T	T		
16519	C	C	T	D-loop	