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Mitochondrial Dysfunction In Hereditary Optic Neuropathies

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Esame finale anno 2009

My one regret in life is that I am not someone else.

Allen W.

INDEX

introduction

aim

results & discussion

materials & methods

references

introduction

1 Mitochondria

Structure

Mitochondria are characterized by a double system of membranes delimitating four “mitochondrial spaces” where different metabolic pathways take place: the outer membrane, the inner membrane, the intermembrane space and the matrix.

The outer mitochondrial membrane (OMM) is permeable to ions and small proteins (MW<10 kDa) because of the abundance of a large conductance channel, known as mitochondrial porin or voltage-dependent anion channel (VDAC) [1]. Hence the intermembrane space composition is very similar to the cytoplasmic one in terms of metabolites and ions concentrations. Other proteins active in the OMM are involved in import of both proteins synthesized in the cytosol and activated fatty acids into the matrix.

The intermembrane space contains about 6% of total mitochondrial protein and the most abundant member is represented by cytochrome *c*, a component of the **oxidative phosphorylation** (oxphos) system. Also pro-apoptotic proteins as well as enzymes, which are involved in the energetic metabolism (adenylate kinase and creatine kinase), in reactive oxygen species removal and in the membrane organization, are localized in the intermembrane space.

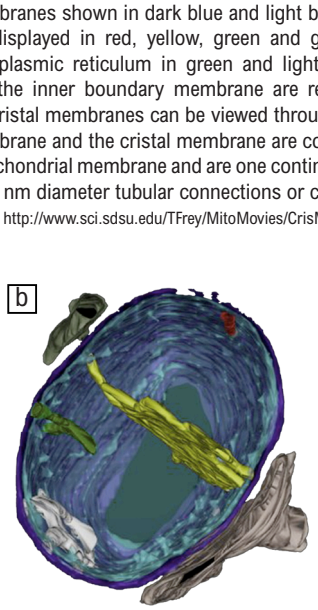
Respect to the OMM the inner mitochondrial membrane (IMM) has a higher ratio protein: phospholipids (3:1 vs 1:1 in the OMM) and it is rich in cardiolipin that seems to play a pivotal role not only in the maintenance of membrane structure but also in the interaction between transmembrane proteins [2,3]. The inner membrane is organized in numerous invaginations, called **cristae**, protruding into the matrix. Mitochondrial cristae contain all complexes forming the oxphos system and since oxphos is the major source of cellular ATP cristae extension and organization are dependent on energetic demand of cell. According to their different metabolic requirements, muscle cells mitochondria usually contain more cristae than liver cells mitochondria indicating a greater

figure 1

a) A three-dimensional model of chick cerebellum mitochondrion. The outer membrane is displayed in translucent dark blue and the cristae in yellow.

b) A three-dimensional model of the tomogram in with the outer and inner boundary membranes shown in dark blue and light blue respectively. Four different cristae are displayed in red, yellow, green and grey along with two segments of endoplasmic reticulum in green and light brown. The outer membrane and the inner boundary membrane are rendered translucent so that the cristal membranes can be viewed through it. The inner boundary membrane and the cristal membrane are components of the inner mitochondrial membrane and are one continuous surface joined at 30 nm diameter tubular connections or crista junctions.

[5 and http://www.sci.sdsu.edu/TFrey/MitoMovies/CrisMitoERMovie.htm]



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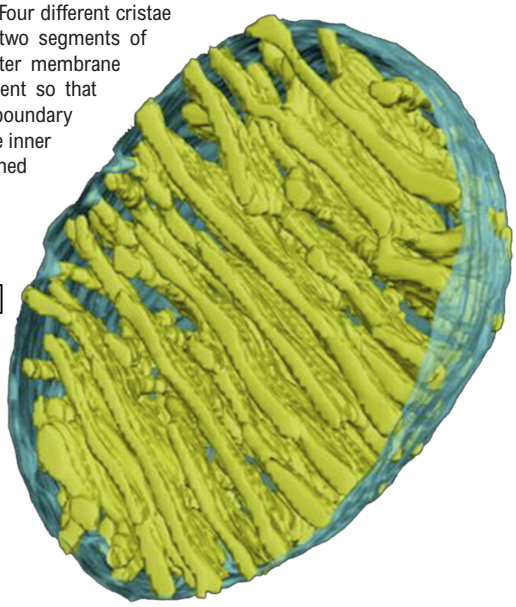
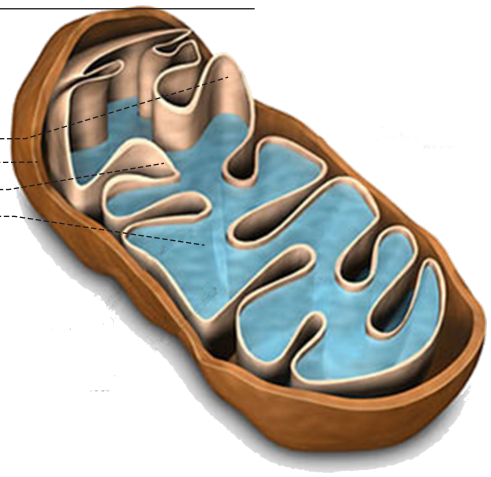


a

figure 2
Mitochondrion: schematic representation



content in the oxidative phosphorylation complexes [4]. Besides oxphos complexes IMM contains several ion channels, proteins involved in the import of nuclear-encoded proteins and the ADP/ATP exchanger that allows to mitochondrial-synthesized ATP to reach the cytosol.

The IMM is actually organized in two distinct domains: the **inner boundary membrane**, facing the OMM and delimitating the intermembrane space, and the **cristae membrane** protruding into the matrix.

The application of electron microscopy tomography (EMT) has provided very realistic model of the internal structure of mitochondria. This technique is based on collecting and merging several views from a relative thick specimen allowing the generation of reliable 3D renders. EMT analyses of a variety of mitochondria, isolated and *in situ*, have provided overwhelming evidence that cristae are not simply random folds in the inner membrane but rather internal compartments formed by invagination of the membrane [5,6]. These invaginations originate at narrow sites where the inner boundary membrane and the **cristae membrane** form tubular connection. The width of these dynamic structures, called **cristae junctions**, might determine the existence of a fifth "mitochondrial space": the **intracristae space** [4]. The application of EMT to mitochondria study has showed the high variability of cristae morphology, not only among different organism but also within a single organelle *[fig.1]*.

Finally, **contact sites** are defined as tight connection regions between OMM and IMM: in these regions the two membranes interact to allow the import of nuclear-encoded proteins or the export of newly synthesized ATP.

Mitochondrial matrix contains a very highly concentrated mixture of several proteins; this is the site of fatty acids *β-oxidation* and *Krebs cycle*. Along with enzymes and cofactors of catabolic metabolism, the matrix contains also all the components of mitochondrial protein synthesis machinery and several copies of mitochondrial DNA molecules as well as antioxidant enzymes like manganese superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxin.

Mitochondria are usually represented as rounded individual organelles but it is now well known that images obtained by electron microscopy analysis *[fig. 2 and fig. 3]* have been actually interpreted incorrectly. The development of fluorescence microscopy along with mitochondrial fluorescence probes has allowed to obtain a more accurate and realistic view of mitochondrial organization in living cells. As shown in

figure 3
Mitochondrion: electron micrograph of sectional view

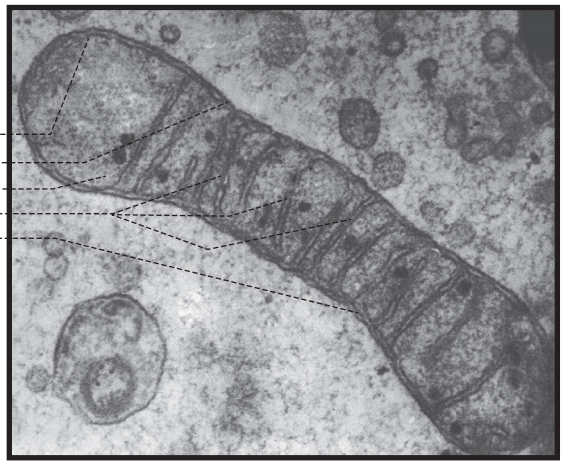
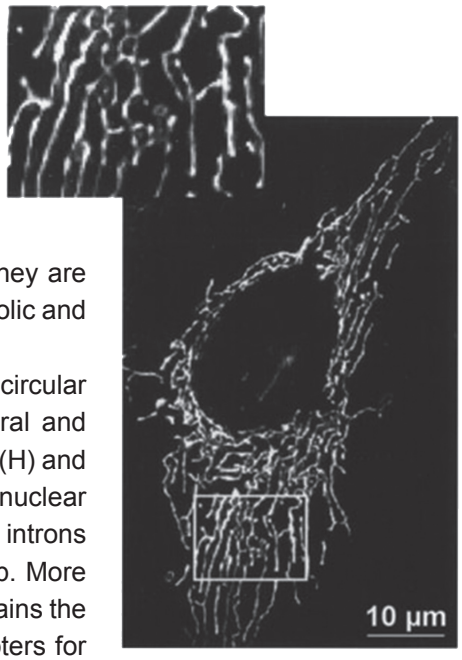


fig. 4 mitochondria appear more like a continuous tubular network rather than single entities and it is now clear that electron microscopy images are actually transversal section view of a single mitochondrial tubule [4]. The relevance and dynamic of mitochondrial network organization will be discussed in *Fission & Fusion* paragraph.

Genome

figure 4
Mitochondrial network imaged by fluorecence microscopy



According to the evolution theory, mitochondria derives from an ancestral symbiotic relationship between an aerobic bacteria and a primordial eukaryotic cell. After the symbiotic event, part of the proto-mitochondrion genome, initially providing all the information needed by a free-living organism, has been transferred to the nucleus [7].
Even if mitochondria in the lower and higher eukaryotes have preserved quite the same type of information in their own DNA, they are still completely dependent on the nuclear genome in terms of catabolic and energetic metabolism, protein translation and DNA replication [8].
In mammalian mitochondrial DNA (mtDNA) is a double-stranded circular molecule formed by 16568 base pairs with very peculiar structural and functional characteristics. The two strands are referred to as heavy (H) and light (L), reflecting their behavior in density gradients. Unlike the nuclear DNA (nDNA) mitochondrial genome is highly compact: there are no introns or spacers between genes that actually may in some case overlap. More there is only one non-coding region of approximately 1 Kb that contains the replication origin for leading strand synthesis (OH) and the promoters for transcription of the H- and L-strands.
Since the formation of fungal-animal lineage mitochondrial genetic code began to drift so that mitochondrial genes cannot longer be interpreted by the nuclear system. For this reason, since then, the mtDNA set has remained relatively constant through the evolution [8].
In Mammalian mtDNA molecule retains only 37 genes: the 12S and 16S rRNA and the 22 tRNAs representing the mitochondrial translational machinery along with 13 structural genes *[fig. 5]*. Gene transcription occurs symmetrically starting from two promoters, one for the G-rich heavy strand (P_H) the other for the C-rich light strand (P_L), and it progresses around the entire molecule. The polycistronic message is then processed to give the final product: the tRNA genes, which are interspersed between many of the protein-coding genes, are cleaved out, then mRNAs are polyadenylated [8].
The 13 mtDNA-encoded proteins are structural subunits of oxidative phosphorylation system. They include: seven (ND1-ND4, ND4L, ND5, ND6) of the 46 peptides of Complex I, one (cytochrome *b*) of the eleven peptides of Complex III, three (COI, COII, COIII) of the 13 peptides of Complex IV and two (ATP6, ATP8) of the 16 peptides of Complex V.
nDNA encodes all other mitochondrial proteins including all four subunits of Complex II, the mitochondrial DNA polymerase γ subunits (POLG), the mitochondrial RNA polymerase components, mitochondrial transcription and translation factors and the other mitochondrial metabolic enzymes [8].
The high mutational rate that distinguishes mtDNA from nDNA is the probable reason for the poor residual genetic content in mtDNA, while the maintenance of some oxphos gene in mitochondrial genome might be justified by the

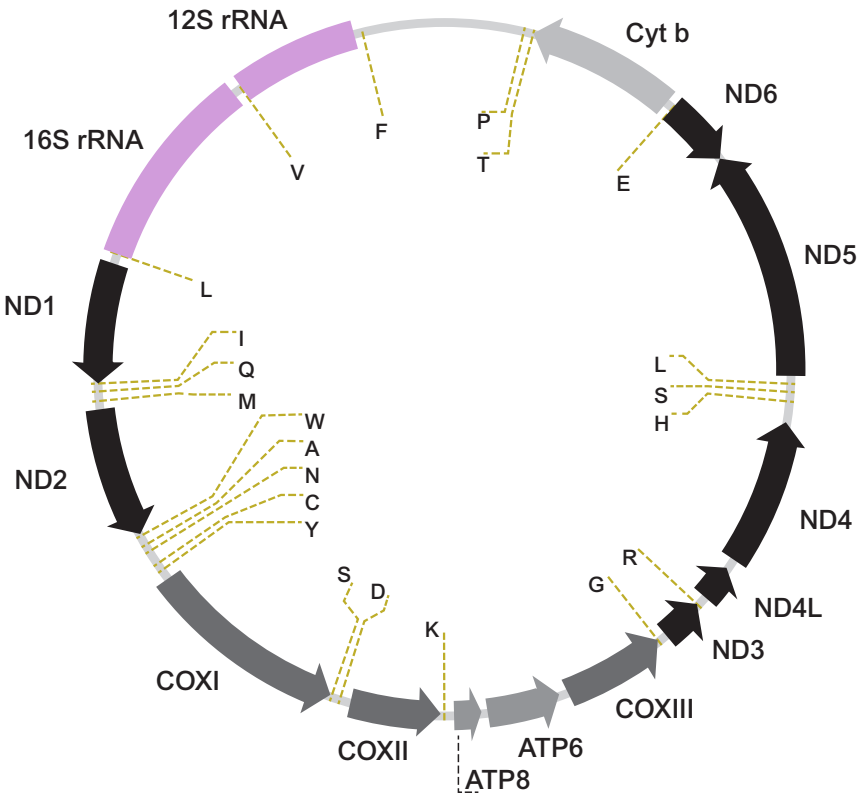
necessity of a tight connection between nucleus and mitochondria in order to match mitochondrial function with cell requirements.

Each somatic cell contains several copies of mtDNA, in the range of 10^3-10^4. mtDNA is organized in association with proteins in a structure called **nucleoid** at approximately 2–10 copies per nucleoid [9]. In higher eukaryotes mitochondrial nucleoid is reported to contain TFAM, a mitochondrial transcription factor, SSBP1 (single-stranded binding protein1), Twinkle (a helicase), and LON protease [10,11]. The nucleoids-associated proteins are supposed to be involved not only in processing but also in the maintenance of mtDNA. Lon is an ATP-dependent protease whose substrates are represented by oxidatively modified and unfolded peptides [12]. Interestingly, this protease shows the capability to specifically bind mtDNA and has been shown to co-immunoprecipitate with polymerase γ and Twinkle [12]. Since substrate binding facilitates ATP hydrolysis and proteolysis while inhibiting DNA binding, Lon has the potential to be a regulator of mitochondrial homeostasis - controlling both proteins and mtDNA - by acting as a sensor of the mitochondrial environment[13].

Mitochondrial genome duplicates independently from cell cycle in mitotic as well as in post mitotic cells. Individual mtDNA molecules can duplicate more than once or not at all in a single cell cycle [14] and at cell division they are distributed randomly into the daughter cells, according to the mechanism known as **Random Segregation**.

Since each cell is provided with multiple copies of mtDNA molecules, the presence of a mutation can result in two different genetic conditions defined as **Homoplasmy** and **Heteroplasmy**. We refer to homoplasmy when all mtDNA molecules in a cell or in an individual are identical, i.e. all mutated or all wild type. On the other hand, in the heteroplasmic status mutated and wild-type molecules coexist in the same cell or in the same individual. If this is the case, because of the random segregation, at each cell division the proportion of mutant and wild type mtDNA can potentially drift toward homoplasmic mutant or homoplasmic wild type state. The notion of heteroplasmy can be

figure 5
Human mitochondrial DNA map. Capital letters indicate tRNA genes.

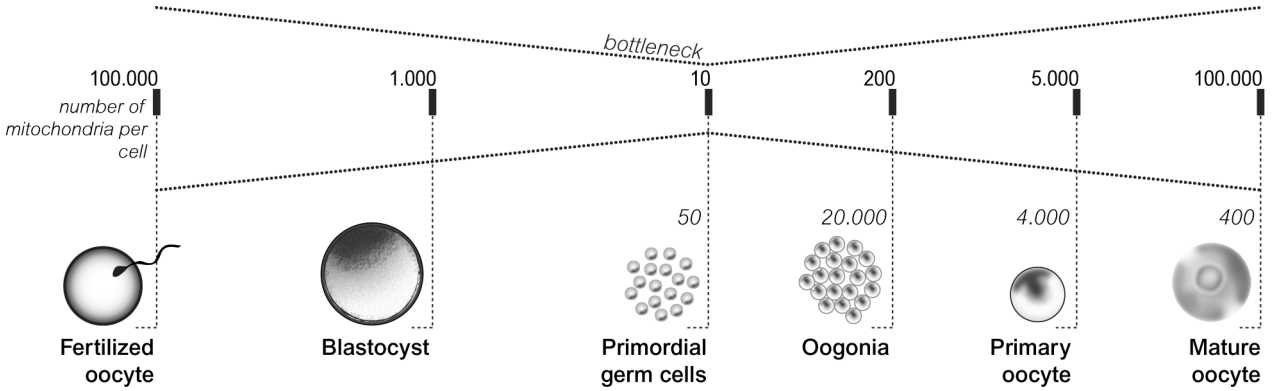


extended at the entire organism where only few tissues may be characterized by the presence of a mtDNA mutation. As general rule silent mtDNA mutations exist in the homoplasmic status while heteroplasmy is the typical condition of pathogenic mutations.

mtDNA is exclusively transmitted from the maternal lineage through the egg cytoplasm. In the zygote the paternal mtDNA is indeed eliminated from the fertilized egg probably because along their way to the oocyte sperm cells are subjected to a high risk of oxidative damage. Despite the high number of mtDNA copies in mature oocytes (10^5), only a few of them are transmitted during meiosis because of the phenomenon known as *bottleneck* [14]. In the *fig. 6* are represented the changes in the number of mitochondria during the development of the female germ line: the mitochondrial *bottleneck* occurs in

figure 6

Diagrammatic representation of changes in the number of mitochondria during development of the female germ line. An estimate of the number of mitochondria is indicated at each stage of germ line development. A genetic bottleneck for the transmission of mtDNA occurs in the primordial germ cells. Development to the mature oocyte involves at least a 10,000 fold increase in the number of mitochondria. [9]



the primordial germ line while a massive increase in cellular mtDNA copies number occurs during the formation of mature oocyte. During one or both of these steps, in presence of mtDNA mutations, the genomic variants could be "selected" causing the genetic shift between generations, likewise the mitochondrial random segregation during the mitosis can cause a genetic shift during the life of a mtDNA mutation carrier.

This pattern could be further complicated by the possibility that not all mtDNA mutations are equally treated in the germ line or during duplication, which means that some mutations could actually have a replicative advantage. Different studies have focused on this issue with different results depending on the mutation considered, but so far the more probable hypothesis is that, in mammals, the transmission of polymorphic and pathogenic mtDNA sequence variants is primarily a stochastic process [9].

Oxphos

Mitochondria, performing the last steps of cellular catabolism, are the primary source of energy for the cell. Reduced equivalents deriving from oxidation of carbohydrates, proteins or lipids are indeed utilized by these organelles to produce adenosin triphosphate (ATP) the major energetic molecule in the cell. Pyruvate, aminoacids and fatty acids deriving from cytosolic catabolic pathways are further oxidized in mitochondria matrix through Krebs cycle or β-oxidation to produce NADH (nicotinamide adenine dinucleotide, reduced form) and $FADH_2$ (flavine adenine dinucleotide, reduced form), as substrates

of the respiratory chain.
Since the inner membrane is freely permeable only to O_2, CO_2 and H_2O_2 and it lacks of NADH transporters, electrons deriving from cytosolic NADH are transferred into mitochondrial matrix through shuttle systems. Cytosolic oxaloacetate is reduced to malate that can cross the inner membrane, once in the matrix malate is reoxidized to oxaloacetate. The reaction is catalyzed by malate dehydrogenase and involves NAD reduction.
Respiratory chain, also called electron transport chain (ETC), consists of four multi-protein enzymatic complexes embedded in the inner mitochondrial membrane and two mobile electron carriers, coenzyme Q (CoQ), also called ubiquinone, and cytochrome *c*.
ETC complexes transfer electrons deriving from NADH and $FADH_2$ to molecular oxygen producing water. The oxygen reduction occurs through a multi-steps process: **Complex I** *(NADH:Ubiquinone oxidoreductase)* or **Complex II** *(Succinate:Ubiquinone oxidoreductase)* sequentially transfer two electrons deriving from NADH or $FADH_2$ to CoQ giving ubisemiquinone (CoQ•) and then ubiquinol ($CoQH_2$).

Complex II is the only enzyme of respiratory chain that does not show proton-pumping activity but it represents an alternative entry point for electrons and a checkpoint for the coordination between Krebs cycle and oxidative phosphorylation. Complex II [see references in 15 and 16] is composed of four subunits all encoded by nuclear genes: the two largest subunits lie in the matrix space and contain a covalently bound flavine adenine dinucleotide together with three Fe-S clusters. Because of its low midpoint potential the 4Fe-4S cluster is supposed not to be part of the electron transfer pathway but rather might represent a structural or regulatory moiety of the enzyme [16]. The other two subunits, less conserved, are localized in the membrane and contain a type *b* heme and two ubiquinone/ubiquinol binding domains localized on opposite sites of the inner membrane. The large distance between the two quinones would suggest that only the quinone bound near the cytplasmic side of inner membrane is part of the electron transfer chain. The catalytic mechanism consists in the reduction of FAD by the succinate molecule followed by electron transfer to CoQ through the Fe-S clusters chain. The role of heme *b* is not yet clearly understood. Besides its potential structural role, this prosthetic group could take part to redox reactions only during reverse electron transfer, being capable to participate in fumarate reduction but not in succinate oxidation [17].

Ubiquinol is oxidized by **Complex III** *(Ubiquinol:Cytochrome c oxidoreductase)* which reduces cytochrome *c*. Complex III [see references in15 and 18] exists as symmetric dimer where each monomer consists of eleven subunits. The catalytic core is formed by three subunits containing all the redox centers of the enzyme: cytochrome *b*, cytochrome c_1 and an iron-sulfur protein known as Rieske protein. Cytochrome *b* contains two heme groups – b_{565} and b_{560} – and two ubiquinone/ubiquinol binding sites called P center and N center; cytochrome c_1 is the cytochrome *c* electron donor. The redox proteins along with other three subunits (7, 10, 11) span the inner membrane while the remaining subunits are exposed to the matrix or to the intermembrane space. The catalytic mechanism is characterized by oxidation of ubiquinol and reduction of ubiquinone during the Q-Cycle. This process allows the translocation of four protons across the membrane and it occurs through one-electron steps oxidation of ubiquinol. Briefly, one electron deriving from the ubiquinol on the P

center sequentially flows from the high-potential Fe_2-S_2 cluster to cytochrome c_1 and cytochrome *c*. The ubiquinol oxidation is completed through a different pathway: the second electron reduces heme b_{560}, heme b_{565} and finally a molecule of ubiquinone bound at the N center to its ubisemiquinone form. The cycle ends with the binding of a second $CoQH_2$ at the P center: in this case, the first electron is used to reduce a second cytochrome *c* molecule while the other flows through heme b_{565} and b_{560} to reduce the ubisemiquinone at the N center determining the uptake of two protons from the matrix. The complete reaction can be so represented:

$$QH_2 + 2 \text{ cyt } c_{ox} + 2H^+_{matrix} \rightarrow Q + 2 \text{ cyt } c_{red} + 4H^+_{intermembrane\ space}.$$

From cytochrome *c* electrons flow to **Complex IV** *(Cytochrome c oxidase)* that finally reduces molecular oxygen to water. Complex IV [see references in 15, 19] is composed of thirteen subunits; three of them - I, II and III - are encoded by mitochondrial genes and represent the catalytic core of the enzyme. In subunit I are localized a copper atom - Cu_B - and two heme groups, called *a* and a_3. Subunit II contains Cu_A, a binuclear copper center. The catalytic action occurs through Cytochrome *c* oxidation by Cu_A. Heme *a* represents the bridge between Cu_A and the heme-copper center formed by Cu_B and heme a_3 where molecular oxygen is bound and reduced to water. Nevertheless subunit III is well conserved during evolution, its function still remains uncertain: the absence of redox centers excludes its participation to electron transfer. Moreover, studies in *P. denitrificans* have showed that a cytochrome oxidase lacking of subunit III conserves the capacity of translocate protons suggesting that this subunit is not determinant for proton pumping [20].

The oxygen reduction by Complex IV occurs through a multi-steps mechanism in which different iron-oxygen intermediates are formed thanks to the cooperation between heme a_3 and Cu_B. The electron transfer is coupled with the uptake of an equal number of protons from the matrix so that for each complete cycle four protons are vectorially translocated into the intermembrane space. The complex is provided with three potential channels by which proton transport can be accomplished. The so-called K channel allows the access of the four protons to the binuclear site for water formation, whereas D and H channels span the entire membrane layer. These channels are characterized by amino acids with protonable side chain, capable to form hydrogen bonds with nearby amino acids and to create a bridge between the matrix and the intermembrane space. However, site-directed mutagenesis studies, performed with the bacterial enzyme, suggest that the H channel is not involved in proton translocation[21]. The nuclear-encoded subunits exposed ether to the matrix or to the intermembrane space, are not directly involved in the catalytic mechanism and are supposed to have a regulatory function.

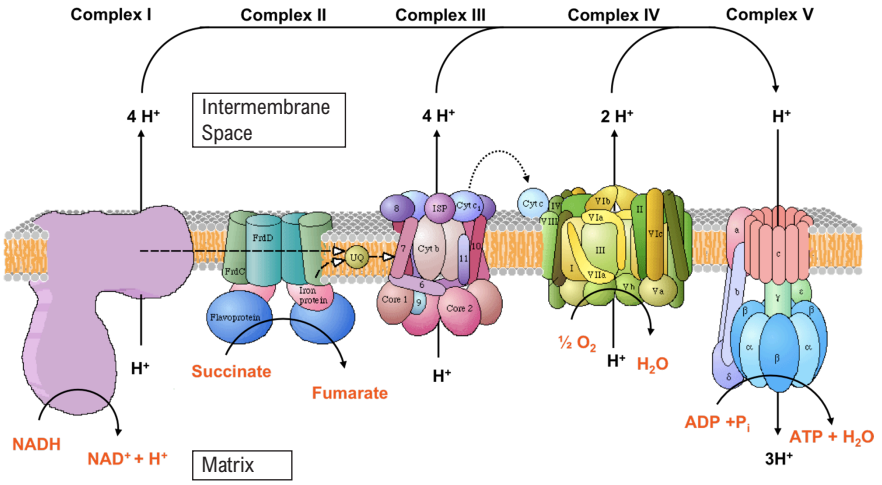
The energy released by the esorgonic electron transfer to the oxygen is then converted in what P. Mitchell, who first proposed the chemiosmotic theory, called **protonmotive force**. Contextually to the redox reactions Complex I, III and IV pump protons from the matrix into the intermembrane space so that through the inner membrane is established an electrochemical gradient (ΔP) consisting of two components: ΔΨ (electric) and ΔpH (chemical).

Complex V *(ATP synthase)* converts the energy stored in ΔP in high-energy phosphate bonds readily available for cell demand. Complex V [see references in 15 and 22] F_0 sector, embedded in the inner membrane, contains a channel that

allows to protons to flow back to the matrix thanks to the electrochemical gradient. The energy released by ΔP dissipation is linked to ATP synthesis by the Complex V soluble portion, F_1, through a tree steps mechanism in which ADP and P_i are bound and condensed to form ATP that is finally released into the matrix. The mammalian F_0 component contains nine subunits (a, b, c_n, d, e, f, g, A6L, F6), while the F_1 hydrophilic component has a α_3, β_3, γ, δ, ε composition where β subunits represent the active sites for the ATP synthesis. In the inner membrane 9-12 c subunits are arranged in a ring connected by a stalk to the catalytic component in the hydrophilic portion. The γ, δ and ε subunits compose the central part of the stalk moiety, while the peripheral stalk, lying to one side of the complex is composed of b, d, F6 and OSCP (oligomycin sensitivity conferring protein) subunits.
Complex V works as a rotary motor in which the protons flow through the F_0 portion modulates the properties of the β subunits. The catalytic subunits exist in three different conformations associated with different affinity for ADP-P_i and ATP, according to the binding-change mechanism proposed by Boyer [23]. The energy deriving from proton gradient dissipation is actually needed to eliminate the strong interaction between the newly synthesized ATP and the catalytic site. The transition between the three different β conformations is driven by the rotation of γ subunit. The b subunit along with other stalk components works as a tether between the F_0 and the α_3-β_3 module inducing the distortion of the β subunits in response to the rotor (c-ring, γ, δ, ε) motion. The proton channel is supposed to be localized in the interface between a and c subunits and the translocation is likely achieved by amino acids provided with carboxylate groups whose elecrostatic interactions drive the rotor.
The IF1 (F_1 inhibitor) protein, active as dimer, is capable to bind F_1 stabilizing the ATP synthase dimeric structure, which has been shown to have a determinant role in the cristae organization. Furthermore, IF1 is active at pH values < 6.5 so that it can works as pH sensor inhibiting the ATP synthase activity in acidic conditions, i.e. when the proton gradient collapses [22].

Thus, the ETC and ATP synthase work together to complete the oxidation of energetic nutrients and produce energy through the oxidative phosphorylation pathway *[fig. 7]*.
ATP is transported out of mitochondria by ANT (adenine nucleotide translocator) a dimeric transporter located in the inner mitochondrial membrane. ANT

figure 7
Schematic representation of oxphos system.



exchanges cytosolic ADP for mitochondrial ATP, the electrogenic antiport being driven by the membrane electrical potential difference. The Pi required in the matrix for the synthesis of ATP is as well imported from the cytosol by a P_i-H^+ symport system [24]. The electrochemical gradient formed by ETC trough the inner mitochondrial membrane thus not only provides the thermodynamic force to drive the synthesis and the release of ATP in the matrix but also drives the import of Complex V substrates (ADP and P_i) from cytosol.

ETC activity is thus tightly coupled to Complex V activity by transmembrane potential so that mitochondrial oxygen consumption rate is indirectly regulated by both matrix ADP concentration and Complex V activity. In absence of ADP, oxygen consumption rate is regulated by proton leakage across the inner membrane, then, in physiological condition, it is very slow. We refer to this condition as *state 4* of respiration. When ADP is available for Complex V protons start to flow through F_0 and the inner membrane becomes depolarized. The depolarization has to be counteracted by ETC that reduces oxygen at a higher rate to restore membrane potential determining the so-called *state 3* of respiration. The presence of uncoupling drugs such as DNP (2,4-dinitrophenol) or proteins as UCP1, 2 or 3 (uncoupling protein) makes the inner membrane permeable to protons, preventing the possibility to maintain the potential. In this condition ETC works at its maximum rate causing a sharp increase in oxygen consumption and determining the so-called *uncoupled respiration*.

The coupling between ETC and Complex V and then the correct control of mitochondrial membrane potential is very important to preserve mitochondria functions; ΔP is in fact required not only for ATP synthesis but also to drive the import of substrates and proteins encoded in the cytoplasm and for ions homeostasis: the electrical component of membrane potential (-180 mV) drives the import of Ca^{2+} into the matrix through a uniporter, while Ca^{2+} efflux is accomplished by Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers [1]. Since several cellular functions, like muscular contraction, neuronal secretion, hormone secretion and apoptosis are controlled by oscillations of cytosolic Ca^{2+} concentration is clear that mitochondrial buffering function need to be strictly preserved to maintain the whole cellular physiology.

For long time oxphos complexes have been supposed to freely diffuse in the inner membrane, with the electron transfer being based on random collision between respiratory chain components. Currently, thank to either kinetic or proteomic studies, the random collision model also defined as “fluid state model” has been substituted with an integrated model where single oxphos complexes would coexist with supercomplexes formed by interaction of Complex I, III and IV organized, in variable stoichiometries, in the so-called respirosome. The supramolecular organization of oxphos complexes has been reported in a wide range of organisms, from bacteria to fungi, yeasts, plants and higher eukaryotes [25]. The functional relevance of the respirosome is suggested by the dependence of Complex I, in terms of assembly and stability, on its interaction with Complex III. Cells harboring a mutation in cytochrome *c* gene leading to impairment in Complex III assembling showed in fact a concomitant reduction of Complex I amount [26]. The same results have been obtained in mitochondria isolated from human skeletal muscle [27] providing evidence that the formation of supercomplexes is essential for Complex I assembly/stability.

Characterization by transmission electron microscopy of bovine heart supercomplexes has confirmed the I_1III_2 and $I_1III_2IV_1$ stoichiometry previously deduced from BN-PAGE (Blue Native Polyacrylamide Gel Electrophoresis)

data [28, 29]. Since after isolation $I_1III_2IV_1$ remains intact and active, whereas a minor fraction of I_1III_2 disintegrate into its individual complexes, Complex IV might play an important role in the stability of the respirosome [29]. According to the integrate model, in physiological conditions individual oxphos complexes exist in equilibrium with supercomplexes so that, depending on metabolic conditions electron transfer in the ETC would occur via substrate channeling of ubiquinone and cytochrome *c* due to supercomplex formation or via random diffusion of these small carriers [30]. One major determinant in oxphos complexes interaction is represented by lipids in the membrane layer. Cardiolipin plays a pivotal role in the assembly and stabilization of functional supercomplexes [31] but other than participating in the association between individual complexes, membrane lipids could be also important in providing the correct environment for CoQ function [25]. The advantage of being organized in supercomplexes could derive primary from substrate channeling: the close proximity of enzymes catalyzing consecutive reactions of the same pathway would in fact increased the metabolic flow resulting in an increased efficiency of the entire system; however, a role of supercomplexes in preventing electrons escaping and then ROS production cannot be excluded [25].

It has been in fact estimated that a low proportion (~0.2%) of the oxygen utilized by mitochondria is converted by partial reduction to reactive oxygen species, a natural by-product of ETC [4]. It is now well known that, at low concentrations, ROS play a role in several signaling pathways through reversible modification of highly conserved cysteines or metionine in several proteins [32], but ROS accumulation due to increased production or inefficient removal may have deleterious consequences for the cell.

Reactive Oxygen Species

Reactive oxygen species (ROS) are a variety of molecules and free radicals - chemical species with one unpaired electron - deriving from partial reduction of molecular oxygen. The reduction of molecular oxygen by one electron at time produces intermediates with different stability and growing oxidative power:

$$O_2 \xrightarrow{e^-} O_2\bullet^- \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} OH\bullet$$

Superoxide anion (O_2•-), the product of a one-electron reduction of oxygen, can dismutate, spontaneously or trough reaction catalyzed by superoxide dismutases, to hydrogen peroxide. This species is not highly oxidant per se but can react with nitric oxide (NO•) in a reaction controlled by the rate of diffusion of both radicals to give peroxynitrite (ONOO) a more powerful oxidant.

Hydrogen peroxide (H_2O_2) is a non-radical relatively stable molecule that may work as messenger in signal transduction pathways integrating mitochondrial function with the rest of the cell.

Reduction of hydrogen peroxide by means of a ferrous anion produces hydroxyl (OH•), one of the strongest oxidants in nature; the presence of O_2•- may propagate this process cycling reducing the transition metal.

Mitochondria are an important source of ROS: ETC works basically with one-electron transfer steps and the redox centers present in oxphos complexes can potentially transfer electrons to molecular oxygen. ROS production in mitochondria depends mainly on both oxygen concentration and redox status

of ETC complexes, and in physiological conditions both parameters are actually determined by tissue metabolic requirements. Complex I and III are the main source of superoxide anion in mitochondria.

The poor knowledge of Complex I structure has not permitted to precisely locate the site of electron leak and all major cofactors have been proposed as site of oxygen reduction: FMN [33], iron-sulfur clusters N2 [34] and N1a [35] and the semiquinone radical formed upon ubiquinone reduction [36]. However, the electron leak from one or more of these sites results in superoxide anion radicals (O_2•-) production in the matrix [37].

One-electron reduction of oxygen in Complex III is accomplished by ubisemiquinone and increases in presence of antimycin, an inhibitor that binds one of the CoQ reduction sites; the release of superoxide anion from Complex III may occur on both side of inner membrane [37].

Anion superoxide production by Complex I reaches its maximal rate during reverse electron transfer (RET). RET occurs when electron supply, i.e. from succinate, reduces CoQ that in presence of a significant high ΔP could give electrons back to Complex I leading to formation of NADH from NAD^+ through FMN; the role of Complex I in this process has been confirmed by use of rotenone. The RET-associated superoxide production is deeply dependent on ΔP since it could be completely abolished by even small decreases in the proton electrochemical potential achieved through addition of ADP or uncoupler [38]. Moreover, it has been shown to be more sensitive to changes in the ΔpH, than in the ΔΨ component [39]. Increase in Complex I prosthetic groups reduction may also occurs in presence of increased NADH/NAD^+ ratio that may arise when respiratory chain activity is inhibited. Thus, it is clear that alterations either in Complex I activity and structure or in the whole oxphos system might likely lead to an overproduction of ROS that could reflect in a non-reversible oxidative modification of lipids, proteins and nucleic acids. Although it has been demonstrated that inhibition threshold for Complex I (30%) or Complex III (70%) must be reached before to observe a significant ROS production[40], in a context of partial energetic impairment due to mutations in mtDNA genome also minimal shifts in the redox equilibrium might represent a further input of stress for the cell.

Indeed, every molecule in the cell can be a target of ROS action: membrane lipids are particular vulnerable due to the presence of double-bounds, proteins may be oxidized by ROS at thiol groups of cysteine or metionine while tyrosine residues may undergo nitrosilation by nitric reactive species whose metabolism is linked to that of ROS. Besides being the major source of ROS, mitochondria are also highly sensitive to oxidative damage: the presence of metals in the prosthetic groups of ETC complexes makes oxphos system an easy target of hydrogen peroxide.

An interesting issue is provided by the “mitochondrial theory of aging” where mtDNA is supposed to play a pivotal role in the aging process [41]. Probably for a minor efficiency in their repair system machinery mitochondrial DNA molecules seem in fact to be particularly prone to oxidative damage. mtDNA oxidative modification will lead to altered or reduced protein synthesis and so to incompetent mitochondrial metabolism through a vicious cycle leading in turn to energy deprivation, redox imbalance and cell dysfunction. According to this, there are evidences that hallmarks of ROS-mediated nucleotide damage, such as 8-hydroxy-deoxyguanosine, are more prevalent in aged tissues [42]. The mitochondrial aging theory is also supported by several reports showing the accumulation of oxidative modified proteins in neurodegenerative

pathologies, very common among old people, such as Parkinson's disease [43] or Alzheimer's disease [44].

Because of the potential deleterious effect of a natural by-product of mitochondrial metabolism aerobic cells evolved efficient systems for ROS scavenging. The ROS-scavenging system must be highly regulated to rapidly answer to even slight changes in the redox state of cell. Since all genes implied in maintaining the correct balance between ROS production and scavenging are nuclear encoded, a proper mitochondria-nucleus cross-talk is of primary importance to counteract oxidative stress risk.

Superoxide dismutase catalyzes dismutation of superoxide anion in hydrogen peroxide. In mammalian three genes encoding superoxide dismutases with different localization have been found [45]. SOD1 and SOD3 encode two enzymes containing copper and zinc in their catalytic site (CuZnSOD) but showing different localization: SOD1 product is localized in cytoplasm, nuclear compartments, mitochondrial intermembrane space and lysosomes of mammalian cells while SOD3 product exists as a homotetramer and is located in the extracellular environment. The expression pattern of SOD3 is highly restricted to specific cell type and tissues where its activity can exceed that of SOD1 and SOD2. A third isoform of SODs has manganese as a cofactor (MnSOD) and is localized in mitochondrial matrix of aerobic cells where it is active in the tetrameric form [45].

Glutathione peroxidase (GPX) contains selenium in the catalytic site and catalyzes reduction of hydrogen peroxide by glutathione; different isoforms are present in cytoplasm and mitochondria and are differently expressed depending on tissues [11].

Peroxiredoxins (Prdx) are thiol-dependent enzymes working as hydrogen peroxide scavenger; isoform 3 is exclusively localized in mitochondrial matrix [46].

Catalase catalyzes hydrogen peroxide conversion in H_2O and O_2, is a tetramer containing heme as prosthetic group and is localized in peroxisomes, in cytosol and in heart mitochondria [4].

Non-enzymatic systems like glutathione, thioredoxines and vitamins (E, C) which can participate to catalytic action of antioxidant enzymes as cofactors, are as well fundamental in maintaining the balance in the redox state of cells.

2 Mitochondrial Pathologies

Mitochondrial pathologies are a heterogeneous group of clinical manifestations characterized by oxidative phosphorylation impairment. At the beginning of their recognition mitochondrial pathologies were regarded as rare disorders but indeed they are more frequent than originally thought. A correct estimation of the incidence of these disorders seem to be not so straightforward probably because of the great variability of age of onset, but they are usually referred as the most frequent pathologies among the inborn errors metabolism group [47].

Mitochondrial diseases manifest usually as multisystem syndromes in which tissues or organs most commonly involved are those characterized by high-energy demand (skeletal muscle, nervous system and heart) so that encephalo-myopathies or encephalo-cardio-myopathies are classic phenotypes. However, also other tissues can be affected and cases of single tissue involvement are not rare. The onset and the course of mitochondrial pathologies can vary greatly, ranging from early to late-onset, slowly or rapidly progressive. The outcome can be lethal in the childhood or highly disabling.
Because of the unique mitochondria peculiarities mitochondrial pathologies can arise from different kind of genetic alterations: since these organelles are deeply dependent on nDNA nevertheless they preserve part of their own DNA, mitochondrial disorders can be caused by mutations in both genomes.

mtDNA mutations

The first evidence of a pathology linked to a mutation in mtDNA dates back to 1988 when Wallace and coworkers found a point mutation in the gene encoding subunit 4 of Complex I in a family with **Leber's hereditary optic neuropathy** (LHON) [48]. So far more than hundred mtDNA mutations have been associated to several disorders and the list seem to be not still complete[49].
Pathogenic mtDNA point mutations have been mapped along all mitochondrial genome and may occur in structural genes as well as in rRNA or tRNA genes; pathologies caused by these mutations are maternally transmitted. Conversely, sporadic pathologies have been associated to mtDNA rearrangements that usually occur spontaneously: the most common deletion is found in one third of patients and involves a 5 Kbp segment [14] *fig. 8* morbidity map. Unlike point mutations affecting structural genes, mtDNA deletions determine lack of several gene functions; a similar effect is caused by rRNA and tRNA genes point mutations that lead to a general impairment of mitochondrial protein translation machinery affecting most of the oxphos complexes.
Since mtDNA contains only 13 structural genes - all encoding subunits of oxphos system - and RNA genes required for synthesis of those proteins, pathologies caused by mtDNA genetic defects share the common characteristic of a deficient oxidative phosphorylation pathway. Nevertheless more or less evident oxidative phosphorylation impairments represent the common link among mitochondrial pathologies clinical phenotypes arising from mtDNA mutations are deeply heterogeneous. The complexity of the picture is supplemented by the lack of a straightforward relation between genetic defect and clinical manifestation: mutations in different positions can lead to the same clinical phenotype as well as a single mutation may be associated to different pathologies.

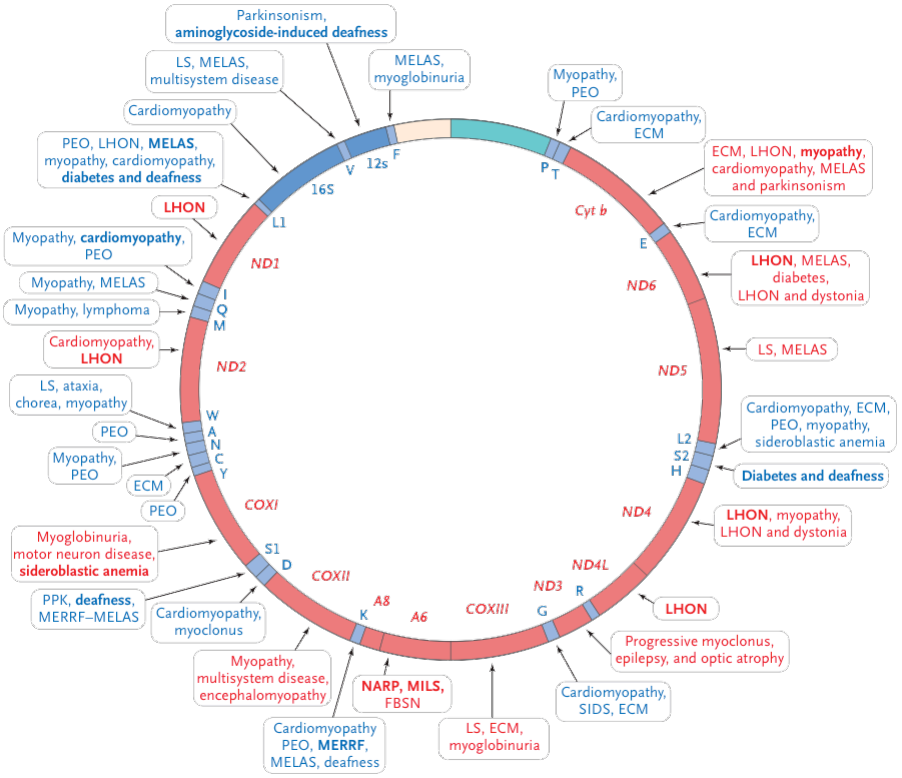
A3243G mtDNA mutation, which lies within the $tRNA^{Leu}$ gene, may be linked to **MIDD** (Maternal Inherited Deafness and Diabetes) characterized by pancreatic β cell dysfunction or to **MELAS** (Myopathy Encephalopaty Lactic Acidosis and Stroke-like episodes) the most common mitochondrial encephalo-myopaty [42]. Although most of the mitochondrial pathology manifests with neurological symptoms, every syndrome is characterized by a peculiar set of clinical signs: as MELAS also **MERRF** (Myoclonic Epilepsy with Ragged Red Fibers) is associated with a point mutation in mitochondrial gene encoding the tRNALys but in this case the effects of mutation are completely different. Immunohistochemical evidence suggest that MELAS mutation is abundant in the walls of cerebral arterioles [50], while MERRF mutation is abundant in the olivary nucleus of the cerebellum [51], suggesting that the different sympotoms may be due to a different distribution of mutated mtDNA. However, these data fail to explain what directs each mutation to a particular area of the brain and how mutation correlates with clinical syndrome.

Part of the variability associated to these pathologies can be attributed to the peculiar rules of mitochondrial genetics; most of the pathogenic mtDNA mutations are in fact heteroplasmic in cells so that a high heterogeneity may exist also among tissues.

Most of the informations regarding pathologies associated to mutations in mtDNA derive from studies on **cybrids** (cytoplasmic hybrids) [52]. Cytoplasmic hybrids are obtained by fusing an immortalized cell line depleted of its own mtDNA (ρ^0 cells) with cells, usually obtained from patients, depleted of

figure 8

Disorders that are frequently or prominently associated with mutations in a particular gene are shown in boldface. Diseases due to mutations that impair mitochondrial protein synthesis are shown in blue. Diseases due to mutations in protein-coding genes are shown in ed. ECM denotes encephalomyopathy; FBSN familial bilateral striatal necrosis; LHON Leber's hereditary optic neuropathy; LS Leigh's yndrome; MELAS mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes; MERRF myoclonic epilepsy with ragged-red fibers; MILS maternally inherited Leigh's syndrome; NARP neuropathy, ataxia, and retinitis pigmentosa; PEO progressive external ophthalmoplegia; PK palmoplantar keratoderma; and SIDS sudden infant death syndrome.



nucleus. The resulting cybrids will carry nuclear genetic information deriving from the immortalized cell and mitochondrial genetic information arising from the patient, so that the mitochondrial mutations effects can be analyzed in a context of “neutral” nuclear background. Furthermore, this model allows to select cell lines with different percentage of heteroplasmy providing a useful system to study the correlation between mtDNA mutation load and biochemical phenotype.

Analysis of different mtDNA mutations in cybrids has allowed to enlighten the existence of a **threshold effect** for the expression of biochemical phenotype. The threshold effect indicates that a minimal quantity of mutated mtDNA is required to determine a clinical manifestation and this value may vary depending on mutation pathogenicity, tissue metabolism and cell metabolic status determining part of the unpredictability that characterizes these pathologies. The existence of a threshold for the clinical phenotype is actually determined by the existence of a biochemical threshold: there must be a minimal amount of mutated protein, or RNA in order to establish impairment in mitochondrial function.

A good example of threshold effect is given by mtDNA point mutations in ATP6 gene at nucleotide 8993: this mutation is associated with either **NARP** (Neuropathy Ataxia and Retinitis Pigmentosa) when tissue heteroplasmy is lower than 90-95% or with lethal childhood Leigh syndrome, a more severe progressive encephalopathy, when it is present at higher percentage [53].

The existence of a threshold implies that, physiologically, rRNAs, tRNAs and oxphos complexes are more than it is required to permit a “sufficient” oxidative phosphorylation; this reserve of molecules may represent a protective mechanism providing a safety margin against the effects of deleterious mutations [54]. Also at biochemical level the presence of wild type mtDNA molecules may support physiological cell requirements and compensate for the deleterious effect of a mutation until the number of normal mtDNA decreases under a threshold value or until the metabolic status of the cell, i.e. its energy requirements, becomes more challenging for mitochondria. The networked mitochondrial organization represents another potential compensative system against pathogenic mutations: mitochondria fusion results in mixing of not only the outer and the inner membrane but also of mitochondrial matrix molecules; mitochondria fused together might exchange genes products so that the overall mitochondrial function in cell can be preserved also if mutated DNA molecules are present. Potentially also mtDNA can mix after fusion: although nucleoids movement seems to be restricted compared to that of matrix proteins, evidence of mitochondrial DNA recombination has been reported [55]. Therefore, the dynamic organization of mitochondria in presence of mutated mtDNA molecules may lead to a condition where all mitochondria in a single cell contain a mixture of mutated and normal mtDNA molecules; a consequence of this homogeneity is that rapid drifts in mutant/wild type mtDNA ratio during mitotic event are avoided, and the likelihood of producing homoplasmic cell is reduced [42].

However, as for almost all “biological rules” also in this case there are exceptions that cannot fit in the threshold effect explanation. Heteroplasmy and threshold effect can not explain peculiarities of Leber hereditary optic neuropathy: mtDNA point mutations associated with this neuropathy are indeed homoplasmic in all tissues but clinical symptoms do not manifest until adulthood, the only “tissue” affected is the optic nerve and the presence of a pathogenic mutation does not necessarily lead to pathology. Mechanisms

different from the oxphos impairment directly caused by these mutations must modulate the manifestation of the LHON clinical phenotype.
Nevertheless the wide variability of mitochondrial pathologies manifestations there are clinical signs that recur in many patients: increased concentration of lactic acid in blood, urine, cerebrospinal fluid, as well as in tissues is a typical sign of oxphos deficiency. Increased lactic acid production may result from pyruvate accumulation under conditions where Krebs cycle and oxphos system are inhibited and may lead to lactic acidosis resulting in a global disturbance of cellular pH [56]. Another typical "mitochondrial" hallmark, very helpful in diagnosis, is the presence of ragged-red fibers in muscle biopsies, index of massive subsarcolemmal proliferation of mitochondria typically negative for cytochrome c oxidase activity. In this context the increased proliferation might represent an attempt to overcome mitochondrial defect and achieve a sufficient energy production into the cell.

nDNA mutations

mtDNA encodes only thirteen of more than thousand proteins present in mitochondria [57]. All proteins required for mtDNA maintenance and expression, oxphos complexes assembly and synthesis of cofactors such as heme and Fe-S clusters are nuclear encoded and imported into mitochondria. Consequently, mitochondrial function is deeply dependent on nDNA and mutations in this genome may also lead to "mitochondrial pathologies". Indeed, most of patients (80-90%) with oxidative phosphorylation deficiency harbor a nuclear mutation. nDNA mutations associated with mitochondrial pathologies can be classified in different categories and may affect oxidative phosphorylation in a direct or indirect way *[tab1]*, these mutations are transmitted according to Mendelian rules and may lie in autosomic genes, either in recessive or dominant condition, or in chromosome X.
Pathogenic mutations have been found in nuclear genes encoding oxphos subunits of all complexes although mutations affecting Complex III, IV and V are less frequent indicating a more likely incompatibility with life [58]. Mitochondrial pathologies may also be due to mutations in nuclear genes encoding for "ancillary" proteins of oxphos system: these proteins are involved in assembly of the various mtDNA and nDNA encoded subunits, together with their prosthetic groups. Mutations in nuclear genes may lead to mtDNA qualitative (deletions) or quantitative (depletions) alterations resulting in impairment or deficient synthesis of mitochondrial oxphos complexes. Impairment in the mtDNA integrity may be due to mutations either in nuclear genes directly involved in mtDNA replication and maintenance or in genes involved in the control of nucleotides pool in mitochondria. Mitochondrial dysfunction has also been linked to alterations of the cytosolic protein import system as well as of proteins involved in mitochondrial motility and homeostasis. Nevertheless nuclear mutations are not subjected to peculiar rules of mitochondrial genetics, apparently overlapping genetic defects can as well lead to a broad range of clinical manifestations.

table 1
Classification of mitochondrial pathologies associated to nuclear DNA mutations. For detailed information see Mitomap.[49]

Structural Respiratory Chain Defects	Gene/Protein	Affected Function
Leigh and Leigh-like syndrome	NDUFS4, NDUFS7, NDUFS8	Complex I
Hypertrophic cardiomyopathy and encephalomyopathy	NDUFS2	Complex I
Macrocephaly, leucodystrophy and myoclonic epilepsy	NDUFV1	Complex I
Leigh and Leigh-like syndrome	FP(SDHA)	Complex II
Severe psychomotor retardation and extrapyramidal signs	uqcrb	Complex III
Non-Structural Respiratory Chain Defects		
Autosomal dominant progressive external ophtalmoplegia (adPEO)	ANT1	ADP/ATP exchange
Mitochondrial neurogastrointestinal encephalomyopathy	TP	Pyrimidine metabolism
Assembly Defects		
Leigh syndrome	SURF1	Complex IV
Cardioencephalomyopathy	SCO2	Complex IV
Neonatal onset hepatic failure and encephalopathy	SCO1	Complex IV
Metabolic acidosis, tubulopathy encephalopathy and liver failure	BCS1L	Complex III
Homeostasis and Import		
Friedrich's ataxia	Frataxin	Fe-S cluster metabolism
Hereditary spastic paraplegia	Paraplegin	Proteolytic processing of mitochondrial proteins
Autosomal dominant optic atrophy	Opa1	Mitochondrial fusion
Progressive external ophthalmoplegia	POLG ANT1	mtDNA replication, ADP/ATP exchange

Therapeutic Approaches

Nevertheless is now well known that mitochondrial pathologies result from impairment of energetic metabolism or in the general mitochondrial physiology the pathogenic mechanisms leading to a so broad spectrum of clinical manifestations are still puzzling. The poor knowledge of pathways linking a mutation to one pathologic phenotype, that can actually be highly variable also within the same family, has not allowed a real development of the "mitochondrial medicine". In spite of the improvement in the diagnostic skills, therapeutic treatments are still inappropriate. In a review published in 2006[59], after having analyzed the results of several trials of pharmacological and non-pharmacological treatments, the authors conclude that there is currently no clear evidence supporting the use of any intervention in mitochondrial disorders and that further research is needed to establish the role of a wide

range of therapeutic approaches.

Usually patients with mitochondrial pathologies are subject to symptomatic therapy: anticonvulsant are utilized against seizure episodes, diabetes responds to dietary or pharmacological treatment while cardiac failure can be treated with pacemaker implant. Since neuronal and muscular tissues are the main targets of mitochondrial pathologies exercise intolerance and weakness are very common and also muscular training has been regarded as a possible treatment. It is well known that endurance-training induces mitochondria biogenesis in skeletal muscle, but in presence of a mutation it may actually increase both mutant and wild type mtDNA [60]. On the other hand, resistance training induces transfer of mitochondrial templates from satellite cells to mature muscle: since satellite cells have usually a higher content in wild type mtDNA this may lower the threshold level below phenotypic expression of the mutation [61].

Pharmacological therapies are focused on removal of toxic metabolites. Lactic acidosis is one of mitochondria pathologies hallmarks; excess of lactic acid can be buffered with bicarbonate or more specifically treated with dichloroacetate (DCA). DCA acts as inhibitor of pyruvate dehydrogenase (PDH) kinase thus, in presence of dichloroacetate, PDH remains in the active form favoring the decarboxilation of pyruvate. However, the peripheral nerve toxicity of DCA does not allow long periods of treatment in patients already prone to develop peripheral neuropathy [58].

A quite diffuse strategy to bypass block in respiratory chain complexes due to mutations in structural genes is based on administration of artificial electron donors/acceptors: CoQ or analogues, ascorbate or NADH and FADH2 precursors have been used in different pathologies but the results seem highly dependent on type of disorder and benefic effects do not seem so promising. MELAS patients treated with creatine have shown an improvement of high-intensity activity but not of lower aerobic exercise [63]. Cytochrome *c* oxidase deficiency due to mutations in SCO2, a nuclear gene encoding a copper chaperone, may be rescued *in vitro* by copper supplementation indicating a potential treatment for the severe form of infantile encephalo-cardio-myopathy associated with this mutation [64, 65].

Since reactive oxygen species are a natural by-product of respiratory chain, defects on the oxphos system may also lead to oxidative stress conditions for the cell other than to impaired energy supply. Attempting to reduce the potential toxic effect of ROS several oxygen radical scavengers have been utilized in treatment of mitochondrial pathologies. Vitamin E and in particular CoQ and idebenone, its analogue, have been largely used in diseases associated to mtDNA mutations especially for the lack of negative side effects but data from controlled trial in large cohort of patients are required to demonstrate the reliable effect of these molecules [58].

The most promising, and most hard to set up, therapeutic strategy for mitochondrial pathologies treatment is gene therapy. The goal is to force a shift in heteroplasmy promoting replication of mtDNA wild type molecules or to replace the impaired protein function. Different approaches may be used to achieve the genetic control of mitochondrial metabolism. Nucleic acids derivatives have been used *in vitro* to selectively inhibit replication of mtDNA mutant molecules in A3844G cells [66]. Moreover, in cells harboring mutations in the mitochondrial $tRNA^{Lys}$ gene, that are associated to MERRF, mitochondrial function in both immortalized cells and primary fibroblasts has been partially restored by a yeast tRNA derivative capable to enter mitochondria [67].

Effects of alterations in structural genes may be mitigated by means of allotopic expression, xenotopic expression or import of restriction endonucleases.

The correct nucleotide sequence of a mutated mitochondrial gene may be inserted in the nuclear genome upon its linking to a mitochondrial targeting sequence to drive import into the organelles. This approach has been utilized to correct, *in vitro*, biochemical defect in cybrids harbouring the T8993G NARP mutation [68] and the G11778A LHON mutation [69].

Rescue of protein activity may be also achieved transfecting affected mammalian cells with mitochondrial or nuclear genes deriving from other organism but encoding homologue proteins (xenotopic expression). Expression of ATP6 gene deriving from *Chlamydomonas reinhardtii*, a green alga, has been shown to overcome deficiency in both viability and ATP synthesis in cybrids with T8993G NARP mutation [70].

Restriction endonucleases may be used to specifically destroy mutant mtDNA molecules: transient expression of the SmaI gene fused to a mitochondrial targeting sequence in NARP cybrids showed specific elimination of the mutant mtDNA followed with repopulation by the wild type mtDNA, resulting in restoration of both the normal intracellular ATP level and normal mitochondrial membrane potential [71]. The same approach has been shown to be effective also *in vivo*: in mice carrying two different mtDNA haplotypes Bayona-Bafaluy and co-workers have shown that expression of a selective restriction endonuclease results in a significant shift in mtDNA heteroplasmy in muscle and brain [72].

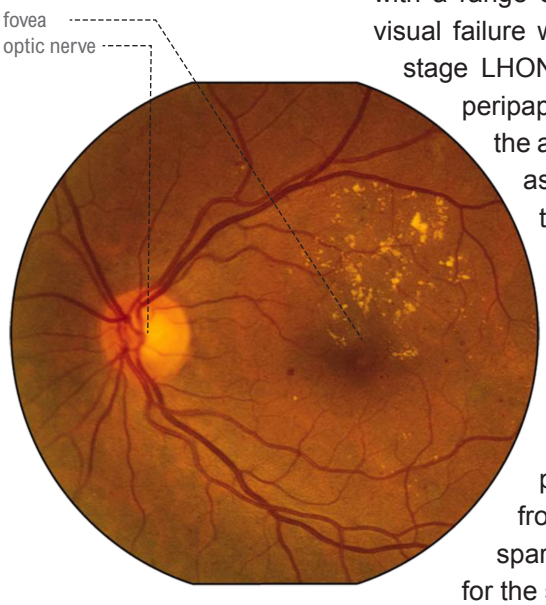
Nevertheless numerous theoretically effective genetic strategies have been proposed, experimental data are still limited to the *in vitro* testing. Several problems have to be overcome before *in vivo* tests and trials may occur: concerning mtDNA mutations the therapeutic wild type gene products (tRNA, rRNA or protein) have to be selectively targeted to mitochondria and specifically addressed to the affected tissue. The limitations of genetic counseling caused by the high unstableness of mitochondrial genome further increase the necessity of really understanding how mitochondrial function can modulate so deeply and differently cell physiology in order to develop adequate therapeutic strategy for patients affected by these highly disabling pathologies.

3 Leber Hereditary Optic Neuropathy

Overview

Leber hereditary optic neuropathy (LHON – OMIM # 535000) was first described in 1871 by the German ophthalmologist Theodore Leber as a form of optic neuropathy maternally transmitted and prevalently affecting young males. The endpoint of the pathology is usually optic nerve atrophy with bilateral loss of vision; the mean age of onset of pathology is between 27 and 34 years with a range of 1 to 70 years [11] but 95% of carriers who will experience visual failure will do so before the age of 50 years [73]. At presymptomatic stage LHON manifests with signs of ocular vascular alteration such as peripapillary telangiectatic microangiopathy; visual loss takes place in the acute stage affecting either synchronously both eyes at onset or asymmetrically first one eye and then the other with an average time interval between affected eyes of 1.8 months [74]. At the end stage the retinal nerve fiber layer degenerates leading to optic atrophy in few months.

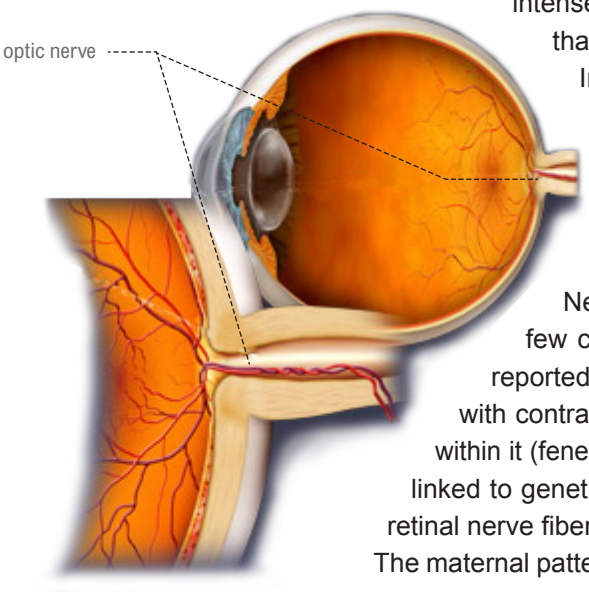
figure 9
Human eye. The fovea, also known as the fovea centralis, is a part of the eye, located in the center of the macula region of the retina.



One of the striking characteristics of LHON is the apparently absolute involvement of **retinal ganglion cells** (RGC) whose axons form the optic nerve. RGC are neuronal cells distributed in a non-linear way in the inner surface of retina where they are particularly concentrated in the perifoveal macula *[fig 9]*. Further from fovea the number of RGC decreases becoming relatively sparse in the periphery. Other than for distribution RGC differ also for the size: those near fovea are smaller with smaller caliber axons.

The axon of each retinal ganglion cell travels through nerve fiber layer (NFL) toward the optic nerve head. There, the fibers make an orthogonal turn and pierce lamina cribrosa to form optic nerve. Only posterior lamina cribrosa the axons become myelinated *[fig 10]* acquiring a greater efficiency respect to unmyelinated portion that has a much slower conduction velocity and requires greater energy to restore the electrical potential [75].

figure 10
The optic nerve is a continuation of the axons of the ganglion cells in the retina.



Mitochondria distribution matches the energy and functional requirements of these axons: NFL and prelaminar-laminar RGC axons are characterized by intense COX staining, index of intense oxidative phosphorylation, that decreases with the appearance of myelin [76].

In optic nerve degeneration observed in LHON patients temporal fibers of papillo-macular bundle (PMB) characterized by small caliber and thin myelin sheath are the first and most severely affected whereas the nasal fibers seemed to be partially spared in late stage of the disease [76].

Nevertheless loss of central vision is mostly permanent, a few cases of spontaneous recovery of visual acuity have been reported. Vision may improve progressively or sometimes suddenly, with contraction of scotoma or reappearance of small islands of vision within it (fenestration) [76]. The probability of visual recovery seems to be linked to genetic background and age of onset as well as to thickness of retinal nerve fiber layer [77].

The maternal pattern of LHON inheritance was confirmed on 1972 by Erickson

who proposed the presence of a mutation in the mitochondrial DNA [78] but only in 1988 Wallace and colleagues identified the first mitochondrial pathogenic point mutation in nine pedigrees with a clinical diagnosis of LHON: this mutation is located at nucleotide position 11778 of mtDNA and converts an highly conserved arginine to histidine at codon 340 in the NADH dehydrogenase subunit 4 (ND4) of Complex I of the respiratory chain [48].

Since then a plethora of mtDNA mutations has been found in different LHON patients but now it is well established that at least 90% of LHON families harbor one of the three so called primary mutations all affecting mitochondrial genes encoding subunits of Complex I of the respiratory chain: **G11778A ND4** [48], **G3460A ND1** [79], **T14484C ND6** [80].

Although the majority of LHON families carry the mtDNA pathogenic mutations in the homoplasmic condition, i.e. all mtDNA molecules in all tissues are mutated, not all maternally related individuals develop the pathology. Therefore, the presence of a LHON mtDNA mutation is necessary but not sufficient for LHON clinical manifestation.

Penetrance of the disease seems dependent on the point mutation: only half of the G11778A index patients had a history of similar affected relatives while the proportion is higher for G3460A (71%) and T14484C (100%) patients [64].

An intriguing feature of LHON is the gender bias: 30-50% of males but only 5-10% of females who harbor one of the three primary mutations actually develop the optic neuropathy [74]. The incomplete penetrance and the gender bias imply that additional genetic and/or environmental factors must modulate the phenotypic expression of LHON. However, the gender bias could also result from a combination of subtle anatomical, hormonal and physiological variations between males and females [73].

The existence of further genetic determinants in LHON has been largely investigated. Because of male prevalence chromosome X has been analyzed for putative LHON-related genes. A linkage analysis in an extended Brazilian family carrying the G11778A LHON mutation as well as a large study in LHON European families revealed the existence of X-linked loci [81, 82] but the real involvement of chromosome X in LHON penetrance still need to be elucidated.

Genetic polymorphisms of nuclear genes involved in oxidative stress response and in apoptosis have been as well investigated in LHON G11778A patients. LHON patients carrying an oxidative-stress related polymorphism in the EPHX1 gene - encoding a microsomal epoxide hydrolase - or an apoptosis-related polymorphism in the TP53 gene - encoding the onco-protein p53 - developed pathology earlier than did those without these genetic trait [73] suggesting that increased vulnerability to oxidative stress or to apoptosis might contribute to exacerbate the pathology.

Mitochondrial genetic background has been also considered as a possible risk factor for LHON penetrance modulation. Haplogroups are defined as mitochondrial DNA variants characterized by several common polymorphisms reflecting the evolutionary history of mtDNA differentiation: the “neutral” mutations contained in a specific haplogroup could modulate mitochondrial diseases expression either exacerbating or protecting from mutations deleterious effect.

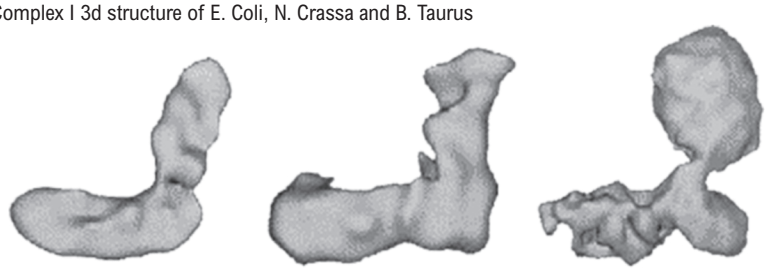
Several studies have been focused on the possible correlation between LHON and mtDNA haplogroups. Analyzing LHON pedigrees haplogroups of different European origin a higher frequency of haplogroup J was observed in patients carrying the G11778A and T14484C mutations with respect to controls.

Haplogroup J is characterized by polymorphisms in two genes encoding Complex I subunits: T4216C in ND1 gene and G13708A in ND5 gene.
Even though these genetic variants do not further impair the respiratory chain activity in G11778A cell the hypothesis is that their presence might be related to alterations in the structure or function of Complex I that could cooperate in disease manifestation. Unlike the other two primary LHON mutations G3460A is distributed in all haplogroups at frequencies similar to the control population [76, 77].
The last factor that could modulate LHON expression is the environment. Five pairs of monozygotic twins harboring a primary LHON mutation have been reported in the literature and in two cases the twins have remained discordant. Although there is always the possibility that the unaffected sibling will lose vision later on his life, the existence of discordant monozygotic twins strongly suggests that non-genetic factors also contribute to LHON penetrance [73].
The contribution of environmental factor is supported by the fact that clinical manifestations of toxic and nutritional neuropathies are similar to that observed in LHON [77].
Several studies have as well been focused on tobacco and alcohol consumption but the results are controversial. Conflicting conclusion have also been reached regarding nutritional deprivation, exposure to industrial toxins, antiretroviral drugs, psychological stress or acute illness.
The role of environmental risk in LHON still remains unclear: in order to obtain reliable epidemiological data there is the necessity to collect sufficient number of subjects. Moreover environmental factors might have different impact on different LHON pedigrees depending, for example, on the mtDNA background of the family.
However, besides the genetic and environmental issue, the principal unanswered question in LHON is the pathogenic mechanism that link mutations in Complex I subunits with a specific pattern of retinal ganglion cells degeneration in a context of highly variable penetrance and gender bias.

Complex I

Complex I in bovine heart is composed of 46 subunits for a molecular weight of about 1000 KDa, representing the biggest and the less known complex of the respiratory chain. This multi-subunit complex catalyzes electron transfer from NADH to CoQ through a flavoprotein and a Fe-S clusters series. Contextually, Complex I drives a vectorial proton transport from the matrix to the intermembrane space contributing to the electrochemical gradient mostly used by ATP synthase to form ATP from ADP and inorganic phosphate.
In eukaryotes Complex I has a dual genetic origin: in mammalian seven subunits (ND1-ND6, ND4L) are indeed codified by mitochondrial DNA while the remaining subunits are nuclear gene products.
Due to its size and hydrophobicity the enzyme has not yet been crystallized but some information about its structure and function derives from electronic microscopy analysis and homology studies. Complex I is a L-shape multi-protein complex composed of an hydrophobic arm embedded in the inner mitochondrial membrane and an hydrophilic arm exposed to the matrix. The L-shape is well conserved from prokaryotes to eukaryotes *[fig 11]*.
In bacteria a simple form of proton pumping NADH:Ubiquinone oxidoreductase is composed of fourteen subunits yielding a complex of 550 KDa [83]. In eukaryotes the corresponding “core”, representing the functional unit of the

figure 11
Complex I 3d structure of E. Coli, N. Crassa and B. Taurus



enzyme, consists of all the seven mitochondrial encoded subunits localized in the hydrophobic arm and seven nuclear encoded subunits containing all the known redox prosthetic groups, namely one molecule of flavine adenine-mononucleotide (FMN) and eight bi- or tetranuclear iron-sulfur clusters (N1a,N1b,N2-N6a,N6b) [84] *[tab 2]*. The remaining subunits are defined "accessory" and are supposed to be involved in assembling and/or regulation of the complex activity. However, a novel role for "non-core" subunits as regulatory molecules outside the mitochondrial compartment is now emerging. Moreover some accessory subunit could take part to additional metabolic routes as suggested by the presence of a mitochondrial acyl carrier subunit and a NADPH binding site in the 39 KDa subunit [85]. In this context a good example is given by NDUFA13 subunit, a complex I subunit localized in the matrix arm. NDUFA13 is also known as GRIM-19 (gene associated with retinoid-interferon-induced mortality 19). The gene was indeed cloned for the first time during a screening for genes required for interferon-beta- and retinoic acid-induced cell death in a mammary carcinoma cell line. Only later the protein was found as part of mitochondrial Complex I [11] showing a dual function for GRIM-19: one in the oxphos system as structural component of Complex I, the other as part of the apoptotic pathway. Complex I subunit as GRIM-19 might represent the actual link between mitochondria and nucleus: in this case Complex I structure/activity would not be limited to energy production but will be involved in the overall cell physiology as "sensor" of cell wellness.

Some information about Complex I structural organization comes from fractionation experiments that have shown that the mitochondrial NADH:Ubiquinone oxidoreductase enzyme can be separated in three major component. The hydrophilic arm is represented by subcomplex **Iλ**, which contains the Fe-S clusters and the flavoprotein fraction. The membrane arm can be fractionated in an hydrophobic module called **Iβ** and in a connecting module called **Iγ**. The **Iγ** module contains the mitochondrial encoded subunits ND1, ND2, ND3, ND4L and the nuclear encoded subunit KFYI [86, 87]. In a previous study the bovine Complex I fractionation resulted in two fragments: **Iα** and **Iβ**. **Iα** represents the hydrophilic arm of the Complex plus the fourteen subunits of **Iγ**, while **Iβ** corresponds to the hydrophobic arm [87] *[tab 2 and fig. 12]*.
The "stalk" connecting the two arms on Complex I has been characterized in *E. Coli*. This portion is supposed to be involved in electrons transfer from the NADH binding site in the hydrophilic arm to the CoQ binding site in the membrane [88]. The stalk moiety in *E.Coli* is composed by NuoB, NuoCD and NuoI subunits corresponding to PSST, 49+30 KDa and TYKY subunits in bovine [89]: TYKY contains Fe-S clusters N6a and N6b while Fe-S cluster N2 is supposed to be localized in the PSST subunit [90].
As the molecular structure also the electron transfer and the proton pumping mechanisms in Complex I are not yet completely understood. The Complex I substrate NADH reduces FMN; from FMN electrons flow through Fe-S clusters

Human Gene	Bovine homologue	Subcomplex
ND1	**ND1**	**Iγ**
ND2	**ND2**	**Iγ**
ND3	**ND3**	**Iγ**
ND4	**ND4**	**Iβ**
ND4L	**ND4L**	**Iγ**
ND5	**ND5**	**Iβ**
ND6	**ND6**	**Iα**
NDUFV1	**51 kDa**	**Iγ**
NDUFV2	**24 kDa**	**Iγ**
NDUFV3	10 kDa	Iγ
NDUFS1	**75 kDa**	**Iγ**
NDUFS2	**49 kDa**	**Iγ**
NDUFS3	**30 kDa**	**Iγ**
NDUFS4	18 kDa	Iγ
NDUFS5	15 kDa	Iα
NDUFS6	13 kDa	Iλ
NDUFS7	**PSST**	**Iλ**
NDUFS8	**TYKY**	**Iλ**
NDUFA1	MWFE	Iα
NDUFA2	B8	Iλ
NDUFA3	B9	Iα
NDUFA4	MLRQ	nd
NDUFA5	B13	Iλ
NDUFA6	B14	Iα
NDUFA7	B14.5a	Iλ
NDUFA8	PGIV	Iα
NDUFA9	39 kDa	Iα
NDUFA10	42 kDa	Iα (loosely)
NDUFA11	B14.7	Iα
NDUFA12	(DAB13) B17.2	Iλ
NDUFA13	(GRIM-19) B16.6	Iλ
NDUFB1	MNLL	Iβ
NDUFB2	AGGG	Iβ
NDUFB3	B12	Iβ
NDUFB4	B15	Iα & Iβ
NDUFB5	SGDH	Iβ
NDUFB6	B17	Iβ
NDUFB7	B18	Iβ
NDUFB8	ASHI	Iβ
NDUFB9	B22	Iβ
NDUFB10	PDSW	Iβ
NDUFB11	(NP17.3) ESSS	Iβ
NDUFAB1	SDAP	Iα & Iβ
NDUFC1	KFYI	Iγ
NDUFC2	B14.5b	Iβ
??	(10.6 kDa)	

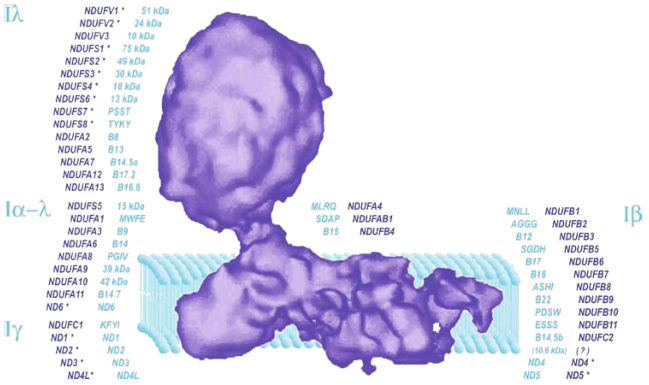
with an unknown sequence. The CoQ electrons donor seems to be the Fe-S cluster N2 even though it seems localized quite far from the CoQ binding site. Two options have been proposed to solve the problem of the distance between CoQ and N2 cluster: 1) in the connecting region there would be an hydrophobic or amphipathic ramp that consent to CoQ to diffuse out of the membrane arm to reach its reductant, 2) large scale conformational changes could bring cluster N2 closer to the membrane domain and then closer to CoQ binding site [85, 91].

During the years different hypotheses have been proposed to explain Complex I proton pumping activity but most likely the redox chemistry drives proton translocation via a conformational mechanism. Since the redox reactions take place exclusively in the hydrophilic arm the energy for proton translocation must be transduced to the proton pumping moiety in the membrane arm via conformational coupling [85, 91]. Likely one or more of the mitochondrial-encoded subunits (ND1-ND6 and ND4L) are part of the proton pumping machinery. Subunits ND2, ND4 and ND5 show some homology to Na^+/H^+ antiporters and have therefore be proposed as involved in proton translocation [91]. Several mutational studies show that dysfunctions in ND1, ND4L, ND4 and ND6 [93, 94, 95, 96] subunits affect ubiquinone reductase activity indicating a contribution of hydrophobic subunits to ubiquinone binding or simply reflecting the tight coupling between redox chemistry and proton pumping activity [85].

The complexity of this enzyme is further increased by several mechanisms by which its activity could be regulated. Complex I is regulated by post-translational modifications: the 18-kDa IP subunit, encoded by NDUFS4 gene localized in chromosome 5, is involved in the normal assembly of the complex and mediates, through its phosphorylation by PKA, regulation of complex activity by the cAMP cascade [97].

A pivotal role in Complex I function may be played by mitochondrial membrane lipids: a tightly bound cardiolipin

table 2 and figure 12
Subunit composition of mammalian Complex I.
The subunits that compose the core unit of the complex are indicated in bold [87].



may be required for structural integrity of the complex, an maybe for the supramolecular organization, while the amounts of the more weakly bound phosphatidylcholine and phosphatidylethanolamine seem modulate the catalytic activity [98].

As previously reported, Complex I is also the major source of mitochondrial superoxide anion. Nevertheless in physiologic conditions the electron leak from Complex I redox centers is very low, there are evidences showing enhanced ROS production in presence of defective Complex I, suggesting that structural modification of the enzyme may play a crucial role in inducing oxidative stress in cell [99]. Complex I defects are indeed associated with a wide group of clinical disorders, ranging from neonatal to adult onset neurodegenerative diseases: besides LHON, cardio- and encephalomyopathies as well as some form of Parkinson's disease can derive from Complex I deficiencies that seem to play a main role also in aging process [100, 101].

The physiological role of additional functional aspects of Complex I like ROS production or apoptosis regulation is even less understood than the redox or proton pumping activity but these aspects are of great interest because of the role of Complex I in several human diseases.

LHON Biochemical features

LHON primary pathogenic mutations affect mitochondrial genes encoding subunits located in the membrane sector of Complex I. The **G3460A** transition in the mitochondrial **ND1** gene converts the modestly conserved alanine 52 to a threonine (A52T). The **ND4 G11778A** mutation changes the highly conserved arginine at amino acid 340 to a histidine (R340H) while the **ND6 T14484C** mutation changes the weakly conserved methionine at amino acid 64 to a valine (M64V).

Several other mutations have indeed been associated to LHON or LHON plus dystonia phenotypes and most of them localized in ND1 and ND6 genes that are considered mutational "hot spots" [102 ,103]. ND1 mutations have also been reported in Alzheimer's disease and Parkinson's disease [11] further confirming that Complex I alterations play a pivotal role in neurodegeneration.

The function of the mutated Complex I subunits in LHON patients is not completely understood. Some study has shown that ND4 and ND6 subunits are essential for the assembly of other ND subunits into the complex [104], the same could be for ND1 subunit which mutations may lead to a severe reduction of holocomplex I levels [87]. As above-mentioned homology of ND4 subunit with bacterial antiporters indicates that it might play a role in the proton pumping activity.

Because of the genetic origin of the pathology, the first and most investigated hypothesis regarding LHON pathogenic mechanism has been the bioenergetic failure. Alterations in Complex I activity might indeed reflect in a decreased ATP production leading to a condition of reduced energy availability for the cell.

Neurons are indeed highly depending on ATP supply not only for neurotrasmission but also for correct localization of mitochondria: in retinal ganglion cells as in other nervous cells mitochondrial biogenesis takes place only in the soma from where mitochondria need to be addressed along the axons through an ATP-dependent mechanism [4]. Impairment in ATP production may thus reflect also in altered distribution of mitochondria: in RGC axons this phenomenon may be highly relevant in the non-myelinated portion of optic nerve.

Different cellular models have been used to study the energetic status in LHON and conflicting results about the extent of oxphos impairment associated to LHON mutations have been produced.

In cybrids carrying each of the three LHON primary mutations only the Complex I-driven ATP synthesis and oxygen consumption rate have been reported to be consistently affected while the succinate-driven ATP synthesis and respiration were not impaired [105]. Interestingly these cells may effectively compensate the oxphos failure through ATP synthesis sustained by alternative pathways such as glycolysis [105].

According to this, *in vivo* results obtained using the 31P magnetic resonance spectroscopy (MRS) in LHON patients show a defective ATP synthesis in skeletal muscle and/or brain [106, 107].

However, lymphocytes from patients harboring the G11778A mutations does not show differences from controls in terms of energy charge, i.e. the relative proportion between ATP, ADP and AMP [108] and in fibroblasts harboring the G3460A mutations the Complex I-linked ATP synthesis was found to be normal [109].

In LHON cybrids shows that the total cellular ATP content has been shown to decreas respect to controls only when the cells are grown in metabolic stressed conditions, i.e. in galactose medium [110], suggesting that the biochemical effect might arise only in conditions of high energy demand.

The discrepancy of results obtained studying the oxphos system in different models might be not so unexpected: all experiments have indeed been performed in cellular models different from the real target of the pathology and the energy demand and consequently the pathogenicity of the mutation might be completely different from that of retinal ganglion cells (RGC). Moreover, the observed variability could further confirm the existence of the above-mentioned genetic or environmental modulating factor that reduce the chance to obtain according results.

Besides the controversial role of energy failure, an important issue about LHON pathogenesis is the apoptotic death that almost simultaneously hits retinal ganglion cells leading to optic nerve atrophy. The programmed death mechanism hypothesized by the absence of inflammation signs at histopathological exam has been also confirmed by biochemical analysis.

Different studies on LHON cybrids grown in galactose medium have shown that these cells are more prone to apoptotic death respect to control. Under this culture condition, the slow metabolism of galactose to glucose-1-phosphate is not sufficient for the cell to synthesize the bulk of ATP requirement by glycolysis, and cells are forced to rely on respiratory chain for ATP synthesis.

The increased release of cytochrome *c* into the cytosol in LHON cybrids grown in absence of glucose indicates that mitochondria are involved in the activation of apoptotic pathway [111]. Moreover, the apoptotic cell death of LHON cybrids grown in the same culture conditions has been shown to be caspase-independent and mediated by AIF (Apoptosis Inducing Factor) and endonuclease G [110]. However, although it has been established that degeneration of RGC in LHON occurs through apoptotic pathway, the connection between Complex I dysfunction and apoptotic commitment still remains not understood.

In the last years, several reports have shown the likely existence of a third factor involved in LHON pathognesis: there is in fact mounting evidence that increased ROS production and oxidative stress may have a pivotal role in optic nerve degeneration.

Complex I is the major source of superoxide anion and alterations in its function

might be reasonably linked to an increased production of $O_2^{\bullet -}$ and to an unbalance in the redox status of the cell. The possibility that LHON mutations affect the interaction of Complex I with CoQ [76] further supports the role of oxidative stress in pathogenic mechanism: CoQ partially reduced molecules are actually another potential source of reactive oxygen species [112].

In a cellular model similar to RGC it has been shown that these cells might differ from other kind of neurons in the production of superoxide. Moreover, mitochondria from RGC-like cells show a minor content of ETC complexes, in particular Complex I, and a minor content of MnSOD respect to brain mitochondria [113]. Although in the work superoxide production is deduced from hydrogen peroxide production still the author has demonstrated that RGC-like cells have a minor content of mitochondrial ROS with respect to brain and neuroblastoma mitochondria supporting the hypothesis that retinal ganglion cells might be very vulnerable also to slight shift in the ROS production/scavenging equilibrium. Several other papers demonstrate the importance of oxidative stress in this particular optic neuropathies. Lymphocytes from LHON patients treated with the oxidizing agent 2 deoxi-D-ribose showed a significant increase in the percentage of apoptotic cells with respect to controls [114] while in mice inoculation into the eyes of a rybozime that reduces MnSOD mRNA and protein level induces loss of axons and myelin in the optic nerve, the hallmarks of optic nerves examined at autopsy of LHON patients [115].

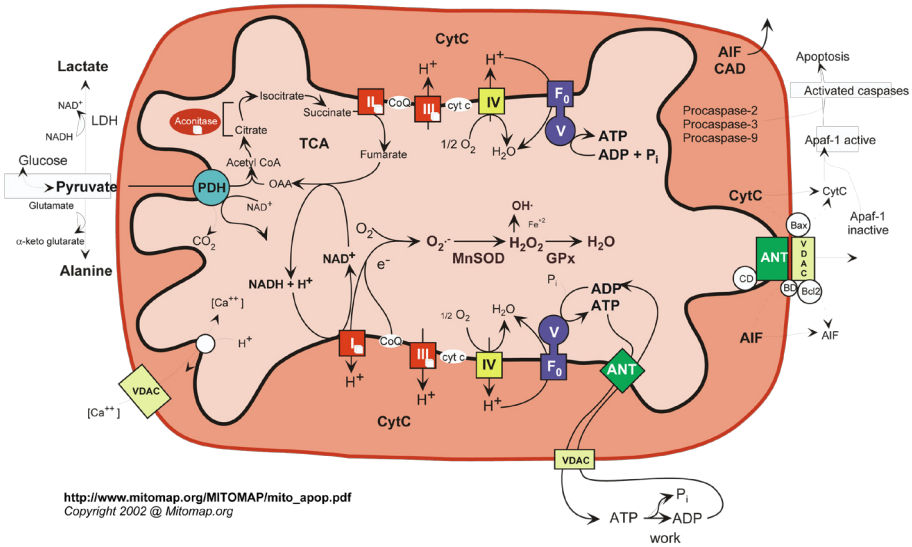
LHON cybrids obtained by a neuronal precursor cell line have shown higher ROS production with respect to controls only upon differentiation in neuronal mature cell indicating that LHON mutations might require a differentiated neuronal environment in order to induce oxidative stress [116].

Concerning the male prevalence in LHON interesting information has been brought by a study carried out in rats: female mitochondria have been reported to be less prone to oxidative stress respect to male mitochondria thanks to a higher expression of antioxidant enzymes that may be modulated by estrogens [117]. These results might represent the effective link between oxidative stress and gender bias in LHON providing another evidence of ROS relevance in disease pathogenic mechanism.

At present time, the plethora of information regarding LHON biochemical features allow to hypothesize a pathogenic mechanism resulting from a complex combination of energy production deficiency, oxidative stress and apoptosis that is supported by the several interconnecting points that link these three processes *[fig 13]*.

figure 13

Diagram of the mammalian mitochondrion showing the relationship between energy production, ROS generation, and regulation of apoptosis [49].



4 Autosomal Dominant Optic Atrophy

Overview

Autosomal dominant optic atrophy (ADOA), also known as Kjer's disease (OMIM # 165500) is the most common form of inherited optic atrophy, with a frequency of 1:12000 to 1:50000 [118]. The disease is characterized by moderate to severe loss of visual acuity with insidious onset in early childhood, temporal optic disc pallor, impairment in the color vision and central scotoma [119]. The disease progression may be quite variable also within the same family, ranging from mild cases with visual acuity that stabilizes in adolescence, to slowly but constantly progressing cases, to cases with sudden, step-like decreases of visual acuity. Despite the remarkable difference of the clinical evolution, the endpoint of the pathological process in ADOA is clinically indistinguishable from LHON. Histopathological and electrophysiological studies suggest that also in this form of neuropathy the underlying defect is retinal ganglion cell degeneration associated with optic atrophy. Although LHON has a sudden onset of visual loss in both eyes and appears at a later age (27-34 years), in atrophic phase it is difficult to distinguish LHON from ADOA without a family history: in both pathologies optic nerve shows axonal loss and demyelination, with the smallest fibers of papillo-macular bundle being the most vulnerable, and absence of inflammatory reaction [120].

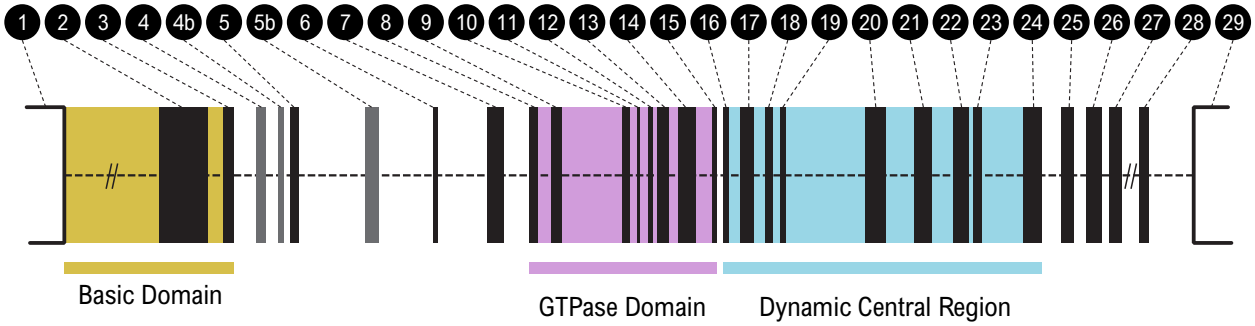
ADOA is transmitted according to Mendelian rules in an autosomal dominant manner; the majority of families are linked to the chromosome 3q28-29 locus where in 2000 a new gene, encoding a dynamin-related protein that is localized in mitochondria (**OPA1**), has been found to segregate with the disease [121 122].

Thus, ADOA represents the second case of pathology in which a single cellular type, the same of LHON, is affected. As for Complex I in LHON, also the nuclear gene linked to ADOA encodes a mitochondrial protein ubiquitously expressed: while most abundant in the retina, the OPA1 mRNA and protein are in fact widely distributed in several tissues [121].

About a hundred OPA1 gene mutations associated to ADOA have been described so far: most of them are nucleotide substitution, but also deletions and insertions are spread throughout the coding sequence of the gene, although the majority is localized in the GTPase domain and in the 3′ end [118, 119] *[fig14]*.

Almost 50% of the OPA1 mutations cause premature truncations and nearly 40% are located within the GTPase domain. They are therefore supposed to

figure 14
OPA1 gene structure. The exons are indicated by numbers.



lead to the loss of protein function providing indications that haploinsufficiency is the cause of the disease [123]. Although haploinsufficiency is, so far, the more likely effect of OPA1 mutations a dominant negative effect cannot be ruled out: indeed, dynamins with deficient GTPase activity can form oligomers with the wild type protein interfering with its GTPase activity [124]. Olichon and co-workers studying the effect of different OPA1 mutations have proposed that those occurring in the GTPase domain would exert a dominant negative effect, while the C-terminal truncations, by removing the potential GED domain and thus abolishing the oligomerization-stimulated activity of the dynamin, would cause haploinsufficiency [118].
Besides the poor knowledge of the pathomechanisms leading to impairment of Opa1 function in ADOA as well as in LHON the link between genotype and phenotype is not clear: the variable penetrance suggest that other still unknown genetic or environmental factors might play a role also in the phenotypic expression of OPA1 gene mutations.

OPA1

OPA1 (optic atrophy-1) gene encodes a dynamin-related protein showing GTPase activity and well conserved during evolution: Mgm1 and Msp1 represent the Opa1 orthologues in S. cerevisiae and in S. pombe, respectively, as demonstrated by complementation of Msp1 loss by OPA1 expression[125].
In human the gene structure comprises 31 exons and consists of a N-terminal mitochondrial targeting sequence followed by short hydrophobic stretches, a coiled-coil domain, a GTPase domain, a middle domain and a C-terminal coiled-coil domain known as GTPase Effector Domain (GED) [126] *[fig.15]*.
The gene expression may result in 2-5 different electrophoretic forms of Opa1 deriving from different proteolytic cleavage; at translational level eight alternative splicing mRNAs derive from different combination of exons 4, 4b and 5b [11, 126]. Two intramitochondrial proteases, the rhomboid-like PARL (presenilin-associated rhomboid-like protein) and the paraplegin, have shown to be involved in Opa1 proteolytic cleavage, either to control the apoptotic process or the mitochondrial morphology [127, 128]. The proteolytic processing of Opa1 has a counterpart in yeast: Mgm1 is as well proteolytically processed into long (l-Mgm1) and short forms (s-Mgm1) by a rhomboid-like protease. l-Mgm1 is integrated in the inner mitochondrial membrane, while s- Mgm1 is released in the intermembrane space; these two forms of Mgm1 seem play overlapping as well distinct roles in maintaining mtDNA, remodeling cristae and fusing mitochondria [129].
Moreover, has been reported that decreased mitochondrial ATP levels, either generated by apoptosis induction, $\Delta\Psi$ dissipation or ATP synthase inhibition, is the common and crucial stimulus that controls OPA1 processing suggesting that the Opa1 ATP-dependent cleavage plays a central role in correlating the energetic metabolism to mitochondrial dynamic [130]: cells might use this

figure 15
Schematic diagram of the domains within dynamin-like protein. Dynamin consists of five distinct domains: the GTP hydrolysis domain (GTPase), a middle domain, a pleckstrin homology domain (PH), a GTPase effector domain (GED), and a highly basic C-terminal proline-rich domain (PRD).

mechanism to monitor the ATP level and use this functional readout to inhibit the fusion of defective mitochondria.

Nevertheless the sub-mitochondrial localization of this protein has long been subject of controversy it is now well accepted that Opa1 is active in the intermembrane space although its relationship with the mitochondrial membranes is not still completely clear. Evidences show that Opa1 co-sediments with both membranes and interacts more or less tightly with the inner membrane [130, 131]. The discrepancies regarding Opa1 localization might be justified by the existence of several isoforms that could have different sub-localizations and functions.

OPA1 is expressed in the myelinated regions of the human optic nerve, which show a decreased number of mitochondria as compared to the soma of the RGC, where biogenesis of mitochondria occurs, and to the fiber layer containing unmyelinated axons before the lamina cribosa [133].

Information regarding Opa1 function derives basically from *in vitro* experiments suppressing or over-expressing the protein; Opa1 is involved in mitochondrial fusion and in inner membrane remodeling, a process of primary relevance in regulation of apoptosis but the real mechanisms underlining its functions need to be still clarified.

Modulation of OPA1 expression may have different consequences depending on the cellular type: overexpression in cells with tubular mitochondria induces fission of mitochondrial network while in cells with physiologically punctuated mitochondria increased OPA1 expression induces mitochondrial fusion [134].

In yeast, the absence of Mgm1, the Opa1 orthologue, determines profound distortion of cristae shape, lack of growth in presence of aerobic substrates and formation of “petite” colonies characterized by mtDNA loss or rearrangements[135]. Mgm1 acts in concert with Fzo1 and Ugo1 to counteract mitochondrial fission and although the mammalian homologue of Ugo1p has not yet been identified, Fzo1p orthologues have been characterized in mitofusin1 and 2 (Mfn1, Mfn2). The same mechanism seems to be also preserved in mouse fibroblasts. In fact in Mfn1 deficient cells Opa1 cannot promote mitochondrial fusion, the defect being complemented by reintroduction of Mfn1 but not Mfn2, unequivocally identifying outer membrane Mfn1 as an essential functional partner of Opa1 [136]. Furthermore, it has been shown that cells lacking both Mfn1 and Mfn2 completely lack mitochondrial fusion and show severe cellular defects, including poor cell growth, widespread heterogeneity of mitochondrial membrane potential, and decreased cellular respiration [137]. Disruption of OPA1 by RNAi blocked as well mitochondrial fusion and resulted in similar cellular defects [137].

A recent study aimed to determine the function of the different Opa1 isoforms has partially clarified the role of OPA1 exons that undergoes alternative splicing. The presence of exon 4 in Opa1 determines functions associated with both $\Delta\Psi$ maintenance and fusion of mitochondrial network while exons 4b and 5b do not contribute to these processes [126]. Overexpression of Opa1-5b induces the classic apoptotic cascade with complete cytochrome *c* release before nuclear fragmentation while silencing of Opa1 variants containing exons 4b or 5b induces apoptosis without relevant release of cytochrome *c* and without mitochondrial network fission or major modification of the cristae structure [126]. There are other evidences suggesting that Opa1 apoptosis regulation might be not linked to its pro-fusion activity. In mouse fibroblasts has been demonstrated that oligomerization of two forms of OPA1 - one soluble and intermembrane space-located and the other one embedded in the inner

membrane - controls the tightness of cristae junctions preventing cytochrome *c* release and protecting from apoptotic death; interestingly, mitofusins seem not required for the antiapoptotic effect of OPA1 [138].
Recently has been reported that mutations in OPA1 can also be responsible for an ADOA "plus" phenotype with sensorineural deafness, ataxia, axonal sensory-motor polyneuropathy, chronic progressive external ophthalmoplegia and mitochondrial myopathy with cytochrome *c* oxidase negative and ragged red fibers [139]. These particular patients, all harboring missense mutations in OPA1, are characterized also by multiple deletions of mitochondrial DNA in their skeletal muscle, thus revealing a new role of the Opa1 protein in mtDNA stability [139], strongly recalling one of Mgm1 functions in yeast.
Mitochondrial function has been analyzed in fibroblasts deriving from ADOA patients carrying different OPA1 mutations all leading to a truncated form of protein: only in galactose medium, i.e. under forced oxidative metabolism, a significant impairment in mitochondrial ATP synthesis driven by Complex I substrates was found. Furthermore, growth in galactose medium has been shown to inhibit mitochondrial fusion in ADOA fibroblasts, but not in control cells [140], indicating that impairment in the energy supply might be involved in the pathomechanism of ADOA.

Fission & Fusion

Mitochondria do not exist as free-floating vesicles in cytoplasm but as a filamentous network that extends throughout the cell. Mitochondrial network is a dynamic entity capable to respond to variations in cell metabolic state and to reorganize depending on tissues energy requirements.

Currently, understanding the impact of organelle morphological changes on the ATP production, or the role of fusion and fission of the mitochondrial network on control of oxidative phosphorylation represents an important challenge in bioenergetics: the discover that two hereditary neurodegenerative disorders such as autosomal dominant optic atrophy and Charcot-Marie-Tooth syndrome are associated to mutations in genes involved in the mitochondrial fusion regulation (OPA1 and MFN2) has further increased the interest in this field, revealing that the control of mitochondrial morphology may have a profound impact on cell physiology. The peculiar structure of mitochondria makes the fusion mechanism a singular issue: from *in vivo* studies in yeast the fusion of the outer and inner membrane appears temporally linked and also in human cells mitochondrial fusion implies a synchronous involvement of outer and inner membranes leading to matrix mixing; the question of how these two sets of compositionally distinct membranes are spatially and temporally coordinated and accurately fused is intriguing [141].
Changes in mitochondrial organization are associated to changes in cell physiological state: mitochondrial network fragmentation could occur synchronously with the cell cycle in human fibroblast [142] indicating that probably mitochondria need to dissociate to correctly segregate in the daughter cells or simply reflecting different energy requirements during mitotic steps. Mitochondria fragmentation is as well a crucial step in the apoptotic cascade; rearrangements in the membranes organization might be actually linked to cytochrome *c* release. The association between mitochondrial morphology and metabolism is supported by the large and tissue-dependent diversity in mitochondrial shape and organelles organization, with an important variability

of organellar section profile, intracellular localization and shape heterogeneity [4]. Differences between tissues concern also the number of cristae and the matrix density [143] further supporting the tight relationship between mitochondrial structure and function.

As demonstrated in lymphocytes migration [144] redistribution of mitochondria in cytoplasm is accomplished as well by shape rearrangements. Indeed, nevertheless the mitochondrial network occupies a vast portion of cytosol the predominant location is near the nucleus or the plasma membrane but the relation with microtubules could permit to mitochondrial tubule to reorganize and accumulate in high-energy demanding cell areas [4]. Subcellular distribution of mitochondria could be relevant also to control the availability of oxygen that is differently is more or less, i.e. closer or further the plasmatic membrane.

The dynamic of mitochondrial network is determined by the equilibrium resulting from the opposite action of fission and fusion, two processes that need to be carefully regulated to properly meet cell requirements. Proteins regulating mitochondrial morphology belong to a large group of conserved dynamin-related GTPase; the signature protein of this family, dynamin, plays an important role in endocytosis and synaptic vescicle recycling through membrane remodeling. The characterization of these processes in lower eukaryotes has allowed to identify the homologue proteins in mammalian and to hypothesize the probable mechanisms involved in the reorganization of mitochondrial network.

In yeast, Dnm1 (dynamin 1) and Fis1 (fission 1) interact to induce fission [145] suggesting that also hFis1, the Fis1 homologue, might be implied in the human Drp1 (dynamin-related protein 1) recruitment on outer mitochondrial membrane. siRNA experiments have shown that Drp1 absence induce a decreased ATP synthesis and an essential increase in mitochondrial membranes fluidity [4] suggesting that also membrane structure is relevant in energy production. Interestingly, in yeast Dnm1 seems to localize in specific regions of mitochondrial outer membrane characterized by reduced diameter and corresponding to separation sites [146]; the proposed mechanism concern the formation of Dnm1 oligomers that wrap around the mitochondrial tubule leading to constriction and separation of mitochondria [147].

The study of mitochondrial dynamic has allowed to discover unexpected non-apoptotic functions for Bcl-2 family members, revealing, as often occurs in biology, surprisingly connections between different pathways. Upon apoptotic signal Drp1 migrate from cytosol to mitochondrial membrane co-localizing with the proapoptotic proteins Bax and Bak, which are responsible for mitochondrial outer membrane permeabilization [148]. In C. elegans CED-9 (Bcl-xL homologue) interacts with Fzo1 (mitofusin homologue) promoting mitochondrial clustering and dramatic reorganization of mitochondrial networks [149] suggesting that anti- or proapoptotic proteins might as well act regulating the assembly of mitochondrial fusion/fission proteins. Several other papers have reported the relation between apoptosis and mitochondrial dynamic but it remains to be understood how the components of the fission and fusion machinery are functioning in coordination with members of the Bcl-2 family, although it can be inferred that modifications in mitochondrial membranes topology may control different pathways promoting or preventing the interaction between transmembrane and intermembrane space proteins [148].

Besides apoptosis, mitochondrial morphology has been found to be also related to the motor proteins system. Efficient fission of mitochondria facilitates the transport of these organelles by anterograde motors: modulation of

dynein-dynactin system, involved in microtubules organization, impact on Drp1 localization promoting a more interconnected mitochondrial network and, consequently, a poorer anterograde transport [4]. Since mitochondria localization is fundamental particularly in neuronal cell, impairment in the equilibrium between fission and fusion leading to incorrect mitochondria distribution might have catastrophic consequences in high energy demanding nervous system sites as nodes of Ranvier.

In the context of mitochondrial pathologies mitochondrial dynamic might fit with the notions of threshold effect and complementation. The existence of a biochemical threshold indicates that there must be a minimal inhibition of a single oxphos complex to affect the overall oxidative phosphorylation: the existence of a threshold might be caused by the presence of an excess of inactive enzymes or reduced intermediates (cytochrome *c* and CoQ) stored in specific region and mobilized only if required.

This hypothesis is supported by the apparently uneven proteins distribution between inner boundary membrane and cristae membrane as well as by the capability of these proteins to redistribute between these domains upon changes of the physiological state of the cell [150]. Moreover, it is probable that one of the primary function of fusion is to enable the exchange of contents between mitochondria: matrix fusion allow proteins exchange and, at a less extent, redistribution and eventually recombination of mtDNA molecules in nucleoids representing a possible way to rescue non-functional mitochondria.

On the other hand, fission could control the isolation of “old” or impaired mitochondria promoting their commitment to authophagy and/or have a role in the distribution of mitochondria in daughter cells during mitosis, becoming a very relevant phenomenon in presence of mutated mtDNA molecules.

aim

1 Leber Hereditary Optic Neuropathy

LHON has been the first mitochondrial pathology to be associated to a point mutation in mtDNA [48]. The loss of bilateral vision, which represents the outcome of the pathology, is caused by optic nerve atrophy determined by specific degeneration of retinal ganglion cells with preservation of the other retinal structures. Genetically, LHON patients are characterized by mutations affecting mitochondrial genes encoding subunits of Complex I of the respiratory chain; several mutations have been identified in different LHON families but most of the reported clinical cases are linked to one of the three so called primary mutations: G3460A at ND1 gene, G11778A at ND4 gene and T14484C at ND6 gene.

Although the first reported LHON case dates back to late 1800 and at present the pathology is well characterized from the histopathological point of view, the basic traits of this neuropathy are still poor understood.

Unlike most of the pathogenic mtDNA mutations, the three LHON primary mutations are usually found in homoplasmic condition, i.e. each cell of every tissue contains only mutated mtDNA molecules. The homoplasmy represents one of the unresolved questions concerning LHON: currently, we do not know yet how a homoplasmic mutation presents in all tissues and affecting an ubiquitously expressed gene may result in the specific involvement of a single cellular type.

LHON is a late-onset pathology, which first symptoms manifest after 20-25 life years. However, interestingly, the course is very short: in few months from the first signs complete blindness occur in both eyes, indicating simultaneous and bilateral degeneration of retinal ganglion cells.

Although homoplasmy is a common condition for LHON mutations not all carriers develop the pathology: the penetrance of LHON mutations is incomplete and highly variable also aong members of the same family members; moreover, LHON penetrance appears to be sex-linked: only 5-15% of women harboring a LHON mutation develop the pathology while in males the frequency reaches 30-50% values [74].

The incomplete and sex-linked penetrance observed in LHON has driven the researchers to look forward putative genetic or environmental factors that might play a role in pathology modulation. The true involvement of X-linked loci still needs to be elucidated, while there could be a correlation with nuclear gene polymorphisms involved in oxidative stress or in apoptosis [73]; however, the effective role of these genetic traits in LHON pathogenesis has not yet been totally clarified. Mitochondrial DNA haplogroup J, containing polymorphic variants at ND1 and ND5 genes, seems to increase the penetrance in presence of G11778A and T14484C mutations [77], while no concordant data have been collected regarding the potential role of environmental factors. Few cases of vision rescue in LHON patients further increase the dark sides of this pathology.

Currently, the pathological mechanism leading to optic nerve atrophy in LHON is still unknown. The bioenergetic failure hypothesis has been tested in several cellular models leading often to discordant conclusions. The gene localization of LHON mutations necessarily predicts an involvement of oxidative phosphorylation but it is yet not clear how Complex I alterations can induce the neuropathy.

Optic nerve degeneration in LHON is due to a wave of apoptotic death that synchronously hits all RGCs: several reports have demonstrated that different LHON cellular model are more prone to apoptotic death with respect to control cells [110, 111, 113] indicating that programmed cell death is the final consequence of Complex I alterations deriving from the presence of primary mutations.
Rising evidence suggests that a pivotal role in LHON pathogenesis is played by reactive oxygen species [113, 115, 116]. Oxidative stress is actually a common trait of different neurodegenerative diseases and the probability that optic nerve atrophy in LHON is the consequence of a complex interaction between energy failure, oxidative stress and apoptosis is very high.
The results reported in this dissertation derive from a project focused on the relative contribution of energy failure and oxidative stress in LHON pathogenesis: the potential role of the polymorphism of a nuclear gene (SOD2) involved in ROS production/scavenging has been analyzed in relation with the impact of one of the three primary mutations on the oxidative phosphorylation system.

SOD2

SOD2 is a nuclear gene encoding MnSOD (Manganese superoxide dismutase), a mitochondrial matrix enzyme catalyzing the dismutation of superoxide anion in hydrogen peroxide. The sequence and the genomic organization of SOD2 gene show striking similarity among mouse, rat, bovine and human, indicating that its function has been well conserved during evolution. The absence of TATA and CCAAT boxes in the promoter and the presence of GC boxes suggest that SOD2 is a “housekeeping” gene [45], ubiquitously expressed. The human gene contains putative NF-kB transcription regulatory element located in the 3′-flanking region of the gene [151]; in the promoter region of all four species are also present multiple copies of Sp-1 and Ap-2 consensus sequences. Sp-1 is a transcription factor with three zinc-fingers motifs essential for DNA-binding; its binding activity is impaired *in vitro* by hydrogen peroxide, thiol-modifying reagents and low levels of reduced glutathione (GSH) [32] indicating that the activity of this protein is regulated in response to modifications of the redox state of the cell. Ap-2 interacts with the 5′-flanking sequences of SOD2 gene: this transcription factor down-regulates the expression of human SOD2 gene via its interaction with Sp-1 within the promoter region [152]. Regulation of SOD2 expression may also occur at translational level by the action of an mRNA-binding protein whose activity is dependent on the cAMP phosphorylation cascade [153].
After cytoplasm translation **MnSOD** monomers are imported into mitochondrial matrix where the enzyme is active as tetramer. Import of nuclear-encoded proteins is a complex process that requires the coordinated regulation of proteins active in the cytosol, in both mitochondrial membranes and in the matrix.
The targeting information of precursor proteins addressed to mitochondrial matrix resides in an amino-terminal sequence extension, the **mitochondrial targeting sequence** (MTS), which is cleaved out by a specific peptidase after import. The major characteristics of MTS are a high content of basic amino acids and amino acids with hydroxyl side chains, the virtual absence of acidic amino acids and the capability to form amphipathic helices in solution [154, 155].
Mitochondrial proteins encoded by nuclear genes cannot be translocated

across the mitochondrial membranes in a folded conformation; they must be in fact at least partially unfolded to fit the import channels. After crossing the outer membrane through the general import pore (TOM), imported proteins are directed to one of two translocases of the inner membrane (TIM21, TIM23). All proteins with a N-terminal MTS, including the matrix proteins, are directed to the TIM23 complex, which forms a channel across the membrane and allows to proteins to be driven into the matrix thanks to membrane potential.
The import of nuclear-encoded proteins is then accomplished by an ATP-dependent molecular system; the core protein of this system is represented by mitochondrial heat shock protein 70 (mtHsp70). The mitochondrial processing peptidase (MPP) terminates the process cleaving off the presequence, and the protein - assisted by folding helpers, such as Hsp70, Hsp60 or peptidyl-prolyl cis/transisomerases - folds into its active form [156].
In human, MnSOD is targeted to mitochondria thanks to a N-terminal 24 amino acids long sequence; as for other matrix proteins MnSOD monomers are imported trough the translocase of the outer membrane and the translocase of the inner membrane (TIM23) in a ΔΨ-dependent manner. Once in the mitochondrial matrix, the MTS is cleaved by mitochondrial processing peptidase and monomers are assembled in the active tetramer containing one manganese atom for each monomer [157].
MnSOD plays a critical role in the detoxification of mitochondrial superoxide anion and its activity is fundamental for cell life as demonstrated by knock out experiments. SOD2 homozygous mutant mice die within the first 10 days of life with a dilated cardiomyopathy, accumulation of lipid in liver and skeletal muscle, and metabolic acidosis [158]. According to Lebovitz et al. [159]SOD2 knockout mice has been shown to survive up to 3 weeks of age but exhibiting several pathologic phenotypes including severe anemia, degeneration of neurons in the basal ganglia and brainstem, and progressive motor disturbances characterized by weakness, rapid fatigue, and circling behavior. These papers clearly provided evidence of the fundamental role played by MnSOD in cell physiology.

Ala16Val dimorphism

In 1996 Shimoda-Matsubayashi reported a T to C substitution in -9 condon of the the SOD2 gene which changes the aminoacidid at position 16 in the mitochondrial targeting sequence from valine (GTT) to alanine (GCT) [160]. In the allele containing alanine the residues 10-17 are predicted to organize in an amphiphilic α-helix structure preceded by a β-sheet segment formed by 4-9 residues. The substitution of the alanine with the valine would determine the disruption of the helix that would be substituted by a long stretch of β-sheet spanning through 4-20 residues. This report shows also that, in Japanese population, the allele containing alanine is more frequent in patients with Parkinson's disease respect to control population.
The *in vitro* import of the human SOD2 alleles in rat mitochondria has been studied to evaluate the effect of MTS conformational change on protein function. The results obtained support the idea that the predicted conformational change modulates the import of MnSOD monomers into mitochondrial matrix. Probably because of its β-sheet conformation, the valine-containing variant is in fact characterized by a slower crossing of inner mitochondrial membrane with respect to the alanine-containing variant [157]. The authors proposed a two

steps model for the import of the valine-containing allele: the partial stalling in the inner membrane would retain the precursor in the intermembrane space for a longer time allowing its partial refolding. The necessity of unfolding the precursor in order to complete the import into the matrix slows down the entire process and it results in a minor efficiency of active homotetramer with respect to the alanine containing variant, as demonstrated by non-denaturing gel activity assay.

Sutton and co-workers [161] has then studied the impact of SOD2 MTS dimorphism on mRNA stability in human cells transfected with both SOD2 alleles. The data show that transfection of Ala-MnSOD allele leads to a four-fold higher levels of the transfected active protein in the matrix with respect to the Val-MnSOD allele; moreover, the confirmed slower import of valine variant has been shown to be associated with a minor stability of the transcript in cytoplasm and with a higher proteasome-processing of the precursors.

Since the great relevance of MnSOD function in cell physiology and, therefore, the necessity of a fine regulation of MnSOD activity, SOD2 dimorphism has been considered as a potential risk factor for different pathological states.

The higher activity of SOD2 Ala variant has been associated with elevation of prostate cancer risk in Caucasians [162] and increased risk of breast cancer among Chinese women with high levels of oxidative stress or low intake of antioxidants [163]. Interaction between the SOD2 Ala/Ala genotype and environmental factors inducing oxidative stress in breast cancer risk has been also confirmed in American women [164] suggesting that an increased superoxide dismutase activity in mitochondria stressed by ROS exposure may further alter the redox balance of cell. In the same way, alcohol abusers with Ala homozygote phenotype show increased susceptibility to severe alcoholic liver disease [165], moreover, the presence of at least one Ala MnSOD allele increases the risk for developing cirrhosis in French alcoholics, the rates of hepatocellular carcinoma development and death in cirrhotic patients [166]. SOD2 dimorphism might as well be associated with development of type 2 diabetes among Japanese-Americans, in this case the valine homozygotes would be exposed to a higher risk [167].

These and several other reports clearly indicate that either high or low superoxide dismutase activity in mitochondria, particularly in presence of stress input, might exacerbate latent pathologies.

Interestingly, the study of SOD2 dimorphism has unearthed a link between MnSOD activity and iron metabolism. Transfection with Ala-MnSOD in hepatoma cells has been shown to modulate the expression of several proteins involved in the uptake, extrusion, storage, and cellular distribution of hepatic iron, and lead to intracellular iron accumulation; accordingly, alcoholic cirrhotic patients with two Ala-MnSOD-encoding alleles frequently exhibit hepatic iron accumulation [168, 169]. An impaired regulation of iron metabolism could have detrimental effects increasing the risk of oxidative stress - see Fenton reaction - other than possibly interfere with the assembly of respiratory chain complexes, which function is highly dependent on Fe-S clusters.

The demonstrated relevance of MnSOD dimorphism in several pathological conditions and the highly probable role of oxidative stress in LHON pathogenesis prompted us to consider the nuclear gene SOD2 as putative regulator of LHON mutations pathogenic potential.

Analyzing the distribution of SOD2 genotypes in an Italian cohort has shown that heterozygous genotype (AV) is more frequent in LHON patients than in control population, while the homozygous valine genotype shows the opposite trend

[fig.1]. The same analysis has been conducted on a large Brazilian pedigree characterized by the 11778/ND4 LHON mutation and by the mitochondrial J haplotype; according to the previous result, heterozygous genotype (AV) appears to be more frequent in LHON affected patients with respect to carriers *[fig.2]*. (Unpublished data kindly provided by Dr. Valerio Carelli).
The uneven distribution of SOD2 genotypes in LHON patients suggests that the SOD2 dimorphism might modulate the manifestation of LHON in subjects genetically predisposed.
Supported by the results of population genetic analysis results we decided to investigate the relatioship between SOD2 genotypes (AA/AV/VV) and MnSOD content and activity in LHON patients harboring the 3460 ND1 LHON mutation as well as in a control population. Subsequently, the investigation has been carried on in order to elucidate if SOD2 can modulate the effect of Complex I LHON pathogenic mutations dysfunction on mitochondrial dysfunction.

figure 1
Distribution of SOD2 genotypes in an italian population cohort (sex matched).

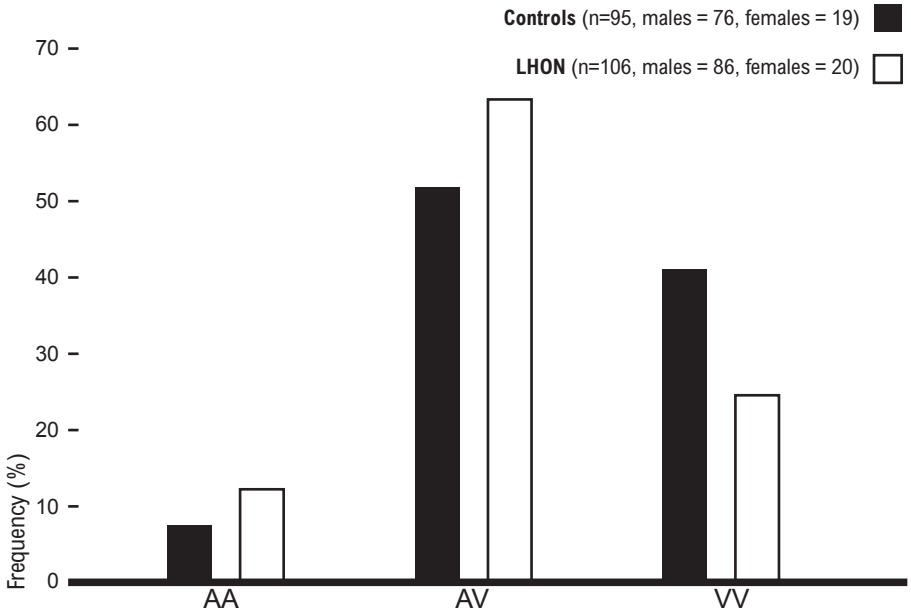
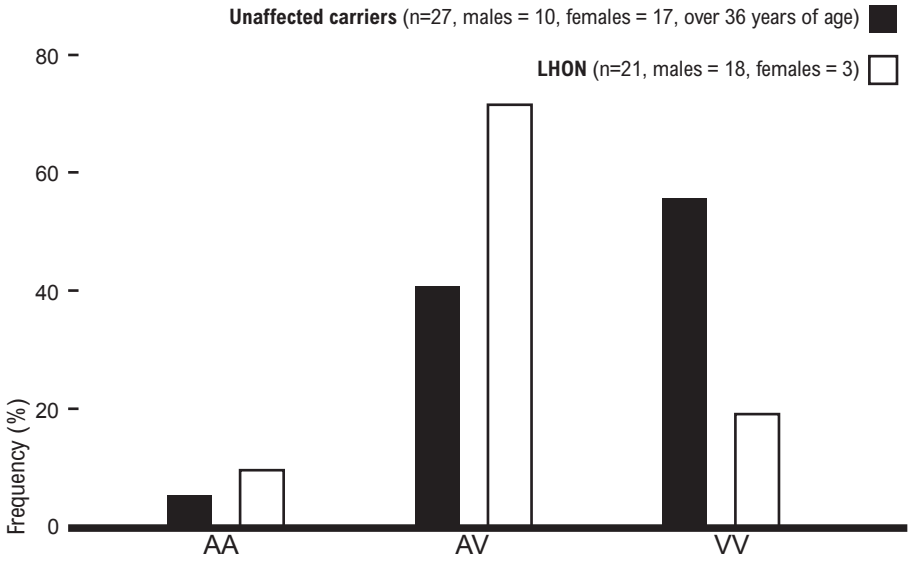


figure 2
Distribution of SOD2 genotypes in a brazilian LHON family.



2 Autosomal Dominant Optic Atrophy

ADOA, or Kjer's disease, is a pathology associated to mutations in the nuclear gene OPA1. Although there is a remarkable difference in timing, with LHON being mainly acute and ADOA slowly progressive, the clinical endpoint of both optic neuropathy is identical. Also in this case patients manifest loss of central vision with selective and early involvement of the small, thinly myelinated fibers of papillo-macular bundle. Besides the common specificity for retinal ganglion cells, LHON and ADOA share also the incomplete penetrance and the variability of clinical manifestation suggesting that also pathologies related to mitochondrial protein encoded by nuclear gene might derive from the cooperation of different genetic/environmental elements.

OPA1 gene encodes a dynamin-related protein showing GTPase activity. Alternative splicing and proteolytic cleavage determine the existence of several isoforms that potentially could have different functions and be differently tissue expressed. The final localization of Opa1 is the mitochondrial intermembrane space: the protein function is not yet well understood although modulation of OPA1 expression in yeasts and higher eukaryotes has shown that its activity is involved in mitochondrial fusion and in cristae remodeling. Several reports have shown the existence of multiple links between Opa1 activity and apoptosis: by a mechanism apparently independent on mitochondrial fusion Opa1 may regulate cytochrome c release from intermembrane space gaining a role also in the intrinsic apoptotic pathway and supporting the notion that nuclear-encoded proteins targeted to mitochondria work as active sensor in the crosstalk between these organelles and the rest of the cell.

Particularly in neuronal cells mitochondrial network dynamic might have a profound impact on mitochondria distribution and energy supply because.

Since most of the OPA1 mutations associated to ADOA lies in the GTPase domain of the protein, haploinsufficiency is the more probable cause of Opa1 dysfunction, although a dominant negative effect has been proposed for mutations lying in the oligomerization domain.

Fibroblasts grown in galactose medium with OPA1 mutations predicted to generate a truncated Opa1 protein have been reported to show impaired oxidative phosphorylation with mitochondrial fragmentation and increased susceptibility to apoptosis [140]. Moreover, the association of multiple mtDNA deletions with 'ADOA plus' phenotypes due to OPA1 missense mutations has suggested a putative role of OPA1 in mtDNA integrity maintenance [139]. However, the relative contribution of these alterations to "classical" ADOA pathogenesis as well as their mutual relationships has not yet been unraveled. Therefore, as LHON also ADOA is a peculiar model of selective neurodegeneration induced by mitochondrial dysfunction through a still unknown pathological mechanism.

In order to investigate the biochemical mechanisms leading to optic nerve atrophy and the cause-effect relationship between Opa1 dysfunction and energy production, we analyzed mitochondrial function and morphology in ADOA patients characterized by a novel mutation in the OPA1 GTPase domain. This project has been developed in collaboration with the research group of Prof. L. Vergani, University of Padova, Italy.

The patients here reported are females belonging to the same family affected by prominent bilateral chronic progressive optic atrophy with childhood-onset.

Two of them manifested also extraocular signs. In one case, the decrease in compound motor and sensory action potentials, a slightly reduced conduction velocity in the lower extremities and initial denervation signs at the tibialis anterioris bilaterally indicate a moderate-severe predominantly axonal sensory-motor peripheral neuropathy at the lower extremities. The second showed moderate muscle deficit at the right tibialis anterioris and winging scapulae. Electromyography disclosed mild decrease of motor and sensory conduction velocities in the lower extremities and increased distal latencies, indicating a predominantly demyelinating peripheral neuropathy at the lower extremities [170].

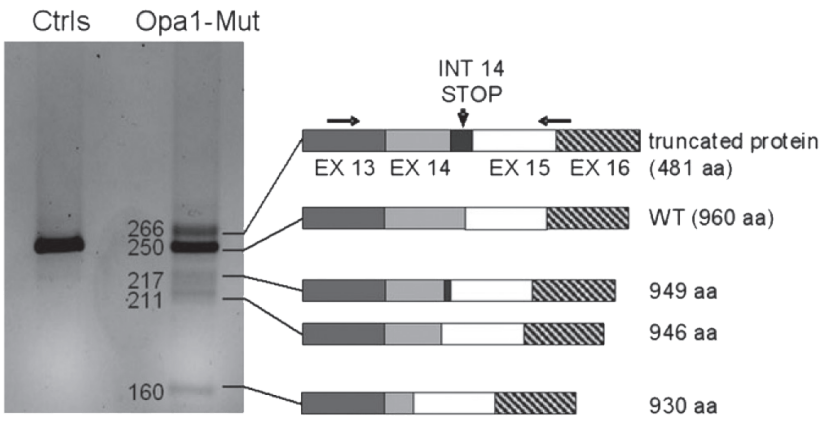
Complete sequencing of OPA1 demonstrated a new 38-base-pair deletion (c1410_1443+4del38) spanning exon and intron 14 in the catalytic GTPase domain, with a predicted splice-site defect [170].

Amplification of cDNA extracted from patient fibroblasts revealed the presence of five different transcripts, one produced by the wild-type allele, the others resulting from the activation of cryptic splice sites in exon 14 or intron 14. The most abundant transcript encodes a truncated protein through the presence of an in-frame UAA termination signal in the retained intronic sequence, 11 codons after the deletion breakpoint, with a predicted protein product of 481 amino acids. The three shorter transcripts, which are much less abundant and maintain the correct reading frame, are predicted to produce proteins lacking 11, 13 or 30 amino acids within the GTPase domain. It is not clear if these polypeptides are stable; however, it should be noted that the longest transcript accounts for the vast majority of aberrant transcripts in patient fibroblasts (>75% on densitometric analysis). Consistent with the molecular data is the reduced abundance of full-length Opa1 protein in patient fibroblasts obtained by immunoblotting (47% of controls) [170]. *[fig. 3]*

The interest in the OPA1 mutations derives not only from the several merging features shared by ADOA and LHON but also by the emerging necessity of a careful study of a relatively new aspect of mitochondrial function: the ability to fuse or fission possibly in response to particular metabolic requirements.

figure 3

Opa1 in patient fibroblasts. Analysis of cDNA in ADOA fibroblasts shows four altered transcripts, resulting in three shorter mRNA (217, 211 and 160) and one longer (266) than normal allele (250), owing to cryptic splice encompassing exon 14 and/or intron 14. Sequence analysis indicated the produced proteins, schematically represented at the side.



3 Protection of cells with energy deficiency

Whatever is the genetic defect, mitochondrial pathologies usually share the common features of a defect in the oxidative phosphorylation system that can lead to a mild or severe reduction of ATP synthesis, resulting in a possibly insufficient energy supply for the cell. In order to overcome the energy deficiency created by oxphos complex deficiency, respiratory chain substrates (CoQ, ascorbate, NADH or $FADH_2$ precursors) supplementation has been tested in patients with defects of oxphos system. Unfortunately, up today results from different tests have not been so promising and more extensive trials are necessary in order to clarify the real efficacy of this kind of treatments.

Although it has not been extensively analyzed in humans, experimental works in animal cells exposed to hypoxia/reoxygenation cycles and mutant yeast have shown that an increased substrate-level phosphorylation could generate enough ATP to correct energy defects caused by different mechanisms [171, 172].

These results suggest that "forcing" substrate-level phosphorylation to work overtime may be a viable approach to remedy the energy crisis due to oxphos impairment. The reported data in these papers bode well for application to patients. To optimize this strategy to human cells, we have chosen exogenous substrates possibly capable of stimulating the Krebs cycle flux and at the same time of removing the excess of reduced pyridine nucleotides (NADH) in oxphos-deficient cells. In Krebs cycle **α-ketoglutarate** is converted by α-ketoglutarate dehydrogenase in succinyl-CoA with concomitant reduction of NAD; succinyl-CoA is then converted in succinate by succinyl-CoA synthetase (A-SCS): the reaction results in the formation of an high energy phosphate bond in form of ATP or GTP that can be transferred by the nucleoside-diphosphate kinase to one ADP molecule to give ATP. The aspartate is added as indirect source of NAD in order to allow to α-ketoglutarate dehydrogenase to keep working.

To verify the validity of this approach in human cells *in vitro*, we have used both experimental models (in which cells are "poisoned" with a specific inhibitor) and "natural" models (cybrids harboring pathogenic mutations).

As experimental model we chose resting fibroblasts because the most severely affected tissues in mitochondrial diseases are slowly proliferating post-mitotic tissues, such as brain and muscle. Carrying out experiments under conditions of high-energy demand in resting fibroblasts better approximates the vulnerability of post-mitotic tissues to mitochondrial dysfunction.

To achieve the condition of impaired energy production in high-energy demand cells we added gramicidin and oligomycin to the culture medium of resting fibroblasts. Gramicidin is an ionophore capable to increase the membrane permeability to monovalent cations: the cell is supposed to respond to gramicidin by increasing the ATP consumption in order to maintain the Na^+/K^+ plasmembrane gradient. Oligomycin specifically binds to the F_o domain of the ATP synthase; the presence of oligomycin inhibits ATP synthase by blocking the proton translocation. Therefore, oligomycin reduces cell capacity of producing energy by oxidative phosphorylation hypothetically miming the effect of mutated subunits in the complex.

The "natural" model is represented by cybrids carrying T8993G or T8993C mutations in the mitochondrial gene encoding the ATPase 6 subunit of ATP

synthase. These mutations are associated with NARP or with the more severe **MILS** (Maternally Inherited Leigh Syndrome) depending on the mutation load, although the T8993C causes milder clinical phenotypes. Clinical features of NARP may variably include peripheral neuropathy, retinitis pigmentosa, cerebellar atrophy, mental retardation, and less frequently epileptic seizures, cardiomyopathy, deafness and optic atrophy. MILS is dramatically characterized by bilateral subacute lesions of the basal ganglia, which extending to the brainstem lead to death of the patient. As previously reported [53], the different severity of T8993G and T8993C mutations is linked to the different effect on mitochondrial metabolism. Both mutations lead to energy deprivation and ROS overproduction however, the relative contribution and the severity of the two pathogenic components is different depending on the mutation considered. The T8993G change mainly induces a dramatic energy deficiency, whereas the T8993C mutation favors a significantly increase of ROS production.

The supplementation of α-ketoglutarate and aspartate has been tested also in cybrids harboring the A8344G mutation affecting the mitochondrial gene encoding the $tRNA^{Lys}$. This mutation is associated to MERRF, a form of Myoclonic Epilepsy with Ragged Red Fibers in which the $tRNA^{Lys}$ mutation alters the translation of mtDNA encoded genes.

The effect of metabolites sustaining substrate-level phosphorylation in resting fibroblasts and in NARP and MERRF cybrids has been tested in a glucose-free growth medium where the carbon source has been substituted by galactose: in this condition the cells are forced to use oxidative phosphorylation to produce energy because the slow metabolism of galactose to glucose-1-phosphate does not allow to satisfy the cellular energy demand by glicolysis. Cell viability and ATP content have been considered as parameters to assay the capability of exogenous substrate to rescue energy failure in view of a potential alternative pharmacological treatment for mitochondrial pathologies.

results & discussion

1 Leber Hereditary Optic Neuropathy

Nevertheless LHON is a long time known pathology, the molecular mechanism that links mutations in Complex I subunits to optic nerve atrophy is still not understood. The energy failure hypothesis has been tested in several cellular models and the results obtained are greatly variable depending on the used experimental model. In our laboratory it has been shown that in LHON cybrids, homoplasmic for either one of primary mutations, the rate of ATP synthesis measured in the presence of nicotinamide adenine dinucleotide (NAD)–dependent substrates was strongly reduced, with the 11778/ND4 mutation having the milder effect. These results were also confirmed by polarographic experiments. However the measurement of cell ATP content did not show any difference between LHON and wild type cybrids, suggesting that LHON cybrids may compensate the mitochondrial defect with glycolysis [105].

In this study we focused on the probable role played by the nuclear gene SOD2 in the modulation of LHON pathogenesis. We tested the relationship between the Ala16Val dimorphism in the mitochondrial targeting sequence of SOD2 gene and the oxphos efficiency to verify if a particular SOD2 genotype could modulate the effect of the 3460/ND1 LHON mutation on cell dysfunction.

In the present work, the impact of the 3460/ND1 mutation on the oxidative phosphorylation system and the putative role of MnSOD gene polymorphism in the incomplete and variable penetrance of LHON disease has been evaluated in primary human cells of patients.

Controls and patients have been recruited by Dr. Valerio Carelli's laboratory at Neurological Department of Bologna University where genotype characterization - for both LHON and SOD2 variants - has been carried out.

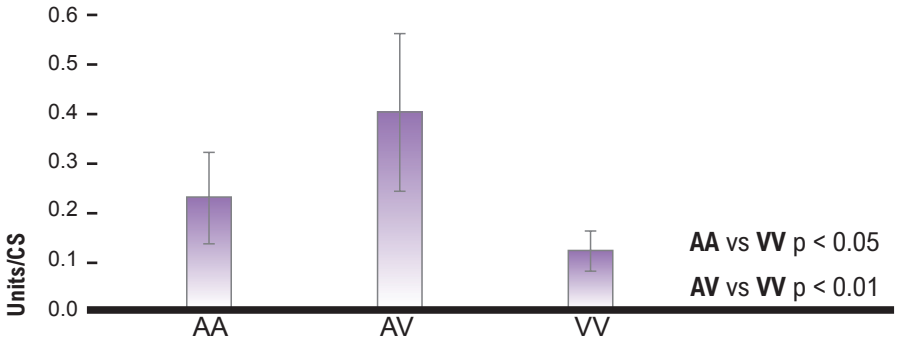
Effect of SOD2 dimorphism on MnSOD activity and content in human platelet enriched fraction

The effect of mitochondrial targeting sequence dimorphism in SOD2 gene has been investigated analyzing the MnSOD activity and its mitochondrial content in a control group composed by 24 individuals as reported in the materials and method section. Previously analysis of this SOD2 dimorphism has shown that in mitochondria and cells transfected with Ala and Val alleles the dimorphism is associated with a different import efficiency resulting in a different content of active enzyme in mitochondrial matrix. The presence of a valine residue in the 16 position of the MTS determines a lower stability of the transcript and a β-sheet conformation of the precursor protein; the consequence of these variations is a slower import of monomers associated to a lower superoxide dismutase activity [157, 161].

In the control group, for each individual, the superoxide activity has been measured in the soluble fraction obtained from HPBF (leukocytes and platelets) while the MnSOD content has been determined by western blot in the mitochondrial fraction extracted from the same cell preparation.

As shown in *Fig.1* our results are in agreement with that obtained in transfected systems. Samples deriving from individuals with SOD2 heterozygous genotype are characterized by a significantly higher MnSOD activity with respect to both Ala homozygotes (-43%, $p < 0.05$) and Val homozygotes (-70%, $p < 0.01$).

figure 1
MnSOD activity in HPBF of controls with either one of three SOD2 genotypes.
Because the enzyme is active in the mitochondrial matrix, activity values were normalized by citrate synthase to take account of the mitochondrial mass in different cells.



The enzyme activity, that is expressed as units per mg of cellular protein (U/mg), has been determined considering the protein quantity capable to induce 50% inhibition of the NBT (Nitroblue Tetrazolium) reduction rate as "MnSOD activity unit". Moreover the activity values have been normalized to citrate synthase activity, that is an index of mitochondrial mass, in order to prevent errors due to the possibly different purity of analyzed samples.
The CuZnSOD activity, calculated on difference between the total superoxide dismutase activity and the MnSOD activity, does not result significantly different among the three control groups, suggesting the absence of "compensatory" effect involving SOD1 product *[fig.2]*.
Concomitantly, we evaluated the mitochondrial content of MnSOD to assess if the higher MnSOD activity was actually determined by a different import of monomers into the mitochondrial matrix. MnSOD protein was quantified in mitochondria isolated from control cells by SDS-PAGE and Western blot followed by immunodetection. The amount of MnSOD protein band has been determined and expressed as percentage of the mitochondrial pyruvate dehydrogenase complex E1α subunit-band, that has been used as protein standard. As shown in *fig.3* the percentage of MnSOD monomer was found to be about 72 %, 85% and 51 % in control samples carrying the AA, AV and VV MnSOD genotype, respectively. More the amount of MnSOD monomer detected in heterozygous samples was statistically higher with respect to both SOD2 homozygous genotypes samples ($p < 0.05$ vs Ala homozygotes and $p < 0.01$ vs Val homozygotes); in this case the observed differences result statistically significant also between the two homozygous genotypes groups ($p < 0.01$).
The ratio between the amount of MTS-MnSOD (26 KDa) and MnSOD (23 KDa) monomer was quantified in the soluble fraction obtained from sonicated

figure 2
CuZnSOD activity.
The enzymatic activity in HPBF of controls with either one of three SOD2 genotypes is calculated on difference between the total superoxide dismutase activity and MnSOD activity.

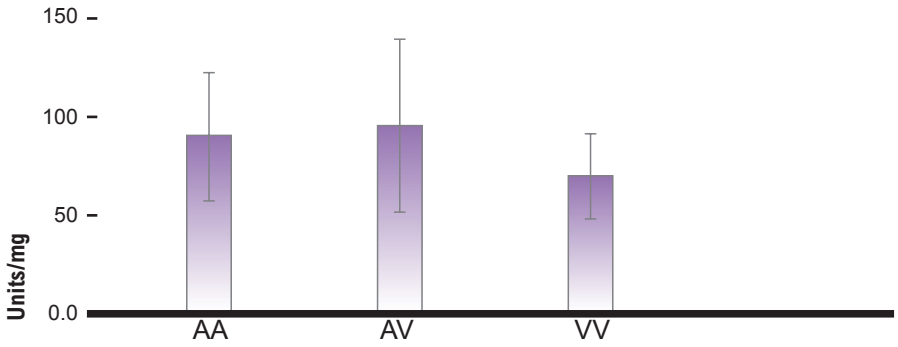
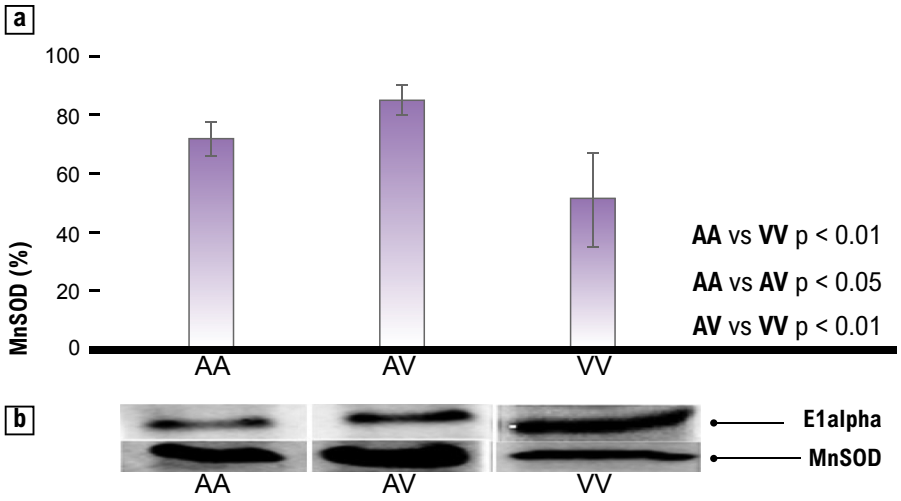


figure 3

MnSOD content in HPBF of controls with either one of three SOD2 genotypes.

a) MnSOD protein is expressed as the percentage ratio between densitometric values of MnSOD and E1α, considered as internal control.

b) Chemoluminescent spots of MnSOD monomers and E1α subunits in three SOD2 genotypes.



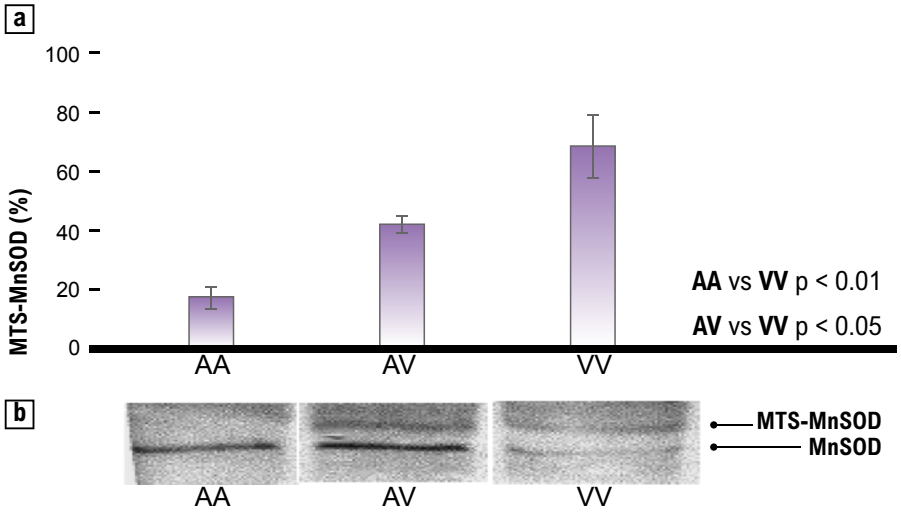
controls by SDS-PAGE (using a 16-19% polyacrilamide gradient) and western blot *[fig.4a]*. By densitometric analysis the percentage mean value of the targeted mitochondrial protein was about 18%, 42% and 68% in the protein samples extracted from cells carrying AA, AV, VV MnSOD genotype, respectively *[fig 4b]*. According to previous published data [157, 161], our results indicate that the Val-containing monomers, retained for a long time in the cytoplasm, are less efficiently imported into the mitochondrial matrix and then assembled into active enzyme.

Our results clearly show that the SOD2 dimorphism affecting the mitochondrial targeting sequence can modulate the MnSOD activity of controls being the highest MnSOD activity of heterozygotes well related with the MnSOD monomer protein content in mitochondrial matrix. Interestingly, as shown by statistical analysis of the SOD2 genotypes, the presence of both MTS-MnSOD alleles (AV genotype) seems increase the probability of developing LHON in

figure 4

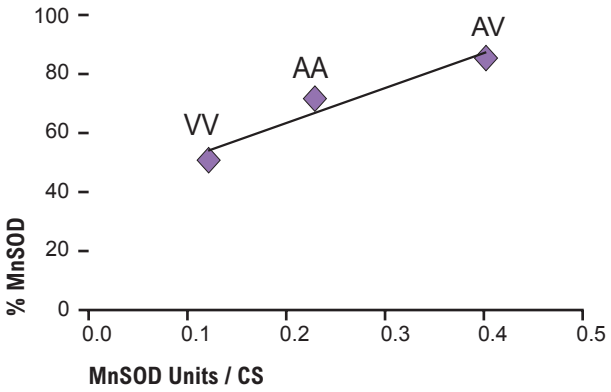
MTS - MnSOD content.

a) Ratio between unprocessed and mature MnSOD in whole cell of controls with different SOD2 genotypes. b) After the proteolytic cleavage in the mitochondrial matrix the monomer shows a molecular weight of 23 KDa. The unprocessed protein, still containing the mitochondrial targeting sequence, shows a molecular weight of 26 KDa. Tubulin was used as internal control.



individuals harboring a LHON primary mutation.
Indeed, our results shows that the highest MnSOD activity observed in heterozygotes is strictly and actually associated with the content of active enzyme in mitochondria. In the correlation graph *[fig. 5]* are reported the MnSOD activity and quantity average values determined in the three control groups carrying different SOD2 genotypes.

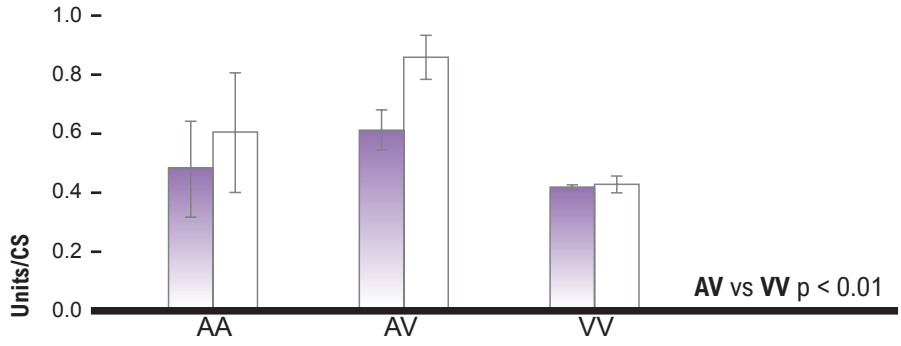
figure 5
Correlation graph.
In the graph the MnSOD quantity and activity' average values of the control group are represented.



Effect of SOD2 dimorphism on MnSOD activity and content of controls and LHON patients lymphocytes

Supported by the results obtained in the control population group we decided to analyze the effect of SOD2 gene polymorphism in LHON patients lymphocytes (twelve individuals total) harboring the 3460/ND1 mutation. The results obtained measuring the MnSOD enzyme activity are in agreement with what we have previously observed in the controls population and it might support the hypothesis that SOD2 dimorphism plays a role in modulation of LHON penetrance. In *fig. 6* it is reported the MnSOD activity normalized on citrate synthase activity (Units/CS), measured in different experimental groups. In controls as well in LHON patients SOD2 heterozygous genotype is associated

figure 6
MnSOD activity.
MnSOD activity in control (purple) and 3460/ND1 LHON (white) lymphocytes in different SOD2 genotypes. As previously reported, activity values were normalized by citrate synthase to take account of the mitochondrial mass in different cells.



with the highest activity of MnSOD. In the control group the enzyme activity differences (AV 0.61 ± 0.07 Units/CS, AA 0.49 ± 0.16 Units/CS, VV 0.42 ± 0.01 Units/CS) result statistically significant only between heterozygotes and valine homozygotes ($p < 0.01$). Interestingly, in LHON patients the presence of the Ala allele in both heterozygous and homozygous combination is associated with an increase of MnSOD activity with respect to controls. However, the statistical significance is reached only comparing the enzyme activity of LHON heterozygotes vs the one of control heterozygotes ($p < 0.01$). Also in LHON lymphocytes protein quantification by western blot has confirmed an increase of the MnSOD content in patients carrying both SOD2 gene alleles *[fig. 7]*.
As expected, the high superoxide dismutase activity observed in SOD2 heterozygotes seems to be a peculiar feature of MnSOD: indeed CuZnSOD activity does not seem to be affected by SOD2 genotype and it does not show significant differences between LHON patients and controls *[fig. 8]*.
The evaluation of catalase activity has been carried out in order to assess if SOD2 genotypes are associated with "reorganization" of antioxidant enzyme systems other than the MnSOD. As well as for CuZnSOD activity, also catalase activity does not vary between different groups *[fig. 9]*, suggesting that the increased MnSOD activity observed in SOD2 heterozygotes is not associated with a parallel increase of other antioxidant enzyme activities.

figure 7
MnSOD content.
a) MnSOD content in 3460/ND1 LHON lymphocytes with either one of SOD2 genotypes. Reported values derive from the ratio between MnSOD and E1α quantity, considered as internal control.
b) Chemiluminescent spots of MnSOD monomers and E1α subunits in three SOD2 genotypes of LHON lymphocytes.

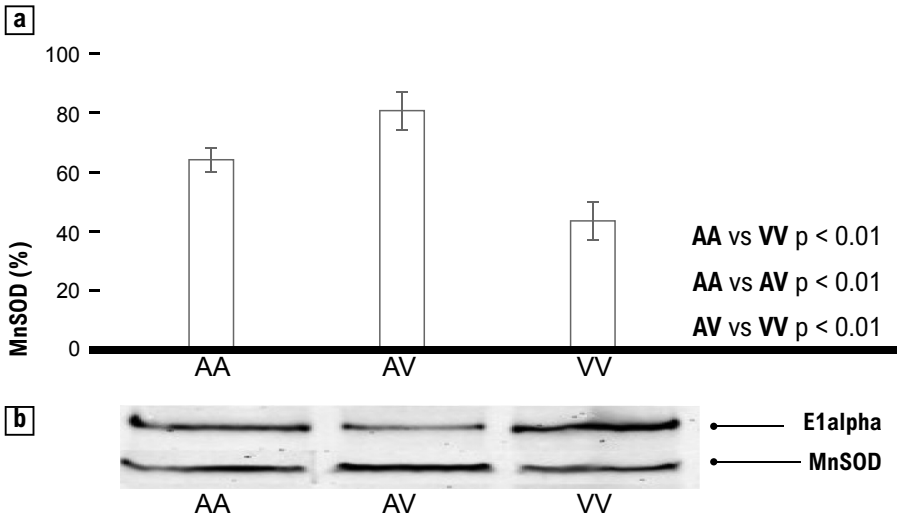


figure 8
CuZnSOD activity.
CuZnSOD activity in control (purple) and 3460/ND1 LHON (white) lymphocytes in different SOD2 genotypes.

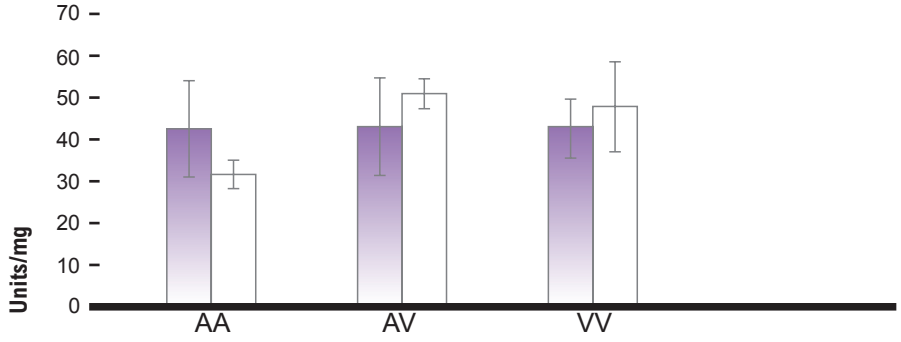
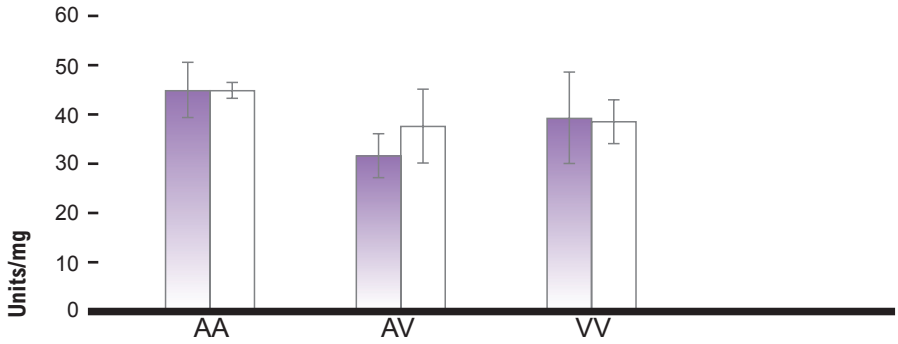


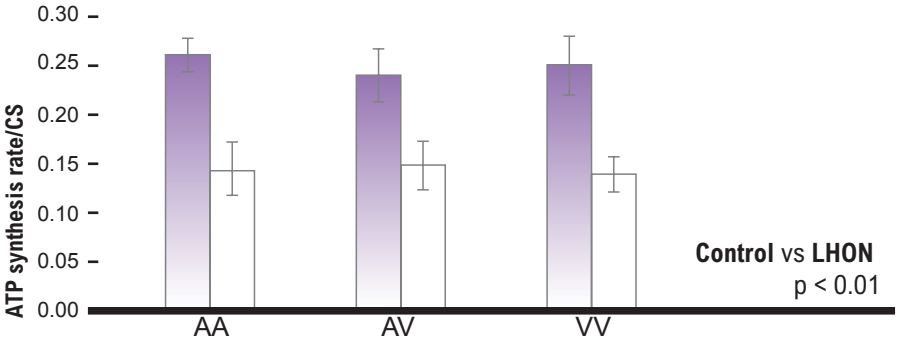
figure 9
Catalase activity.
Catalase activity in control (purple) and 3460/ND1 LHON (white) lymphocytes in different SOD2 genotypes.



ATP synthesis rate in lymphocytes from controls and LHON patients

Mitochondrial function has been evaluated in lymphocytes isolated from peripheral blood of LHON patients and controls. The ATP synthesis rate has been considered as reliable parameter for testing the effect of 3460/ND1 LHON mutations on oxphos, it means on cellular energetic capability. Controls and LHON patients lymphocytes data were matched depending on SOD2 genotype in order to highlight the potential association between the mitochondrial MnSOD activity and the pathogenic power of mutation. We measured either the Complex I-driven ATP synthesis in the presence of malonate, an inhibitor of succinate dehydrogenase complex, or the Complex II-driven ATP synthesis in the presence of rotenone, a specific NADH dehydrogenase complex inhibitor. After inducing the *in vitro* ATP synthesis for 3 minutes, ATP extracted from samples was quantified with a chemiluminescent method based on luciferin-luciferase reaction using a known amount of ATP as standard.
As shown in *fig.10* independently of the SOD2 genotype lymphocytes deriving from LHON patients are characterized by a reduced capability to synthesize ATP in presence of Complex I substrates ($p < 0.01$). The extent of reduction - 45% in Ala homozygotes (AA), 39% in heterozygotes (AV), 46% in Val homozygotes (VV) - suggests that the effect of LHON pathogenic mutations on oxphos function could be not significantly dependent on SOD2 genotype. The evaluation of Complex II-driven ATP synthesis has confirmed the exclusive impairment of Complex I function in LHON patient cells: there are no significant differences between the succinate-driven ATP synthesis rate of

figure 10
Complex I-driven ATP synthesis rate.
ATP synthesis rate in presence of NADH-dependent substrates in control (purple) and LHON (white) lymphocytes. Values were normalized by citrate synthase activity considered as index of mitochondrial mass.



LHON patients and controls lymphocytes and it does not result affected by SOD2 genotypes *[fig. 11]*.

The ATP synthesis rate values have been reported after normalization to citrate synthase activity of each analyzed sample. Although there are no significant differences between LHON patients and controls, lymphocytes of both Ala homozygotes and heterozygotes show a trend toward an increased citrate synthase activity with respect to SOD2 genotype matched controls *[Fig. 12]*. Because of the citrate synthase activity results we hypothesized that in LHON patients the presence of an Ala allele in the SOD2 mitochondrial targeting sequence could be associated with an increased mitochondrial content. To address this hypothesis the mtDNA copies number of controls and LHON patients lymphocytes has been determined. Although a trend toward an increased mtDNA copies number is observed in Ala homozygotes, in the control group the presence of different SOD2 genotypes does not significantly affect the number of mtDNA molecules. Interestingly, only in LHON patients the presence of at least one Ala allele in the SOD2 gene, i.e. in both homozygosis and heterozygosis, is associated with a significant higher quantity of mitochondrial DNA with respect to Val homozygotes *[fig.13]*.

figure 11

Complex II-driven ATP synthesis rate.

ATP synthesis rate in presence of succinate in control (purple) and LHON (white) lymphocytes. Values are normalized by citrate synthase activity considered as index of mitochondrial mass.

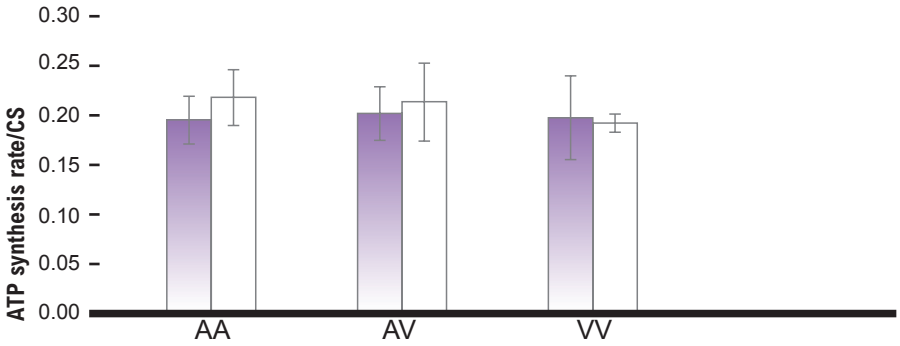


figure 12

Citrate synthase activity.

Citrate synthase activity in control (purple) and LHON (white) lymphocytes.

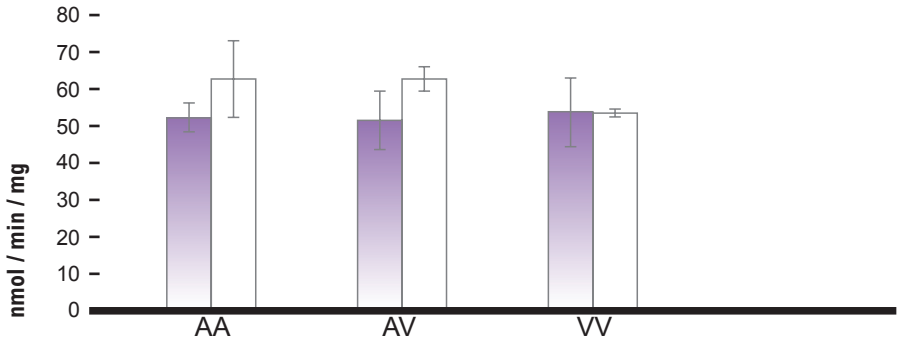
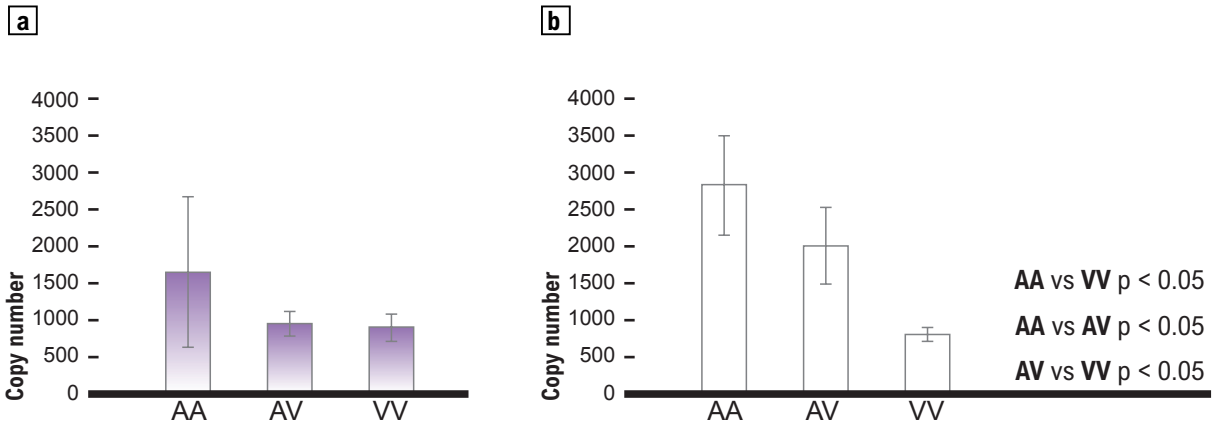


figure 13
Mitochondrial DNA copy number.
Mitochondrial DNA copy number in control lymphocytes (a) and in 3460/ND1 LHON lymphocytes (b). While in control population the presence of different SOD2 genotypes does not affect the number of mtDNA molecules, LHON patients with at least one Ala allele show an increased number of mtDNA molecules respect to valine homozygotes.



Discussion

MnSOD is the main antioxidant enzyme that scavenges superoxide and could be considered the first line of defense against O_2•- generated in the mitochondrial matrix by electron transport chain. In mice, complete ablation of MnSOD causes early postnatal death with severe oxidative damage to mitochondria and increased sensitivity to hyperoxia [158,159]. The study of the SOD2 Ala16Val dimorphism has demonstrated that MnSOD genetic variants represent a risk factor for different kind of pathologies. The presence of Ala allele seems to increase the risk of cancer and liver diseases in predisposed subjects [162,163,164], while the Val allele in homozygosis increases the risk for type 2 diabetes [167]. Indeed, it is clear that MnSOD activity need to be excellently regulated in order to maintain cell redox equilibrium: surprisingly, in presence of preexisting stress factors, even a too high superoxide dismutase activity in mitochondria could be deleterious for cell physiology [164, 165].
The variable penetrance of LHON disease and the notion that, besides energy failure, oxidative stress plays a pivotal role in LHON pathogenesis have prompted us to study the frequency of SOD2 genotypes in order to verify if the MTS variants are evenly distributed in LHON cohorts and in control population. As reported in the previous section, either comparing LHON versus controls or, in the same LHON family, affected individuals versus carriers the SOD2 heterozygous genotype (AV) seems increase the probability of manifesting the pathology. These results prompted us to study the effect of SOD2 dimorphism on MnSOD activity in order to investigate the molecular mechanism by which SOD2 genotype could eventually modulate the pathogenic potential of LHON mutations.
The results obtained show that, as hypothesized, the Ala16Val dimorphism in the MTS of MnSOD monomer is linked to a different import efficiency of the protein: in a control population the presence of the Ala allele in the SOD2 genotype determines a higher mitochondrial content of MnSOD monomer with respect to controls carrying the VV SOD2 genotype; moreover it results in an increased MnSOD enzyme activity. Interestingly, the highest MnSOD quantity and activity are found in controls harboring the heterozygous SOD2 genotype.
The same trend is also found in patients harboring the 3460/ND1 LHON

mutation but in LHON with SOD2 heterozygous genotype the MnSOD activity is significantly higher than in SOD2 genotype matched controls.
Concerning the bioenergetic defect, we demonstrated that in lymphocytes the 3460/ND1 LHON mutation induces alterations in Complex I catalytic activity that determine a reduction of cell capability to synthesize ATP through the oxidative phosphorylation. Moreover, as demonstrated by measurements of the Complex II driven ATP synthesis rate, the energy failure of LHON patient cells is strictly and only dependent on Complex I dysfunction.
The extent of the ATP-synthesis rate reduction detected in LHON lymphocytes is independent on patients SOD2 genotypes, i.e. none of the MnSOD allele is capable to mitigate or exacerbate the effect of LHON mutation on the mitochondrial energy metabolism. Thus, the modulation of LHON mutation pathogenicity by MnSOD might occur through mechanisms that do not directly involve Complex I and oxidative phosphorylation impairment.
Our data further support the theory regarding the impact of Ala16Val dimorphism on the peptide secondary organization: the presence of a valine determines a β-sheet conformation of the SOD2 mitochondrial targeting sequence that induces a partial stall of the monomer during its translocation into mitochondria [157,160] determining a minor content of the valine-containing peptide in the matrix. Interestingly, the MnSOD variants have a different behavior not only during the import process: the slowly imported Val variant is actually more prone to proteasomal degradation and its mRNA shows a minor stability, possibly due to impaired cotranslational import [161].
Based on this evidence, it can be inferred that SOD2 variants could as well being differently regulated at transcriptional level: the valine allele, showing poor import efficiency, could determine an up-regulation of SOD2 expression. In case of heterozygosity the up-regulation would have the major effect because the overexpressed alanine allele would easily reach the mitochondrial matrix causing an accumulation of monomers and, thus, of active enzyme. If two valine alleles were present, the up-regulation would have no effect because of the instability of monomers and mRNA in cytoplasm and because of the partial stall during the inner mitochondrial membrane crossing.

The different regulation of MnSOD expression in individuals carrying different MnSOD2 genotypes would explain why we found a higher quantity and activity of MnSOD in heterozygotes with respect to both AA and VV homozygotes. The higher superoxide activity is specifically due to the SOD2 gene product as demonstrated by the absence of differences in the CuZnSOD activity between our experimental groups.
In order to verify if the different MnSOD activity affects expression and/or regulation of other antioxidant enzymes we have measured the activity of catalase, which acts as hydrogen peroxide scavenger. Interestingly, catalase function seems not dependent on SOD2 genotype: indeed all samples tested show a very similar enzymatic activity.
As previously reported, MnSOD catalyzes the dismutation of anion superoxide in mitochondrial matrix. The reaction involves the oscillation of Mn oxidation state and the formation of one molecule of hydrogen peroxide from two superoxide anion molecules. Although superoxide anion radical spontaneously dismutes to O_2 and H_2O_2 quite rapidly, superoxide dismutase activity is biologically necessary because superoxide reacts even faster with NO radical to form peroxynitrite, a very powerful oxidant. To counteract the accumulation of this toxic molecule the cell has then evolved an efficient system, whose reaction rate is limited only by diffusion. Besides the powerful action of MnSOD as O_2•-

scavenger, it must to keep in mind that the product of its catalytic process is a non-radical molecule that belongs to reactive oxygen species and that could have a toxic effect. Hydrogen peroxide, other than working as messenger in signal transduction pathways at low concentration, can in fact irreversibly modify proteins or react with iron to give hydroxyl radical, a highly reactive oxidant molecule capable to induce modifications also in DNA. Thus, the activity of MnSOD needs to be finely regulated in order to maintain the correct balance between superoxide anion and hydrogen peroxide concentrations in mitochondria.

Based on our results, it can be suggested that, in absence of increased hydrogen peroxide scavenging capacity, in SOD2 heterozygotes the high MnSOD activity might determine an increase of the hydrogen peroxide cellular content.

A steady state characterized by a higher concentration of hydrogen peroxide could explain the possible effect of SOD2 heterozygous genotype in LHON pathogenesis: an unbalance of the hydrogen peroxide production and scavenging determined by the increased MnSOD activity, in the absence of compensatory mechanisms, could create a more sensible mitochondrial environment to the effect of LHON mutations. The reduction of Complex I activity in a context of increased oxidative stress, although not necessarily leading to energy failure in the whole cell [105], could anyway have deleterious effect on the regulation of the overall mitochondrial function that, particularly in neuronal cells, must be finely controlled to readily reply to different requirements in terms of energy metabolism, calcium buffering, apoptotic commitment and mitochondria localization along the different axons regions. The increased content of mtDNA in LHON patients characterized by at least one Ala allele of SOD2 gene confirms that there must be some tight link between MnSOD activity and pathogenic effect of LHON mutations. The prominent effect of oxidative stress could contribute to explain the gender bias observed in LHON: women could be less prone to develop the pathology because of estrogens that, particularly in retinal tissue, could have a protective function. Bovine and rat retinas have been shown to express both estrogen receptor isoforms (ERα and ERβ) throughout the retinal thickness but particularly in the ganglion cell layer, and also in humans ERβ protein has been localized in the ganglion cell layer [117, 173]. Other than as signals molecules - acting stimulating the expression of antioxidant enzymes - estrogens can achieve a neuroprotective function working directly as potent antioxidants by quenching free radicals and terminating their propagation [117, 173]. Moreover, 17β-estradiol has been shown to be involved in brain metabolism regulation stimulating glucose import and catabolism through an increased expression of glycolytic enzymes and pyruvate dehydrogenase subunits [174]. Furthermore, 17β-estradiol may as well regulate mitochondrial metabolism stimulating Complex IV subunits expression resulting in an enhancement of energetic efficiency [174]. The double effect of estrogens in oxidative stress protection and energy metabolism stimulation might represent a discriminating factors contributing to the increased penetrance of LHON in males.

2 Autosomal Dominant Optic Atrophy

Although the relationship between mitochondrial morphology and function has not yet been elucidated, evidence shows that defects in mitochondrial morphology could be associated with alteration of the energy production system. To better understand this relationship we have studied, in collaboration with Professor Lodovica Vergani of Padova University, the mitochondrial function in fibroblasts isolated from patients carrying a novel mutation in the OPA1 gene. The mutation generates a truncated form of Opa1 protein and is associated with a particular ADOA phenotype, characterized by muscular atrophy and optic nerve degeneration. Skeletal muscle biopsies analysis has shown the presence of subsarcolemmal and intermyofibrillar aggregates of mitochondria with increased size variability and focal cristae abnormalities suggesting that the mutation deeply affect mitochondrial morphology. On the contrary, unlike reported for a different OPA1 mutation [139], the mitochondrial DNA content was similar in ADOA compared to controls. Mitochondrial morphology has been further studied through confocal microscopy analysis in fibroblasts and in myotubes differentiated from myoblast isolated from muscular biopsy. The use of mitochondria targeted red fluorescent protein (mtRFP) has shown that a significant proportion of OPA1-mutated fibroblasts presented fragmented mitochondria, appearing as spheres or very short rods compared to interconnected tubules seen in control fibroblasts. Aberrant mitochondrial shape has been observed also in differentiated myotubes that showed a tubular mitochondrial network alternated with areas of fragmented mitochondria, even in the same cell. Moreover, spatial distribution of mitochondria was irregular in ADOA myotubes with areas of abnormal mitochondrial clustering while other areas were almost completely devoid of mitochondria [170].

To verify whether the observed organelle shape changes were a primary consequence of OPA1 pro-fusion activity loss, we performed a mitochondrial functional analysis in fibroblasts isolated from two ADOA patients [170], in order to verify if energy failure could contribute to the pathogenic mechanism that cause the optic nerve degeneration associated to Opa1 mutant form.

ATP synthesis rate

To study the role of OPA1 mutation on mitochondrial function we measured the ATP synthesis rate driven by Complex I in resting fibroblasts grown under different metabolic conditions. Besides the standard growth medium containing glucose *[fig.14a]* we tested the oxphos efficiency substituting glucose with galactose, in presence of pyruvate, to force cells to rely on oxidative phosphorylation for energy production. Galactose induces a down regulation of the glycolytic pathway because it is very slowly converted to glucose-6-phosphate *[fig.14b]*. In this experimental condition the presence of gramicidin further stress the mitochondrial metabolism: indeed, this ionophore forms selective cations channels inducing a higher energy-demand to maintain cell homeostasis activating the plasma membrane Na^+/K^+ ATPase *[fig.14c]*.

Although in all culture conditions no statistically significant differences have been found between ADOA and controls fibroblasts, our results indicate that the higher is the energy produced by oxphos, the lower is the ATP synthesis

figure 14
Complex I-driven ATP synthesis rate.
Complex I-driven ATP synthesis rate measured in fibroblasts of both controls and ADOA patients cultured in 25 mM glucose-110 mg/l pyruvate medium (a), 5 mM galactose-110 mg/l pyruvate medium (b) and 5 mM galactose-110 mg/l pyruvate+40 ng/ml gramicidin medium (c).

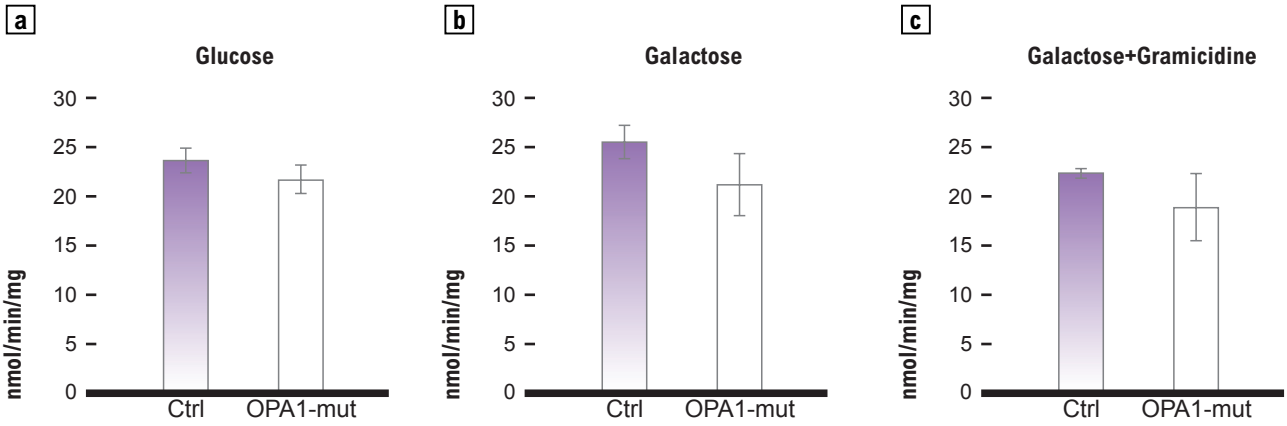
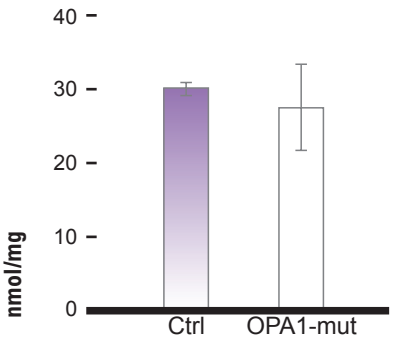


figure 15
ATP content.
ATP content in fibroblasts of both controls and ADOA patients cultured in 5 mM galactose-110 mg/l pyruvate medium for 72h.



rate in ADOA compared to controls. As shown in *fig.15* the slight difference observed in ATP synthesis rate in fibroblasts grown in galactose medium does not determine decreased energy availability in ADOA fibroblasts that show a similar ATP content compared to controls.

Mitochondrial membrane potential

Dysfunctions of both mitochondrial respiratory chain and ATP synthase, and integrity of the inner mitochondrial membrane were also investigated in both state 3 and 4 respirations using rhodamine-123. The mitochondrial membrane potential (ΔΨ) was measured by the fluorescence quenching of the potentiometric probe. In galactose/pyruvate medium ADOA cells show a slightly increased ΔΨ that is not statistically significant either during active oxidative phosphorylation (state 3) or when the ATP synthase is inhibited by oligomycin (state 4) *[fig. 16 a,b]*.
Moreover the mitochondrial membrane potential in different respiratory conditions has been evaluated with TMRM using fluorescence microscopy. Confirming the results obtained with the spectroflourimetric method, no significant difference between controls *[fig. 17 a, b]* and patients *[fig. 17 a1, b1]* was observed in any tested culture condition.

figure 16

Mitochondrial membrane potential.

RH-123 steady-state fluorescence quenching (F.Q.) mean values measured in galactose-grown fibroblasts of both controls and ADOA patients under state 3 (a) and 4 (b) respiratory conditions.

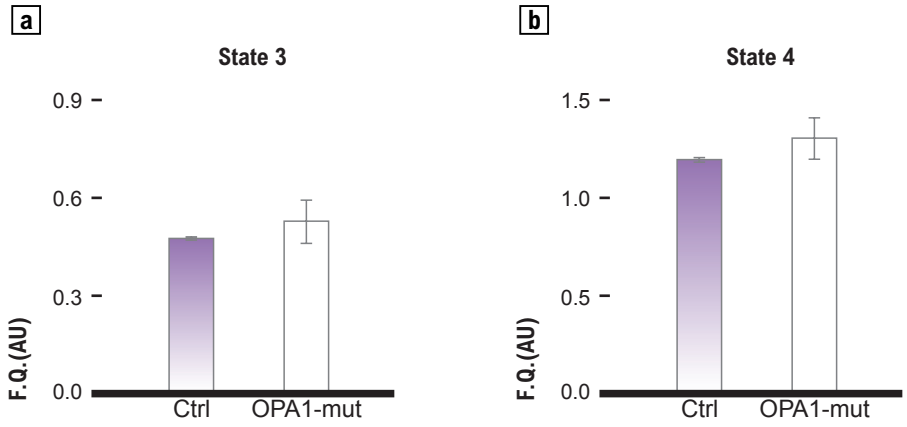
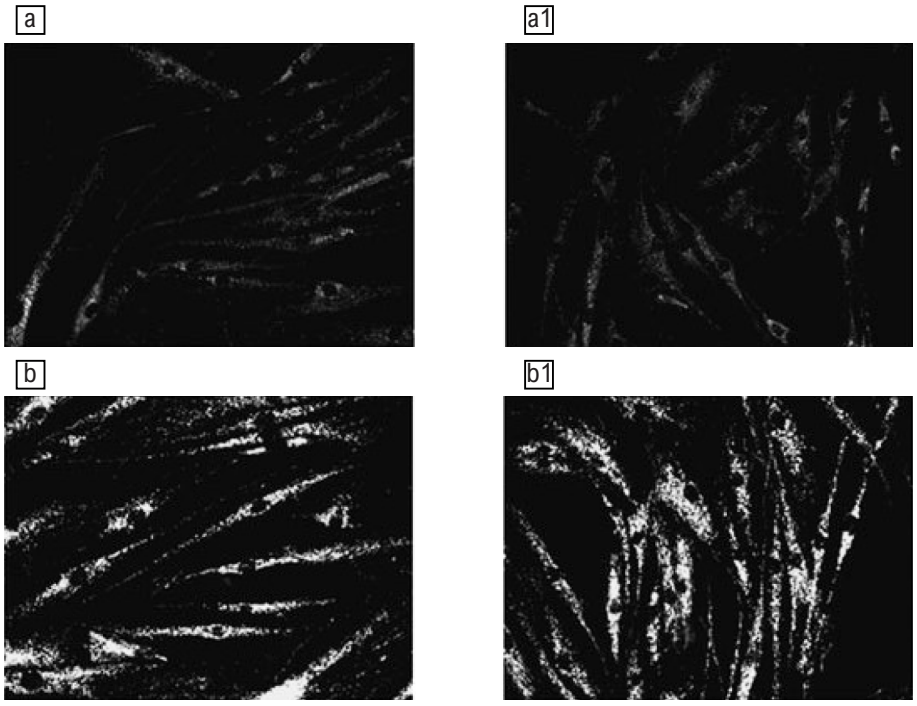


figure 17

Mitochondrial membrane potential.

Images of fluorescence microscopy of control and ADOA fibroblasts maintained in galactose/pyruvate medium. Mitochondrial membrane potential was measured by means of TMRM probe under different metabolic state. State 3 of respiration: a) control , a1) ADOA fibroblasts. State 4 of respiration b) control, b1) ADOA fibroblasts.



Discussion

The results reported concern an ADOA family whose members harbor a new OPA1 deletion affecting the GTPase domain. Patients studied developed the "classic" ADOA, characterized by childhood onset and slow progression, but a depth muscular examination showed subclinical neuromuscular involvement. Based on the mitochondrial function analysis we concluded that, in these patients, the primary defect is determined by mitochondrial network disruption without any appreciable impairment of the oxidative phosphorylation system.
In order to rule out also latent deficiency in the mitochondrial energy metabolism we evaluated ATP synthesis rate and membrane potential in standard growth medium and in media determining a more challenging environment in terms of cellular energy demand.
Regarding ATP synthesis rate we did not find significant differences between ADOA and control fibroblasts even though a trend toward increased reduction in ADOA samples is observed when fibroblasts are grown in absence of glucose or in the medium containing gramicidin. These results indicate that under stressing metabolic conditions, it means when high mitochondrial efficiency is required, cells containing an altered form of Opa1 are probably more vulnerable and this could contribute to explain the particular susceptibility of retinal ganglion cells to these genetic defects.
To confirm these results we also evaluated the mitochondrial membrane potential in different cellular metabolic conditions. In order to detect also latent mitochondrial dysfunction oligomycin has been used during membrane potential monitoring. Cells with impaired respiratory chain complexes might maintain the electrochemical mitochondrial membrane potential by the reversal function of ATP synthase that uses the energy deriving from ATP hydrolysis to pump proton into the intermembrane space. In this case, blocking the ATP synthase proton channel with oligomycin results in a drop of membrane potential as opposed to the expected increase that follows ATP synthesis inhibition in healthy mitochondria. Notably and accordingly with our results, in both control and patients fibroblasts and myotubes, oligomycin treatment resulted in an increase in both TMRM and RH-123 fluorescence, indicating that also in presence of this OPA1 mutation membrane potential is actively maintained by proton pumping of fully functional respiratory chain complexes.
Unlike reported by Amati-Bonneau *et al.* [139], which found mtDNA multiple deletions in patients with a particular syndromic ADOA phenotype characterized by neurological and myophatic signs, in our case the OPA1 mutation does not affect mtDNA stability in skeletal muscle. Our results are in disagreement with data reported by Zanna *et al.* [140] showing decreased Complex I-driven ATP synthesis in ADOA fibroblasts grown in glucose-free medium. The different results obtained studying OPA1 mutations affecting different gene region could be due to the possible capability of Opa1 to regulate mitochondrial functions in different ways and through interaction with different partners.
Likely, different OPA1 mutations could differently affect mitochondrial capability to produce energy as well as mitochondrial DNA stability: on the basis of this hypothesis the possible involvement of tissues different from the optic nerve - the classical target of ADOA - could also be explained in ADOA patients. Anyway, energy failure cannot justify the standard and most common ADOA phenotype in which retinal ganglion cells are specifically affected. Therefore, it could be hypothesized that dysfunctions in Opa1 activity primary act impairing

mitochondrial functions that are not directly linked to energy production.
Indeed, in fibroblasts and myotubes grown in standard medium, this new OPA1 mutation induces a collapse in the mitochondrial network providing the clear indication that abnormal mitochondrial morphology and distribution could have a basic role in ADOA pathogenesis. Interestingly, in myotubes but not in fibroblasts, OPA1 mutation leads also to altered mitochondrial distribution: the different behavior could be due to relative Opa1 abundance or isoforms expression other than to different mitochondrial network regulation in different tissues.
Particularly in optic nerve, altered mitochondria distribution per se could be cause of degeneration. The peculiar anatomy of optic nerve requires in fact an uneven mitochondrial distribution to match the different energy requirements in myelinated and non-myelinated regions. In retinal ganglion cells - whose axons form the optic nerve - a non-controlled distribution of even well functioning mitochondria could locally lead to insufficient energy supply, progressive axonal impairment and apoptotic cell death, while other tissues could be not so sensitive to alterations in mitochondrial morphology that are not directly linked to energy production defects.
The fact that mutations in mitofusin 2, another dynamin-related protein, are associated to type 2 Charcot-Marie-Tooth syndrome characterized by severe axonal peripheral neuropathy, sometimes with subacute optic neuropathy confirms that fusion protein activity is fundamental for neuronal cells function and could support the hypothesis that mitochondrial morphology could, in specific tissues, represent a basic factor for cell physiology.
Our results indicate that different OPA1 mutations may differently affect the protein function and suggest that an accurate exam of ADOA patients might reveal subclinical signs in different tissues, helping the understanding of the pathogenic mechanism that lead to slow degeneration of optic nerve.
Apparently, the mutation found in the pedigree analyzed acts primary impairing the mitochondrial fusion: the pathogenic mechanism might therefore derive from an excessive fission, a lack of complementation between mitochondria or from the inhibition of the interaction with motor protein involved in mitochondria subcellular distribution. Because of the slow progression that characterizes the pathology it can be inferred that the mutation in the OPA1 gene does not directly induce cell death but, more probably, a slight, chronic impairment of mitochondrial function that, with time, results in the apoptotic commitment of retinal ganglion cells.
The parallel study of LHON and ADOA could contribute to better understand the physiology of optic nerve, possibly favoring the development of therapeutic strategies aimed to mitigate or prevent the effect of these diseases.

3 Protection of cells with energy deficiency

As shown in the previous paragraphs defects on mitochondrial metabolism may have different and deleterious effects on cell physiology. Mitochondrial dysfunctions are acquiring great attention from the clinical area because of their recognized relevance not only in aging but also in age-related pathologies including cancer, cardiovascular diseases, type II diabetes, and neurodegenerative disorders [8].

The involvement of mitochondria in such detrimental pathologies enhances the necessity of standardization of therapeutic strategies capable of rescuing the normal mitochondrial function. However, primarily because of the poor knowledge of pathogenic mechanisms underlying these disorders, therapy for both primary mitochondrial diseases and late-onset neurodegenerative diseases is woefully inadequate.

Advances in mitochondrial biology and in pharmacology have facilitated the design of drugs targeted to mitochondria [62]. Although promising, this is still a young field, and there are concerns with “mitochondrial drugs” mainly due to our ignorance of the potential long-term toxic effects and our inability to regulate drug delivery to target tissues [59]. A promising gene therapy approach is the so-called “heteroplasmic shifting”, aimed at lowering the mutant mtDNA below the pathogenic threshold by different ingenious techniques, such as allotopic expression of the normal gene, but its applicability to humans appears remote.

Experimental evidence suggest that stimulating substrate-level phosphorylation to work overtime may be a viable approach to remedy the energy crisis due to oxphos impairment in yeast and mammalian cells exposed to ischemia and reperfusion [171]. Based on these data and in order to test a potential therapeutic approach for mitochondrial pathologies we applied this strategy to human cells. We evaluated the effect of exogenous substrates capable of stimulating the Krebs cycle flux while at the same time removing the excess of reduced pyridine nucleotides (NADH) in oxphos-deficient cells.

Thus, in order to propose an alternative treatment for energy deficiency-disorders, we tested the effect of substrates capable to stimulate the mitochondrial substrate-level phosphorylation on cell viability and energy availability in different experimental cell models, that were grown under different metabolic conditions. In fibroblasts, the energy defect was achieved by culturing normal cells in presence of oligomycin, a selective inhibitor of the ATP synthase complex.

Rescue of energy-deficient resting fibroblasts

We chose resting fibroblasts as experimental model in order to better mimic the metabolic state of slowly proliferating post-mitotic tissues, such as brain and muscle, that are most severely affected in mitochondrial diseases.

By maintaining human resting fibroblasts up to 72 h in glucose- and FBS-free medium, in which galactose (+ pyruvate) substituted for glucose, anaerobic glycolysis was almost abolished [175] but cell viability was fully preserved even when cells were exposed to gramicidin and then subjected to a metabolic condition of increased energy consumption [176] *[fig. 18 a, b]*.

In conditions in which signs of cellular distress are absent the addition of 5 mM α-ketoglutarate + 5 mM aspartate (from now on referred to as "substrates") to the medium does not show detectable effects.
In contrast, and as expected, adding to the medium low concentrations of oligomycin, the specific inhibitor of the F_0 component of ATP synthase complex, caused a sharp decrease of viable fibroblasts, about 95% of which were dead at 72 hours *[fig. 18 c]*. In the same culture condition the presence of substrates clearly show protective effect, reducing the mortality to less than 30%.
The protective effect of substrates was confirmed by the morphological analysis of cells cultured for 72h: resting fibroblasts in the galactose medium and forced to increased the rate of ATP synthesis by exposure to gramicidin (as above) showed normal morphology, in accord with the viability data *[fig. 19 a,b]*. However, when energy demand was increased and ATP synthase was inhibited by oligomycin *[fig.19 c]*, the fibroblasts showed marked morphological changes, including shrinkage and detachment from the dish, typical features of cell death. The supplementation of the growth medium with 5 mM α-ketoglutarate + 5 mM aspartate resulted in fibroblasts normal morphology retention *[fig. 19 d,e,f]*.

figure 18
Substrates rescue cell death in oligomycin-treated fibroblasts.
Cells were incubated in DMEM-enriched medium (a), in the same medium plus 40 ng/ml gramicidin (b), or in the same medium plus 40 ng/ml gramicidin and 0.6 nM oligomycin (c), as detailed in "Materials and Methods." Dotted lines (----) denote presence of the substrates in the culture medium.

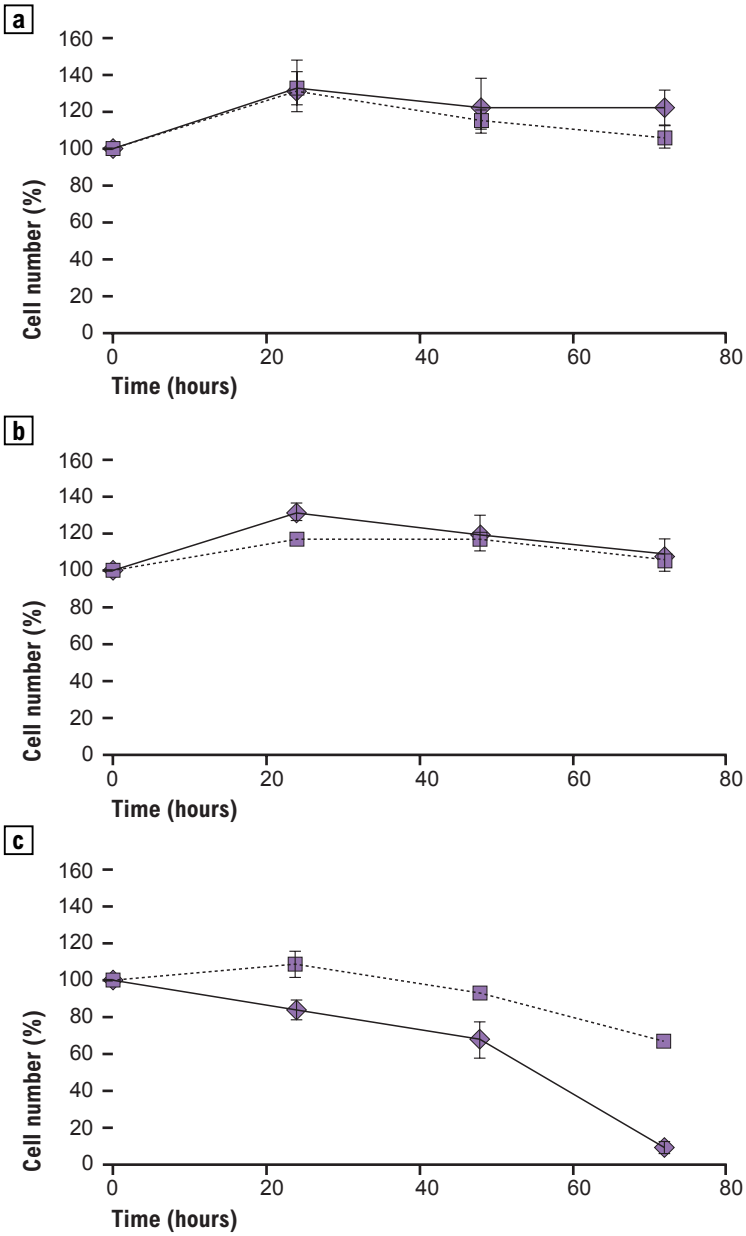


figure 19
Microscopical evaluation of fibroblasts viability.
Cells were incubated for 72 hours in DMEM-enriched medium (a) or in the same medium plus 40 ng/ml gramicidin (b) or 40 ng/ml gramicidin + 0.6 nM oligomycin (c), in the absence (a, b, c) or in the presence (d, e,f) of the substrates.

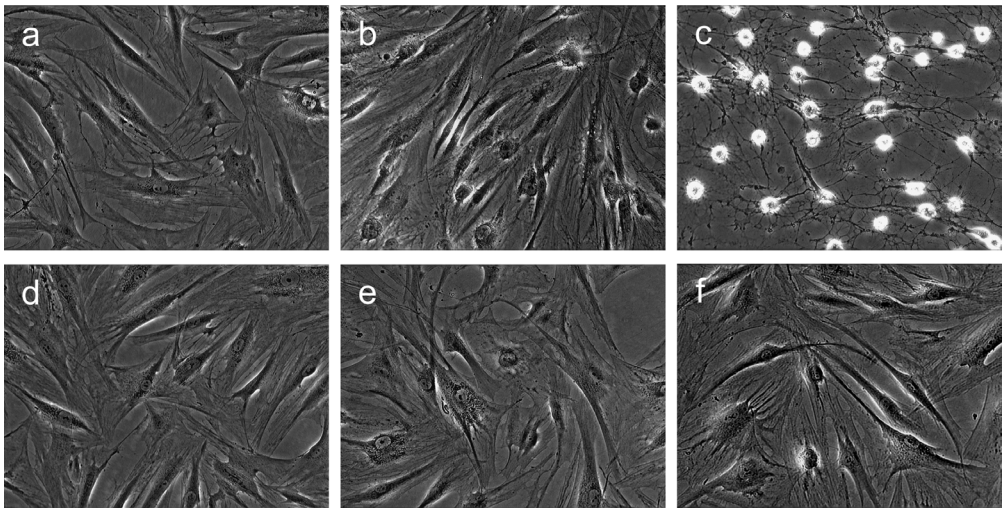
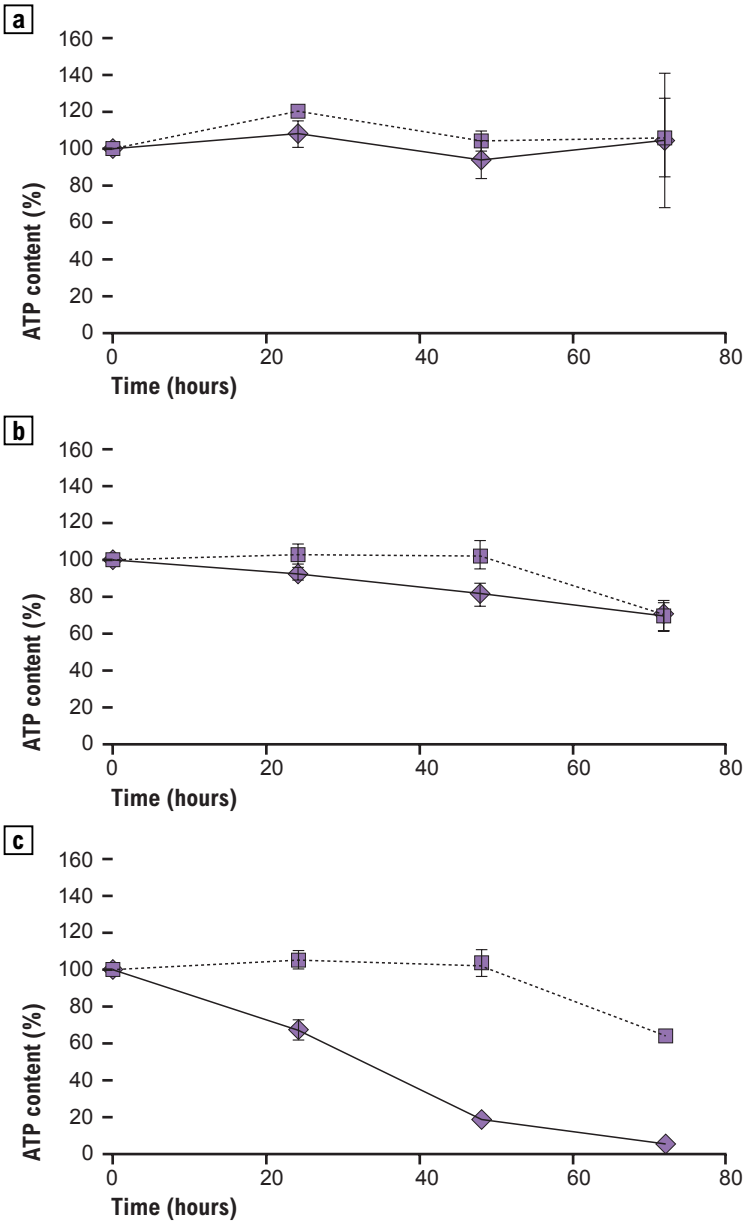


figure 20

ATP levels in resting oligomycin-treated fibroblasts.
Cells were incubated in DMEM-enriched medium (a), in the same medium plus 40 ng/ml gramicidin (b), or in the same medium plus 40 ng/ml gramicidin and 0.6 nM oligomycin (c). Total ATP levels were assayed every day by using a luminescent-based assay (see Materials and Methods). Dotted lines (----) denote presence of the substrates in the medium. At zero time, the ATP content of the fibroblasts was 18.56 +/- 2.50 nmol/mg protein.



To verify whether the protective action of substrates was due to their ability to supply ATP, we measured the total ATP cellular content and found that the addition of α-ketoglutarate and aspartate acts stimulating the mitochondrial substrate-level phosphorylation. ATP level was found sufficiently high for cell viability *[fig. 20]*. This was particularly evident in cells challenged by addition of both gramicidin, which stimulates the Na^+/K^+ ATPase, [176] and oligomycin *[fig. 20 c]*. Interestingly, the decrease of ATP preceded cell death, thus indicating cellular stress even when cell count and morphology were still normal *[fig. 18 c and fig. 20 c]*.

Effect of the substrates on cybrids homoplasmic for NARP T8993G and T8993C mutations

As addition of the substrates to the growing media showed a protective effect in fibroblasts with an artificially induced defect of ATP synthase, we investigated whether they could also prevent the death of homoplasmic cybrids carrying either one of two mtDNA mutations in the ATP 6 gene associated with NARP. NARP cybrids were grown in galactose-pyruvate containing medium in order to push the oxidative phosphorylation.

Substantial protection was observed only in the NARP T8993G cybrids *[fig. 21a and b]*. Time course showed, independently by substrates presence, normal growth of wild type cybrids. Conversely, after 3 days, about 75% of the T8993G cybrids survived in substrates-enriched medium whereas only about 10% survived in the absence of the substrates. Significantly, the decrease in ATP level of the T8993G cybrids paralleled cells death: residual ATP was nearly 10% after 3 days in the absence of substrates, whereas it remained above 80% when cells were grown in the presence of substrates *[Fig. 21c and d]*. In contrast, the growth curves of wild type and mutant homoplasmic NARP T8993C cybrids were similar *[Fig. 22a and b]*, indicating that also in a challenging metabolic environment the presence of the T>C point mutation does not affect significantly cell viability. Accordingly, the ATP content was also similar in wild type and mutant cybrids *[Fig. 22c and d]*. Thus, both growth and ATP content of wild type and NARP T8993C cybrids were unaffected by addition of the substrates to the culture medium.

figure 21

Viability and ATP content in wild type and homoplasmic T8993G mutant cybrids.

Viability (a,b) and ATP content (c,d) in wild type (a,c) and homoplasmic T8993G mutant (b,d) cell lines grew in DMEM-enriched medium. The dotted line (----) indicates presence of the substrates. At zero time, the ATP content was 9.70 +/- 0.99 nmol/mg protein in wild-type cells and 8.07 +/- 0.23 nmol/mg protein in homoplasmic mutant cells.

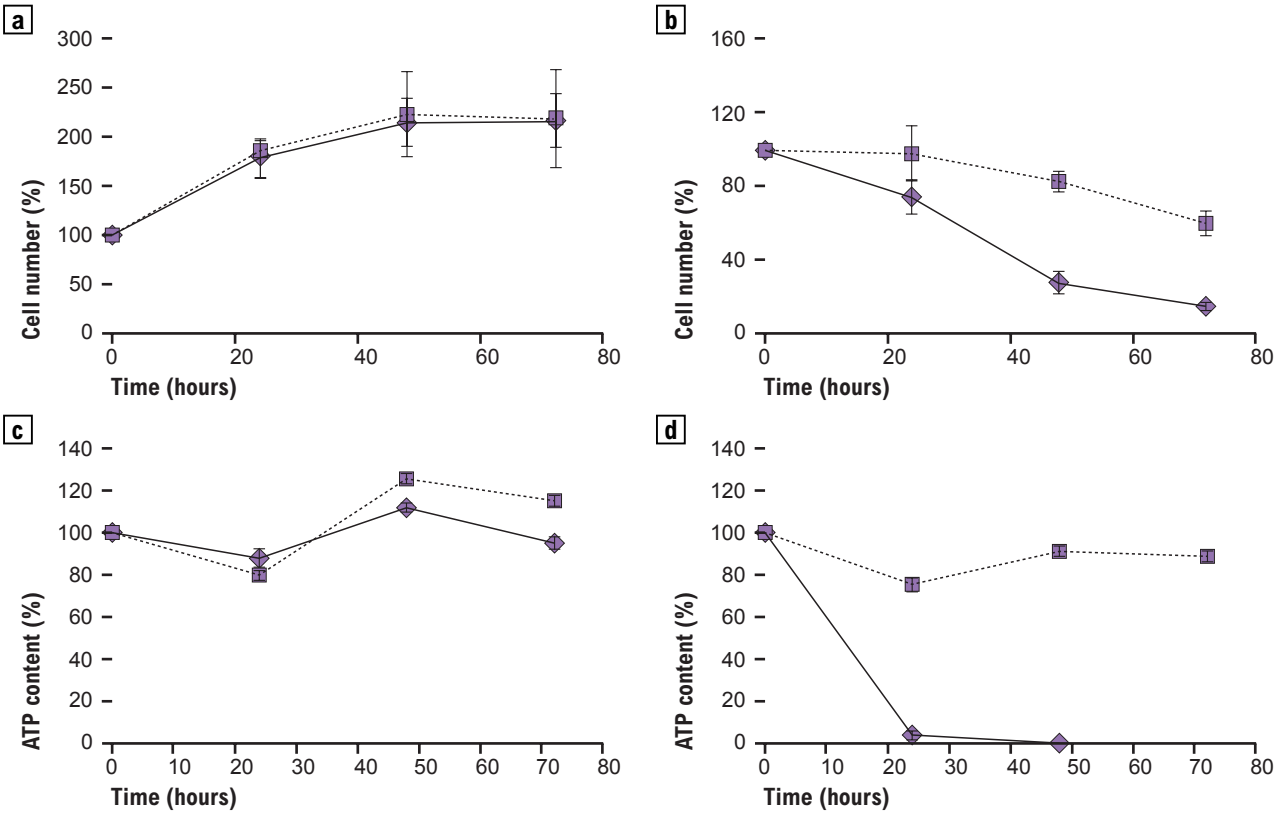
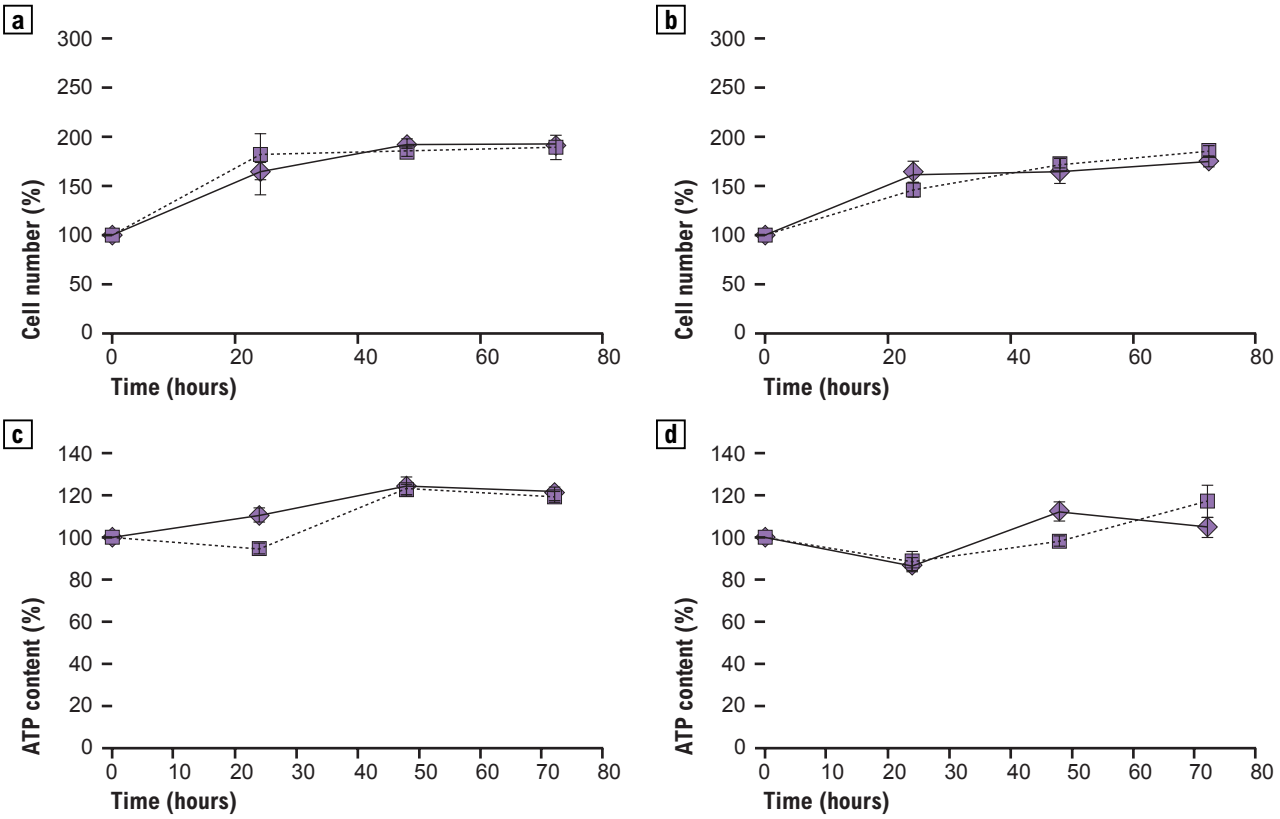


figure 22

Viability and ATP content in wild type and homoplasmic T8993C mutant cybrids.

Viability (a,b) and ATP content (c,d) of wild-type (a,c) and homoplasmic T8993C mutant (b,d) cell lines grew in DMEM-enriched medium. The dotted line (----) indicates presence of the substrates. At zero time, the ATP content was 8.33 +/- 0.64 mmol/mg protein in wild-type cells 8.25 +/- 1.39 nmol/mg protein in homoplasmic mutant cells.



Discussion

This study is based on the notion that cells with dysfunctional oxphos and energy deficiency have to maintain themselves on substrate-level phosphorylation in order to compensate for the mitochondrial defect. The limit of this potential rescue mechanism is that the substrate-level phosphorylation cannot supply sufficient ATP when energy demand is high. However it may supply a significant contribution to rescue oxphos-dysfunctional cells since the only glycolytic flux, if too high, will result in an increased ATP production through substrate-level phosphorylation that induces lactic acidosis, further impairing cell metabolism. It is thus clear that mitochondrial defects cannot be compensated simply potentiating the anaerobic glycolysis.

In Krebs cycle α-ketoglutarate stimulates the substrate-level phosphorylation enhancing the production of succinyl-CoA whose conversion in succinate results in phosphorylation of a GDP molecule that can, in turn, give an ATP molecule thanks to the action of the nucleoside-diphosphate kinase.

The beneficial action of α-ketoglutarate in a context of energy impairment would have limited effect. Cells with impaired ATP synthase are indeed characterized by an increased mitochondrial membrane potential that induces a reduction of the respiratory chain: the expected final result is an increase in the NADH/NAD^+ ratio, which would inhibit the α-ketoglutarate dehydrogenase reaction and possibly render the addition of the substrates useless. The presence of aspartate would prevent this stimulating the reoxidation of NADH, according to the following mechanism: α-ketoglutarate is imported into the mitochondrial matrix in exchange for malate, which derives from reduction of oxalacetate through NADH oxidation. Aspartate represents a source of oxalacetate and prevents the accumulation of NADH that can be consumed by malate dehydrogenase: the final result would be a stimulation of the α-ketoglutarate import into the mitochondrial matrix *[fig. 23]*.

Our results show that the presence of these exogenous substrates has beneficial effects in cells with energy deficiency deriving from ATP synthase impairment.

We tested the effectiveness of this strategy on resting fibroblasts cultured in a glucose-free medium in which energy deficiency was induced by growing cells in presence of oligomycin. Once demonstrated that the beneficial effect of substrates on cell survival was specifically due to an increased production of ATP through an anaerobic pathway, we evaluated the rescuing approach also in cybrids homoplasmic for either one of the ATP 6 mutations associated with NARP: T8993C and T8993G.

The different effect showed *[fig. 21 and 22]* by NARP mutations could be easily explained by the different impact that T8993C and T8993G mutations have on ATP synthase function. A previous paper published by our laboratory demonstrate that the T>C mutation causes only a marginal energy deficiency but a relatively high increase in ROS production [53], which might damage cells structure, induce the mitochondrial transition pore, and lead to apoptosis [177]. A similar correlation between severity of the mutation and pathogenic mechanism (impaired ATP synthesis vs increased ROS production) has been documented in cultured cells harboring mutations in coenzyme Q10-synthesizing enzymes [178]. Unlike T>C, T>G mutation acts primary through ATP depletion so that an increased substrate-level phosphorylation represents a reliable rescue mechanism for cells harboring this genetic variant of ATP 6 gene.

The lack of benefic effects observed in cybrids homoplasmic for the A8344G

tRNALys *[fig.24]* might further confirm that exposure to α-ketoglutarate/ aspartate achieves benefic effects only if the primary defect in cells is determined by impairment in energy production.

The pathogenic mechanism associated to mutations in mitochondrial tRNA genes is completely different form that of mutations in structural genes: alterations in tRNAs structure will indeed have deleterious effect on the entire mitochondrial translational machine leading to a general impairment of mitochondrial metabolism whose consequences certainly overcome the energy production ability. The short resistance showed by these cells in glucose-free medium - 100% mortality was reached in only 24h - is a clear index of how detrimental this mutation can be for mitochondrial activity.

Therefore, our results suggest that patients suffering for some forms of ATP synthase deficiency might benefit from dietary or pharmacological treatment based on supplementation of α-ketoglutarate and aspartate.

However, it cannot be ruled out that administration of α-ketoglutarate and aspartate would have positive effects also in patients with defects not directly affecting the ATP synthase: pyruvate dehydrogenase- and cytochrome c oxidase-related Leigh syndrome [58] or defects of complex II [179], resulting in

figure 23

Metabolic pathway for anaerobic ATP production by the supplemented substrates.

α-ketoglutarate (α-KG) is transferred by the oxoglutarate carrier to the mitochondrial matrix (32), where it enters the tricarboxylic acid cycle. Here, it is converted first to succinyl-CoA, then to succinate by the succinyl-CoA synthetase (A-SCS), with generation of ATP. Aspartate (Asp) enters the mitochondria (33), is transaminated to oxaloacetate (OAA), which is reduced to malate (reducing equivalents as NADH are removed from the matrix and NAD+ is regenerated to support the reaction catalyzed by the α-ketoglutarate dehydrogenase). After leaving the mitochondrial matrix, malate contributes to shuttling α-KG into the organelle. IMM = inner mitochondrial membrane.

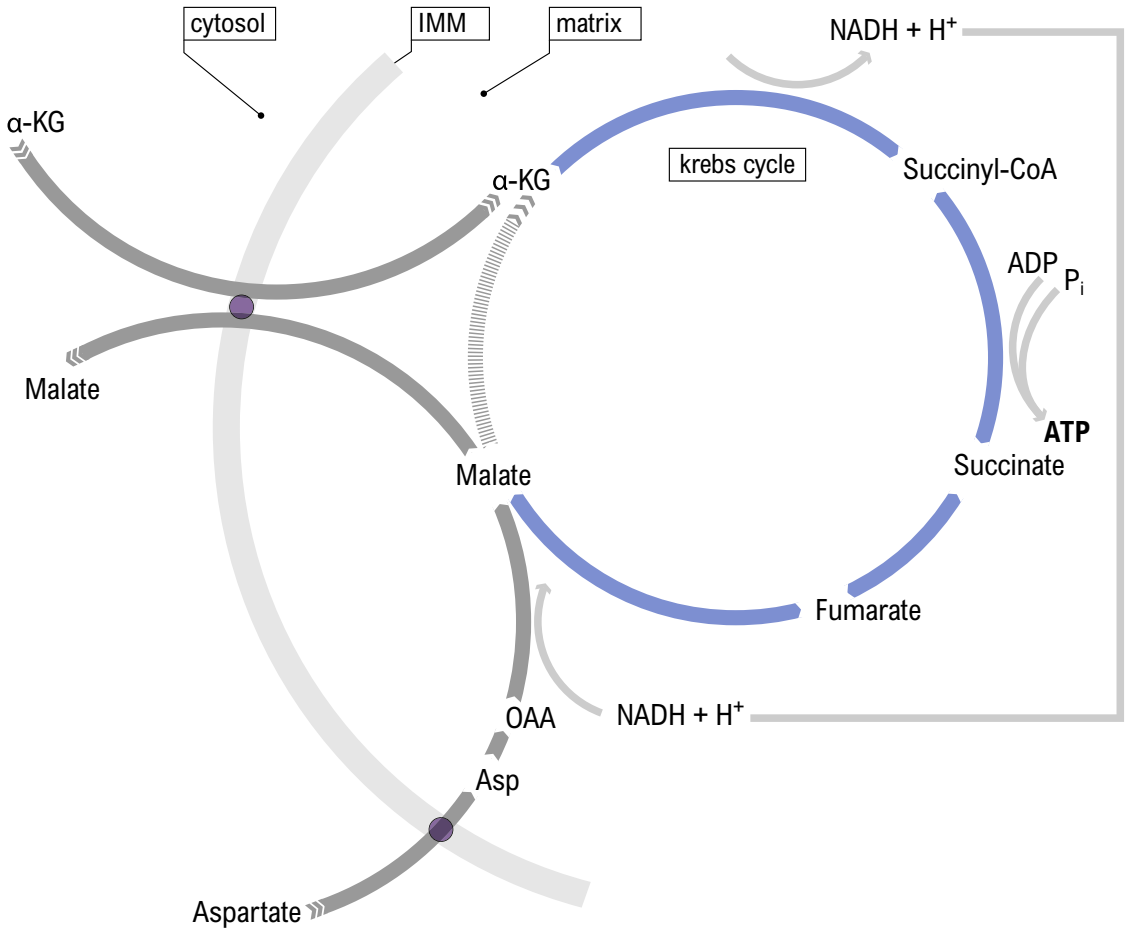
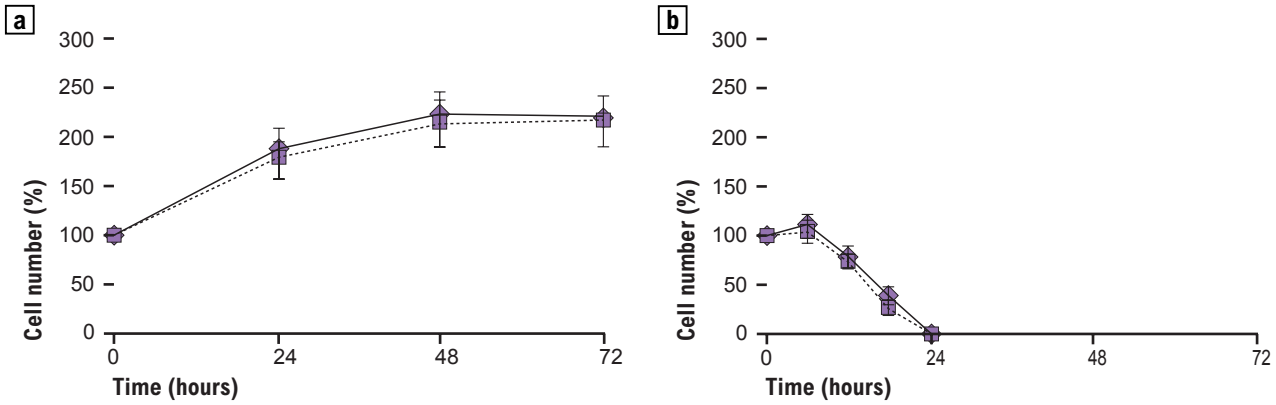


figure 24

Viability of MERRF cybrid cell lines.

Viability of wild type (a) and homoplasmic A8344G mutant (b) cell lines grew in DMEM-enriched medium. The dotted line (----) indicates presence of the substrates.



energy failure might as well benefit from this kind of treatment. Increasing the energy availability to the optic nerve cells could delay or mitigate the optic nerve atrophy, therefore the efficacy of substrate-level phosphorylation stimulation might also be evaluated in LHON patients, possibly in combination with an antioxidant therapy.

materials & methods

Projects Reference:

L *Leber Hereditary Optic Neuropathy*
A *Autosomal Dominant Optic Atrophy*
P *Protection of cells with energy deficiency*

Patients

L The characterization of the relation between SOD2 genotypes and MnSOD activity in control population has been carried out in 24 individuals aged from 20 to 50 years characterized for all SOD2 allelic combinations: seven alanine homozygotes (AA), ten heterozygotes (AV) and seven valine homozygotes (VV).
The same analysis, has been carried out also in two different experimental groups composed of four controls for each SOD2 genotype and four 3460/ND1 LHON patients for each SOD2 genotype.

A Patients reported in the ADOA chapter have been described in the AIM section.

Cells

L After the first biochemical characterization of SOD2 genotypes in control group we decided to proceed with the same analysis looking for a possible relationship between SOD2 genotypes and the biochemical effect of LHON primary mutations on oxphos efficiency.
We chose lymphocytes as experimental model for this study. PBMCs (Peripheral Blood Mononuclear Cells) were obtained by Ficoll-Hypaque Plus centrifugation of EDTA-treated blood, washed twice in PBS (Gibco) and resuspended in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 50 IU/ml penicillin and 50 mg/ml streptomycin. PBMCs (1×10^6 cells/ml) were cultured in T-75 flasks for 3 h at 37 °C in a humidified incubator. Lymphocytes (non-adherent cells) were then harvested, pelleted, resuspended (2×10^6 cells/ml) in fresh medium and cultured for 48 h.
Cells resuspended in 50 mM KH_2PO_4 pH 7.8 containing 0.1% bovine serum albumin and the protease inhibitor cocktail were exposed to sonic oscillation for 45 s, in 15 s burst, at 4°C. The total superoxide dismutase activity was then measured in the supernatant obtained after centrifugation at 100000 g for 30 minutes.

A For the biochemical characterization of OPA1-mutated fibroblasts cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose, 110 mg/l pyruvate and 4 mM glutamine supplemented with 15% Fetal Bovine Serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (standard medium) and incubated at 37°C in 5% CO_2 atmosphere.
Mitochondrial membrane potential was measured in fibroblasts grown in a glucose-free medium supplemented by 5 mM galactose, 110 mg/L pyruvate and 15 % dyalized FBS. ATP synthesis was measured in fibroblasts grown in standard medium, in galactose medium and in galactose medium in presence

of 40 ng/ml gramicidin devoid of dyalized FBS for 48h .

P We studied the effect of exogenous substrates in human fibroblasts and cybrid lines containing mtDNA derived from NARP and MERRF [180] patients, as previously described [181]. Control fibroblast cell lines were established from normal subjects with informed consent using standard techniques. Briefly, skin biopsies were seeded in Dulbecco modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 110 mg/l pyruvate and 4 mM glutamine supplemented with 20% fetal bovine serum (FBS), 100 Units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B, and were incubated at 37 °C in the presence of 5% CO_2 until fibroblasts grew out of the biopsies. Cell lines were then expanded in complete medium supplemented with 15% FBS. NARP and MERRF cybrids were grown in DMEM containing 4.5 g/l glucose, 110 mg/l pyruvate and 4 mM glutamine supplemented with 10% FBS, 100 Units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B, and only for MERRF cybrids 50 µg/ml uridine, incubating the cells at 37 °C in the presence of 5% CO_2.

Extraction of human platelet-enriched blood fraction (HPBF)

L Erythrocytes from venous peripheral blood were sedimented for one hour at in 5% dextran 250.000, 0.12 M NaCl, 10 mM EDTA pH 7,4. The fraction containing leukocytes and platelets was then centrifuged at 5500 rpm for 10 minutes. The pellet was subjected to hemolysis and centrifuged at 10500 rpm for 10 minutes.

Extraction of soluble fraction from HPBF

L The pellet obtained was suspended in phosphate buffer (KH_2PO_4 50 mM pH 7,8, 0.3% BSA free fatty acid, Sigma proteases inhibitors cocktail), homogenated in electric homogenizer and exposed to sonic oscillation for 5 minutes, in 15 s burst. After sonication samples were centrifuged at 45000 rpm for 60 minutes and the supernatant collected for measurement of superoxide dismutase activity.

Mitochondria extraction from HPBF

L For mitochondria extraction the pellet obtained after hemolysis was suspended in HEPES-buffer (210 mM mannitol, 70 mM saccharose, 1 mM EGTA, 5 mM HEPES, 0,3% BSA free fatty acid, proteases inhibitors mix pH 7) and incubated with digitonin. From permeabilized sample, mitochondria were obtained by differential centrifugations. The discarded pellet, containing nuclei and membrane fragments, was used for genotypic characterization while the mitochondrial fraction (10-20 mg/ml in mitochondria buffer) was conserved at -80°C until use.
All isolation steps were carried out at 4°C.

Superoxide dismutase activity assay

L The activity of both MnSOD and CuZnSOD was measured in sonicated control soluble fraction of HPBF and in lymphocytes according to the nitroblue tetrazolium-based assay reported by Oberley and Spitz [182] at 560 nm and 25°C. The MnSOD activity was evaluated in presence of cyanide in order to avoid the contribution of CuZnSOD. CuZnSOD activity was obtained subtracting the MnSOD activity from the total activity measured in absence of cyanide. Since the MnSOD is a mitochondrial enzyme, comparison of its activity in control and in patient cells was done following normalization with respect to the activity of citrate synthase, which is a general marker for mitochondrial volume in cells.

Catalase activity assay

L The catalase activity was measured spectrophotometrically at 240 nm following the hydrogen peroxide decomposition according to Aebi et al. [183]; the cyanide-sensitivity of the enzyme was assessed in the presence of 0.2–0.4 mM KCN, as suggested by Heck et al. [184].

ATP synthesis

L A Cellular ATP synthesis rate was measured by the highly sensitive luciferin/luciferase chemiluminescent method. In order to permeabilize cells and minimize ATP synthesis by biochemical pathways other than oxidative phosphorylation, lymphocytes (20×10^6 cells/ml) were incubated for 3 minutes at room temperature with 60 µg/ml digitonin, 2 mM iodoacetamide, and the adenylate kinase inhibitor, P1, P5-di(adenosine-5′) pentaphosphate penta-sodium salt (25 µM), in 10 mM Tris (pH 7.4), 100 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA (ethylene glycol tetraacetic acid), 3 mM EDTA (ethylenediaminetetraacetic acid) and 2 mM $MgCl_2$. Complex I driven ATP synthesis was induced by adding 10 mM glutamate, 10 mM malate, 2 mM malonate and 0.5 mM ADP to the sample. Complex II driven ATP synthesis was induced by adding 20 mM succinate, 4 µM rotenone and 0.5 mM ADP to the sample.

The reaction was carried out at 30°C and after 3 min it was stopped by the addition of 80% DMSO (dimethylsulphoxide). Synthesized ATP extracted from the cell suspension was assayed by the luminometric method as previously described [185]: as a blank a sample that was not energized with glutamate/malate or succinate, but containing both 18 µM antimycin A and 2 µM oligomycin and 0.5 mM ADP, was used.

ATP synthesis rate in OPA1-mutated fibroblasts was measured in 1-2 x 10^6 cells grown in standard and energy-challenging medium as above reported

L Western Blot

Mitochondria were lysed in β-Mercaptoetanolo-SDS buffer and boiled for 2 minutes. Proteins were separated by SDS-PAGE in 8-16% gradient gel and

then blotted into a nitrocellulose membrane. The membranes were saturated overnight in blocking solution (KH_2PO_4 1 mM, NaCl 150 mM, NaH_2PO_4 3 mM pH 7,4 containing 0.05% Tween-20, 2% non-fat dry milk and 2% bovine serum albumin).
Primary (E1α rabbit polyclonal IgG-SantaCruz, MnSOD sheep polyclonal IgG-Calbiochem) and secondary (horse-radish peroxidase conjugated from SantaCruz and Calbiochem) antibodies were diluted in blocking solution and incubated for one hour at room temperature.
Non-bound antibodies were removed by washing in PBS-0.05% Tween-20 solution. Protein bands were detected by chemiluminescence method (ECL-GE Healtcare) with the Fluor-S MultImager System (BioRad). Spots were than analyzed by densitometry with Quantity One software (BioRad).
E1α subunit of mitochondrial pyruvate dehydrogenase complex has been considered as loading control for normalization of MnSOD monomers quantity in order to minimize differences in the extraction procedures. The ratio between the amount of MTS (mitochondrial targeting sequence)-MnSOD (26 KDa) and MnSOD (23 KDa) was quantified in the soluble fraction obtained from sonicated control HPBF by SDS-PAGE (16-19% gradient) and western blot.

A Mitochondrial membrane potential measurement

Fibroblasts were harvested after trypsinization, washed in PBS (1 mM KH_2PO_4, 0.15 M NaCl, 3 mM Na_2HPO_4) and resuspended in 0.25 M Saccharose, 10 mM Hepes, 100 μM EGTA, 2 mM $MgCl_2$, 4 mM KH_2PO_4 pH 7.4, in order to obtain a protein concentration of about 2-3 mg/ml. For each measurement protein estimation with Lowry method was carried out.
The steady state mitochondrial electrical potential of permeabilized fibroblasts was assessed at 25°C by means of the fluorescent cationic dye Rhodamine-123 (RH-123), which distributes electrophoretically into the mitochondrial matrix in response to the electrical potential across the inner mitochondrial membrane [186].
Briefly, cell suspension were incubated with an ADP regenerating system (5 U/ml hexokinase and 10 mM glucose), 33 nM cyclosporine A in order to minimize loss of membrane potential through permeability transition pore, 2 mM malonate, 0.4 mM ADP, 10 mM glutamate/10 mM malate and 50 nM Rhodamine-123 [187].
RH-123 fluorescence quenching (λ_{exc} = 503 nm; λ_{em} = 527 nm) was measured at 25°C after permeabilization with 20 μg/ml digitonin (state 3 respiratory condition). State 4 and uncoupled respiratory condition were induced by adding 0.4 μM oligomycin and 0.2 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) respectively.
Fluorescence micrographs (20x) of adherent permeabilized fibroblasts were obtained incubating the cells at 37°C for 7 minutes in respiratory buffer (0.25 M Saccharose, 20 mM Tris, 4 mM $MgSO_4$, 0.5 mM EDTA, 10 mM KH_2PO_4 pH 7.4) containing 20 μg/ml digitonin, 10 mM glutamate, 10 mM malate, 2 mM malonate, 0.4 mM ADP and 0.2 μM TMRM (tetramethylrodhamine methyl ester) in absence (state 3 respiration) and in presence of 0.4 μM oligomycin (state 4 respiration).

Cell survival

P Cell survival was assessed after growth in DMEM-enriched medium supplemented or not with substrates for 72 h. Cells were washed with PBS, trypsinized, collected, and, if necessary, diluted to 1×10^6 cells/ml. The cells were then incubated in air at room temperature with an equal volume of trypan blue dye and counted by three independent investigators (mean ± SD). The number of viable cells was expressed as percentage of survival relative to the number of cells counted at the beginning of the experiment.

Morphological analysis

P Fibroblasts were plated in 60 mm Petri dishes and cultured for 72 h. Petri dishes were washed once with PBS and the cells were immediately visualized with an inverted microscope (Olympus IX50 equipped with a monochrome CCD camera). Multiple high-power (20x) images were acquired, and cells were scored as dead if they appeared smaller or shrunken and brighter, indicating release from the dishes.

Measurement of ATP content

A P ATP content was determined by measuring the light emitted during the oxidation of D-luciferin catalyzed by luciferase in the presence of ATP [185] (ATP bioluminescent assay kit CLS II; Roche, Switzerland). Briefly, 30 µg of cell protein were suspended in 100 mM KCl, 10 mM Tris, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM EDTA, 2 mM $MgCl_2$, pH 7.4, and ATP was extracted in DMSO. ATP levels were quantified as nmol/mg protein and expressed as percentage of cellular ATP content at the beginning of the experiment (t = 0).

Citrate synthase assay

L A The citrate synthase activity was assayed essentially as described in Trounce et al. [188] by incubating samples in 125 mM Tris pH 8 with 0.2% Triton X-100, and monitoring the reaction at 30°C by measuring spectrophotometrically the rate of free coenzyme A (90 µM) release at 412 nm.
The citrate synthase activity has been considered as a general marker for mitochondrial volume in cells and its value was utilized for normalization of other mitochondrial activities.

Protein Content

L A P Sample protein content was assessed spectrophotometrically (λ= 750 nm) by Lowry's method [189] in presence of 0.3% (w/v) sodium deoxycholate. Bovine serum albumin was used as standard.

Statistical analysis

L A P All results are expressed as mean +/- standard deviation. Statistical significance of differences between sample populations was evaluated using one-way ANOVA followed by Bonferroni *post hoc* test.

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Abbreviations

ADOA	autosomic dominant optic atrophy
ANT	adenine nucleotide translocator
ATP, ADP, AMP	adenosine-5'-triphosphate, -diphosphate, -monophosphate
BN-PAGE	blue native polyacrylamide gel electrophoresis
DCA	dichloroacetate
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMT	electron microscopy tomography
ETC	electron transport chain
FADH2	flavine adenine dinucleotide, reduced form
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FMN	flavin mononucleotide
HPBF	human platelet enriched blood fraction
IMM	inner mitochondrial membrane
LHON	Leber hereditary optic neuropathy
MELAS	myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy with ragged red fibers
MIDD	maternal inherited deafness and diabetes
MILS	maternal inherited Leigh syndrome
mtDNA	mitochondrial deoxyribonucleic acid
MTS	mitochondrial targeting sequence
nDNA	nuclear deoxyribonucleic acid
NADH	nicotinamide adenine dinucleotide, reduced form
NARP	neuropathy ataxia and retinitis pigmentosa
NBT	nitroblue tetrazolium
OMM	outer mitochondrial membrane
PBS	phosphate buffered saline
RET	reverse electron transfer
RGC	retinal ganglion cell
RH-123	rhodamine-123
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
SSBP1	single-stranded binding protein1
TMRM	tetramethylrodhamine methyl ester
UCP	uncoupling protein
VDAC	voltage dependent anion channel

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