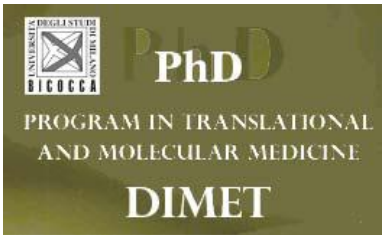

DARIA DIODATO

Matr. N°. 745002

Identification and functional validation of new mtDNA and nuclear gene variants responsible for mitochondrial disorders

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A mia madre Francesca e mio padre Ernesto, che stimo immensamente e senza i quali tutto questo non sarebbe stato possibile.

A Luca, che ha meravigliosamente cambiato la mia vita.

Table of Contents

Chapter 1: General Introduction

CHAPTER 1

General introduction: Mitochondria and mitochondrial medicine

1.1 Mitochondria

Mitochondria are double-membrane organelles ubiquitous in eukaryotes and essential for survival. They are responsible for cell energy supply and have also been shown to play an important role in cell signaling, particularly in the apoptotic process (Letai et al, 2006). Mitochondria host different metabolic pathways, including β-oxidation, the Krebs cycle, and lipid and cholesterol synthesis. Their primary function is to produce adenosine triphosphate (ATP), the energy currency of the cell, through the Oxidative Phosphorylation System (OXPHOS).

Given their essential role in the human body, dysfunctions eventually occurring in different aspects of their functioning can result in a multitude of clinical conditions, often

configuring very severe disease phenotypes (Schapira, 2006).

1.1.1 Mitochondrial structure

Origin of mitochondria date back to primitive bacteria-like organism, which developed an endosymbiotic relationship with eukaryotic cells. Their capacity to generate ATP through OXPHOS made them the main intracellular energy source (Margulis et al. 1976).

Mitochondria density varies between different tissues depending on their energy demand. Thus, neurons, cardiac and skeletal muscle cells have a high density of mitochondria, and this explains their sensitivity to energy-dependent defects caused by mitochondrial dysfunctions. Mitochondria are surrounded by a double-membrane; the outer membrane is porous and allows diffusion of low molecular

weight substances between the cytosol and the intermembrane space (IMS). They have always been considered as non-communicative organelles, but recently an opposite true is emerging: they are involved in frequent fission and fusion events and in exchange of mtDNA (Margineantu et al, 2002).

The inner mitochondrial membrane (IM) separates the IMS from the mitochondrial matrix and contains the complexes of the mitochondrial respiratory chain (MRC); it functions also as a barrier to ionic diffusion, thus allowing the formation of the proton gradient necessary to produce ATP. The mitochondrial matrix hosts several enzymes involved in different pathways (i.e. tricarboxylic acid-TCA- cycle and oxidation) and the mitochondrial deoxyribonucleic acid (mtDNA) (Fig.1).

1.1.2 Mitochondrial genetics

Mitochondria contain their own DNA, a circular double-strand molecule of 16569 base pairs found in multiple copies within the mitochondrial matrix. MtDNA is much smaller than most nuclear genes and extremely compact, containing no introns and small non-coding regions; it codes for 13 protein of the OXPHOS system and for 2 ribosomal and 22 transfer RNAs, necessary for the mitochondrial protein synthesis (Fig.1). Although human mtDNA encodes the rRNAs and tRNAs for protein synthesis, it completely depends upon the nucleus for the provision of the majority of structural MRC proteins and the enzymes for replication, repair, transcription and translation.

Recently has been shown that mtDNA co-localizes with various proteins responsible for

their maintenance in structures called nucleoids, thought to provide the perfect microenviroment to protect and repair the mtDNA molecules from insults (Garrido et al, 2003).

Fig.1 Mitochondrial structure (from Encyclopaedia Britannica, 1998)

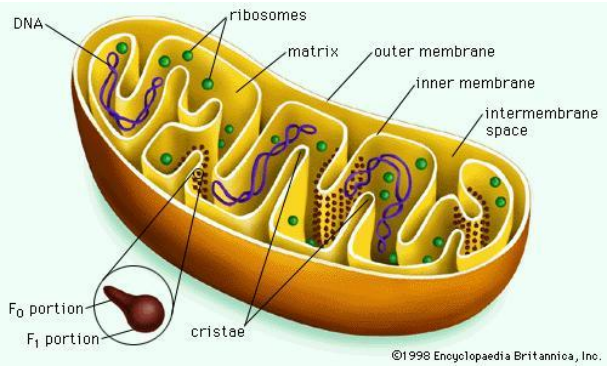
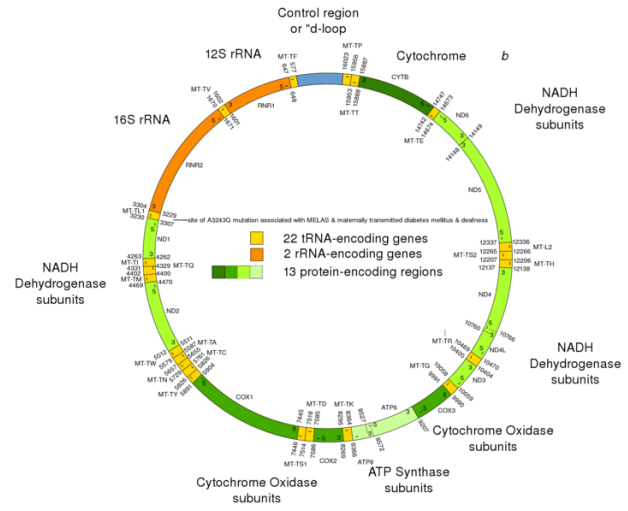


Fig.2 Mitochondrial DNA (from www.ganfyd.org)



MtDNA replicates continuously, in an independent manner from the nucleus, in dividing as well as in non-dividing cells (Chinnery and Samuels, 1999). It is transcribed polycistronically and translated by mitochondrial ribosomes.

MtDNA replication requires several nuclear encoded factors such as DNA polymerase gamma (POLG), mitochondrial transcription factor A (mtTFA), mt single-strand binding protein (mtSSB) and enzymes important for the supply of deoxynucleotides, such as thymidine kinase 2 (TK2) and deoxy-guanosine kinase (dGUOK).

After fertilization, sperm mtDNA is degraded; therefore mtDNA is inherited exclusively from the mother. Only in one case has been reported a paternal inheritance of a microdeletion in a mtDNA complex I gene (Schwartz and Vissing, 2002) but this possibility has been studied and excluded in several further studies (Filosto et al, 2003; Taylor et al, 2003).

There are thousands of mtDNA molecules per cell; these copies can be identical (a condition called *homoplasmy*) or differ for the presence

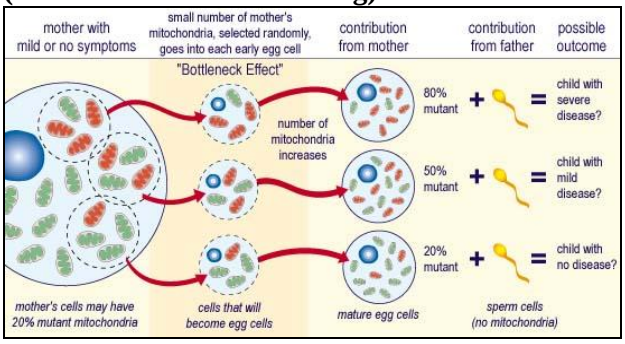
of one or more variants, inherited or accumulated during the ageing process (*heteroplasmy*). The percentage of a mutant variant (level of heteroplasmy) can vary between different cells and tissues, because mitochondria are randomly segregated at every cell division. A bottleneck effect is active in primordial female germinal cells (primary oogonia) when wild type and mutant mtDNA molecules are randomly partitioned between oocytes and drastically reduced. This means that in the final oocyte population some cells would carry a high percentage of mutation, others intermediate or low percentages, with important implications for disease severity in the offspring.

Neutral mtDNA variants (polymorphisms) are usually homoplasmic, whereas pathogenic changes are usually, but no invariably,

heteroplasmic. The percentage of mutated mtDNA will result in a pathogenic phenotype after exceeding a certain threshold, related to the energy demand of different tissues and organs (Schapira 2006, DiMauro and Schon, 2001) and variable amongst different mutations (Fig.3).

Leber hereditary optic neuropathy (LHON), the first disorder associated to mitochondrial DNA mutation (Wallace et al. 1988), is caused by homoplasmic mtDNA mutations.

Fig. 3 Mitochondrial mtDNA transmission (from www.mitoresearch.org)



1.1.3 Oxidative Phosphorylation System

Mitochondria main role is to generate ATP trough the oxidative phosphorylation system (OXPHOS). This is carried out by the MRC composed of five multiheteromeric complexes located in the IMM (complex I, CI; complex II, CII; complex III, CIII; complex IV, CIV or cytochrome c oxidase, COX; complex V, CV, or ATP synthase), and two mobile electron shuttles, ubiquinone (Coenzyme Q, CoQ), a lipoidal quinone, and cytochrome c (cyt c), a heme-containing small polypeptide (Fig.4). The electron flow through these components generates an electrical gradient ($\Delta\Psi$) and a pH gradient across the IMM. These gradients constitute the driving proton motive force for the phosphorylation of ADP to ATP, operated by CV, and for several other processes, such as

Ca^{++} import inside mitochondria, heat production and protein translocation.

Complex I (NADH-ubiquinone oxidoreductase) is the largest complex in the OXPHOS system, composed of ≈45 subunits with a total molecular mass of 1000kDa (Carroll et al. 2006). Seven subunits (ND1-ND6, ND4L) are encoded by mtDNA, the others by nuclear genes. It catalyzes the oxidation of NADH, derived from the oxidation of pyruvate, fatty acids, and amino acids, by ubiquinone (CoQ).

Complex II (succinate dehydrogenase ubiquinone–ubiquinol reductase) is the smallest MRC complex and the only membrane-bound member of the tricarboxylic acid (TCA) cycle. It is composed of four subunits all encoded by nuclear genes (SDHA, SDHB, SDHC, and SDHD). CII acts as a

succinate dehydrogenase (SDH) and catalyzes the oxidation and dehydration of succinate to fumarate; it takes part in the MRC by coupling this reaction to the reduction of ubiquinone to ubiquinol thus transfer electrons to CIII.

Complex III or ubiquinol-ferricytochrome c oxidoreductase holds one subunit, *cytochrome b*, encoded by the mitochondrial genome and 10 subunits encoded by the nuclear genome. CIII catalyzes the electron transfer from reduced $CoQH_2$, (ubiquinol) to cytochrome c.

Cytochrome c oxidase (COX, complex IV) is composed of thirteen subunits: the three largest ones are encoded by mtDNA genes (MTCOI, MTCOII, MTCOIII), while the remaining subunits are encoded by nuclear genes. CIV is the terminal component of MRC and transfers electrons from reduced cytochrome c to molecular oxygen. The

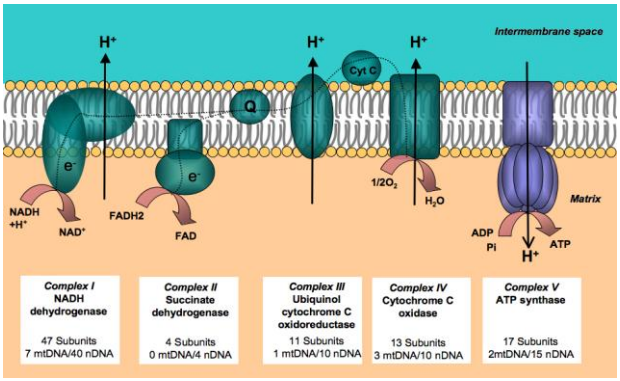
electron flow through CI, CIII and CIV is coupled to proton pumping across the IMM.

Complex V or ATP synthase converts the electrochemical gradient in ATP. It is composed of an integral membrane structure, Fo and a peripheral matrix-facing structure, F1 that contains the catalytic ATP synthase domain. F0 and F1 are physically connected to each otherby two additional structures: a centrally located, asymmetrical stalk and an externally tethered stator. All F1 subunits (a, b, g, d, e), and most of the Fo subunits (b, c, d, e, f, g, OSCP and F6) are nuclear encoded (Collinson et al. 1996). Only two proteins (MTATP6 and 8) are encoded by mtDNA (Boyer, 1993). Both MTATP6 and ATP8 are part of the Fo, connecting it to the stator.

Metabolic fuels feed reducing equivalents to the respiratory chain via glycolysis, fatty acid

or aminoacid oxidation. A key point in carbohydrate oxidation is pyruvate, which stands at the crossroads of glycolysis, gluconeogenesis and OXPHOS, and goes into TCA cycle via the pyruvate dehydrogenase complex (PDHc) that generates acetyl-CoA. In the mitochondrial matrix fatty acids are oxidized by β-oxidation, which again generates acetyl-CoA for the TCA cycle (Fig.). Reducing equivalents generated during the oxidation of the primary substrate or from the TCA cycle are transferred to OXPHOS as NADH (at the level of CI), or reduced flavins (entering at cII or cIII).

Fig. 4 Mitochondrial respiratory chain (from www.bioscience.org)



1.1.4 Other mitochondrial functions: ROS production and apoptosis

Complex V ultimately leads to the condensation of ADP and Pi into ATP and water. The stoichiometry between the two arms of OXPHOS, namely, respiration (oxidation) and phosphorylation (ATP synthesis) is indeed highly flexible and

controllable, varying according to the energetic demand of mitochondrion and cell. This notion is important to understand why ATP is just one of the options that mitochondria are capable to use to exploit the energy liberated (and stored) by respiration; other options are fundamental biological phenomena such as the production of heat, the storage and release of important ions, such as Ca^{++}, Mg^{++} and Fe^{++}, the biosynthesis of nucleotide precursors, e.g. uridine, and the generation of reactive oxygen species (ROS). During respiration a small percentage (1-2%) of molecular oxygen is not fully reduced to water but instead is reduced to the superoxide anion, O_2^-(or to hydrogen peroxide, H_2O_2), which can be converted to the highly reactive species hydroxyl radical (OH^-); together these oxygen derivatives are called reactive oxygen

species, ROS (Balaban et al. 2005). ROS are mainly generated at two sites in OXPHOS: CI and CIII. ROS can be harmful by damaging proteins, lipids and DNA through oxidation. To counteract the potential harmful effects of ROS, cells are provided with an anti-oxidant machinery, including enzymes such as the mitochondrial manganese superoxide dismutase and glutathione peroxidase (Nelson et al. 2004, Zeviani & Lamantea 2006), and anti-oxidant substances (Fig.5).
However, ROS also act as powerful signals that trigger a host of homeostatic adaptive mechanisms and metabolic pathways, thus playing an important role in the cell.

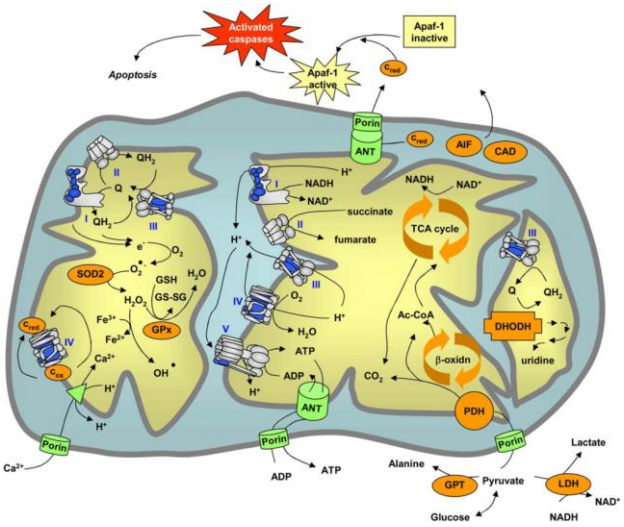
One of the most significant research advances in mitochondrial biology was the discovery in the past decade that mitochondria play an

important role in apoptosis, a fundamental biological process by which cells die in a programmed manner. This is an active process that, after permeabilization of mitochondrial membranes, leads to the release of potentially toxic proteins and to alterations of the mitochondrial physiological vital functions (Liu et al. 1996).

Several nDNA-encoded pro-apoptotic proteins including cytochrome c, endonuclease G, apoptosis inducing factor (AIF), and smac/DIABLO normally reside in the mitochondria and once these protein factors are released from mitochondria, they trigger a series of biochemical events that activate the apoptotic signaling cascades. Possibly the most well characterized apoptotic cascade is the activation of caspases by citochrome c and

ApafI (Yuan et al, 2003; Danial & Korsmeyer, 2004).

Fig.5 Mitochondrial energy metabolism



Schematic representation of mitochondrial energy metabolism and related pathways. I, II, III, IV, and V, complexes I-V. Complexes II, III, and IV are shown as functionally active dimers. b-oxidn, b-oxidation; PDH, pyruvate dehydrogenase complex; Ac-CoA, acetyl-CoA; DHODH, dihydroorotate dehydrogenase; GPT, glutamic pyruvic

transaminase; LDH, lactate dehydrogenase; AIF, apoptosis inducing factor; CAD, caspase-activated DNase; Q, coenzyme Q (ubiquinone); QH2, reduced coenzyme Q (ubiquinol); c-red and c-ox, reduced and oxidized cytochrome c. MtDNA-encoded subunits are shown in blue, membrane transporters in green, other enzymes and pathways in orange. Different metabolic pathways are depicted in different areas of the mitochondrial matrix and inner membrane compartments purely for illustrative purposes. The left-hand area shows Ca2+ transport and the respiratory chain enzymes that are involved in the production of ROS. The middle area shows the main mitochondrial energy pathways. The right-hand area shows the action of DHODH.
Components of the mitochondrial apoptotic pathway (AIF, cytochrome c, CAD) are located in the intermembrane space and, upon pro-apoptotic signalling, are released in the cytosol. (from Smeitink et al. 2006)

Mitochondria activate apoptosis also in a caspase-independent manner via the apoptosis-inducing factor; AIF that once released from mitochondria into the cytosol goes to the nucleus and induces chromatin condensation and DNA fragmentation through activation of

nucleases (Cande et al. 2002, Lipton & Bossy-Wetzel 2002).

1.2 Mitochondrial disorders

The Garrod's paradigm 'one protein, one enzyme, one defect' proposed for inborn errors of metabolism finds a remarkable exception in mitochondrial disorders because of the intricacy of the OXPHOS machinery. In fact, only a minor part of OXPHOS abnormalities are ascribed to primary mutations in the MRC complex subunits, whereas the majority is due to defects in the several pathways involved in RC formation, regulation or turnover.

In 1998 Wallace et al. described the first evidences of direct link between mitochondria and disease; they found an mtDNA mutation

causing Leber's hereditary optic neuropathy (Wallace et al. 1988). In the same year Holt et al. identified deletions in mtDNA causing myopathy (Holt et al. 1988).

Combined, genetic defects of OXPHOS have an incidence at approximately 1:5000 (Schaefer et al. 2004, Thornburn et al. 2004, Uusimaa et al. 2007, Mancuso et al. 2007), or even more (Cree et al. 2009). Therefore they have not to be considered "so rare" diseases.

Given the complexity of mitochondrial genetics and biochemistry, the clinical manifestations of mtDNA disorders are extremely heterogeneous (Di Mauro and Davidson 2005). The maxim "any tissue, any symptom, any age" well recapitulates all these concepts (Munich et al. 1996). They range from lesions of single tissues or structures, such as the optic nerve in Leber's hereditary

optic neuropathy (LHON), or the cochlea in maternally inherited non-syndromic deafness, to phenotypes characterized by broader and more severe presentations with multi-organ involvement including myopathies, encephalomyopathies, cardiopathies, or multisystem syndromes (Table 1). Concerning the onset, mitochondrial disease can present at every stage of life from the neonatal period to very old age.

The spectrum of mitochondrial diseases in children is wide, usually different from that found in adults, and new clinical features are continuously reported (Debray et al. 2008).
In infants and children, the most common clinical and neuropathological presentation is Leigh syndrome (LS). Affected patients show severe psychomotor delay, lactic acidosis,

respiratory abnormalities, dystonic features, cerebellar and pyramidal signs, abnormal ocular movements and often recurrent vomiting. Magnetic resonance imaging (MRI) shows a typical neuropathological pattern that characterizes this condition: lesions are present in the brainstem, thalami and posterior columns of the spinal cord (Leigh, 1951). Basal ganglia and posterior fossa structures may be also simultaneously involved. Ragged-red fibers are consistently absent in muscle biopsy. Leigh disease is a genetically heterogeneous entity, being variably caused by either mtDNA or nuclear gene mutations; in few cases can be X-linked, being caused by mutations in *PDH1a*, the gene coding the E1a subunit of pyruvate dehydrogenase complex. In general, childhood presentations of mitochondrial disease tend to be more severe

than those with adult onset. Renal disease is a prominent clinical feature of pediatric mitochondrial disorders, evident in mitochondrial depletion syndrome (Bourdon et al, 2007), complex III deficiencies (BCS1L mutations) (De Lonlay et al, 2001; De Meirleir et al, 2003) or mitochondrial aminoacyl-tRNA synthetase defect (Belostotsky et al, 2011). Hepatic dysfunction and haemopoietic stem cell failure are unusual features of adult-onset mitochondrial disease, whereas are more often present in children.

Generally, the diagnostic hypothesis of a mitochondrial disorder is made when signs of a multisystem involvement are present, like psychomotor delay or regression, deafness, optic neuropathy or peripheral neuropathy, cardiomyopathy, renal failure and so on (Table 1). Diagnostic criteria exist to classify clinical

cases as having probably, possibly or unlikely mitochondrial disease. Patients usually undergo to an extensive clinical and biochemical investigation; nevertheless the genetic defect often remains unknown.

Adult patients usually show signs of myopathy associated with variable involvement of the central nervous system (CNS) (ataxia, hearing loss, seizures, optic neuropathy or polyneuropathy, pigmentary retinopathy and, more rarely, movement disorders and cognitive deterioration). Other patients complain only of muscle weakness or wasting with exercise intolerance (Zeviani and Carelli 2003).

There has been a series of reports about clinical, biochemical and genetic characterizations of mitochondrial diseases; moreover an involvement of mitochondria has

become evident in many other clinical conditions including neurodegenerative disorders (Swerdlow 2009), cancer (Carew & Huang. 2002) and ageing (Reddy 2008). All these considerations led to the use of the term "mitochondrial medicine" to underline the central role of the mitochondrion in all biologic processes.

Table 1. Clinical and biochemical findings of mitochondrial disease in children (adapted from Debray et al. 2008)

(A) Signs and symptoms highly suggestive of a mitochondrial disease	**(B) Signs and symptoms compatible with mitochondrial disease**
Neurologic Episodic or progressive mental regression. Episodic neurological symptoms of unknown cause Cerebral stroke-like episode with nonvascular distribution of lesions Unexplained brainstem dysfunction (oculomotor changes, altered level of	**General** Failure to thrive; short stature Fatigue **Neurologic** Progressive or static developmental delay; encephalopathy Cerebral atrophy Seizures, especially myoclonic

consciousness, hypothermia or hyperthermia, hypotension or hypertension)
Brainstem involvement in MRI (Leigh syndrome-like)

Muscular
Myopathy with presence of ragged red fibres

Cardiovascular
Unexplained hypertrophic cardiomyopathy
Arrhythmia of unknown cause: heart block, and others

Ophthalmologic
External ophthalmoplegia with or without ptosis
Sudden or insidious optic neuropathy

Gastroenterologic
Unexplained liver failure (especially if valproate-related)
Severe intestinal dysmotility, chronic pseudoobstruction

Clinical biochemistry
Persistent elevation of blood lactate
Episodes of acidosis, ketosis or hyperlactatemia, exceeding the expected

Peripheral (usually axonal) neuropathy; unexplained spinal muscular atrophy
Cerebellar ataxia
Extrapyramidal movement disorders
Hypotonia or progressive spasticity
Leukodystrophy
Exercise intolerance with or without rhabdomyolysis
Migraine

Other
Ophthalmologic (optic atrophy, cataracts), pigmentary retinal degeneration
Sensorineural hearing loss; aminoglycoside-induced deafness
Sideroblastic anemia
Dermatological (hypertrichosis, pili torti, subcutaneous lipomas)
Endocrine (hypoparathyroidism, glucose intolerance, diabetes)
Dilated cardiomyopathy
Recurrent vomiting
Unexplained liver disease (fatty liver, hepatocellular lysis, cirrhosis)
Pancreatic insufficiency
Renal (tubular acidosis; renal Fanconi syndrome; unexplained glomerulopathy; nephrotic

physiological concentration; postprandial ketosis Characteristic MRS spectra (lactate)	syndrome)

1.2.1 Diagnosis of mitochondrial disorders

Since mitochondrial diseases are so clinically and genetically heterogeneous, their diagnosis could be quite tricky for the general practitioner or some clinicians. Multi-organ involvement, a hallmark of mitochondrial disease, may not be clear at the onset and tissue-specific forms of mitochondrial disease can progress very slowly, finally involving other systems in a protracted period of time. A defined genotype-phenotype correlation is not applicable to mitochondrial disorders, because different mutations in mtDNA or nuclear genes can result in the same phenotype, whereas the same mtDNA mutation, for

example the m.3243A>G, may give rise distinct phenotypes like MELAS (mitochondrial encephalomyopathy and lactic acidosis), MIDD (maternally inherited diabetes and deafness) and chronic progressive external ophtalmoplegia (CPEO).

Given their extreme clinical and genetic complexity, mitochondrial disorders require extensive investigations.

Clinical investigations: an accurate general and neurological examination is essential to detect brain, muscle and/or peripheral nerve damage, the presence of retinopathy and/or optic neuropathy, sensorineural deafness, renal or cardiac alterations. Blood tests are essential to detect abnormalities in blood cells count, diabetes, increase of liver enzymes or signs of renal failure. Furthermore is important to

assess possible endocrine dysfunctions and check thyroid or sexual hormones levels. A wide spectrum of abnormalities is present at the Magnetic Resonance Imaging (MRI) but often typical patterns of involvement, like lesions in the basal ganglia and brainstem, are seen. Sometimes Computerized Tomography (CT) scan is useful to detect basal ganglia calcifications.

Respirometry is an objective measure of respiratory muscles involvement and cardiac evaluation (electro- and eco-cardiogram) is extremely important to disclose conduction defects and/or signs of cardiomyopathy (hypertrophic or dilated). Electroencephalogram (EEG) is also important in the clinical assessment of epileptic conditions and electroneuromyography (ENMG) is a very

useful tool in the evaluation of neuromuscular involvement.

Lactic acidosis is a hallmark of mitochondrial dysfunction; acidosis derives from the reduction to lactate of pyruvate, which accumulates for the block of respiration. However the increase of lactate levels in blood may be absent in clear mitochondrial disease or present only during stress. Hyperlactatemia can also be caused by shock, hypoxia, sepsis, hypoperfusion, cardiac failure and inborn errors of metabolism, including some organic acidemias, disorders of gluconeogenesis and fatty acid oxidation. Liquoral or cerebrospinal fluid (CSF) lactate level is a more reliable diagnostic marker than blood lactate, above all in patients with brain involvement (Brown et al. 1988, Finsterer. 2001). Another reliable

tool is the proton magnetic resonance spectroscopy (H^+MRS) which allows non-invasive detection of elevated cerebral lactate and other relevant compounds (Haas et al. 2008).

Muscle histological and histochemical examination: analysis of muscle biopsy remains a very important step for the detection of mitochondrial disease, especially in adult patients. A typical morphological alteration is the presence of "ragged red fibers" (RRF). RRFs are characterized by the accumulation of abnormal mitochondria under the sarcolemmal membrane. The same aggregations can also be seen using succinate dehydrogenase (SDH) assay, that can be even more useful because complex II (subunits of which are encoded only by nuclear genome) is completely

unaffected by abnormalities of mtDNA. This assay can be combined with the COX reaction; a common finding in mitochondrial disorders is the presence of muscle fibers that stain negative to this histochemical reaction. In patients where only a very small number of fibers are COX deficient, sequential COX-SDH histochemical reaction is very useful to identify abnormal fibers that might otherwise be overlooked; COX-deficient fibers appear dark blue and are easily distinguishable from brown COX-positive fibers (Fig. 6). A global decrease in COX activity is seen in patients with nuclear DNA mutations affecting either COX subunits or one of the COX assembly factors such as SURF1 (Zhu et al, 1998). However, these typical "mitochondrial" findings may be absent in otherwise demonstrated mitochondrial disorders. This is

the case of LHON or Neuropathy Ataxia and Retinits Pigmentosa (NARP), and it is also true for many pediatric cases.

Biochemical investigations: in the suspect of a mitochondrial disorder measures of respiratory chain function should be carried out in muscle or cultured skin fibroblasts. Skin biopsy is a minimally invasive procedure and allows detecting biochemical defects in 50% cases (De Paepe et al. 2006, Janssen et al. 2007). If the biochemical readout of fibroblasts is negative, analysis should be carried out in muscle or liver biopsies. Both mtDNA and nuclear-determined respiratory chain defects may be tissue-specific because of the heteroplasmy of mtDNA mutations and tissue-specific levels of nuclear gene products (Antonicka et al. 2006). Although

measurement of mitochondrial enzyme activities is a key element in the diagnostic process, unfortunately protocols are often not standardized between different laboratories. Careful attention to rapid freezing or, wherever available, to rapid testing of fresh tissue, reduces artifactual decreases of respiratory chain activities.

Genetic and molecular analysis: the dual genetic inheritance (mtDNA and nuclear DNA) of mitochondrial diseases makes molecular investigation of these conditions complex and requires an accurate consideration of the clinical, histochemical and biochemical picture. Pediatric mitochondrial disorders are commonly due to recessive mutations in nuclear genes. MtDNA mutations account for about 25% of pediatric cases, but

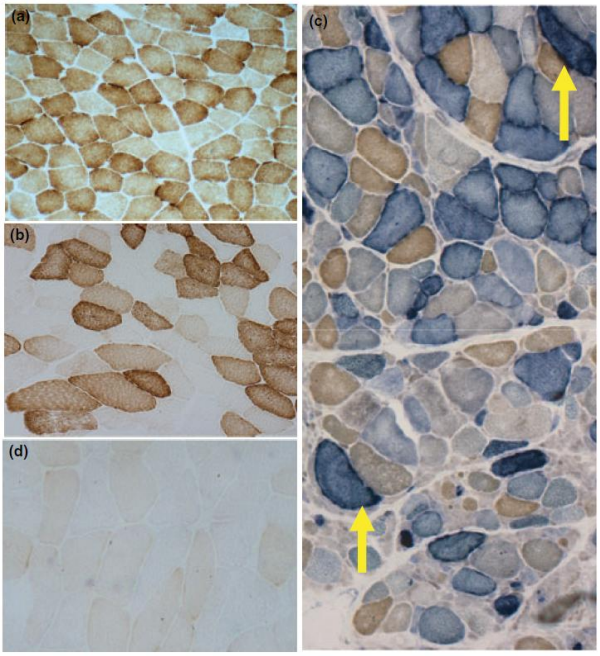
atypical presentations may lead to frequent misdiagnosis of this group.

Nuclear genetic defects are usually investigated in freshly extracted DNA from peripheral white blood cells. Blood could be less useful for detecting mtDNA mutations, because the level of heteroplasmy could be too low to be detected.

Skeletal muscle is the tissue of choice for molecular genetic analysis of mtDNA. This is because skeletal muscle is often an affected tissue, samples may be already available for enzymatic assays, and for some mutations the levels of heteroplasmy in skeletal muscle reflect those in other affected post-mitotic tissues such as the brain (Oldfors et al. 1995). Southern Blot analysis carried out in muscle DNA allows to identify single deletion, multiple deletions or depletion of mtDNA;

sometimes this technique fails to identify low levels of deletions and Long-Range PCR is useful in this case (He L et al, 2002).

Fig. **6** Muscle histochemistry in mitochondrial disorders



(a) COX activity demonstrates a normal variation between type 1 (oxidative) and type 2 (glycolytic) muscle fibers reflecting their relative density of mitochondria and principal

metabolic activity. (b) Both fiber types demonstrate a reduction in COX activity, but this is not uniform and results in mosaic pattern of dark and light brown fibres with some fibers barely visible. These extremely pale fibers are more readily observed when stained sequentially for COX then succinate dehydrogenase (SDH) activity (c). The latter produces a blue stain that is lost in the presence of adequate COX activity. Consequently COX-deficient fibers appear vivid blue on the dual COX⁄SDH stain. This mosaic pattern is typical of heteroplasmic mtDNA mutations affecting complex IV. Nuclear DNA or homoplasmic mtDNA mutations can result in a uniform reduction in COX activity (d) and this can be a useful diagnostic guide for further genetic studies (from Mc Farland and Turnbull, 2008).

1.2.2 Mitochondrial disorders due to mtDNA mutations

About 10-25% of mitochondrial diseases are caused by mutations in mtDNA (Mancuso et al. 2007, Mcfarland et al. 2004).

MtDNA accumulates mutations at a faster rate compared to nuclear DNA. In analyzing mtDNA variants we should take into account the fact that mtDNA is maternally inherited and two populations of mitochondrial genome

can coexist (heteroplasmy). A pathogenic mtDNA variant can be present at a variable percentage in different tissues, as a consequence of the bottleneck effect, and its phenotypic expression depends on the energetic threshold.

Mutations of mtDNA can be classified into large-scale rearrangements (deletions or duplications) and point mutations. MtDNA mutations can further be divided in those that affect mitochondrial protein synthesis (the ones that encompass rRNA or tRNA genes) and those that affect protein-coding genes. Both groups have been associated with well-defined clinical syndromes. While large-scale rearrangements are usually sporadic, point mutations are usually maternally inherited.

Large-scale rearrangements of mtDNA

Single, large-scale rearrangements of mtDNA comprise single partial deletions, or partial duplications. Deletions can vary in size from few bases to several kilo bases and be located in any part of the molecule. Rearranged molecules, lacking a portion of the mitochondrial genome, can be detected as an independent mtDNA species (single mtDNA deletion) or joined to a wild-type molecule or a mixture of the two rearrangements co-exists in the same cell (Zeviani et al. 1988; Poulton et al. 1989). The most common deletion is 5Kb long, and affects a region containing tRNAs and protein-coding genes.

Phenotypic presentation of single, large scale mitochondrial DNA disease can be divided into three main presentations: Kearns-Sayre syndrome (KSS, OMIM#530000**)** (Kearns and

Sayre, 1958), a multi-system childhood or teenage onset syndrome; chronic progressive external ophthalmoplegia (CPEO; Moraes et al., 1989), a milder presentation that involves mainly extraocular muscles with ptosis, but often can cause also widespread muscular weakness; and Pearson Syndrome (OMIM#557000) (Pearson et al., 1979), an often fatal infantile-onset disorder characterized by sideroblastic anaemia and exocrine pancreatic dysfunction, which may develop into KSS in individuals who survive infancy. This classification is often imprecise because the spectrum of mtDNA deletion disease is wide and variable: a mild progressive external ophtalmoplegia (CPEO) stay at the mild side of the spectrum, whereas severe clinical picture of "CPEO+multisystem" or KSS at the other

(Aurè et al, 2007). Different studies have tried to correlate mtDNA deletion size and position with age at onset and phenotype severity. Two recent studies found that the location of the mitochondrial DNA deletion was partly predictive of phenotype or age at onset, though with contrary conclusions (Yamashita et al., 2008; Lopez-Gallardo et al., 2009). In another recent paper (2013) Grady et al showed a significant correlation between mtDNA deletion features and disease severity.

Kearns–Sayre syndrome is characterized by the onset of chronic progressive ophthalmoparesis and pigmentary retinopathy before the age of 20 years. Other clinical features include cerebellar ataxia, proximal myopathy, heart conduction defects, cardiomyopathy, endocrinopathies, short

stature and an elevated CSF protein. In KSS and PEO, diabetes mellitus and hearing loss are frequent additional features, which may occasionally precede, even by years, the onset of neuromuscular symptoms (Shoffner et al. 1989). RRFs are invariably present at muscle biopsy.

Life expectancy is often considerably reduced, but with supportive care and early treatment of cardiac complications, long-term survival is still possible.

Some patients have duplication of mtDNA, which although might not be pathogenic themselves, could be an intermediate step toward the generations of deletions.

mtDNA point mutations

These mutations can be substitutions of single bases or micro-insertions/micro-deletions in the mtDNA molecule.

Mutations affecting mitochondrial protein synthesis are located in rRNA or in tRNA genes. Clinical experience has shown that these mutations are usually associated with multisystem disorders, lactic acidosis, and massive mitochondrial proliferation that appears as the presence of RFF in the muscle biopsy (Engel and Cunnigham, 1963). These fibers react intensively with the SDH staining but are usually COX-negative. The only exception to this rule is the **Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke Like Episodes** (**MELAS**, OMIM #540000). This syndrome is typically due to the A3243G mutation in the gene encoding

$tRNA^{Leu}$ *(MTTL1)*. Other point mutations associated with MELAS have been reported, although they are much rarer than the A3243G (Taylor & Turnbull 2005). MELAS syndrome is characterized by stroke-like episodes caused by focal cerebral lesions, often localized in the parieto-occipital regions of the brain, increased lactic acid levels in blood (and cerebro-spinal fluid, CSF) and "ragged-red" fibres in the muscle biopsy. Stroke-like episodes are usually paroxysmal events with no clear precipitant, but the role of recurrent focal seizures may be crucial and it remains unclear whether (subclinical) seizure activity invokes, or results from, a stroke-like episode. Other common signs are mental deterioration, recurrent migraines with "cerebral" vomiting, focal or generalized epilepsy, neurosensorial

deafness, short stature and endocrine dysfunctions.

The m.3243 A>G mutation is also associated to a condition known as **Maternally Inherited Diabetes and Deafness** (MIDD, OMIM#520000). MIDD can be also associated with the m.14709T>C mutation in the gene encoding the $tRNA^{Glu}$ (*MTTE*) or with the m.8396A>G mutation in the RNA^{Lys} gene (*MTTK*).

Patients with the A3243G mutation interestingly show a gradual progression towards insulin requirement irrespective of the level of heteroplasmy identified in urine or muscle (Whittaker RG et al, 2007). The m.3243 A>G mutations has been reported associated also with a pure myopathy phenotype and a MERFF/MELAS overlap syndrome.

Myoclonus Epilepsy with Ragged-Red Fibers (**MERRF**, OMIM#545000) is mainly due to the A8344G transition in the gene encoding $tRNA^{Lys}$. Numerous other point mutations of mtDNA have been associated with different clinical phenotypes in single patients or in a few families (Zeviani & Di Donato 2004). MERRF is typically characterized by myoclonus, epilepsy, muscle weakness, motor incoordination (ataxia) and sometimes, mental deterioration. Clinical manifestations can vary greatly even within the same family. This phenotypic variability is attributed to the level of heteroplasmy and to tissue distribution of the mutation.

The m.8344 A>G mutations has been associated also with Leigh syndrome.

Leber's Hereditary Optic Neuropathy (**LHON**, OMIM#535000) is a juvenile-onset disease affecting mostly males. Theodor Leber first reported the clinical condition in 1871, describing it as a 'familial neuro-ophthalmic disease' and 117 years later Wallace et al. could demonstrate that the majority of LHON families harbor the same mtDNA mutation (m.11778G>A). Numerous mtDNA mutations have subsequently been described in association with LHON, but three mutations (m.11778G>A, m.3460G>A and m.14484T>C) are present in at least 95% of families (Man PY et al, 2003). These mutations are located in complex I (NADH: ubiquinone-oxidoreductase) encoding genes *MTND4*, *MTND1* and *MTND6*, respectively. LHON is characterized by acute or subacute, painless loss of central vision due to rapidly

progressive optic atrophy. Vision loss can be partial or complete, usually permanent, and is the only consistent manifestation of the disease which, more rarely, may also include alterations in cardiac rhythm. The muscle biopsy does not show evidence of RRFs and is not recommended for the diagnosis of the disease. Other mutations, all present in complex I mtDNA genes, have been identified (Valentino et al. 2002; Valentino et al, 2004). All these mutations are usually homoplasmic or in high mutant heteroplasmic proportions.

Neurogenic muscle weakness, ataxia, retinitis pigmentosa (**NARP**, OMIM#551500) is a mitochondrial disorder characterized besides symptoms that forms the acronym of this disorder, also by epilepsy, and sometimes mental deterioration. NARP is

associated with mutation T8993G in the gene encoding subunit 6 of mitochondrial ATPase (MRC complex V). In patients presenting a milder NARP phenotype, a transition T->C in the same position has also been described. Symptoms usually appear in adulthood. Ragged-red fibres are absent in muscle biopsy.

Leigh syndrome (**MILS**, maternally inherited Leigh syndrome **OMIM**#256000). The T8993G mutation when present in >90% of heteroplasmy, leads to the dramatic phenotype of Leigh syndrome or Subacute Necrotizing Encephalomyelopathy. The onset of this condition is in the early infancy or childhood and the clinical picture is characterized by signs of CNS involvement like cerebellar and pyramidal signs, dystonia, seizures, respiratory abnormalities, incoordination of ocular

movements, and recurrent vomiting. Developmental delay and regression are prominent but nonspecific clinical features of this disorder; when they occur in conjunction with raised CSF lactate and are supported by the typical MRI pattern, the diagnosis of Leigh syndrome can be made.

Exercise intolerance is a common feature in patients with mitochondrial disorders, but it is often overshadowed by other symptoms and signs. We have learnt that exercise intolerance, myalgia, and myoglobinuria can be the unique presentation of respiratory chain defects. These can be caused by complex I, complex III, or complex IV defects, although they seem to be more commonly associated with complex III deficiency (Andreu et al., 1999).

1.2.3 Nuclear gene mutations

Nuclear gene defects related to mitochondrial disorders can be classified into four groups:

1) Defects of genes encoding structural or assembly factors of respiratory chain complexes.

2) Defects of genes altering the stability of mtDNA.

3) Defects of genes encoding factors involved in metabolic pathways influencing the biogenesis of mitochondria, including OXPHOS.

4) Defects of genes encoding factors involved in the biosynthesis of lipids and cofactors

In the first three groups are disorders that are clearly associated with a specific biochemical defect of one of the respiratory chain complexes. The fourth group includes protein products that play a role in different mitochondrial metabolic pathways indirectly associated with OXPHOS.

1.2.3.1 Defects of genes encoding structural or assembly factors of respiratory chain complexes

Complex I deficiencies

Complex I deficiency is the most commonly identified biochemical defect in the majority of centers, accounting for 25 to 30% of all mitochondrial disorders presenting in childhood. Mutations in mtDNA genes are responsible for 20 to 25% of CI deficiencies. Mutations have been reported in 13 of the 38

nuclear-encoded subunits causing in another 20-25% of individuals with CI deficiencies. The other 50% is believed caused by mutations in nuclear genes encoding for CI assembly factors. To date eight CI assembly factors have been described (Saada et al. 2008, Pagliarini et al. 2008, Saada et al 2009, Calvo SE et al 2010).

The clinical presentation of CI deficiency is usually a progressive neurological disorder, often Leigh syndrome, occasionally complicated by cardiomyopathy, or multisystem involvement.

Mutations in CI assembly cause a wide range of clinical disorders, ranging from lethal neonatal disease to adult-onset neurodegenerative disorders. Phenotypes include macrocephaly with progressive leukodystrophy, nonspecific encephalopathy,

cardiomyopathy, myopathy, liver disease, Leigh syndrome (Loeffen et al. 2000, Pitkanen and Robinson, 1996).

Complex II deficiencies

Complex II deficiency is a rare disorder, representing 2-8% cases of respiratory chain deficiency (Munnich et al. 2001, Ghezzi et al. 2009).

Mutations in *SDHA*, the gene encoding the flavoprotein subunit of SDH complex, have been associated frequently with Leigh syndrome, but have been shown also to cause infantile isolated cardiomyopathy or late onset neurodegenerative disorder with optic atrophy, ataxia and proximal myopathy (reviewed in Zeviani and Klopstock, 2001). Moreover different mutations in CII subunits (*SDHB*,

SDHC and *SDHD*) have been associated to tumor forms, such as paragangliomas and phaeochromocytomas. Recently, mutations in *SDHD* have been associated to an autosomal recessive encephalomyopathy with CII deficiency (Jackson et al, 2013).

Two assembly factors specific for CII have been identified, *SDHAF1* and *SDHAF2*, for SDH Assembly Factor 1 and 2. SDHAF1 mutations were identified in infants affected by a leukodystrophic encephalomyopathy and accumulation of succinate in the white matter (Ghezzi et al., 2009; Ohlenbrush et al, 2012), whereas a germ line missense mutation in SDHAF2 has been reported in two families with hereditary, multiple head and neck paraganglioma (PGL2, Hao et al, 2009; Bayley et al, 2010).

Complex III deficiencies

CIII defects are less frequent than CI or CIV deficiency, and are usually caused by mutations in mtDNA encoded CYB. Mutations in nucleus-encoded CIII subunits have been reported in *UQCRB*, also known as *QP-C*, *UQCRC2* and *UQCRQ* genes. A rearrangement in *UQCRB* has been associated with metabolic crises after fasting (reviewed in Fernandez-Vizarra et al., 2009). Mutations in *UQCRC2* have been found in three patients from a consanguineous Mexican family presenting with neonatal onset of hypoglycemia, lactic acidosis, ketosis, and hyperammonemia. A homozygous mutation in *UQCRQ* has been associated with severe infantile Leigh-like syndrome and profound CIII deficiency. Mutations in *BCS1L* gene, a cIII assembly factor, have been associated

with a broad spectrum of disease, ranging from the severe GRACILE syndrome (OMIM#603358) characterized by growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death to neonatal proximal tubulopathy, hepatic involvement and encephalopathy (DeLonlay et al. 2001, Fernandez-Vizarra et al. 2007). Recently two new CIII assembly factors have been identified: *TTC19* mutations have been reported associated with phenotypes ranging from progressive neurodegenerative disorder with onset in late infancy, to subacute and rapidly progressive neurological failure and severe olivo-ponto-cerebellar atrophy with psychosis or spinocerebellar ataxia (Ghezzi et al. 2011, Nogueira et al, 2013; Morino et al, 2014); a mutation in *LYRM7* has been recently reported in a baby patient with early onset,

severe encephalopathy, lactic acidosis and profound, isolated cIII deficiency in skeletal muscle (Invernizzi et el, 2012).

Complex IV deficiencies

CIV deficiency is clinically heterogeneous, ranging from isolated myopathy to severe multisystem disease, with onset from infancy to adulthood. In 2001 Shoubridge gave a comprehensive review of CIV deficiencies and noted that they are inherited mostly as autosomal recessive disorders caused by mutations in nuclear genes, whereas mutations in the mtDNA-encoded COX subunit genes are relatively rare. COX6B1 is the only nuclear encoded complex IV subunit reported to carry a mutation in a kindred with early onset leukodystrophic encephalopathy,

myopathy and growth retardation (Massa et al, 2008).

Almost all the nuclear-gene defects of COX identified are due to mutations in assembly factors of the enzyme, including *SURF1* (Tiranti et al. 1998), *SCO1* (Valnot et al. 2000a), *SCO2* (Papadopoulou et al. 1999), *COX10* (Valnot et al. 2000b), *COX15* (Antonicka et al. 2003), *LRPPRC* (Mootha et al. 2003), *FASTKD2* (Ghezzi et al, 2008), *COA5* (Huisglott et al, 2011), *COX14* (Weraarpachai et al, 2012), *COX 20* (Szklarczyk et al, 2014).

These autosomal recessive COX deficiencies are usually associated with severe early onset Leigh-like phenotypes variably presenting with lactic acidosis, respiratory distress, metabolic dysfunction, cardiomyopathy, ataxia, psychomotor delay. These conditions

are often lethal at a very early age, but mutations in *TACO1* and *FASTKD2* have been found in affected members with an early onset but slowly progressive neurodegenerative disorder.

Complex V deficiencies

Four disease-causing nuclear genes have been identified so far, two encoding assembly factors (*ATPAF2*, *TMEM70*), whereas the other two (*ATP5E*, *ATP5A1*) encoding structural subunits of the F1 domain. In other CV deficient cases the genetic cause is still unknown (Sperl et al. 2006). Jonckheere et al (2013) identified *ATP5A1* as the disease causing gene in two sibs with fatal infantile mitochondrial encephalopathy and complex V deficiency. A homozygous missense mutation

in *ATP5E* has been associated with neonatal-onset lactic acidosis, 3-methylglutaconic aciduria (3-MGA), mild mental retardation, and peripheral neuropathy (Mayr et al, 2010). Mutations in *TMEM70* have been reported in infants with cardiomyopathy, 3- methyl-glutaconic aciduria (MGA), respiratory distress, intra-uterine growth retardation and dysmorphic features (Cizkova et al, 2008; Spiegel et al, 2011). To date, only one case of complex V deficiency has been referred to a homozygous missense *ATPAF2* mutation associated with degenerative encephalopathy, connatal lactic acidosis and MGA (De Meirleir et al, 2004).

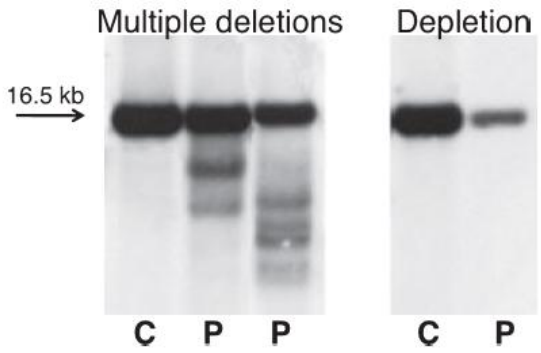
1.2.3.2 Defects of genes altering the stability of mtDNA

Mitochondrial DNA replicates itself independently from the cell cycle that regulates nuclear DNA replication, thanks to a number of proteins forming an autochthonous mitochondrial replisome apparatus. Some of these factors have been identified in humans and are: the DNA polymerase gamma (POLG), Twinkle helicase (PEO1), mitochondrial single-stranded DNA binding protein (mtSSB), and enzymes necessary for supply of deoxy-nucleotides triphosphate (dNTPs).

Structural defects of the DNA-processive enzymes are often associated with mtDNA mutagenesis and multiple mtDNA deletions (qualitative alterations), whereas defects affecting the dNTP pool usually cause mtDNA

depletion, that means reduction of mtDNA copy number/cell (quantitative alterations) (Fig. 7).

Fig. 7



Southern blot analysis of linearized mitochondrial DNA (mtDNA) samples containing multiple mtDNA deleted and mtDNA depleted species. C, control; P, patient (From Spinazzola and Zeviani, 2009).

Defects of dNTPs pool

Ant1. In humans, the mitochondrial ANT or adenine nucleotide transporter 1 exists in three isoforms (ANT1, 2 and 3), different for tissue distribution and kinetic properties. ANT1 protein is a homodimer of 30-kD subunits embedded in the mitochondrial inner membrane and forms a gated pore through which ADP moves across the inner membrane into the mitochondrial matrix and ATP moves from the matrix into the cytoplasm (summary by Neckelmann et al, 1987). ANT1 mutations are responsible for a relatively benign, slowly progressive, and extremely rare form of autosomal dominant form of PEO. Two patients with recessive mutations of ANT1 have been reported so far (Palmieri et al. 2005, Echaniz-Laguna et al, 2012). They both presented with hypertrophic cardiomyopathy,

mild myopathy with exercise intolerance, lactic acidosis but no ophthalmoplegia. RRFs and mtDNA multiple deletions were found in muscle.

p53 controlled RR (p53R2 or RRM2B.) The *RRM2B* gene encodes the small subunit of *p53 inducible ribonucleotide reductase*, a heterotetrameric enzyme responsible for de novo conversion of ribonucleoside diphosphates into the corresponding deoxyribonucleoside diphosphates, essential for DNA synthesis. Mutations in this gene may cause: 1) severe autosomal recessive infantile mtDNA depletion syndrome with proximal renal tubulopathy and multiple MRC defect in muscle (mitochondrial DNA depletion syndrome type 8A: Bourdon et al, 2007;

Kollberg et al, 2009); 2) juvenile-onset MNGIE or adult-onset myopathy with PEO, antibiotics-induced deafness, peripheral neuropathy (mitochondrial DNA depletion syndrome type 8B: Shaibani et al, 2009); 3) autosomal dominant PEO and multiple mtDNA deletions (Tyynismaa et al. 2009).

Deoxy-guanosine kinase (*dGK or dGUOK)* In mammalian cells, the phosphorylation of purine deoxyribonucleosides is mediated predominantly by two deoxyribonucleoside kinases: cytosolic deoxycytidine kinase (dCK) and mitochondrial deoxyguanosine kinase (dGK). Mutations in *dGUOK* are associated with a severe autosomal recessive hepatocerebral form of mtDNA depletion. Symptoms include lactic acidosis, failure to thrive, hypotonia and hypoglycaemia,

persistent vomiting, hepatomegaly associated with progressive neurological symptoms. Liver dysfunction is usually progressive, evolving from microvescicular steatosis into cirrhosis and chronic liver failure (Saada et al. 2001, Freisinger et al. 2006). In two families with hepatocerebral syndrome and mtDNA depletion also cystathionuria has been described (Tadiboyina et al, 2005).
MRC complex activities are variably decreased in muscle and histology can be normal (Mendel et al, 2001).

Succinyl-CoA synthetase (*SUCLA2 and SUCLG1)* Succinyl-CoA synthetase (SCS) is a mitochondrial matrix enzyme that catalyzes the synthesis of succinyl-CoA from succinate and CoA. The enzyme is composed of an invariant alpha subunit and a beta subunit that

determines whether the enzyme is GTP-specific (G-SCS) or ATP-specific (A-SCS) (Sanadi et al. 1954). The activity of both SCS-G and SCS-A is present in mitochondria from rat liver, kidney, and heart, but SCS-A, the ADP-forming enzyme, predominates in the brain (Lambeth et al. 2004).

The *SUCLA2* gene encodes the β subunit of the ADP-forming succinyl-CoA synthetase (SCS-A) (Allen & Ottaway 1986). Mutations in *SUCLA2* cause a severe autosomal recessive mtDNA depletion syndrome with encephalomyopathy and mild methylmalonic aciduria, (Carrozzo et al. 2007; Elpeleg et al, 2007). Patients' skeletal muscle has combined MRC complex defects.

The *SUCLG1* (also reported as *SUCLA1*) gene encodes the alpha subunit of SCS. The first 27 amino acids of SUCLG1 are a mitochondrial

targeting sequence. Mutations in this gene have been described in severe forms of encephalomyopathy with methylmalonic aciduria and mtDNA depletion; typical features are psychomotor delay, lactic acidosis, hypotonia, and death in the first days of life (Ostergaard et al, 2007; Rouzier et al, 2010).

Thymidine Phosphorylase (TP or TYMP) *TYMP* is involved in the catabolism of pyrimidines by promoting the phosphorolysis of thymidine into thymine and deoxyribose-phosphate. Defects of TYMP result in systemic accumulation of thymidine and deoxyuridine, which leads to imbalance in the deoxynucleotide pool. This in turn causes accumulation of point mutations, multiple deletions, and partial depletion of mtDNA, mainly in muscle. TP mutations are the cause

of mitochondrial neuro-gastro-intestinal encephalomyopathy (MNGIE), a disease characterized by ophthalmoparesis, peripheral neuropathy, leukoencephalopathy, and gastrointestinal symptoms such as intestinal dysmotility.

Thymidine kinase 2 (TK2) TK2 is a deoxyribonucleoside kinase that phosphorylates thymidine, deoxycytidine, and deoxyuridine. The clinical spectrum associated to *TK2* mutations varies from a severe muscle weakness with marked dystrophic alterations, encephalopathy and seizures (Galbiati et al. 2006) to a milder myopathic phenotype with longer survival, no motor regression and in some patients proximal tubulopathy (Behin et al, 2012; Saada et al. 2001). There is mtDNA

depletion in muscle with multiple MRC complex defects.

Only in a fraction of the reported cases of MDS, a causative mutation has been found. It is likely that other genes of dNTPs regulation or mtDNA maintenance will be found.
For instance a peculiar form of hepato-cerebral MDS is due to mutation in *MPV17* gene, which codes for a protein with still unknown function (Spinazzola et al. 2006).

Defects of the DNA-processive enzymes

Twinkle. Autosomal dominant progressive external ophthalmoplegia (adPEO) with mitochondrial DNA (mtDNA) deletions-3 (PEOA3) is caused by heterozygous mutation in the nuclear-encoded Twinkle that is a helicase involved in mtDNA replication.

Mutations in *PEO1*, the gene that encodes Twinkle, are associated with clinical presentations of variable severity, ranging from late-onset 'pure' PEO to PEO 'plus' syndromes, with proximal muscle and facial weakness, mild ataxia, peripheral neuropathy, sensorineural deafness and cognitive problems (Suomalainen et al, 1992; Hudson et al, 2005; Van Hove et al, 2009). These patients have RRFs and COX deficient fibers at muscle biopsy with multiple mtDNA deletions. A recessive *PEO1* mutation causes infantile onset spino-cerebellar ataxia (IOSCA) (Nikali et al. 2005), a disease characterized by a combination of ataxia, athetosis, muscle hypotonia and severe epilepsy, that can be the most prominent feature (Lonnqvist et al, 2009). Other features such as ophthalmoplegia, hearing loss and optic

atrophy appear later in the disease course. These patients show mtDNA depletion in brain and liver.

POLG1. The mtDNA polymerase, pol-γ, is essential for mtDNA replication and proof-reading based repair. It is composed of a 140-kDa catalytic subunit (pol-γ A, coded by *POLG1*) and a 55-kDa accessory subunit (pol-γ B, coded by *POLG2*), which functions as a DNA binding factor, increasing the processivity of the polymerase holoenzyme. Mutations affecting *POLG1* are one of the most frequent causes of mitochondrial disease and the most frequent cause of autosomal dominant PEO (adPEO or PEOA). In adPEO due to *POLG1* mutations, typical features are severe dysphagia and dysphonia, and occasionally, a movement disorder including

parkinsonism, cerebellar dysfunction or chorea (Luoma et al. 2004). *POLG1* mutations can cause PEO with multiple mtDNA deletions also in an autosomal recessive way of inheritance (arPEO or PEOB) and the phenotype is usually more severe (Agostino et al. 2003). Recessive *POLG1* mutations have been associated with different syndromes such as sensory ataxic neuropathy, dysarthria, and ophthalmoparesis or SANDO (Van Goethem et al. 2003), and in infantile Alpers-Huttenlocher syndrome or Hepatopathic Poliodystrophy, that is an mtDNA depletion syndrome characterized by psychomotor retardation, intractable epilepsy, and liver failure in infants and young children (Naviaux et al. 2004, Ferrari et al. 2005). Some patients with Alpers syndrome may show mild intermittent 3-methylglutaconic aciduria and

defects in mitochondrial oxidative phosphorylation (Wortmann et al, 2009). Recessive mutations have also been reported in atypical PEO with optic atrophy and retinopathy (Milone et al, 2011) and in MELAS-like phenotype (Deschauer et al, 2007).

Mutations in *POLG2* have been reported in adult-onset PEO (PEOA4) or PEO plus (Longley et al. 2006; Young et al, 2011).

Recently mutations in *MGME1* (mitochondrial genome maintenance exonuclease 1), encoding a mitochondrial RecB-type exonuclease that cleaves single-stranded DNA and processes DNA flap substrates, have been found in patients with a multisystem mitochondrial disorder with multiple deletions and combined

MRC complex defect in muscle (Kornblum C et al, 2013).

Mutations in *DNA2* that encodes for a helicase/nuclease family member likely involved in mtDNA replication, have been found in adult-patients with proximal myopathy, in one case associated with depression, and multiple deletions in muscle (Ronchi et al, 2013).

1.2.3.3 Defects of genes encoding factors involved in metabolic pathways influencing the biogenesis of mitochondria, including OXPHOS.

Defects of mitochondrial translation

Mitochondrial translation requires tRNA and rRNA synthesized in situ from the

corresponding mitochondrial genes, and more than a hundred proteins provided by nuclear genes and imported into mitochondria.

It is a four-step process involving nuclear encoded translation initiation (IF2, IF3), elongation (EF-Tu, EF-Ts, EF-G1 and EF-G2), termination (RF1) and ribosome recycling factors (Smits et al, 2010).

Genetic diseases due to defective mitochondrial protein synthesis have so far been associated with mutations in several nuclear genes and are characterized by combined oxidative phosphorylation defect.

Mutations have been found in *GFM1* gene encoding the elongation factor EF-G1 in patients with severe infantile encephalopathy with lactic acidosis and liver failure (Coenen et al, 2004), with a Leigh phenotype (Valente et al 2007, Smits et al 2011).

Mutations in *TSFM* that encodes for the elongation factor EF-Ts have been associated with a phenotype characterized by encephalopathy and cardiomyopathy (Smeitnik et al, 2006).

Only one homozygous mutation was identified in the mitochondrial elongation factor Tu (EF-Tu) in a patient with a severe infantile macrocystic leucodystrophy with micropolygyria (Valente et al. 2007).

Mutations in many other factors involved in mitochondrial translation have been found in a variety of mitochondrial syndromes i.e. in tRNA modifying enzymes like *PUS1*, encoding for pseudouridine synthase 1 (OMIM#608109) or *TRMU* encoding for tRNA methyltransferase (OMIM#610230), in mitochondrial ribosomal protein like *MRPS16* (OMIM#609204) and *MRPS22*

(OMIM#605810), in *C12Orf65* (OMIM#613541) encoding for a termination activator and in translational activator like *LRPPRC* (OMIM#607544) or *TACO1* (OMIM#612958).

Recently mutations in *MTO1*, encoding the mitochondrial-tRNA modifier 1, have been reported in patients with hypertrophic cardiomyopathy, lactic acidosis, and multiple defects of mitochondrial respiratory chain (MRC) activities (Ghezzi et al, 2012; Baruffini et al, 2013).

Mitochondrial aminoacyl tRNA synthetases (mt-aaRSs) These are key enzymes in the translation of the genetic information catalyzing the specific attachment of each of the 20 amino acids (aa) to a cognate tRNA (Ibba and Soll, 2000). Mitochondrial and

cytoplasmic aaRSs are encoded by distinct nuclear genes with the exception of GARS and KARS. The gene for mitochondrial glutaminyl-tRNA synthetase has not been found yet, possibly because mitochondrial glutamyl-tRNA synthetase (EARS2) efficiently mis-aminoacylates mt $tRNA^{Gln}$ to form glutamate charged-$tRNA^{Gln}$ (Nagao et al, 2009). Hence, mt-aaRSs are 19 instead of 20, including the 2 with double localization.

In the last years these enzymes have been emerged as an important cause of mitochondrial disorders and until now 12 genes encoding different mt-aaRSs have been identified as the genetic cause of mitochondrial disorders usually with an early-onset and transmitted as autosomal recessive traits (Table 2). Such a broad clinical spectrum can be partially explained by the multiple and

still unknown functions of mt-aaRSs. However, there is a strict genotype-phenotype correlation for most of these genes, though it is not clear the reason for such specific cellular or tissue damages, since all aaRSs are ubiquitous enzymes and work in the same pathway.

DARS2, encoding the mitochondrial aspartyl-tRNA synthetase (mt-AspRS), was the first *aaRS2* gene reported to cause a human disease, characterized by a peculiar leukoencephalopathy named LBSL (leukoencephalopathy with brain stem and spinal cord involvement, MIM #611105) (Scheper et al, 2007). Atypical presentations including specific MRI pattern associated with no clinical symptoms (Labauge et al, 2011) or no lactate elevation (Sharma et al, 2011) have been described.

Mutations in *RARS2*, the gene encoding for the mitochondrial arginyl-tRNA synthetase (mt-ArgRS), have been reported in clinical forms of progressive atrophy in cerebellum, pons (pontocerebellar hypoplasia type 6, PCH6; OMIM #611523), with cerebral cortex and white matter involvement, causing a severe infantile phenotype characterized by lethargy, seizures, hypotonia and premature death (Edvardson et al, 2007). Later, it has been demonstrated that the subtype 1 of pontocerebellar hypoplasia (MIM#607596) can be also caused by mutation in *RARS2* (Namavar et al, 2011).

YARS2 encodes for the mitochondrial tyrosyl-tRNA synthetase (mt-TyrRS). Only two missense mutations in this gene have been reported till now, associated with myopathy, lactic acidosis and sideroblastic anemia

(MLASA, OMIM #613561) (Riley et al, 2010).

SARS2 encodes the mitochondrial seryl-tRNA synthetase (mt-SerRS) and has been found mutated in patients a multisystem disorder, characterized by prematurity, progressive renal failure leading to electrolyte imbalances, metabolic alkalosis, and pulmonary hypertension, called HUPRA syndrome (HyperUricemia, Pulmonary hypertension, Renal failure, and Alkalosis) (Belostosky et al, 2011).

In 2011 mutations in *AARS2*, encoding the mitochondrial alanine tRNA synthetase have been reported to cause hypertrophic cardiomyopathy and lactic acidosis in infants from two different families. Patients' fibroblasts or myoblasts did not show any OXPHOS defect, whereas heart and muscle

tissues showed multiple MRC defects (Gotz et al, 2011).

Mutations in the mitochondrial methionyl-tRNA synthetase (mt-MetRS or *MARS2*) can cause autosomal recessive spastic ataxia with leukoencephalopathy (ARSAL or Spastic Ataxia 3) in humans and neurodegeneration in flies. Patients' brain MRI showed cerebellar atrophy and white matter alterations, sometimes associated with thin corpus callosum; disease onset was very variable (Bayat et al, 2012).

Mutations in *HARS2*, encoding the mitochondrial histidyl-tRNA synthetase (mt-HisRS), have been associated with Perrault syndrome, a clinical entity characterized by ovarian dysgenesis and sensorineural hearing loss. This recessive syndrome is genetically heterogeneous being caused by mutations in

different genes (*HSD17B4*, *CLPP* beside *HARS2*), and is associated with premature ovarian failure in females and progressive hearing loss in both males and females (Pierce et al, 2011). More recently homozygous mutations in *LARS2*, encoding the mitochondrial leucyl-tRNA synthetase (mt-LeuRS), have been also found in patients with Perrault syndrome (Pierce et al, 2013).

Mutations in the mitochondrial phenylalanine-tRNA synthetase, FARS2 (mt-PheRS) have been identified in siblings with fatal epileptic encephalopathy, liver disease and lactic acidosis. Neuropathological findings together with liver disease filled the criteria for Alpers-Huttenlocher syndrome (Elo et al, 2012).

EARS2 encodes for the mitochondrial glutamyl-tRNA synthetase (mtGluRS) and mutations in this gene have been identified in

patients with a typical MRI pattern described as LTBL (leukoencephalopathy with thalamus and brainstem involvement and high lactate (Steenweg et al, 2012). *EARS2* mutant patients presented a peculiar biphasic clinical course: an infantile disease onset with rapid progression followed by stabilization, and in some cases improvement, and partial recovery of lost skills. MRI displayed abnormal thalami, midbrain, pons, medulla oblongata and alterations of cerebral and cerebellar white matter.

GARS and *KARS*, encoding enzymes, glycine-tRNA synthetase (GlyRS) and lysil-tRNA synthetase (LysRS) respectively, catalyze the reaction in both mitochondria and cytosol.

Dominant *GARS* mutations have been found in patients with Charcot-Marie-Tooth disease type 2D (CMT2D; OMIM#601472) and with

distal hereditary motor neuronopathy type VA (HMN5A; OMIM#600794) (Antonellis et al, 2003).

Mutations in *KARS* have been initially described in a patient with an intermediate form of autosomal recessive Charcot-Marie-Tooth disease (CMTRIB; OMIM#613641) (McLaughlin et al, 2010), developmental delay, self-abusive behavior, dysmorphic features, and vestibular Schwannoma. Very recently, *KARS* homozygous missense mutations have been identified in subjects with nonsyndromic-hearing-impairment phenotype (Santos-Cortez et al, 2013).

Biochemical features of mt-aaRS related disorders is usually a combined OXPHOS defect, but some of them can present with an isolated complex defect in muscle or with no biochemical defect at all.

Transcription of mtDNA requires a small number of nucleus-encoded proteins including a single RNA polymerase (POLRMT), auxiliary factors necessary for promoter recognition (TFB1M, TFB2M) and promoter activation (TFAM), and a transcription termination factor (mTERF) (Scarpulla 2008). No mutation affecting these transcriptional factors has yet been described.

Defects of mitochondria fission and fusion

Mitochondria are not static and isolated organelles but form a complex network. Mitochondrial fusion and fission require conserved protein machineries at the outer and inner membranes that mediate membrane mixing and division events. The proteins that regulate mitochondrial dynamics are

associated with a wide spectrum of cellular functions like integrity and turnover of mitochondria, electrical and biochemical connectivity, segregation and protection of mitochondrial DNA (Okamoto et al. 2005).

GTPases of the dynamin family have a crucial role in both fission and fusion. *OPA1* is a GTPase and member of the dynamin protein family. Mutations in this gene lead to autosomal dominant optic atrophy (ADOA). OPA1 protein is also implicated in apoptosis (Frezza et al. 2006) and oxidative phosphorylation (Zanna et al. 2008), and multiple mtDNA deletions are found in muscle of some *OPA1* mutant patients (Amati-Bonneau et al. 2008, Hudson et al. 2008), suggesting that *OPA1* is also involved in mtDNA maintenance.

Mutations in *MFN2* gene that encodes another dynamin-like enzyme regulating the fission-fusion dynamics, mitofusin 2, are responsible for mitochondrial disorders, autosomal dominant Charcot-Marie-Tooth neuropathy type 2A and 4A (CMT2A, CMT4A) (Zuchner et al. 2004, Zuchner et al. 2005).

Interestingly mutation in the *DLP1* gene, which encodes a dynamin-like protein implicated in mitochondrial and peroxisomal fission, cause a severe phenotype characterized by systemic lactic acidosis, increased plasma very long chain fatty acids, and microcephaly.

Table 2: Clinical and radiological phenotypes associated with different *aaRS2* mutations (modified from Diodato et al, unpublished)

	OMIM (gene)	Protein	Clinical picture	OMIM (phenotype)	Age at onset	MRI pattern	Reported cases
DARS2	*610956	mt aspartyl-tRNA synthetase	Cerebellar ataxia, spasticity, dorsal column dysfunction, cognitive impairment	#611105	Childhood/ adulthood	Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (LBSL)	>30 families
RARS2	*611524	mt arginyl-tRNA synthetase	Encephalopathy with lethargia, hypotonia, epilepsy and microcephaly	#611523	Perinatal	Pontocerebellar hypoplasia, brain stem thinning	>10 families
YARS2	*610957	mt tyrosyl-tRNA synthetase	Myopathy, lactic acidosis and sideroblastic anemia (MLASA)	#613561	Childhood	None	3 families
SARS2	*612804	mt seryl-tRNA synthetase	Hyperuricemia, pulmonary hypertension, renal failure and alkalosis (HUPRA)	#613845	Perinatal	None	4 families

AARS2	*612035	mt alanyl-tRNA synthetase	Hypertrophic cardiomyopathy, delayed motor development, cerebellar ataxia	#612035	Childhood	None	7 families
MARS2	*609728	mt methionyl-tRNA synthetase	Autosomal recessive spastic ataxia	/	Childhood/ adulthood	Cerebellar atrophy and white matter alterations, thin corpus callosum	1 family
HARS2	*600783	mt histidyl-tRNA synthetase	Sensorineural hearing loss and ovarian dysgenesis (Perrault syndrome)	#614926	Childhood/ adulthood	None	1 family
LARS2	*604544	mt leucyl-tRNA synthetase	Sensorineural hearing loss and ovarian dysgenesis (Perrault syndrome)	#615300	Childhood/ adulthood	None	1 family
FARS2	*611592	mt phenylalanine-tRNA synthetase	Epileptic encephalopathy, liver disease and lactic acidosis	#614946	Perinatal	Cerebral and cerebellar, brain stem and basal ganglia atrophy	3 families
EARS2	*612799	mt glutamyl-tRNA synthetase	Global developmental delay or arrest, epilepsy, dystonia, spasticity,	#614924	Early childhood	Leukoencephalopathy with thalamus and brainstem involvement and high lactate	12 families

			and high lactate			(LTBL)	
GARS	*600287	Glycyl-tRNA synthetase	Charcot-Marie-Tooth (CMT) disease 2D or distal hereditary motor neuropathy VA	#601472, #600794	Childhood/ adulthood	None	>8 families
KARS	*601421	Lysil-tRNA synthetase	Autosomal recessive CMT (intermediate, B)	#613641	Childhood/ adulthood	None	4 families

Defects of mitochondrial protein import

Two known mitochondrial diseases are clearly attributable to abnormal protein import.

Mutations in *TIMM8A*, encoding DDP1 (deafness–dystonia protein1), a component of the import machinery for mitochondrial carrier proteins is mutated in X-linked Mohr–Tranebjaerg disease, characterized by deafness, followed by progressive neurological troubles, including dystonia and optic atrophy (Roesch et al. 2002).

Mutation of *DNAJC19*, encoding a putative mitochondrial import protein, causes autosomal recessive dilated cardiomyopathy with ataxia (Davey et al. 2006).

Fe-S defects

Components of oxidative phosporylation machinery need to be equipped with cofactors such as haeme or Fe–S clusters that are needed for their electron transfer activity. A number of assembly factors and enzymes involved in the biosynthesis and incorporation of prosthetic groups are necessary for the proper assembly of functional MRC complexes (Lill and Muhlenhoff, 2006).

Mutations in ABC7, an iron mitochondrial exporter, which controls the generation of cytosolic iron-sulphur proteins, is responsible for X-linked sideroblastic anaemia and ataxia (Allikmets et al 1999); frataxin, a mitochondrial protein which is responsible for Friedreich's ataxia, is also involved in iron handling, heme synthesis and iron-sulphur protein maintenance (Campuzano et al, 1996).

1.2.3.4 Defects of genes encoding factors involved in the biosynthesis of lipids and cofactors

Defects of the membrane lipid milieu

Except for cytochrome *c*, which is located in the intermembrane space, all components of the respiratory chain are embedded in the lipid milieu of the inner mitochondrial membrane, composed predominantly of cardiolipin. Cardiolipin is not merely a scaffold but is essential for proper functioning of several mitochondrial OXPHOS complexes and several mitochondrial carrier proteins (Jiang et al. 2000, Gohil et al. 2004). This is the reason why defects in cardiolipin could cause OXPHOS dysfunction and hence mitochondrial disease. In fact there is an example on this regard, the Barth syndrome

(mitochondrial myopathy, cardiomyopathy, growth retardation, and leukopenia) (Barth et al 1999). The mutated gene in this syndrome, *TAZ* (or *G4.5)*, encodes an acyl–coenzyme A synthetase (*tafazzin*) that has an important role in cardiolipin synthesis, because cardiolipin concentrations are markedly decreased in skeletal and cardiac muscle and in platelets from affected patients (Schlame et al. 2000).

Coenzyme Q deficiency

CoQ10 or ubiquinone is a lipophilic component of the electron-transport chain, which transfers electrons from Complex I or II, and from the oxidation of fatty acids and branched-chain amino acids, via flavin-linked dehydrogenases to Complex III. The CoQ also plays a role as an antioxidant and as a membrane stabilizer. Primary CoQ10

deficiency is a rare, clinically heterogeneous autosomal recessive disorder caused by mutations in any of the genes encoding proteins involved in the synthesis of CoQ. Associated phenotypes are variable and can include encephalomyopathy, seizures, ataxia, cardiomyopathy, renal failure, Leigh syndrome and isolated myopathy (Lalani et al, 2005). Mutations underlying CoQ10 deficiencies have been found in the following genes: *PDSS1* (OMIM#607429), *PDSS2* (OMIM#610564), *COQ9* (OMIM#612837), *COQ6* (OMIM#614647).

Mutations in *APTX* (Quinzii et al. 2006) and *ADCK3* (Lagier-Tourenne et al. 2008) were recently found in patients with ataxia and low levels of CoQ in muscle biopsies, supporting the hypothesis that the ataxic form is a genetically heterogeneous disease in which

CoQ10 deficiency can be secondary (Le Ber et al. 2007).

1.3 Identification of the genetic defect in mitochondrial disorders

Since the double genetic back-ground of mitochondrial disorders, discovering the genetic defect underlying these conditions can be tricky.

An exhaustive family history in order to hypotize the way of transmission (autosomal recessive or dominant, maternal) is essential to drive the molecular diagnostic work-flow. A strong suspicion of an mtDNA syndrome will lead to investigate for hot-spot mutations in candidate genes, whereas evidence of maternal way of inheritance without peculiar clinical

features will lead to sequence the entire mtDNA.

Conventional methods for disease gene discovery include those based on linkage analysis as well as homozygosity mapping (Lander et al, 1986; Lander et al, 1987), in which markers are used to identify recombination events in pedigrees to narrow the candidate genomic regions segregating with affected status. A typical follow-up is then to re-sequence exons within the candidate region(s) to find protein-altering variants such as missense or nonsense single-base substitutions, or small insertions or deletions (indels). These approaches, by the way, require large pedigrees with a simple, "monogenic" and highly penetrant inheritance.

Next generation sequencing (NGS), a technique based on the enrichment by either

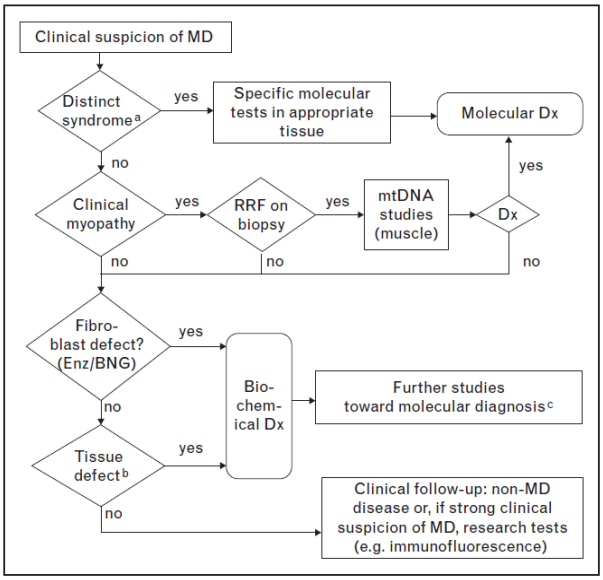
solid-phase or in-solution targeted capture and that can capture the entire protein-coding sequence of an individual (the 'exome', over 30 Mb) for sequencing, has been largely used in the last years in the diagnosis of mitochondrial disorders.

1.3.1 Cybrids and mtDNA mutations

Investigating the epidemiology of mtDNA genetics is complicated by the wide spectrum of clinical presentation, the diverse range of mutations and the high carrier rate, all of which will lead to underestimates of prevalence. Over 100 point mutations associated with human disease have been described in protein coding genes, tRNAs, and rRNAs (Servidei et al, 2004). The criteria for defining pathogenic mtDNA mutations have required revisions as our understanding of

mitochondrial biology and pathology has expanded.

Fig. 8 Diagnostic work-flow in mitochondrial disorders (from Debray et al, 2008)



Because mtDNA mutations are fixed in the population at a much higher rate than are nuclear DNA mutations, the mitochondrial genome harbors large numbers of neutral base changes that are not pathogenic but are usefully employed in anthropological studies (Wallace et al., 1999) and in forensic medicine (Zapico et al, 2013). The abundance of neutral polymorphisms means that the pathogenic role of new mutations associated with human disease needs to be established beyond a reasonable doubt. The following criteria are important in establishing the pathogenicity of a novel mutation: 1) Obviously, the mutation must not be a known neutral polymorphism. 2) The base change must affect an evolutionarily conserved and functionally important site. 3) Deleterious mutations are usually heteroplasmic, although a few pathogenic

mutations, including the first one ever reported, causing LHON (Wallace et al., 1988), are, in fact, homoplasmic. 4) The degree of heteroplasmy in different family members should be in rough agreement with the severity of symptoms.

A useful diagnostic tool is the single fiber PCR (Moraes and Schon, 1996), a method that allows the correlation between mutational load and functional abnormality, by mechanically ``plucking'' from thick cross-sections of muscle histologically normal and abnormal fibers and measuring the abundance of the mutation in each fiber by PCR.

Mitochondrial tRNA mutations account for approximately 50% out of all mutations described in human mtDNA (Ruiz-Pesini et al, 2007) and only 10% of polymorphisms fall within these genes (Helm et al., 2000; Ruiz-

Pesini et al., 2007). Studies about the folding and the amount of free energy of mitochondrial tRNAs showed that some of these changes were not neutral; the ones with more free energy deviation compared to controls were in minor amount suggesting the presence of a negative selection for these variants (Vilmi et al., 2005). Increasingly, researchers accept that mtDNA sequence changes can interact each other, with nuclear genes, or with environment to cause disease. Evidence also suggests that the nuclear background associated with a mtDNA mutation can affect its biochemical expression (Cock et al, 1995).

In the past it has been difficult to identify mtDNA variants and above all to prove their pathogenic role. However, with the development and use of novel cybrid

(cytoplasmic hybrid) models new mtDNA mutations responsible for mitochondrial disorders have been recognized and validated. These cybrid cell lines are created by fusing mitochondrial-free (Rho0) cells with fibroblasts from individuals harbouring a certain amount of a mtDNA variant; the resultant cells will have identical nuclear DNA but vary in the degree of heteroplasmy (Fig. 9).

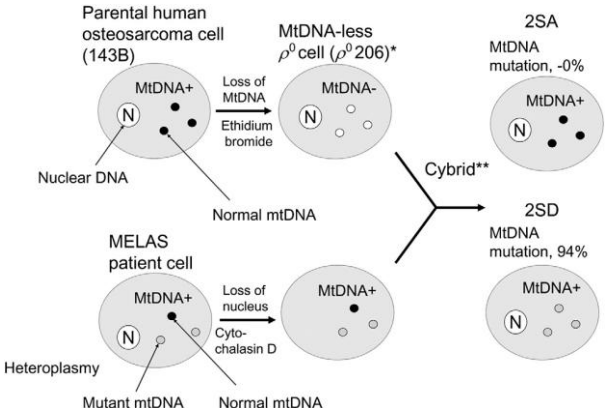
In order to study mitochondrial dysfunction, human mtDNA-less ρ0 cells have been established (King et al, 1989). ρ0 cells are depleted of mtDNA by long-term exposure to ethidium bromide, while maintaining the same nuclear genetic background as the original cells. Then, cybrids were created by transfer of cytoplasts (cells without nucleus but with mitochondria and harboring a specific mtDNA

mutation) from patients to ρ0 cells, and their mitochondrial respiratory functions have been evaluated (Yoneda et al, 1992). Cybrids carrying mutated mtDNA show mitochondrial impairment, reflecting the level of heteroplasmy, when compared to those carrying normal mtDNA; these cells are also a useful in vitro culture model for studying the characteristics of mitochondrial dysfunction, as seen for the 3243A>G mutation in MELAS syndrome (Chomyn et al, 1992).

1.3.2 Exome sequencing and mitochondrial disorders

Human genome comprises about $3x10^9$ bases having coding and noncoding sequences. About $3x10^7$ base pairs (1%) (30Mb) of the genome are the coding sequences.

Fig. 9 Creation of transmitochondrial cybrids harbouring A3243G mutation causing MELAS (from Yoshii et al, 2012)



Less than 10% (about 3Mb) of the whole-genome sequence is well characterized; moreover inconclusive clinical knowledge can be derived from genome sequences (Rizzo et

al, 2012). In addition, it is estimated that 85% of the disease-causing mutations are located in coding and functional regions of the genome (Botstein et al, 2003; Majewski et al, 2011). Consequently sequencing of the complete coding regions (exome) has the potential to uncover the causes of large number of rare, mostly monogenic, genetic disorders as well as to detect predisposing variants in common diseases and cancers. Data resulting from the exome analysis of each individual contain about 10000 non-synonymous variants, depending on ethnicity and calling methods. It is estimated that a normal individual has 50-100 mutations in the heterozygous state that can cause a recessive Mendelian disorder when being homozygous or compound heterozygous (Consortium G.P.A, 2010). Known as massively parallel or 'next-

generation' sequencing (NGS), these technologies have enabled investigators to obtain variant information down to single-base resolution in a rapid, high-throughput fashion on the scale of the whole human exome (WES or whole exome sequencing) or genome. Enrichment by either solid-phase or in-solution targeted capture (Mamanova et al, 2010) can rapidly isolate candidate regions of interest ranging from hundreds of kilobases in size or capture the entire protein-coding sequence of an individual (the 'exome', over 30 Mb) for sequencing.

Since WES conveys to the identification of a large number of nucleotides variants, a filtering strategy capable to highlight the possibly pathological ones is needed. First, WES data are compared with already reported variants in existing databases (Exome Variant

Server, Hapmap and SNP database); then missense or nonsense nucleotide changes are selected and among missense variants, the ones causing a substitution of a highly conserved amino acid residue are prioritized. The main rationale given for this is that these variants tend to be of larger effect than non-coding variants, and also because it is difficult to predict the effects of non-coding and synonymous variants with any certainty. Hence, in order to reduce noise when analyzing possible disease-causing variants, non-coding and synonymous variants are often ignored or greatly down-weighted.

The first report of selective sequencing of whole exome was the identification of the genetic defect causing Freeman-Sheldon syndrome, a rare dominant inherited disorder caused by mutations in *MYH3* (FSS;

MIM193700) (Ng et al. in 2009). Rare and common variants were identified. After assessment of the quality of the exome data, 13347 variants appeared to be novel. Subsequent filtering of these variants against dbSNP or those found in the HapMap samples defined the *MYH3* gene as a disease causing gene in FSS patients. To extend the strategy to recessive disease, shortlisted genes were required to have at least two private protein-altering variants instead of just one. This condition accounts for two situations: the disease mutation should be homozygous, or two different mutations on different haplotypes are expected instead. This approach was applied to a presumed recessive disease, Miller syndrome (Ng et al, 2010) where heterozygous mutations in *DHODH* were identified.

Over 200 nuclear genes have been identified that cause mitochondrial disease, and the list continues to grow as over 1500 genes have been identified controlling mitochondrial structure and function (Calvo et al, 2010; Scharfe et al, 2009). A large number of studies in the last years identified new causing disease genes by WES. Mitochondrial diseases caused by nuclear genes are usually inherited as autosomal recessive trait. This can be useful in making the genetic diagnosis of small suggestive pedigrees where the analysis of WES data must consider homozygous or compound heterozygous variations first. In case of a newly identified variant in a patient or in a small family, a clearly defined genetic diagnosis is difficult to be plausible only on the basis of variant finding. Its absence in population controls must be verified and the

presence of the same and other variants in the same gene in other patients or families with similar clinical presentations are usually used to confirm the new pathogenic variant/s. However, if the disorder is extremely rare, it is hard to find more patients. Further functional experiments are crucial to validate the pathogenic role of any newly determined variant; if the mutated gene has a defined role in a well known pathway related to thedisease, it is acceptable to perform biochemical confirmatory experiments.

In addition to WES, panels containing sets of genes already known to cause mitochondrial disorders have been designed and can be analyzed by NGS fastening the diagnostic process. Diagnosis of mitochondrial disease by WES or NGS of targeted gene panels has been explored over the past years and has proven to

be highly effective in both screening of known disease genes, and identifying new disease genes (Calvo et al, 2010; Vasta et al 2013).

1.4 Treatment of mitochondrial disorders

Management of inherited mitochondrial diseases typically involves general measures, such as the optimization of nutrition and administration of vitamins and food supplements, along with symptom-based management such as eye props or ptosis surgery for patients with CPEO. Rationale for an effective treatment is to correct the genetic and/or metabolic defect since the growing understanding of mitochondrial function made clear that several aspects could be therapeutically targeted. Different approaches

have been tested to maintain and/or restore proper mitochondrial function, such as: 1) preventing transmission of mtDNA; 2) enhancing mitochondrial biogenesis through the regulation of specific transcriptional factors; 3) gene therapy (replacement or repair); 3) altering the balance between wild-type and mutated mtDNA (i.e. exercise training); 4) metabolic manipulation (radical oxygen scavenging, mitochondrial calcium homeostasis); 5) modulation of mitochondria quality control.

Two comprehensive reviews on this topic were written by Koene et al. (2009) and Andreux et al. (2013) with the last new approaches, some of which have yet to be explored in humans (Tab.3).

Anyway, at the moment treatment for mitochondrial diseases caused remains

unsatisfactory and mostly confined to supportive measuresand supplementation of drugs and compounds with generic beneficial effects on mitochondrial metabolisms. Since defects of the respiratory chain result in increased production of free radicals, the use of antioxidants has some sound basis. N-acetylcysteine and coenzyme Q10, both antioxidants, improved OXPHOS function and have been demonstrated to reduce free radical production in cybrids (Mattiazzi et al. 2004). Although coenzyme Q10 has shown some early promise in Parkinson's disease and Friedreich's ataxia, such results can only be regarded as provisional at this stage. CoQ10 has proved to be useful in some forms of CoQ10 deficiency (Salviati et al, 2005) but there have been no large-scale studies to

determine the effectiveness of coenzyme Q10 in primary mitochondrial diseases.

Other compounds can be used in treatment of oxidative damage like tocopherols (Kir et al. 2005), or analogue or Vitamin E and CoQ derivatives which are directly targeted to mitochondria, have been shown to be effective in cultured cell models of OXPHOS disease (James et al. 2005, Distelmaier et al. 2009).

EPI-743, a novel redox compound, has been shown to improve clinical outcome of Leigh patients in phase 2A open label trial (Martinelli et al, 2012).

Creatine, the substrate for the synthesis of phosphocreatine, has been used in different trials in patients with neuromuscular disorders and gave opposite results regarding its efficacy.

Table 3. Recent strategies for mitochondrial therapy

Preventing transmission of mtDNA	Gene therapy	Altering balance between mutated and wt mtDNA	Controlled regulation of transcription factors	Metabolic manipulation	Mt quality control
Oocyte donation	Allotopic expression	Mutation repair (Zinc finger binding proteins)	Upstream sensors: caloric restriction, AMPK, mTOR, NAD^+ boosters, sirtuins	Preventing oxygen damage (scavenging, allotropic expression, Twinkle overexpression)	Fusion and fission
Mutation analysis of chorionic villi	Correction of translational defects (tRNA)	Restriction endonucleases	Downstream sensors: nuclear receptors, NRF1 and TFAM	Calcium modulation	Mitophagy
Preimplantation genetic diagnosis	Expression of nuclear encoded ANT-1 and TFAM	Peptide nucleic acid oligomers	Downstream effectors	Uncoupling proteins	Protein folding homeostasis (mitochondrial UPR)
	Adeno-associated virus mediated	Stimulating satellite cells		Nutritional intervention	
		Transcription activator-like effector nucleases (TALENs)			

An intervention often used in patients with mitochondrial disease is the metabolic therapy. Metabolic acidosis resulting from increased lactate production, a common feature in mitochondrial defects, can be treated, at least in the short-term, buffering with antiacid substances such as sodium bicarbonate. Some drugs, which stimulates pyruvate oxidation (i.e. dichloroacetate), has also been used to lower lactate concentration, but adverse effects preclude its widespread clinical use (Barshop et al. 2004).

In the rare mitochondrial disorder, MNGIE, due to mutations in *TP* gene (Hirano et al. 2005), the problem is the accumulation of dangerous nucleoside precursors, thymidine. A therapeutical symptomatic approach is to try to decrease the blood level of these substances through transfusions or by binding them to a

water-soluble compound facilitating urinary excretion. Recently promising results in MNGIE patients have been obtained with hematopoietic stem cell transplantation (HSTC) (Torres-Torronteras et al, 2011); this procedure has some risks and for this reason must be accurately evaluated (Filosto et al, 2012; Finkenstedt et al, 2013).

Scope of the thesis

My DIMET project has been focused on the identification of the genetic defects responsible for mitochondrial disorders and the characterization of their role.
Diagnosis of mitochondrial disorders that have a double way of inheritance can be difficult and for about 50% of patients with a

clinical/biochemical diagnosis of mitochondrial disorders the genetic defect is still unknown.

Mitochondrial diseases are quite common with an estimated birth prevalence of one in 5000, although recently has been demonstrated that one in 500 children has a pathogenic mtDNA mutation.

The pathogenic role of mtDNA variants need to be verified and proved, since the large amount of mtDNA variants are neutral changes. For the identification of mutations in nuclear genes, next generation sequencing has dramatically improved the discovery rate of pathogenic variants in several inherited diseases, including mitochondrial disorders.

I took part in a project, presented in chapter two, to verify the pathogenic role of a variant

in *MTCOI*, the mtDNA gene encoding the subunit I of complex IV. This variant was identified in a female with a MELAS-like phenotype and studied using cybrid cell models.

The study described in chapter three concerns the description of a new phenotype, characterized by cerebellar ataxia, cognitive problems and primary ovarian failure, associated with mutations in *AARS2* the gene encoding the mitochondrial alanine-tRNA synthetase. Two unrelated probands with a clinical and biochemical phenotype suggestive of mitochondrial disorder were investigated by whole exome sequencing (WES) and were found positive for mutations in *AARS2*. Other 4 patients were then identified on the basis of the clinical features and MRI pattern.

Finally, in chapter 4, I described the identification of the first mutations in two mitochondrial aminoacyl-tRNA synthetases, for valin (VARS2) and threonine (TARS2), as responsible of severe infantile mitochondrial syndromes with OXPHOS deficiency. Mutations in *VARS2* and *TARS2* genes were identified by WES in a subject and two siblings respectively, and then validated with complementation experiments in fibroblasts.

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CHAPTER 2

MELAS-like encephalomyopathy caused by a new pathogenic mutation in the mitochondrial DNA encoded cytochrome c oxidase subunit I

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Abstract

We report a 35-year-old woman presenting a stroke-like episode with transitory aphasia followed by generalized tonic-clonic seizures. She had severe hearing loss and suffered from frequent episodes of migraine. Although a brain MRI disclosed a T2-hyperintense lesion in the left parietal lobe, she had hardly any long-term sequela. Exercise intolerance, myalgias and limb-girdle muscle weakness indicated a slowly progressive myopathy. Extra-neurological features included short stature, and secondary amenorrhea with low gonadotropin levels, indicating secondary hypogonadism. However, she had three mutation-free, healthy children by ovarian stimulation. A muscle biopsy showed ragged-red, cytochrome c oxidase-negative fibers, and an isolated defect of cytochrome c oxidase

activity in muscle mitochondria. Sequence analysis of muscle mtDNA revealed a previously unreported heteroplasmic m.6597C>A transversion in the MTCOI gene, encoding subunit I of cytochrome c oxidase, corresponding to p.Q232K aminoacid change. Analysis on transmitochondrial cybrids demonstrated that the mutation is indeed associated with COX deficiency, i.e. pathogenic.

1. Introduction

Mitochondrial encephalomyopathies are caused by mutations in either mitochondrial DNA (mtDNA) genes, or in nuclear DNA genes encoding proteins required for the formation and activity of the mitochondrial respiratory chain (MRC) complexes. MRC

carries out oxidative phosphorylation (OXPHOS), an essential pathway providing most of the energy to the cell. Cytochrome c oxidase (COX, complex IV, CIV), the terminal enzyme of MRC, couples electron transfer from cytochrome c to molecular oxygen, with proton translocation from the mitochondrial matrix to the intermembrane space, and is thus essential for the energy-providing breakdown of nutrient-derived substrates. Human COX is a multiheteromeric complex composed of 13 subunits [1]. The three largest (MTCOI, MTCOII, MTCOIII), which form the catalytic core, are encoded by mtDNA genes, whereas the others are nuclear-encoded. The majority of genetically defined defects of COX involve nuclear gene products engaged in COX assembly, resulting in autosomal recessive, earlyonset, usually fatal, disorders. A

paradigmatic example is Leigh syndrome associated with COX deficiency, usually caused by mutations in SURF1, a COX early-assembly factor. In contrast, only a few mutations have been reported in the mitochondrial COX subunits, mostly in single patients or families, the clinical presentations varying from acquired idiopathic sideroblastic anemia, to hepatocerebral failure, encephalomyopathy, myopathy with recurrent myoglobinuria, or motor neuron disease [2–7]. Strokelike episodes are the clinical hallmark of one of the most common mitochondrial syndromes, i.e. MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) usually associated with the m.3243A>G mutation in the mtDNA gene encoding mt-tRNALeu(UUR). Rarer MELAS mutations involve other positions of the same

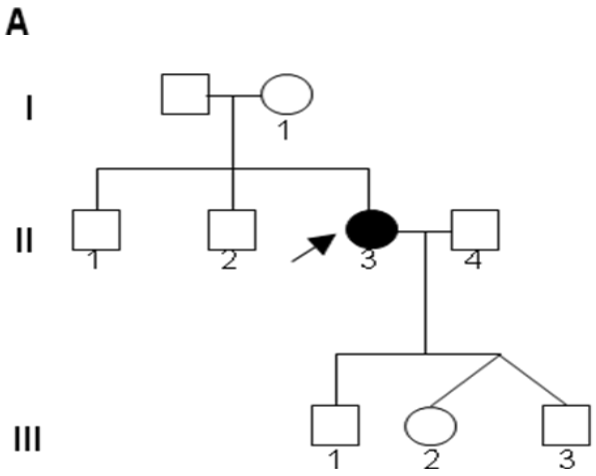
mt-tRNA Leu(UUR), other mt-tRNAs, or specific positions in mtDNA genes encoding structural MRC subunits, particularly the m.12513A>G mutation in ND5 of complex I. To date, in only two MELAS-like patients have MTCO gene mutations been reported. The first patient, harboring a m.6328T>C (p.S142F) mutation in MTCOI, was affected by exercise intolerance since the age of 16 years, complicated by hypertrophic cardiomyopathy diagnosed at 22 years, and by a stroke-like episode at 33 [8]. The second patient, an 11 year-old girl with a m.7023G>A (p.V374M) mutation in MTCOII presented with tonic-clonic seizures, weakness and an acute episode of aphasia during a stroke-like episode. MRI of the cerebral hemispheres showed multiple infarcts [9]. We report here a

third MELAS-like patient carrying a new mutation in the MTCOI gene.

2. Case report

The patient is a 35-year-old Italian woman, born after uncomplicated pregnancy and delivery from healthy unrelated parents. The family history was negative for epilepsy, cerebrovascular events, myopathy, or diabetes. Her two brothers are healthy, as are her three children and both parents (Fig. 1A). She was referred to us after a stroke-like episode characterized by transient aphasia and paresis of the left arm, followed by generalized tonic-clonic seizures. Symptoms regressed within 48 h. A brain MRI performed after 24 h showed a T2-hyperintense lesion in the right parietal lobe (Fig. 1B, panels a, b). The EEG showed the presence of sharp-waves in the same

region. Neurological examination after 48 h disclosed neither mono- nor hemiparesis, nor cerebellar signs, and her speech was normal. However, she was of short stature (146 cm), and suffered of progressive hearing loss starting in infancy. She also referred to the



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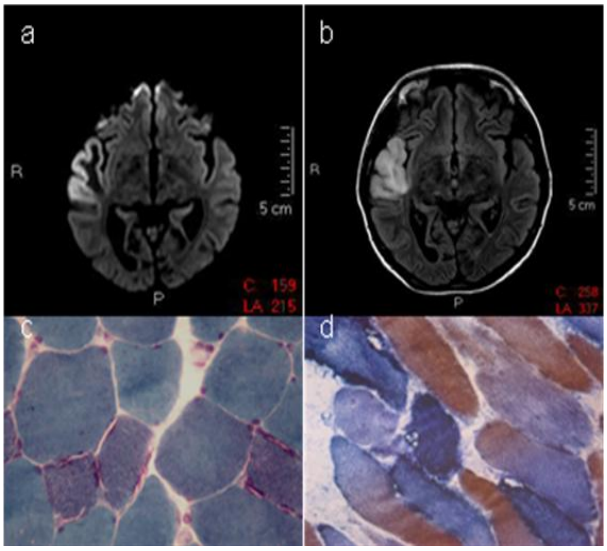

Fig. 1. MRI, Genetic and biochemical findings. (A) Family tree. The proband is indicated by a black symbol and an arrow. (B) T2-weighed MRI consecutive transverse sequences showing moderate cerebral cortical atrophy and a hyperintense lesion in the right temporal lobe. (a–b) Gomori trichrome staining of a muscle biopsy showing RRF. (c) SDH-positive COX-negative muscle fibers, (d) Magnification 20X.

examiner recurrent episodes of muscle pain and cramps, associated with easy fatigability, and had proximal muscle weakness (MRC scale = 4), with mild hypotrophy, indicating a progressive myopathy. The ocular motility was normal. Slight cognitive impairment was noticed. She reported frequent episodes of migraine (once-twice a week), and permanent secondary amenorrhea with low FSH and LH levels since age 15. However, she had three children conceived by ovarian stimulation, again indicating secondary hypogonadism. The following blood tests were negative: lactate at rest, creatine kinase, anti-nucleus antibodies, extractable nuclear antigen (ENA), anti-neutrophilic cytoplasmic antibodies (ANCA), antistreptolysin titer (TSO). Echo-doppler and angio-MRI failed to show haemodynamic alterations in neck and head

districts. The echocardiogram and ECG were normal as well.

3. Results

Informed consent for participation in this study was obtained from the patient and her adult relatives, in agreement with the Declaration of Helsinki and approved by the Ethical Committees of the Fondazione Istituto Neurologico - IRCCS, Milan, Italy. Genetic analysis of the patient's children was performed upon specific, written request signed by both parents. The myopathic signs and symptoms prompted us to perform a muscle biopsy on the left quadriceps. Histological and histoenzymatic analysis [10] documented numerous Ragged-Red Fibres (RRF) by modified Gomori Trichrome staining, that were negative at the

histoenzymatic reaction to COX, but strongly positive at the SDH reaction, as confirmed by COX/SDH double staining (Fig. 1B, panels c, d). Measurement of the MRC complex activities confirmed a severe, isolated COX defect in muscle homogenate, as low as 15% compared to the mean of control values (Fig. 1C), whereas the MRC activities were all in the normal range in cultured skin fibroblasts [11] (not shown). Sequence analysis of mtDNA from muscle showed a new MTCOI missense mutation, m.6597C>A (p.Q232K), on an otherwise normal H mtDNA haplogroup. The Q residue in position 232 of MTCOI is highly conserved through phylogenesis. Quantification by allele-specific RFLP-PCR analysis [12] revealed that the mutation was 95% heteroplasmic in muscle, 70% in urinary-sediment cells, 40% in

cultured skin fibroblasts, and 30% in lymphocytes of the patient (Fig. 1D). The heteroplasmy was not detectable in urinary-sediment cells of the patient's mother, of one brother, and of her three children (Fig. 1D). In order to confirm the pathogenic role of the Q232K mtDNA mutation, we created transmitochondrial cybrids containing the mtDNA from our patient and a heterologous, nuclear genome from 143B human osteosarcoma cells [13,14]. No homoplasmic mutant cybrids could ever be selected. We selected two 80% heteroplasmic mutant mtDNA clones (Fig. 2A) and compared the activities of individual MRC complexes with those of several wild-type cybrid clones derived from the same patient (Fig. 2B). As shown in Fig. 2B, the CIV/CS activity ratio in the two mutant clones was 16 and 19, vs 72 ±

24 in the wild-type clones, clearly indicating mutation-associated COX deficiency.

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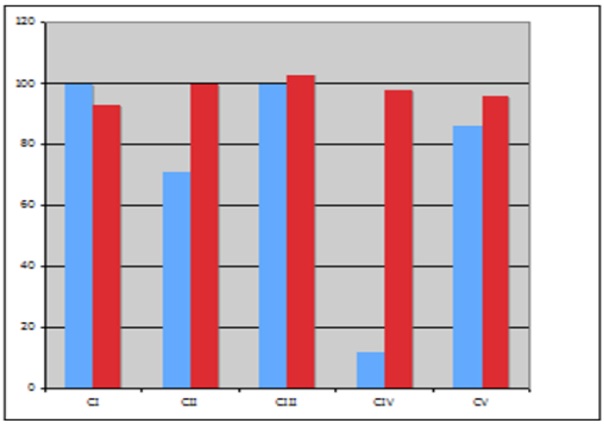

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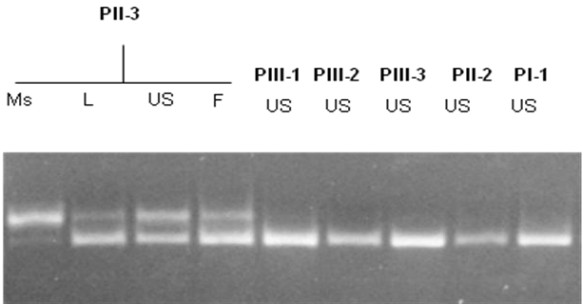


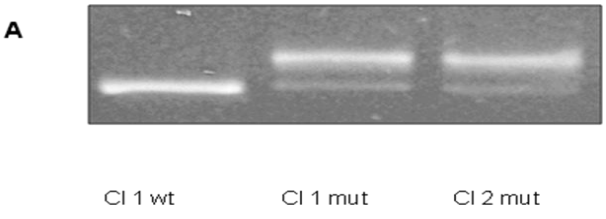
Fig. 1 Fig. 1. MRI, Genetic and biochemical findings. (C) MRC CI, CII, CIII, CIV, CV activities normalized to the activity of citrate synthase in muscle homogenate (blue bars) and fibroblast lysates (red bars) of the patient. Values areexpressed as percentages of the controls' mean, taken as 100%. (D) Allele-specific RFLP analysis. A PCR fragment was amplified using a 41-mer forward primer corresponding to mtDNA region 6555–6596 and a 22-mer reverse primer corresponding to mtDNA position 6886–6908. The forward primer sequence was:50CCTCAACACCACCTTCTTCGACCCCGCCG GAGGAGGAGACCCCATTCGGTAC-30 The underlined GG doublet replaces the TA doublet present in the mtDNA Cambridge sequence. This change creates a diagnostic KpnI restriction site in the PCR fragments carrying the wild type 6597C, while the restriction site is destroyed in the PCR fragments carrying the mutant 6597A. After digestion with KpnI the 353-bp uncut fragment corresponding to mutant mtDNA (top band) was separated from the 312-bp (bottom band) and 41-bp (not shown) fragments from wild-type mtDNA by electrophoresis through a 3% agarose gel. M: muscle, L: lymphocytes, U: urinary-tract cells, F: fibroblasts. Individuals are indicated according to the nomenclature of the family tree.

Contrariwise the activities of CIII/CS and CI/CS were comparable between the two mutant clones and the wild-type ones.

Western-blot immunodetection and densitometric measurement on one-dimension blue-native gel electrophoresis. (BNGE) from cybrid lysates showed that the 80% heteroplasmic mutant clones had about 35% holo-COX content compared to the wild-type clones. An example displaying the results in a mutant clone vs a wt clone is shown in Fig. 2C. We also investigated by one-dimension BNGE Western blot the structure of COX in mutant muscle; the content of the holoenzyme was about 45‑50% that of a control subject, suggesting impaired assembly or stability, whereas the content of other MRC complexes was normal [15,16] (Fig. 2D).

4. Discussion

The neurological history of this relatively young patient was heralded by acute, reversible stroke, for which obvious vascular and cardiac causes had been ruled out. However, the presence of hearing loss, short stature, mild mental impairment, migraine, exercise intolerance, myalgias, cramps, and limb-girdle weakness and hypotrophy led us to suspect a mitochondrial encephalomyopathy, most likely a MELAS or MELAS-like condition.



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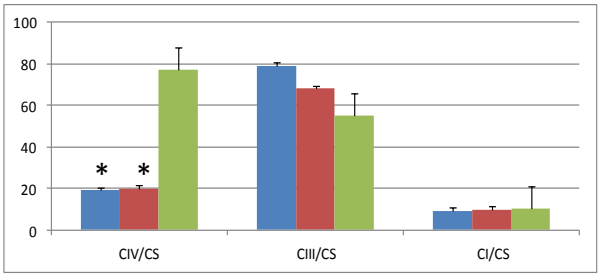

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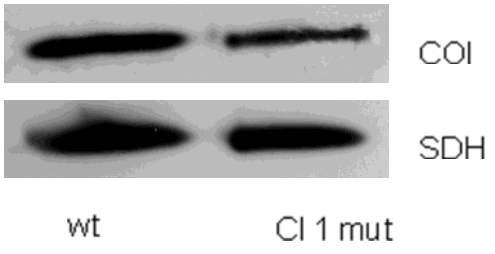

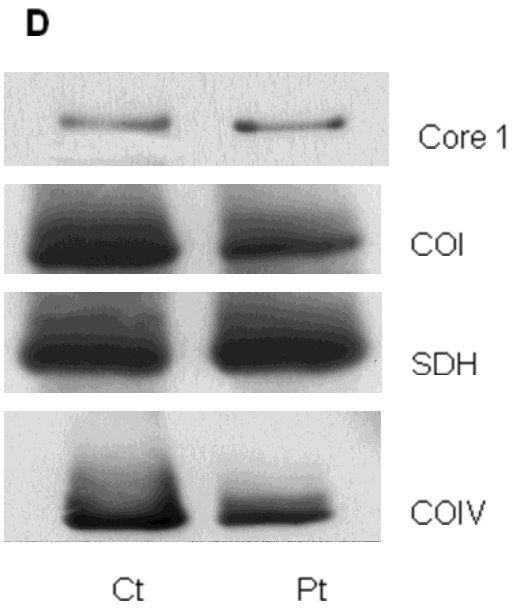

Fig. 2. Characterization of patient-derived cybrids. (A) RFLP-PCR analysis on cybrids clones. (B) CIV/CS, CIII/CS and CI/CS activities in the two mutant clones (light and dark gray bars) compared to the wild type ones (black bars).
(C) One dimension blue native gel electrophoresis (BNGE) from cybrid lysates showing a 35% reduction of holo-COX content in the mutant clone compared to the wt one. (D) One-dimension blue-native gel electrophoresis (BNGE) from muscle homogenate showing a 45‑50% reduction of holo-COX content in the patient compared to a control subject.

This hypothesis was definitely confirmed by the presence of numerous fibres showing mitochondrial alterations (RRF) in a skeletal muscle biopsy. In 90% of the cases, MELAS (MIM 540000) is associated with a specific mutation, m.3243G>A, in the mt-tRNALeu(UUR) gene, or, less frequently, with mutations in other mt-tRNA genes, usually associated with predominant complex I (CI) deficiency. A few mutations in mitochondrial ND genes, particularly ND5, have also been associated with MELAS, again resulting in CI deficiency in muscle. In most of these cases, including the m.3243G>A mutation, the muscle biopsy shows ragged red, COX positive, rather than negative, fibres. In contrast, our patient showed profound and isolated reduction of COX activity in muscle histological sections and homogenate,

suggesting a mutation in CIV. This suspect was eventually confirmed by sequence analysis of mtDNA, which revealed the presence of a missense mutation in theMTCOI gene. In general, mutations in MTCOI are very rare, usually sporadic, and associated with a spectrum of conditions including sideroblastic anemia [3], myopathy with myoglobinuria [6], and, as in our case, stroke-like episodes, leading to severe, drug-refractory epilepsy [8,9]. Our patient had a milder clinical presentation, characterized by a single episode of seizures during a transient stroke-like episode, which did not recur afterwards, on an underlying progressive, albeit moderate, mitochondrial myopathy. In fact, it was the examination of the muscle biopsy that allowed to establish the diagnosis of mitochondrial disease, eventually leading us to identify the

responsible mutation in mtDNA. Interestingly, the patient also showed secondary amenorrhea, clearly caused by hypophyseal insufficiency, supporting the idea that, albeit often overlooked, hypogonadism is not uncommon in mitochondrial disease. The Q232K substitution changes the net charge and steric hindrance of the aminoacid residue. The Q232 residue is highly conserved through phylogenesis (Fig. 3A,B). It is located at the extremity of the 6th transmembrane domain of MTCOI, at the interface between MTCOI and MTCOII, in a region exposed to solvent. From the crystal structure of the Paracoccus denitrificans enzyme, this residue is at the entrance of the channel connecting the surface of the enzyme facing the inter-membrane space with the pouch harboring the prosthetic groups, through which electrons flow from

cytochrome c to molecular oxygen [17,18] (Fig. 3C). Analysis based on the 3D crystal structure of COX predicts several structural and possibly functional abnormalities determined by the Q232K change (Fig. 3C), First, the lateral chain of the Q232 is surrounded by negatively charged and hydrophobic residues, which belong to both MTCOI and MTCOII subunits; the substitution with a positively charged K could electrostatically perturb the region, affect the electron transfer from the CuA center in MTCOII to the heme a of MTCOI, and/or impair the interaction between MTCOI and MTCOII. In addition, since Q232 is next to the proton exit pathway, the K232

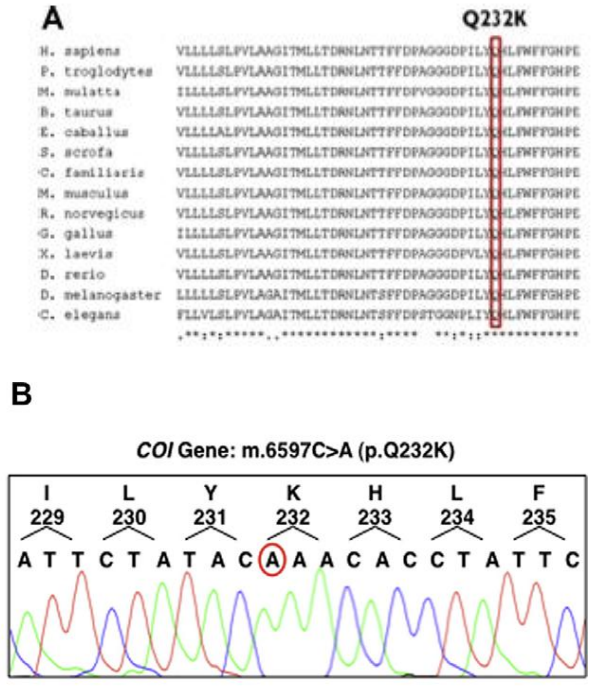

Fig. 3. In silico and in vitro analysis of wild-type and mutant MTCOI gene and protein. (A) Interspecies protein alignment. The red box indicates the Q232 residue. (B) Electropherogram of the MTCOI gene. The mutant A is encircled in red.

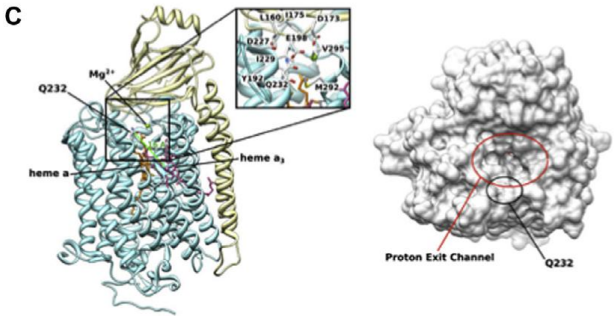

Fig. 3 In silico and in vitro analysis of wild-type and mutant MTCOI gene and protein. (C) Crystal structure of the Paracoccus denitrificans MTCOI (cyan) and MTCOII (yellow) proteins, displaying the 3D position of the Q232 residue, according to a lateral view (left), or a view from the external surface of the inner mitochondrial membrane (right). See main text for details.

mutant residue could also alter the efficiency of proton pumping. Taken together, these observations indicate that the Q232K MTCOI mutation can impair the activity and partly destabilize the structure of COX, in agreement with our own biochemical data and BNGE-

based results. In agreement with the deleterious nature of the mutation, the heteroplasmic load of the m.6597C>A change did segregate with the clinical presentation in the family members: both the mother of the patient, and her brother, who are asymptomatic, showed no mutation, at least in the biological samples that could be examined, i.e. urinary sediment and blood. Likewise, the three sons of the patient are healthy with no mutation, suggesting bottleneck-based selection of mutation-free oocytes in their mutant mother. Accordingly, we consistently failed to obtain homoplasmic mutant cybrids, possibly because this condition is incompatible with cell survival, suggesting that the mutation exerts a toxic role rather than simply reducing OXPHOS proficiency, since rho0 cells, i.e. mtDNA-less, respiration-incompetent cells, do

survive in the same auxotrophic medium, containing uridine and high concentration of pyruvate, used for our cybrids. A similar mechanism could also be active in other MTCOI mutations, which could explain both their rarity and non-transmission.

Acknowledgments

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CHAPTER 3

Unpublished results

Novel (ovario)leukodystrophy related to AARS2 mutations

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ABSTRACT

Objectives: The study was focused on leukoencephalopathies of unknown cause with the aim to define a novel, homogeneous phenotype suggestive of a common genetic defect, based on clinical and MRI findings, and to identify the causal genetic defect, shared by patients with this phenotype.

Methods: Independent next generation exome sequencing studies were performed in two unrelated patients with a leukoencephalopathy. MRI findings in these patients were compared to available MRIs in a database of unclassified leukoencephalopathies; eleven patients with similar MRI abnormalities were selected. Clinical and MRI findings were investigated.

Results: Next generation sequencing revealed compound heterozygous mutations in *AARS2* encoding mitochondrial alanine-tRNA

synthetase in both patients. Functional studies in yeast confirmed the pathogenicity of the mutations in one patient. Sanger sequencing revealed *AARS2* mutations in four of the eleven selected patients. The six patients with *AARS2* mutations had childhood to adulthood onset signs of neurological deterioration consisting of ataxia, spasticity and cognitive decline with features of frontal lobe dysfunction. MRIs showed a leukoencephalopathy with striking involvement of left-right connections and ascending and descending tracts, and cerebellar atrophy. All female patients had ovarian failure. None of the patients had signs of a cardiomyopathy.

Conclusions: Mutations in *AARS2* have been found in a severe form of infantile cardiomyopathy in two families. We present

six patients with a new phenotype caused by *AARS2* mutations, characterized by leukoencephalopathy and, in female patients, ovarian failure, indicating that the phenotypic spectrum associated with *AARS2* variants is much wider than previously reported.

INTRODUCTION

Mutations in genes coding for mitochondrial amino acyl tRNA synthetases, the enzymes that charge a specific tRNA with its cognate amino acid, are recently emerging as a new important cause of mitochondrial disease, and have been associated with a wide spectrum of clinical phenotypes (1-3). However, defects in each amino acyl tRNA synthetase seem to determine rather homogeneous clinical

presentations (1-5).

The use of whole exome sequencing (WES) has markedly increased efficiency and improved the genetic analysis for inherited disorders, allowing gene identification for small groups of patients sharing a phenotype, individual cases with atypical presentation, and for disorders lacking consistent genotype-phenotype correlation (6-8).

Mutations in the mitochondrial alanine tRNA synthetase gene (*AARS2;* MIM*612035) have been found in a severe form of infantile cardiomyopathy in two families (9). In the present study we describe a very different clinical picture determined by *AARS2* genetic defects in six patients affected by a progressive leukoencephalopathy and, in females, ovarian failure, a clinical presentation previously described as

"ovarioleukodystrophy" (10). In two patients the mutations were found by independent WES studies, and were functionally validated by a yeast recombinant model in one patient. Recognition of a specific MRI pattern of leukoencephalopathy led us to identify the other patients and better define the disease phenotype.

PATIENTS AND METHODS

The study was approved by the Ethical Committees of the Foundation IRCCS Istituto Neurologico "Besta", Milan, Italy; the Children's National Medical Center, Washington, USA; and the VU University Medical Center, Amsterdam, The Netherlands, in agreement with the Declaration of Helsinki. Informed consent was signed by the patients.

Patients

Patients 1 (P1) and 2 (P2) were included in two independent next generation sequencing studies aimed at elucidating individual genetic defects. P1 was the only patient in whom muscle and skin biopsies were performed. A detailed description of histo- and biochemical studies is reported in Supplemental data. On the basis of the MRI findings in these two patients, ten patients from nine families were selected from the Amsterdam database, which contains MRIs of more than 3000 cases with a leukoencephalopathy of unknown origin (11) and one patient from the leukoencephalopathy cases of the Istituto Neurologico "Besta" without genetic diagnosis. Common MRI features were frontal and parietal white matter abnormalities with relative sparing of the central posterior-frontal or frontoparietal

region. The anterior part of the corpus callosum and a thin strip in the splenium were affected, with relative sparing of the central region. Most female patients had ovarian failure in addition to the leukoencephalopathy, a combination called ovarioleukodystrophy (10). Two unsolved ovarioleukodystrophy patients previously described (10,12) were also included in the study.

For all patients clinical and general laboratory data were collected retrospectively. All available MRIs were analyzed and abnormalities were scored as previously described (10).

Molecular studies

Total genomic DNA was extracted by standard methods from peripheral blood leukocytes, or muscle biopsies. In P1, WES and variant

filtering were performed, as previously described, using 100 base-pair (bp) paired-end runs (13). For P2, a custom probe library (Roche NimbleGen Inc., Madison, WI, USA) designed to capture coding exons plus 20 bp into the flanking introns of 500 genes known to be related to mitochondrial disorders was used for target gene capture followed by deep sequencing at average coverage depth of 1000 per base. Additionally, clinical WES (Illumina) was performed. Sanger sequencing of the gene identified by WES was performed in all patients (9).

Functional studies

Yeast strains derived from W303-1B were used (14). Detailed methods including respiratory activity, cytochrome spectra, in vivo mitochondrial DNA protein synthesis,

amino acylation of mitochondrial tRNA for alanine, cytochrome c oxidase (complex IV) and complex I+III activities, and primers for the preparation of plasmids used in this study are reported in Supplemental data.

RESULTS

Patients and laboratory findings

The clinical features of the patients in whom we found mutations in the gene mentioned below are summarized in table e-2. A brief description is given here.

P1 is a female, in whom psychomotor developmental delay became evident at 2 years of age. She achieved walking without support at 3 years, but with impaired balance. Her condition remained stable until age 15, when she developed progressive gait ataxia, tremor,

cognitive deterioration and psychosis. At age 18, she developed secondary amenorrhea due to ovarian failure. The latest neurological examination at age 30 revealed severe cerebellar ataxia with nystagmus, dysarthria, intention tremor, and instable gait, because of which she could only walk with support. General physical examination revealed no abnormalities; in particular, there was no evidence of cardiac dysfunction. ECG and cardiac ultrasound were normal.

P2 is a male, who came to medical attention in infancy due to congenital nystagmus. In primary school, mild clumsiness and learning difficulties were a concern. In his early teenage years, he developed mild right-sided hemiparesis and ataxia, which was ascribed to periventricular leukomalacia due to periventricular white matter abnormalities on

MRI. Around age 17, deterioration set in with gait difficulties due to bilateral dystonia and spasticity, dysarthria, and cognitive decline. A viral gastroenteritis was followed by abrupt mental decline, unintelligible speech, inability to eat due to choking, recurrent vomiting, and inability to walk due to worsening of ataxia and spasticity (left more than right). General physical examination revealed no abnormalities, including no evidence for cardiac dysfunction.

P3 is a female, who presented with secondary amenorrhea due to ovarian failure at age 28. At age 33, she developed depression, cognitive deterioration, behavioral problems with signs of frontal dysfunction, and urinary incontinence. Neurological examination revealed down-beat nystagmus, some postural and appendicular tremor of the arms, but

otherwise no motor disability. She developed stereotyped motor behavior and severe apraxia. At age 35 she did not recognize family members any more. Her only means of communication were grunting and facial expression. She had to be tube fed. Antiepileptic medication was started because of epileptic seizures. At age 36, she was bed-ridden and had no interactions with her surroundings.

P4 is a female, who was diagnosed with ovarian failure at age 23. She developed tremor of her hands at age 24. Because of white matter abnormalities on the MRI, she was diagnosed with multiple sclerosis. From age 26, rapid cognitive, behavioral and motor deterioration became evident. She developed progressive dystonia, cerebellar ataxia and spasticity. Neurological examination revealed

that the signs of motor dysfunction were left more severe than right. General physical examination revealed no abnormalities. She lost speech, became bed-ridden and died at age 28.

P5 is a female, who had primary amenorrhea due to ovarian failure. From age 40, she developed depression and rapid cognitive deterioration. Neurological examination revealed no motor dysfunction. General physical examination revealed no abnormalities. She became bed-ridden and died at age 46.

P6 is a female, who was diagnosed with ovarian failure at 20 years of age. At age 22, she presented with gait problems caused by hypertonia of the legs, left more than right. She developed depression bur no cognitive regression. Neurological examination revealed

a spastic paraparesis with ataxic signs. The disease was rapidly progressive. She lost the ability to walk. At latest examination at age 25 she was wheelchair-bound.

Laboratory findings are summarized in table e-3. None of the patients investigated had elevated blood or CSF lactate. Strikingly, all five female patients had primary or secondary amenorrhea due to ovarian failure.

P1 and P6 were the only patients extensively investigated for a possible mitochondrial defect. In P1, the Gomori Trichrome stain and succinate dehydrogenase reaction of a skeletal muscle biopsy were normal, whereas the histochemical reaction to cytochrome c oxidase was diffusely reduced (Figure 1A). Likewise, biochemical assay showed severe isolated cytochrome c oxidase deficiency (15% of residual activity) in muscle

homogenate (Figure 1B), whereas the activities of all respiratory chain complexes, including cytochrome c oxidase, were normal in cultured skin fibroblasts. Oxygraphic studies performed in patient's fibroblasts, grown in either glucose or galactose medium, showed no defect compared to controls (not shown).

Sequencing of mitochondrial DNA in P1 revealed a heteroplasmic variant m.5979G>A (p.Ala26Thr) in *MTCOI*, reported as rare single nucleotide polymorphism (<0.1% in Mitomap database) present in haplogroup H45. The low levels of mutation loads in P1 and her family members and the results obtained from cybrids (not shown) ruled out a primary causative role for this mitochondrial DNA variant.

In P6, histological and histochemical analyses

of a muscle biopsy revealed a diffusely reduced cytochrome c oxidase staining, but no ragged red fibers. Measurement of individual oxidative phosporylation enzyme activities in muscle homogenate showed reduced cytochrome c oxidase (33% of residual activity) (Figure 1B). Mitochondrial DNA sequencing revealed no pathogenic point mutations, but several polymorphisms typical of haplogroup T1a1.

MRI abnormalities

The MRI abnormalities are summarized in table e-4 and are illustrated in Figures 2 and e-1. In all six patients, the cerebral white matter abnormalities were inhomogeneous and patchy, affecting and sparing strips of tissue. In several patients, the signal of small parts of the T2-hyperintense white matter was low on

fluid-attenuated inversion recovery (FLAIR) images, indicating white matter rarefaction. Typically, the signal abnormalities were predominantly present in the frontal and parietal periventricular and deep white matter, sparing a segment of white matter in between. White matter structures were affected in a tract-like fashion, with involvement of left-right connections through the corpus callosum and involvement of ascending and descending connections. Depending on the location of the cerebral hemispheric white matter abnormalities, the frontopontine, pyramidal or parieto-occipitopontine tracts were longitudinally affected.

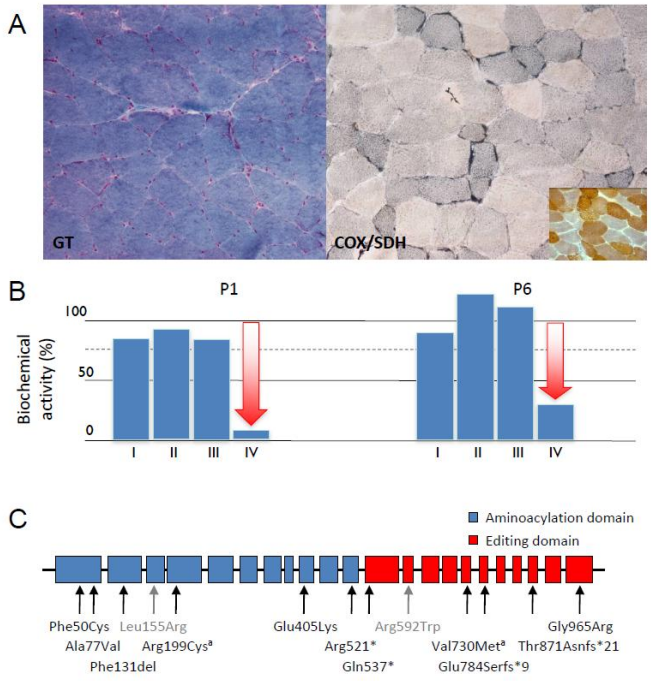

Figure 1. Biochemical and genetic features.

A. Morphological analysis of muscle biopsy from patient P1: Gomori trichrome (GT) staining and cytochrome c oxidase (COX)/succinate dehydrogenase (SDH) histoenzymatic double staining (COX/SDH). Inset shows the reaction in an age-matched control biopsy. B. Biochemical activities of mitochondrial

respiratory chain complexes in patients P1 and P6 muscle homogenates. All enzymatic activities are normalized for citrate synthase activity, and indicated as percentages relative to the mean control value. C. Genomic structure of *AARS2* with exons coding for the amino acylation (blue) and editing (red) domains. The arrows indicate the position of mutations identified in this study (black) or previously reported (grey). [a] The two missense variants p.Arg199Cys and p.Val730Met are on the same allele; the latter (rs35623954) is reported to have a frequency >1% in control populations, suggesting that the former is the pathogenic variant.

The corpus callosum abnormalities were also dependent on the cerebral hemispheric white matter involvement; they could be limited to a single lesion in the splenium, affect a large part of the anterior corpus callosum and only a narrow strip in the splenium, or involve the entire corpus callosum in an inhomogeneous fashion. Diffusion-weighted images showed

multiple small areas of restricted diffusion in the cerebral white matter and corpus callosum. The abnormalities were limited to white matter structures and were progressive over time. No contrast enhancement was observed. Cerebellar atrophy was variable and affected the vermis more than the hemispheres. Cerebral atrophy was at most mild.

Whole exome sequencing and Sanger Sequencing

WES was performed on genomic DNA from P1 (6). After excluding common single nucleotide polymorphisms (>0.1%), the remaining changes were prioritized according to the presence of homozygous or compound heterozygous mutations, as expected for a recessive inherited trait, and for known or predicted mitochondrial localization of the

corresponding protein (15). This filtering procedure led to the identification of a single outstanding gene entry, *AARS2* (NC_000006.11), in which a missense (c.149T>G, p.Phe50Cys) and a nonsense (c.1561C>T, p.Arg521*) heterozygous variant were present (NM_020745.3) (Figure 1C-D, Table 1). The two mutations segregated within the family.

P2 was subjected to a gene panel containing 500 genes known to cause mitochondrial disorders, because MRI findings had raised concern for a mitochondrial leukoencephalopathy. Due to the extremely rapid clinical deterioration, clinical WES was initiated shortly thereafter. With both policies, two missense mutations in *AARS2* were identified (c.2893G>A, p.Gly965Arg and c.1213G>A, p.Glu405Lys).

Sanger sequencing of *AARS2* exons and intron-exon boundaries was performed in all patients. In four of the eleven patients selected on the basis of MRI features *AARS2* mutations were found (Table 1). In the two patients with ovarioleukodystrophy, in whom no mutations in *EIF2B1-5* had been found (10, 12), no *AARS2* mutations were detected. All identified nucleotide substitutions had a frequency <0.01% in public databases and the corresponding amino acid changes were predicted to be deleterious (table e-5).

Functional studies

Because no biochemical readout was detected in cell lines of P1, the possible deleterious impact of the *AARS2* mutations on oxidative phosphorylation was tested in a

Saccharomyces cerevisiae yeast model. Phe50 in human *AARS2* is highly conserved in phylogenesis, including yeast (Figure e-2), in which the ortholog *ala1* gene (NC_001147.6) codes for both the cytosolic and mitochondrial alanyl tRNA synthetase (16). We disrupted *ala1* by homologous recombination and re-expressed the wild-type cytosolic isoform (Supplemental data), thus generating a viable but oxidative phosphorylation incompetent strain ($ala1^{L\text{-}16X}$), which lacked the mitochondrial isoform. In this strain, we expressed either the wild-type *ala1* gene ($ala1^{wt}$), a p.Phe22Cys mutant allele ($ala1^{F22C}$), equivalent to the human p.Phe50Cys variant, or a p.Val500* mutant allele ($ala1^{V500X}$), equivalent to the human p.Arg521* variant.

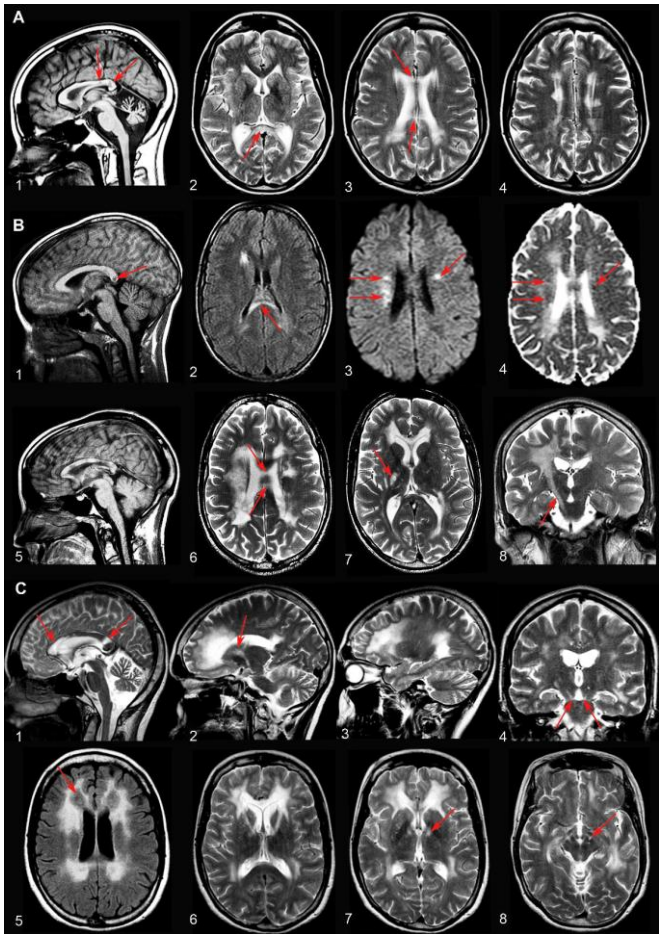
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Figure 2. Brain MRI

A. MRI in P1 at age 28. The sagittal T1-weighted image shows serious cerebellar atrophy and two strips of abnormal signal in the splenium (arrows in image 1). The axial T2-weighted images show inhomogeneous areas of abnormal signal in the periventricular white matter. The areas on the left and right are connected signal abnormalities in the corpus callosum (arrows in images 2-4).

B. MRI in P2 at age 14 (images 1 and 2), age 21 (next two images 3 and 4) and age 23 (images 5-8). At age 14 a lesion is seen in the splenium of the corpus callosum (arrows in image 1) and in the right frontal periventricular white matter. The diffusion-weighted images suggest the presence of multiple small areas of restricted diffusion in the abnormal white matter (arrows in image 3), confirmed by low signal of the corresponding areas on the apparent diffusion coefficient map (arrows in image 4). The most recent MRI shows multiple segments of abnormal signal in the corpus callosum (image 5 and arrows in image 6). More extensive signal abnormalities are seen in the periventricular white matter, especially on the right

(images 6 and 7). Signal abnormalities extend downwards through the posterior limb of the internal capsule and the pyramidal tracts in the brain stem on the right (arrows in images 7 and 8).

C. MRI in patient 3 at age 35. The midsagittal image shows that the anterior part of the corpus callosum is abnormal, while only a strip of signal abnormality is seen in the splenium (arrows in image 1). Images 2 and 3 illustrate that the frontal and parietal white matter is abnormal, while the central white matter in between is normal. The tract involvement is evident (arrows in images 2 and 4). The axial FLAIR image shows that the affected white matter is rarefied (arrow in image 5). The axial T2-weighted images illustrate the involvement of the anterior limb of the internal capsule (image 6) and the frontopontine tracts going down into the brain stem (arrows in images 7 and 8).

In contrast to $ala1^{wt}$, the expression of ala^{V500X} failed to restore growth on non- fermentable sources (i.e. glycerol). The oxidative growth of the strain expressing $ala1^{F22C}$ was similar to

the wild type at 28°C but clearly reduced at 37°C (Figure e-3). We obtained similar results from assays evaluating the oxygen consumption (Figure 3A), the *in vivo* mitochondrial protein synthesis (Figure 3B), the activities of the respiratory chain complexes I+III and IV, and the spectra of mitochondrial respiratory chain cytochromes (Figure e-3). Finally, we evaluated the charging of the tRNA with alanine; the p.Val500* change determined the complete inability to charge the mitochondrial $tRNA^{Ala}$ with its cognate amino acid, whereas the p.Phe22Cys change determined a partial reduction in the amino acid charging at 37°C (Figure 3C). We also analyzed the effects of $ala1^{L125R}$, corresponding to the previously identified mutation p.Leu155Arg (9), and found that in all experiments this missense

mutation behaves as a null allele (Figure 3, Figure e-3). The other known *AARS2* mutation, p.Arg592Trp, could not be tested, because the residue is not conserved in yeast (Figure e-2).

Taken together, the experiments in yeast confirmed that the p.Phe50Cys missense mutation is deleterious only in stress conditions, while the truncated protein due to the nonsense mutation is nonfunctional.

Table 1

***AARS2* mutations found in the patients**

Patient	cDNA	protein	State	Paternal/ Maternal	EVS frequency
1	c.149T>G	p.Phe50Cys	heterozygous	M	ø
	c.1561C>T	p.Arg521*	heterozygous	P	ø
2	c.2893G>A	p.Gly965Arg	heterozygous	M	ø
	c.1213G>A	p.Glu405Lys	heterozygous	P	ø
3	c.1609C>T and c.2350del	p.Gln537* and p.Glu784Serfs*9	heterozygous	M	ø
	c.595C>T and c.2188G>A	p.Arg199Cys and p.Val730Met	heterozygous	P	0.008% and 3.1%
4	c.230C>T	p.Ala77Val	heterozygous	M	0.008%
	c.595C>T and c.2188G>A	p.Arg199Cys and p.Val730Met	heterozygous	P	0.008% and 3.1%
5	c.595C>T and c.2188G>A	p.Arg199Cys and p.Val730Met	heterozygous	M	0.008% and 3.1%
	c.390_392del	p.Phe131del	heterozygous	P	ø
6	c.595C>T and c.2188G>A	p.Arg199Cys and p.Val730Met	heterozygous	M	0.008% and 3.1%
	c.2611dup	p.Thr871Asnfs*21	heterozygous	P	ø

EVS, Exome Variant Server (varianttools.sourceforge.net/Annotation/EVS); Ø, not found

DISCUSSION

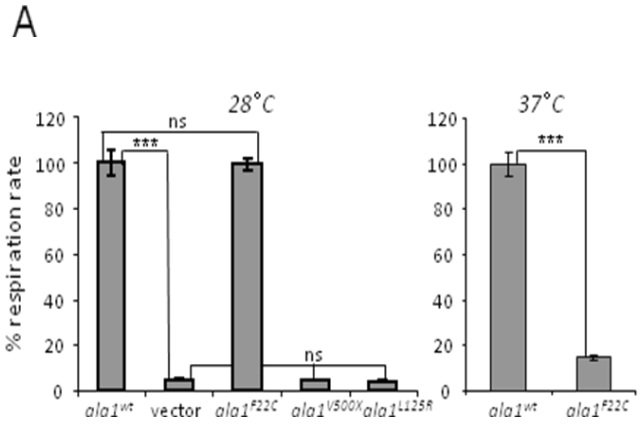
Defects in several different mitochondrial amino acyl tRNA synthetases have been associated with specific clinical phenotypes (1, 3-5). However, these enzymes are ubiquitously expressed and take part in the same process, so this phenotypic segregation is difficult to explain. This conclusion of phenotypic segregation may be based on inclusion bias, because the genes are only analyzed in patients with specific phenotypes. Additionally, the number of patients with mutations in different mitochondrial amino acyl tRNA synthetases is at present too small to provide conclusive evidence of the existence of exclusive genotype-phenotype correlations.

Recently, *AARS2* mutations have been reported in three subjects as the cause for infantile hypertrophic cardiomyopathy, lactic

acidosis, and brain and skeletal muscle involvement, with early fatal outcome (9). Our patients have a very different clinical picture, with signs of neurological dysfunction and a leukoencephalopathy on MRI. None of the patients had signs of a cardiomyopathy.

In P1 and P2 onset was in childhood with initially a stable course or very slow disease progression, while the course after onset of evident deterioration was rather rapid in all cases. The neurological dysfunction comprised motor deterioration, consisting of cerebellar ataxia and spasticity, and cognitive decline with features of frontal lobe dysfunction with poor memory, inactivity and other behavioral changes, as well as depression and other psychiatric features. Comparing the clinical features and MRI findings, it is clear that the clinical signs of the patients depend on which

tracts are affected. P2, P4 and P6 had prominent signs of motor dysfunction and had pyramidal tract involvement on MRI. P3 and P5 mainly had signs of behavioral and cognitive dysfunction and frontopontine tract involvement.



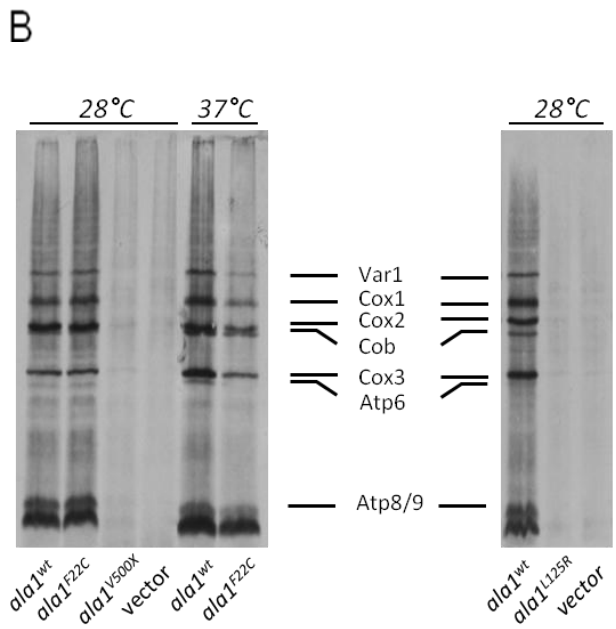


Figure 3. Yeast studies

A. Oxygen consumption rate of the *ala1*$^{L\text{-}16X}$ strain transformed with the *ala1*^{wt} allele, the empty vector, and the mutant alleles *ala1*^{F22C}, *ala1*^{V500X}, *ala1*^{L125R}. Respiratory rates were normalized to the strain transformed with *ala1*^{wt}, for which the respiratory rate was 81.1 nmol min^{-1} mg^{-1} at 28°C and 33.4 nmol min^{-1} mg^{-1} at 37°C. Values are the mean of three independent experiments, each with an independent clone. Two-tail,

paired t test was applied for statistical significance. *: p<0.05;**<0.01:***: p<0.001.

B. In vivo mitochondrial protein synthesis of the *ala1*$^{L\text{-}16X}$ strain transformed with the *ala1*^{wt} allele, the empty vector, and the mutant alleles *ala1*^{F22C}, *ala1*^{V500X}, *ala1*^{L125R}. Mitochondrial gene products were labeled with [^{35}S]-methionine in whole cells in the presence of cycloheximide for 10 min at 28°C or 37°C.

Cox, cytochrome c oxidase; Cob, cytochrome b; Atp, ATP synthase; Var1, small mitochondrial ribosome subunit.

C

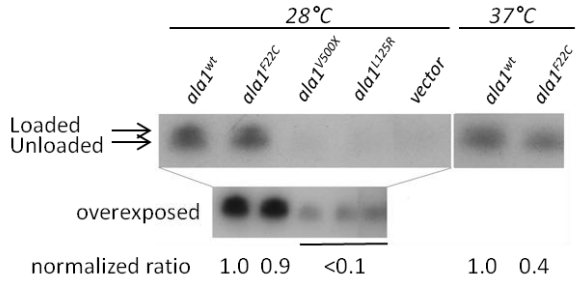


Figure 3. Yeast studies

C. Mitochondrial $tRNA^{Ala}$ loading of the $ala1^{L\text{-}16X}$ strain transformed with the $ala1^{wt}$ allele, the empty vector, and the mutant alleles $ala1^{F22C}$, $ala1^{V500X}$, $ala1^{L125R}$. Signals were quantified with Quantity One (Bio-Rad). For each strain the ratio between the charged $tRNA^{Ala}$ and the uncharged one was calculated and normalized to the $ala1^{wt}$ strain.

The striking white matter tract involvement and the presence of spots of restricted diffusion in the cerebral white matter are features shared by another disorder with mutations in a mitochondrial amino acyl tRNA synthetase: "leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation" (LBSL), caused by mutations in *DARS2* (MIM*610956). In LBSL the white matter spots of restricted diffusion move over time. They are ascribed to temporary myelin

vacuolization, a feature frequently seen in mitochondrial leukoencephalopathies (17).

In P1 and P6, the only patients who underwent detailed histo- and biochemical analyses, we detected a profound, isolated defect of complex IV activity in skeletal muscle, rather than a combination of mitochondrial DNA-related respiratory chain defects, which is the expected consequence of reduced mitochondrial protein synthesis. The pathogenicity of the two variants found in P1 was experimentally proven by a recombinant yeast model. Interestingly, the yeast p.Phe22Cys mutation, equivalent to p.Phe50Cys found in P1, impairs complex IV activity much more than complex I+III activity, whereas p.Leu125Arg, equivalent to human p.Leu155Arg and described in the cardiomyopathy patients (9), decreases them

both, suggesting that the isolated defect of complex IV found in our patients may predominantly depend upon specific missense mutations, possibly influencing the main target tissue (brain versus heart). Variable involvement of different respiratory chain complexes and different degrees of defective activities have also been reported for different mutations in other amino acyl tRNA synthetases (18).

The yeast mutation equivalent to human p.Phe50Cys was only deleterious in stress conditions, while the other tested mutations were highly deleterious in basal conditions, suggesting that the p.Phe50Cys mutant protein maintains a residual activity, that could explain the slowly progressive clinical presentation of our patient, in contrast to the rapidly fatal outcome reported for the

cardiomyopathy patients (9). While our yeast model enabled us to demonstrate the deleterious effect of the p.Leu155Arg mutation, poor conservation between human and yeast made it impossible to validate in the latter the p.Arg592Trp substitution reported in patients with cardiomyopathy (9). As suggested by Götz *et al.* (9), this mutation could impair the editing activity of *AARS2*, leading to increased mistranslation of the alanine codon by serine or glycine. According to this hypothesis, a specific pathogenic mechanism, mistranslation, would lead to cardiomyopathy, while translation deficiency, caused by other amino acyl tRNA synthetase mutations, is usually not associated with heart damage (3). Interestingly, hypertrophic cardiomyopathy has been described in subjects with mutations affecting *MTO1*, an enzyme

responsible for tRNA modifications that increase the accuracy and efficiency of mitochondrial DNA translation (19, 20).

A striking feature was the presence of ovarian failure in all female patients with *AARS2* mutations. Mutations in two other mitochondrial amino acyl tRNA synthetase encoding genes, namely *HARS2* (MIM*600783) and *LARS2* (MIM*604544), have recently been associated with Perrault syndrome, a recessive disorder characterized by ovarian dysgenesis and sensorineural hearing loss. However, Perrault syndrome is genetically heterogeneous; it can also be caused by mutations in *HSD17B4*, encoding a peroxisomal enzyme involved in fatty acid beta-oxidation, and mutations in *CLPP*, encoding a mitochondrial endopeptidase. Strikingly, mutations in *POLG*

(MIM*174763), the gene encoding the DNA polymerase gamma for the replication of human mitochondrial DNA, are associated with a broad spectrum of symptoms including premature menopause (21) and ovarian failure (22).

Of the seven patients included in the present study, who had similar MRI abnormalities, but no *AARS2* mutations, two were male, and two were pre-pubertal females. Of the three adult female patients, one had normal menses until put on contraceptive injections, one had a period of 3 years of amenorrhea, but recently started menstruating again, and on one the information is not available. With this information it is not possible to make a definitive conclusion on the presence or absence of ovarian failure in these patients.

The preferential involvement of brain white

matter and ovaries is shared by vanishing white matter (MIM 603896), a disease caused by mutations in any of the five genes (*EIF2B1* to *EIF2B5*) encoding subunits of the translation initiation factor eIF2B. Female patients often develop ovarian failure, manifest as primary or secondary amenorrhea (12). eIF2B impairment leads to a defect in translation initiation for nuclear genes, not affecting translation of the mitochondrial encoded proteins. Hence, even if both *AARS2* and *EIF2B1-5* mutations are likely associated with block or dysregulation of protein synthesis, their targets and site of action (i.e. mitochondria versus cytosol) are completely separated and, at present, we have no explanation for the common resulting phenotype. The two patients with ovarioleukodystrophy included in our study, as

well as the seven other selected patients with the same or very similar MRI abnormalities, had no *AARS2* mutations, suggesting that they have mutations in untranslated regions of the gene or there could be (an)other gene(s) that, when mutated, are associated with the same selective vulnerability.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Supplemental Data

Supplemental methods: Detailed methods for histochemical, biochemical and yeast studies

Supplemental Table e-1: Primers used in this study

Supplemental Table e-2: Clinical characteristics

Supplemental Table e-3: Laboratory findings

Supplemental Table e-4: MRI findings

Supplemental Table e-5: In silico prediction of pathogenicity for *AARS2* mutations

Supplemental figure e-1: MRI of patients P4 and P5

Supplemental figure e-2: Protein sequence alignment of *AARS2* orthologs

Supplemental Figure e-3: OXPHOS growth and biochemical assays in yeast

Supplemental Methods

Respiratory chain function

Cryostatic cross sections of skeletal muscle biopsies were used for histological and histochemical studies, according to standard techniques. Gomori trichrome staining, succinate dehydrogenase and cytochrome c oxidase activities were performed as previously described (1). The activities of respiratory chain complexes in muscle homogenates and digitonin-treated fibroblasts were measured using standard spectrophotometric techniques. Micro-oxygraphy was used to measure oxygen

consumption rate in fibroblasts, using a SeaHorse FX-96 instrument (2). Fibroblasts from P1 were cultured in DMEM glucose medium, or glucose-free 5mM galactose DMEM medium for 72 hours before the analysis.

Yeast strains, plasmids and media

The W303-1B genotype is *Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100*.Strains were grown in YP complete media (1% yeast extract, 2% peptone ForMedium, Norfolk, UK) or SC synthetic defined media (0.19% YNB without aminoacids and NH_4SO_4 powder ForMedium, Norfolk, UK, 0.5% NH_4SO_4) supplemented with 1gr/L dropout mix without uracil and tryptophan (3). Media were supplemented with various carbon sources at 2% (w/v) (Carlo Erba Reagents,

Milan, Italy), in liquid phase or after solidification with 20g/L agar (ForMedium, Norfolk, UK).

ala1 was cloned under its natural promoter by PCR-amplification with oligonucleotides ALA1C (Supplementary table 1) and cloning of the *Sac*I-*Sph*I-digested *ala1* fragment in pFL36 (4). The plasmid was inserted in W303-1B through the Li-Ac method (5). Since disruption of *ala1* is lethal, we performed disruption of the gene in strain W303-1B harboring pFL36*ala1*. Disruption was performed through one-step gene disruption by PCR-amplification of KanMX4 cassette (6) with primers ALA1D (Additional file 1) and transformation of the former strain, thus obtaining W303-1B *ala1Δ*/pFL36*ala1*.

ala1 fragment was subcloned from pFL36 to pFL38 and pFL39 (4). All the mutations were

introduced through the *Dpn*I-mediated site directed mutagenesis (7) with appropriate primers (Supplementary table 1). pFL38*ala1* was mutagenized to obtain an isoform of *ala1* encoding the cytoplasmic but not the mitochondrial Ala1 isoform, called $ala1^{L\text{-}16X}$, through mutagenesis of codon -16 (respect to the ATG initiator of the cytoplasmic isoform), that was changed to the stop codon TAG, as previously performed by others (8), thus obtaining pFL38$ala1^{L\text{-}16X}$. pFL39*ala1* was mutagenized with appropriate primers (Additional file 1) to obtain mutant alleles pFL39$ala1^{F22C}$, pFL39$ala1^{L125R}$ and pFL39$ala1^{V500X}$.

W303-1B *ala1Δ* pFL36*ala1* was co-transformed with pFL38$ala1^{L\text{-}16X}$, and each variant of pFL39*ala1*, and then pFL36*ala1* was lost through plasmid-shuffling, thus

obtaining strains encoding a cytoplasmic wild type Ala1 isoform and a mutant Ala1 isoform.

Yeast analysis

Respiratory activity and *in vivo* mtDNA protein synthesis were performed as previously described (9, 10). Reduced vs. oxidized cytochrome spectra of yeast cell cultured for 24hrs at 28°C or 37˚C in SC medium supplemented with glucose at non-repressing concentration of 0.6% were recorded (Varian Cary300 UV-VIS Spectrophotometer) at room temperature.

Cytochrome c oxidase and NCCR (complex I+III) activities were measured on a mitochondrial-enriched fraction prepared as previously describe (11, 12) after a cellular growth to a concentration of 1.5-2 OD_{600}/ml in

SC medium supplemented with 2% galactose and 0.2% glucose.

For the in vivo determination of charged Ala-tRNA, a mitochondrial-enriched fraction prepared as above was subjected to total RNA extraction in acid condition as previously described (13) to maintain the acylated tRNAs. Approximately 5 µg of total mitochondrial RNA were loaded in a 20 cm long 12% polyacrylamide (acrylamide:bisacrylamide, 19:1, w/w)/7M urea gel in 0.1M sodium acetate buffer pH 4.6. After approximately 40-hour at 120V at 4°C (partially denaturing conditions), gel was electroblotted onto a Hybond-N+ in TAE buffer (40 mM Tris, 20 mM sodium acetate, I mM EDTA, pH 7.4). Northern blot analysis was performed by standard method by using mt-tRNA^{ala} oligo (Additonal file 1) as a probe,

which was 5'-labelled with EasyTides® Adenosine 5'-triphosphate, [γ-32P] (Perkin Elmer) with T4 Polynucleotide Kinase (NEB) in 20 µl. Signals were acquired after different time exposures with Carestream BioMax MS films.

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Supplemental Table e-1: Primers used for qPCR and yeast studies

Use	Forward	Reverse
Human *AARS2*		
RTqPCR-pair1	TGCTTTTTGTCAATGCGGG	GGATCCACGGTGCCCAG
RTqPCR-pair2	TCCCAAGCCGCACTGC	CCTACAGCCCCAGTACGTAACAG
Yeast *ala1*		
Cloning:ALA1C	ggggGAGCTCgacaatccgggtaatgcacac[1]	ggggGCATGCaacagcaaatcttttgtccttg[1]
Disruption: ALA1D	atcaactagaaccataatgacgatcggtgataagcaaaaatggacGCTTCGTACGCTGCAGGTCGACG[2]	tcatcaactgcatcctttatagcggctggtttatcacccatacctGATATCATCGATGAATTCGAGC[2]
Mutag: L-16X	caactaccggattaaggaactAgactctttctttcaagaagc[3]	gcttcttgaaagaaagagtcTagttccttaatccggtagttg[3]

Mutag: F22C	gtccgtaatacctttctagactattGcaaatctaaagaacacaag[3]	cttgtgttctttagatttgCaatagtctagaaaggtattacggac[3]
Mutag:V500X	caaatacggcagcgccaacTGATaaggtaccattttgaaactgc[3]	gcagtttcaaaatggtaccttATCAgttggcgctgccgtatttg[3]
Mutag:L125R	ggaagctattacttactcatggactAGAttgactgaagtatacggc[3]	gccgtatacttcagtcaaTCTagtccatgagtaagtaatagcttcc[3]
mt-tRNA[ala]	attggagttaatgagacttgaact	

[1] In upper case the restriction sites

[2] In upper case the sequences which amplify the KanMX4 cassette

[3] Mutagenic primers: in upper case the nucleotide changes.

Supplemental Table e-2: Clinical characteristics

Patient	**1**	**2**	**3**	**4**	**5**	**6**
Gender	f	m	f	f	f	f
Year of birth	1981	1991	1977	1982	1962	1988
Siblings (affected / unaffected / otherwise affected)	0 / 1 / 0	0/1/0	0 / 1 / 0	0 / 0 / 1	0 / 1 /0	0/1/0
Consanguinity parents	no	no	no	no	no	no
Pregnancy, delivery, neonatal period	normal	normal	normal	normal	normal	normal
Initial motor & cognitive development	normal	normal	normal	normal	normal	normal
Presentation						
Age at presentation (y)	3	7	33	24	40	22
Signs at presentation	impaired balance and frequent falls	learning difficulties	depression, cognitive decline	hand tremor, beha-vioral changes, cognitive decline	memory problems, depression	gait difficulties and impaired balance

Preceding event	no	no	no	no	no	no
Course over time						
Regression	yes	yes	yes	yes	yes	yes
Signs of regression	motor & cognitive deterioration	cognitive deterioration, dysarthria, dystonia, ataxia and spasticity	cognitive deterioration, urinary incontinence	motor & cognitive deterioration, spasticity and ataxia	cognitive deterioration	motor deterioration, spasticity and ataxia
Psychiatric	psychosis	no	depression	no	depression	depression
Endocrine	secondary amenorrhea due to ovarian failure	none known	secondary amenorrhea due to ovarian failure	secondary amenorrhea due to ovarian failure	primary amenorrhea due to ovarian failure	secondary amenorrhea due to ovarian failure
Epilepsy	no	no	yes	no	no	no
Outcome	dementia, still walking with support	partial improvement and stabilization	bedridden since age 36	death at 28 years	death at 46 y	wheelchair-bound since age 23

Neurological findings						
Age at latest examination (y)	30	21	36	26	43	23
Intelligence	dementia	moderate learning disability	dementia	dementia	dementia	calculation problems
Dysmorphic features	no	no	no	no	no	no
Head circumference	normal	normal	normal	normal	normal	normal
Vision, eye movements	nystagmus	nystagmus	downbeat nystagmus, no tracking	decreased vision, no tracking	normal	nystagmus
Hearing	normal	normal	normal	normal	normal	normal
Dysarthria	yes	yes	no speech	no speech	no	no
Arms						
Tone	normal	increased	decreased	increased	normal	normal
Muscle strength	normal	decreased	normal	decreased	normal	normal
Reflexes	increased	brisk	increased	increased	normal	increased
Spasticity	no	yes	no	yes	no	no
Ataxia	yes	yes	yes	yes	no	no

Extrapyramidal signs	no	dystonia L>R	no	dystonia left hand	no	no
Sensory problems	no	no	no	no	no	no
Legs						
Tone	increased	increased	decreased	decreased	normal	increased
Muscle strength	normal	decreased	normal	normal	normal	normal
Reflexes	increased	brisk	increased	increased	normal	increased
Babinski signs	yes	yes	no	yes	no	yes
Spasticity	yes	yes	no	yes	no	yes
Ataxia	yes	yes	no	yes	no	yes
Extrapyramidal signs	no	dystonia L>R	no	no	no	no
Sensory problems	no	no	no	no	no	no

f, female; m, male; y, year

Supplemental Table e-3: **Laboratory findings**

Patient	**1**	**2**	**3**	**4**	**5**	**6**
Routine hematology and biochemistry	normal	normal	mild leuko- & thrombocytopenia	normal	normal	normal
Endocrine	low estradiol, ↑ FSH & LH	no clinical signs of endocrine dysfunction, n.i.	low estradiol, ↑ FSH & LH	estradiol & prolactine normal, ↑ FSH & LH	↑ FSH, LH and prolactin	low estradiol, normal FSH &LH
Plasma lactate	normal	normal	normal	normal	n.i.	n.i.
CSF lactate	n.i.	normal	normal	normal	n.i.	nomal
Plasma amino acids	n.i.	increased alanine	normal	normal	n.i.	normal
Urinary organic	n.i.	normal	normal	normal	n.i.	normal

acids						
Acylcarnitine profile	n.i.	n.i.	n.i.	n.i.	n.i.	normal
Arylsulfatase A	n.i.	normal	normal	normal	normal	normal
Galactocerebrosidase	n.i.	normal	normal	normal	n.i.	normal
Very long chain fatty acids	n.i.	normal	normal	normal	n.i.	normal
Other		CSF 5-HIAA 47 (nl 67-140), HVA 100 (nl 145-324), neopterin 8 (nl 8-28), tetrahydro-biopterin 11 (nl 10-30), 5-				

		methyltetra-hydrofolate 40 (nl 40-120)				
Genetic testing	no mutations in *FRDA*, *APTX* and *POLG1*; no mtDNA mutations in muscle	whole exome sequencing and nuclear mitochondrial panel	n.i.	fragile X: negative, no mutations in *EIF2B1-5* genes	no mutations in *EIF2B1-5* genes; normal karyotype	no mutations in *EIF2B1-5* genes
Muscle biopsy	isolated COX deficiency	n.i.	n.i.	n.i.	n.i.	isolated COX deficiency
EMG/NCV	n.i.	n.i.	n.i.	n.i.	normal	normal
ECG	normal	normal	normal	n.i.	n.i.	normal
Cardiac ultrasound	normal	n.i.	n.i.	n.i.	n.i.	n.i.

n.i., not investigated; CSF, cerebrospinal fluid; nl, normal; 5-HIAA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; FSH, follicle stimulating hormone; LH, luteinizing hormone; mtDNA, mitochondrial DNA; COX, cytochrome c oxidase; NCV, nerve conducting velocity; EMG, electromyogram; ECG, electrocardiogram

Supplemental Table e-4: MRI findings

Patient	**1**	**2**	**3**	**4**	**5**	**6**
Age first MRI	**18 y**	**14 y**	**34 y**	**26 y**	**42 y**	**25 y**
Predominant location WM abnormalities	periventricular R	periventricular	periventricular, frontal & parietal	periventricular, frontal & parietal	periventricular, frontal & parietal	periventricular
Relative sparing	most WM spared	most WM spared	frontoparietal border area, subcortical WM	subcortical WM	frontoparietal border area, subcortical WM	most WM spared
Aspect WM abnormalities	single small lesion R	confluent, asymmetric (R>L)	largely confluent, partially patchy, symmetric	partially patchy, partially confluent, asymmetric (R>L)	largely confluent, partially patchy, symmetric	confluent, symmetric
Corpus	-	-	+	+, patchy	+, also atrophic	-

callosum genu						
Corpus callosum body	-	-	+, anterior part	+, patchy	+, anterior part	+, focal strip
Corpus callosum splenium	-	+, focal strip	+, focal strip	+, patchy	+, focal strip	-
Internal capsule anterior limb	-	-	+	-	+	-
Internal capsule posterior limb	-	-	+, frontopontine tracts	+, pyramidal and parieto-occipitopontine tracts	+, frontopontine tracts	+
External capsule	-	-	anterior part	anterior part	anterior part	-
WM rarefaction and/or cysts	-	-	+, central frontal and parietal WM	+, central frontal and parietal WM	central frontal WM	-
Cerebral cortex, basal nuclei,	-	-	-	-	-	-

thalami						
Enlargement lateral ventricles	-	-	-	+, mild	+, mild	-
Enlargement subarachnoid spaces	-	-	-	-	+, frontal, mild	-
Cerebellar WM and peduncles	-	-	-	-	-	-
Cerebellar cortex, dentate nucleus	-	-	-	-	-	-
Cerebellar atrophy	+, mild, vermis > hemisph.	-	+, vermis > hemisph.	+, mild	+, mild	+, mild
Midbrain	-	-	+, frontopontine tracts in cerebral peduncles	+, pyramidal and parieto-occipitopontine tracts	+, frontopontine tracts in cerebral peduncles	+, pyramidal tracts

Pons	-	-	-	-	-	-
Medulla	-	-	-	-	-	+, left pyramid
Contrast enhancement	-	n.d.	-	n.d.	-	1
Restricted diffusion	-	-	n.d.	multifocal WM spots, corpus callosum	n.d.	n.d.
MRS lactate	-	n.d.	n.d.	n.d.	n.d.	not evident
Change over time	worsening of WM abnormalities and cerebellar atrophy	worsening of WM abnormalities	worsening of WM abnormalities			

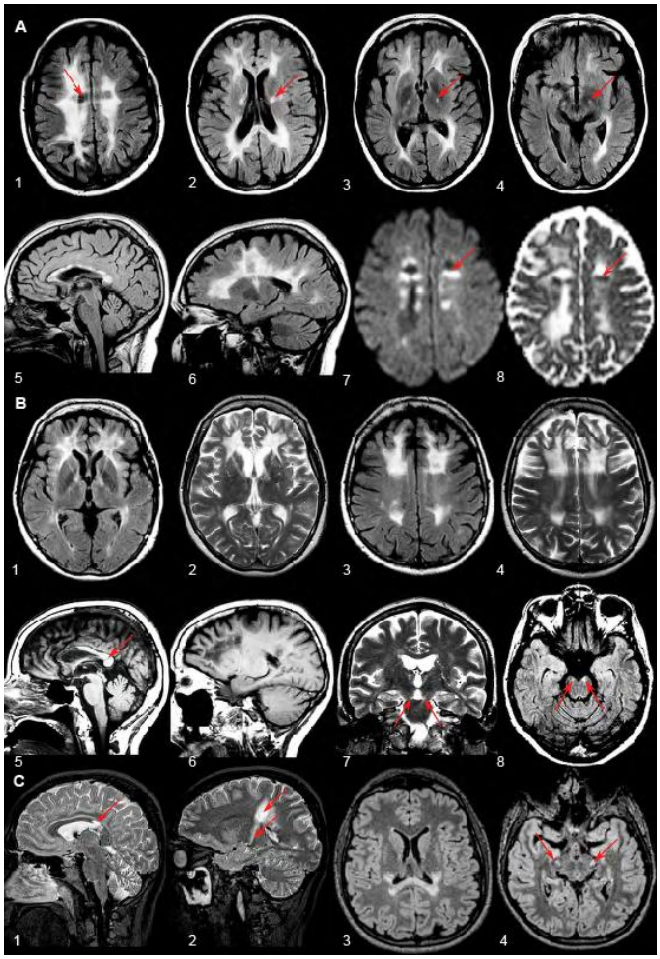
WM, white matter, n.d., not done; hemisph., hemisphere(s)

Supplemental Table e-5: In silico prediction of pathogenicity for *AARS2* mutations

Patient	c.DNA[1]	protein	State	Mutation Taster[2]	SIFT[3]	Polyphen2[4]	Pmut[5]	SNP&GO[6]
1	c.149T>G	p.Phe50Cys	heterozygous	Disease	0.00	0.999	0.6932	Disease
1	c.1561C>T	p.Arg521*	heterozygous	Disease	/	/	/	/
2	c.2893G>A	p.Gly965Arg	heterozygous	Disease	0.00	0.995	0.5952	Disease
2	c.1213G>A	p.Glu405Lys	heterozygous	Disease	0.00	0.999	0.3374	Disease
3	c.1609C>T and	p.Gln537*	heterozygous	Disease	/	/	/	/
3	c.2350del	p.Glu784Serfs*9	heterozygous	Disease	/	/	/	/
3, 4, 5, 6	c.595C>T and	p.Arg199Cys	heterozygous	Disease	0.02	0.964	0.8301	Disease
3, 4, 5, 6	c.2188G>A	p.Val730Met	heterozygous	Disease	0.01	0.998	0.3966	Neutral
4	c.230C>T	p.Ala77Val	heterozygous	Disease	0.02	0.978	0.4018	Disease
5	c.390_392del	p.Phe131del	heterozygous	Disease	/	/	/	/
6	c.2611dup	p.Thr871Asnfs*21	heterozygous	Disease	/	/	/	/

[1] Nucleotide numbering reflects cDNA numbering starting from the A of the ATG translation initiation codon in the reference sequence NM_020745.3.
[2] http://www.mutationtaster.org; Disease: disease-causing variant.
[3] http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html; SIFT probabilities < .05 are predicted to be deleterious.
[4] http://genetics.bwh.harvard.edu/pph2/index.shtml; Humvar pathogenicity scores ranges from 0 (benign) to 1.00 (probably damaging).
[5] http://mmb.pcb.ub.es/PMut; pathogenicity scores ranges from 0 (neutral) to 1 (pathological).
[6] http://snps-and-go.biocomp.unibo.it/snps-and-go; Neutral: Neutral Polymorphism, Disease: Disease-related Polymorphism.

Supplemental Figure e-1: MRI in patients P4, P5 and P6



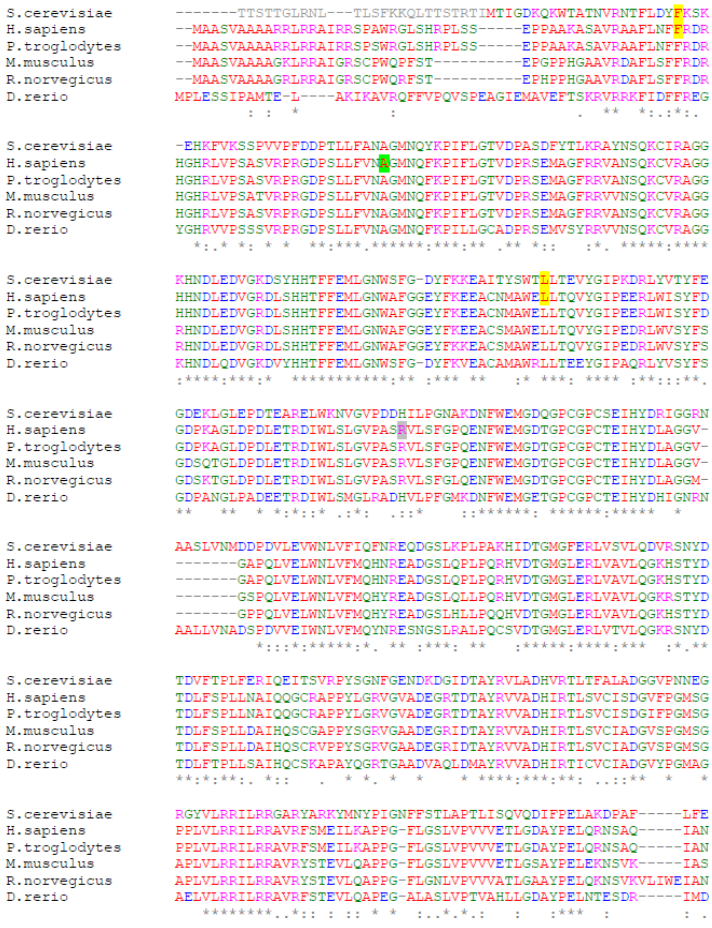
A. MRI in P4 at age 26. The axial FLAIR image shows areas of white matter rarefaction (arrow in image 1). The frontopontine tracts are affected (arrows in images 2-4). The corpus callosum contains multiple lesions (image 5). Note the tract involvement under the rarefied area (image 6). Multifocal areas of diffusion restriction are present (images 7 and 8).

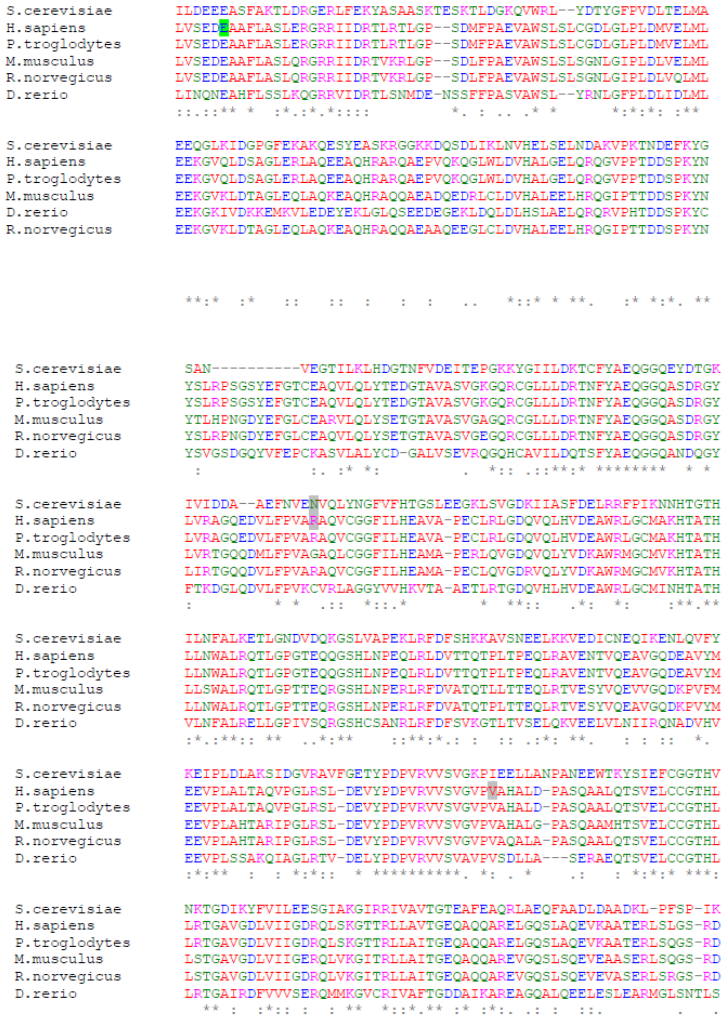
B. MRI in P5 at age 42. The frontal white matter is most prominently affected (images 1 and 2) and partially rarefied (image 1). The middle section of the brain is spared (images 3, 4 and 6). The anterior corpus callosum is affected, the middle part is spared and a strip in the splenium is affected (arrow in image 5). The frontopontine tracts are affected (arrows in images 7 and 8).

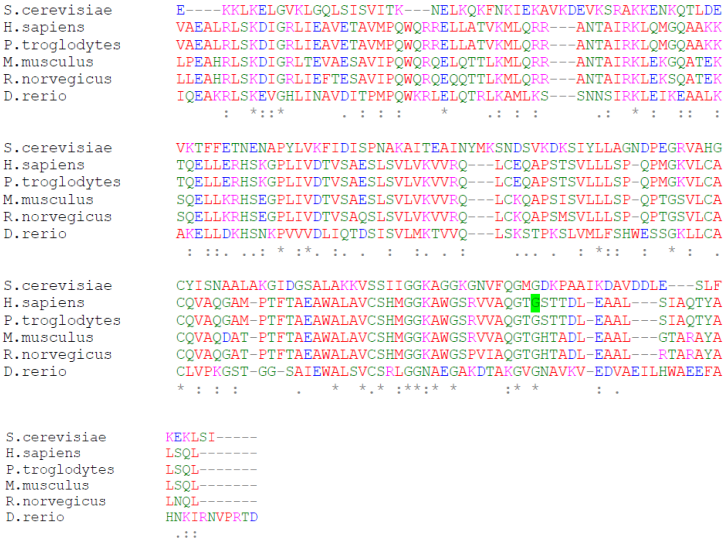
C. MRI in P6 at age 25. The pyramidal tracts are affected from the motor cortex through the posterior limb of the internal capsule (arrows in image 2) into the brain stem (arrows in image 4).

The lesions are connected through the corpus callosum (arrow in image 1 and image 3).

Supplemental Figure e-2: Protein sequence alignment of AARS2 orthologs obtained by Clustal-Omega




S.cerevisiae
H.sapiens
P.troglodytes
M.musculus
R.norvegicus
D.rerio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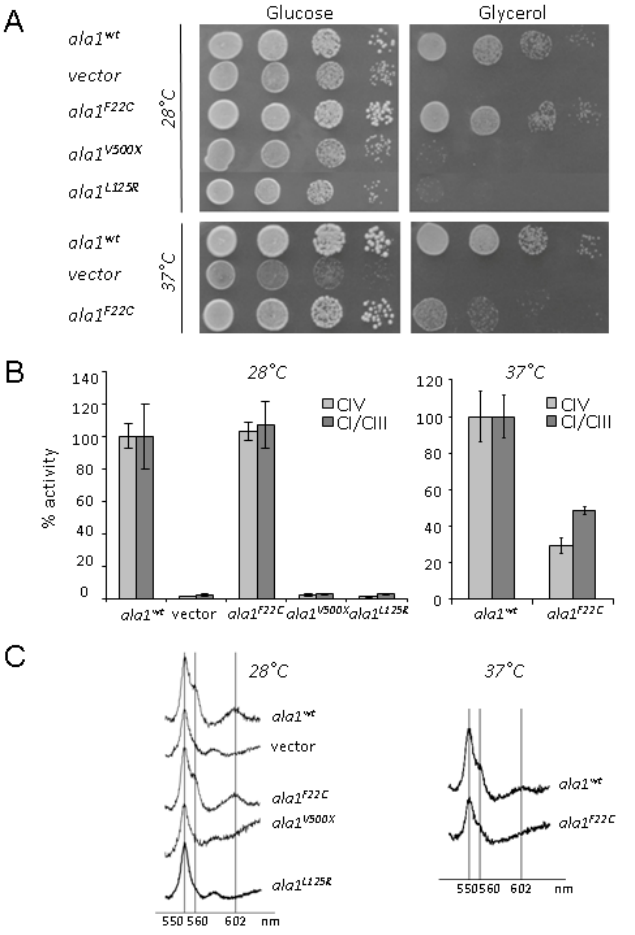
H.sapiens VAEALRLSKDIGRLIEAVETAVMPQWQRRELLATVKMLQRR---ANTAIRKLQMGQAAKK
P.troglodytes VAEALRLSKDIGRLIEAVETAVMPQWQRRELLATVKMLQRR---ANTAIRKLQMGQAAKK
M.musculus LPEAHRLSKDIGRLTEVAESAVIPQWQRQELQTTLKMLQRR---ANTAIRKLEKGQATEK
R.norvegicus LLEAHRLSKDIGRLIEFTESAVIPQWQRQEQQTTLKMLQRR---ANTAIRKLEKSQATEK
D.rerio IQEAKRLSKEVGHLINAVDITPMPQWKRLELQTRLKAMLKS---SNNSIRKLEIKEAALK
VKTFFETNENAPYLVKFIDISPNAKAITEAINYMKSNDSVKDKSIYLLAGNDPEGRVAHG
H.sapiens TQELLERHSKGPLIVDTVSAESLSVLVKVVRQ---LCEQAPSTSVLLLSP-QPMGKVLCA
P.troglodytes TQELLERHSKGPLIVDTVSAESLSVLVKVVRQ---LCEQAPSTSVLLLSP-QPMGKVLCA
M.musculus SQELLKRHSEGPLIVDTVSAESLSVLVKVVRQ---LCKQAPSISVLLLSP-QPTGSVLCA
R.norvegicus SQELLKRHSEGPLIVDTVSAQSLSVLVKVVRQ---LCKQAPSMSVLLLSP-QPTGSVLCA
D.rerio AKELLDKHSNKPVVVDLIQTDSISVLMKTVVQ---LSKSTPKSLVMLFSHWESSGKLLCA
CYISNAALAKGIDGSALAKKVSSIIGGKAGGKGNVFQGMGDKPAAIKDAVDDLE---SLF
H.sapiens CQVAQGAM-PTFTAEAWALAVCSHMGGKAWGSRVVAQGTGSTTDL-EAAL---SIAQTYA
P.troglodytes CQVAQGAM-PTFTAEAWALAVCSHMGGKAWGSRVVAQGTGSTTDL-EAAL---SIAQTYA
M.musculus CQVAQDAT-PTFTAEAWALAVCSHMGGKAWGSRVVAQGTGHTADL-EAAL---GTARAYA
R.norvegicus CQVAQGAT-PTFTAEAWALAVCSHMGGKAWGSPVIAQGTGHTADL-EAAL---RTARAYA
D.rerio CLVPKGST-GG-SAIEWALSVCSRLGGNAEGAKDTAKGVGNAVKV-EDVAEILHWAEEFA
KEKLSI-----
H.sapiens LSQL------
P.troglodytes LSQL------
M.musculus LSQL------
R.norvegicus LNQL------
D.rerio HNKIRNVPRTD

Supplemental Figure e-3: OXPHOS growth and biochemical assays in yeast



A. Growth of the *ala1Δ* strain expressing the wild-type cytosolic isoform (*ala1*$^{L\text{-}16X}$) transformed with the *ala1*^{wt} allele, the empty vector, or the mutant alleles *ala1*^{F22C}, *ala1*^{V500X}, *ala1*^{L125R} on YP medium supplemented with 2% glucose or 2% glycerol. Cells were pre-grown on YP-glucose medium and plated after serial dilutions to obtain spots of $5x10^4$, $5x10^3$, $5x10^2$ and $5x10^1$ cells/spot. Plates were incubated at 28°C or 37° C.

B. Cytochrome c oxidase (CIV) activity and complex I-III activity of the ala1$^{L\text{-}16X}$ strain transformed with the *ala1*^{wt} allele, the empty vector, and the mutant alleles *ala1*^{F22C}, *ala1*^{V500X}, *ala1*^{L125R}. Activities were normalized to the strain transformed with *ala1*^{wt}. Values are the mean of three independent experiments, each with an independent clone. Two-tail, unpaired t test was applied for statistical significance. **: $p<0.01$.

C. Cytochrome spectra profiles of the ala1$^{L\text{-}16X}$ strain transformed with the *ala1*^{wt} allele, the empty vector, and the mutant alleles *ala1*^{F22C}, *ala1*^{V500X}, *ala1*^{L125R}. The peaks at 550, 560 and 602 nm (vertical bars) correspond to cytochromes c, b and aa3 respectively. Cytochromes b is a component of complex III, aa3 of complex IV. The height of each peak relative to the baseline of each spectrum is an index of cytochrome content.

CHAPTER 4

Unpublished results

VARS2 and *TARS2* mutations in patients with mitochondrial encephalomyopathies

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Abstract

By whole-exome analysis we identified: a homozygous missense mutation in *VARS2* in one subject with microcephaly and epilepsy associated with isolated deficiency of the mitochondrial respiratory chain (MRC) complex I; and compound heterozygous mutations in *TARS2*, in two siblings presenting with axial hypotonia and severe psychomotor delay associated with multiple MRC defects. The nucleotide changes segregated within the families, were absent in SNPs databases, and were predicted to be deleterious. The amount of the mutated proteins was reduced in patients' samples; expression of the corresponding wild-type enzymes led to recovery of the biochemical impairment of mitochondrial respiration in immortalized mutant fibroblasts; yeast modelling of the

VARS2 mutation confirmed its pathogenic role. Taken together, these data demonstrate the causative role of the identified variants. Our study reports the first mutations in *VARS2* and *TARS2*, encoding for two mitochondrial aminoacyl-tRNA synthetases, as cause of early-onset mitochondrial encephalopathy.

Introduction

Mitochondrial disorders include widely heterogeneous clinical syndromes, frequently presenting as encephalo- and/or cardiomyopathies, with a broad range of associated causative genes (Ghezzi and Zeviani, 2012). Their biochemical signature is the presence of defective activity in the mitochondrial respiratory chain (MRC) complexes, resulting in faulty Oxidative

Phosphorylation (OXPHOS) which can impair ATP production. Several mutations in genes associated with defects of mitochondrial protein synthesis, affecting either the mitochondrial DNA (mtDNA) or nucleus-encoded genes, have been reported in various forms of mitochondrial disorders (Rotig, 2011).

Translation of the 13 mtDNA encoded, OXPHOS-related proteins takes place within the mitochondrial matrix. This complex process requires ribosomal and transfert RNAs (rRNAs and tRNAs), encoded by mtDNA, and more than 100 proteins, encoded by nuclear genes, translated by cytosolic ribosomes and imported into mitochondria. These proteins include aminoacyl-tRNA synthetases (aaRSs), ribosomal proteins, ribosomal assembly factors, tRNA- and rRNA-modifying enzymes,

initiation, elongation and termination translation factors (Smits et al., 2010). Mutations in any component of the mitochondrial translation machinery can in principle cause inherited mitochondrial disorders affecting MRC complexes containing mitochondrial encoded subunits (cI, cIII, cIV, cV), with the preservation of complex II, the only complex not containing mtDNA-encoded proteins.

An increasing number of mitochondrial disorders due to faulty protein synthesis is caused by mutations in genes encoding mitochondrial (mt-) aaRSs (Konovalova and Tyynismaa, 2013), which catalyze the ligation of specific amino acids to their cognate tRNAs, a crucial process for faithful protein synthesis. Mitochondrial and cytoplasmic aaRSs are encoded by distinct nuclear genes,

with the exception of GARS and KARS, which are present in both cellular compartments; *aaRS2* indicates the gene coding for the mitochondrial enzyme. Mutations in *aaRS2* genes have been associated with diverse clinical presentations, usually with an early-onset and transmitted as autosomal recessive traits. A relatively tight genotype-phenotype correlation has been reported for most of these genes, albeit the basis of cell- or tissue-specific damage remains unclear, since all mt-aaRS are ubiquitous enzymes operating in the same pathway (Rotig, 2011).

We report here the identification by whole-exome sequencing (WES) of the first described mutations in *VARS2* and *TARS2* in patients with clinical presentations compatible with mitochondrial disorders, and OXPHOS

deficiency. All identified variants were not present in SNPs databases, were predicted to be deleterious, and segregated within the families; moreover their pathogenic role was proven using complementation assays on immortalized fibroblasts from patients and, for the *VARS2* mutation, a specific recombinant yeast model.

Patients and Methods

Informed consent, approved by the Ethical Committee of the Foundation IRCCS Istituto Neurologico "C.Besta", Milan, Italy, in agreement with the Declaration of Helsinki, was signed by the parents of the patients.

Patient 1 (P1)

P1 is an 8 year old patient, born at the 37th week of gestation by normal delivery. Early after birth, he showed psychomotor delay,

facial dysmorphisms and microcephaly. He never achieved autonomous deambulation. At 4 years of age onset of partial seizures was characterized by episodes of hypotonia and tonic-clonic movements involving the right and subsequently the left arms; critical episodes characterized by facial movements resembled versive seizures. In several occasions seizures evolved into status epilepticus.

Brain MRI displayed hyperintense lesions in the periventricular regions, the insula and fronto-temporal right cortex (Figure 1A-C). Spectroscopy revealed an increase of lactate peak in the frontal white matter. EEG was irregular with abnormal waves in the fronto-temporal and occipital derivations of both sides (R>L). Muscle computerized tomography showed bilateral quadriceps

hypotrophy but no evident histological alteration was observed in the muscle biopsy.

Patient 2 and 3 (P2, P3)

P2 and P3 were two siblings (Figure 1E) presenting a syndrome characterized by axial hypotonia with limb hypertonia, psychomotor delay and high levels of blood lactate. They both died a few months after birth of a metabolic crisis.

The brain MRI of P2 at 5 months of age showed a thin corpus callosum and hyperintense lesions of the pallida (images not available); no clear alterations were present in the MRI of P3, taken at 3 months of age. Autoptic examination was carried out on P2 and revealed subsarcolemmal lipofuscin-positive deposits at the trichrome Gomori staining of muscle, cerebral spongiosis and hepatic steatosis.

Biochemical studies

Biochemical measurement of OXPHOS complex activities was performed by standard spectrophotometric assays (Bugiani et al., 2004) in muscle homogenate and digitonin-treated skin fibroblasts. Oxygen consumption rate was measured using a SeaHorse FX-96 apparatus (Bioscience) (Invernizzi et al., 2012) in fibroblasts grown either in glucose-rich or in 5mM galactose, glucose-free DMEM media for 72h.

Molecular analysis

Total genomic DNA was extracted by standard methods from peripheral blood lymphocytes or muscle biopsies. Southern blot analysis of muscle mtDNA and sequencing of the entire mtDNA was performed, as described. (He et

al., 2002). Whole-exome next-generation sequencing (WES) and variant filtering were performed as described (Haack et al., 2012). Exons and exon-intron boundaries of human *VARS2* (NM_001167734.1; NP_001161206.1) and *TARS2* (NM_025150.4; NP_079426.2) were amplified using primers listed in Supp. Table S1, and analyzed by Sanger sequencing. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. Total RNA was isolated from cell pellets using the RNeasy Mini Kit (Qiagen) and reverse transcribed to cDNA using the GoTaq® 2-Step RT-qPCR System (Promega), following manufacturer recommendations. *VARS2* and *TARS2*

expression in DNase-treated cDNA samples was determined using reverse transcription quantitative PCR with specific amplicons and SYBR-green chemistry (Supp. Table S1).

Western blot analysis

Approximately 10^6 cells from patients and controls were trypsinized, pelleted, sonicated, and solubilized, as described elsewhere (Tiranti et al., 1999). SDS-polyacrylamide gel of 50 μg protein/lane and western blot analysis were performed using antibodies against VARS2 (Mitosciences LLC), TARS2 (GeneTex), HSP60 (Abcam).

Lentiviral transduction

The wt cDNAs from *VARS2* or *TARS2* was cloned into the pLenti6.3/V5-TOPO Vector (Invitrogen), and virions were obtained as previously described (Zhang et al., 2009). Fibroblasts were immortalized with pRNS-1 by a lipofectin transfection (Litzkas et al., 1984). Mutant and wt immortalized fibroblasts were infected with viral supernatant and selected upon exposure to 2μg/ml Blasticidin (Invitrogen).

Yeast studies

A detailed description of media, plasmids and strains used for yeast studies is reported in the supporting information online, with primers listed in Supp. Table S2. Yeast growth, respiratory activity and *in vitro* mtDNA protein synthesis were performed as

previously described (Barrientos et., 2002; Goffrini et al., 2009). Division time and respiration were measured after growth in SD medium without valine or with valine 40 µg/ml, whilst mtDNA protein synthesis was measured after growth on SC medium supplemented with 2% galactose and 0.2% glucose.

Results

Biochemical findings

Measuring of MRC complex activities in P1 revealed an isolated complex I deficiency (25% of residual activity) in muscle homogenate (Fig 1D), but no enzymatic defects in fibroblasts. However, oxygen consumption performed in P1 fibroblasts cultured in either glucose or galactose medium

showed defective respiration rate compared to control (Supp. Figure S1).

Biochemical analysis of P2 showed multiple deficiency of the MRC complex activities in muscle homogenate (Figure 1F) whereas no defect was observed in fibroblasts; P3 muscle showed the same multiple defect, whereas the MRC complex activities were all normal in a post-mortem liver specimen. Oxygen consumption rate of P2 fibroblasts was in the control range in glucose growth medium, but was lower than normal in galactose, compared to control cells (Supp. Figure S1). No fibroblasts were available from P3.

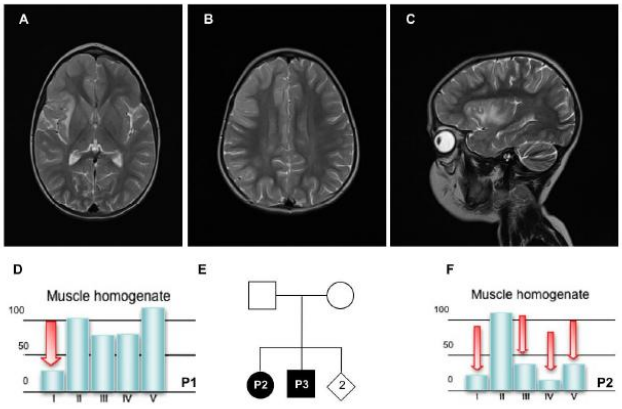


Figure 1: Patients' phenotypical, biochemical and mutational features

A-C: Axial (A, B) and sagittal (C) T2-weighted images of Patient 1, taken at 4 years of age. Note T2-hyperintense lesions in the fronto-temporal right cortex (A). There are also signal abnormalities in the semioval center (B) and the insula (C).

D: Bar graphs with MRC complex activities in P1 muscle homogenate. I, II, III, IV and V correspond to the activities of complex I, II, III, IV and V, respectively, normalized for the citrate synthase activity.

E: Pedigree of Patient 2 and 3. Black symbols designate affected subjects.

F: Bar graphs with MRC complex activities in P2 muscle homogenate. I, II, III, IV and V correspond to the activities of complex I, II, III, IV and V, respectively, normalized for the citrate synthase activity.

Mutation detection

In all patients mtDNA sequencing revealed no pathogenic mutations and Southern Blot was negative for mtDNA deletions or depletion. We then looked for mutations in nuclear genes by WES, performed on genomic DNA from P1 and P3. After filtering to exclude common SNP (>0.1%), the remaining non-synonymous/splice site (NS/SS) changes and microinsertions/deletions (INS/DEL) were prioritized according to the presence of homozygous or compound heterozygous mutations, as expected for recessive transmission suggested by the structure of our

pedigrees, and for known or predicted mitochondrial localization of the corresponding protein (Elnster et al., 2008, Haack et al., 2012, Ghezzi et al., 2012).

In P1 the filtration strategy revealed the presence of a homozygous missense mutation (c.1100C>T, p.Thr367Ile, Figure 2A) in *VARS2*, the gene encoding the mitochondrial valyl tRNA-synthetase. Thr367 is conserved in several species, including yeast (Supp. Figure S2), and the change Thr367Ile has high scores for pathogenicity according to different bioinformatic tools (Supp. Table 3). The mutation was present in heterozygous state in both parents.

Following identical procedures for P3 analysis, we identified two variants in *TARS2*, encoding the mitochondrial threonyl tRNA-synthetase: a missense mutation (c.845C>T, p.Pro282Leu)

and a nucleotide change in position +3 of intron 6 (c.695+3A>G) (Figure 2B-C). Both changes were present also in his affected sister P2, and are predicted to be deleterious (Supp. Table 3). Father and mother were heterozygous carriers for the splice site and the missense mutation, respectively; one healthy sibling was heterozygous for the splice site mutation, while the other was negative for both. Pro282 is conserved in mammals and bird, and present in the yeast Ths1 (Supp. Figure S2), corresponding to the cytosolic, and possibly also to a mitochondrial, threonyl-tRNA synthetase. All the identified nucleotide substitutions were not reported in public databases, including dbSNPs and NHLBI Exome Sequencing Project.

Analysis of *VARS2* and *TARS2* transcripts and proteins

As expected, the homozygous missense mutation didn't alter the *VARS2* transcript levels in P1 fibroblasts (Supp. Figure S3). However, the amount of the VARS2 protein detected by Western blot analysis was strongly reduced compared to control fibroblasts, suggesting instability of the mutant protein (Figure 2E).

In order to evaluate the effect of the intronic variant on *TARS2* transcript, we amplified cDNAs extracted from P2 and control fibroblasts. In P2 we did not detect any aberrant species but the band corresponding to the full-length transcript was found to contain only the mutated nucleotide T in position c.845, suggesting that the intronic mutation in the splice site determines an aberrant and

highly unstable *TARS2* transcript (Supp. Figure S3). Quantitative RealTime-PCR analysis revealed reduced expression of *TARS2* (Supp. Figure S3) and Western Blot analysis showed a clear decrease of TARS2 protein in P2 fibroblasts compared to controls, confirming the hypothesis on the null contribution of the allele with the intronic mutation (Figure 2F).

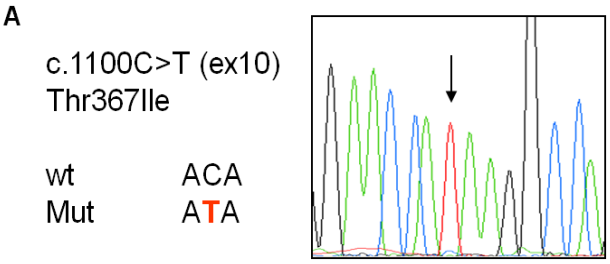
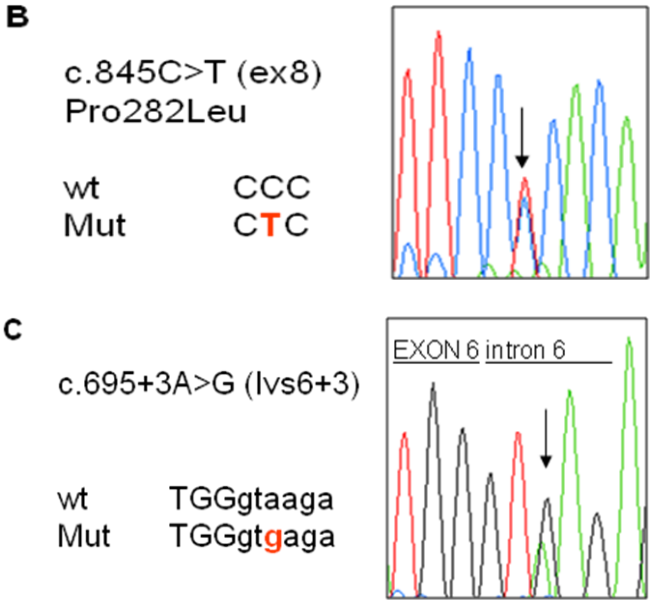
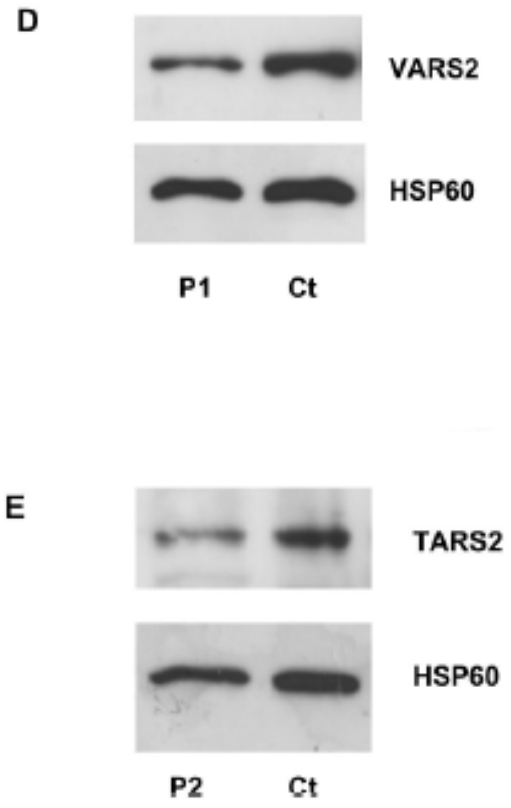


Figure 2: Molecular and protein characterization of VARS2 and TARS2 mutations

A: Electropherogram of the *VARS2* genomic region encompassing the c.1100C>T nucleotide substitution in patient 1.



B-C: Electropherograms of the *TARS2* genomic regions encompassing the c.845C>T (B) and c.695+3A>G (C) nucleotide substitutions in patient 3.



D: Western Blot analysis of fibroblasts from Patient 1 (P1) and control fibroblasts (Ct) using antibodies

against VARS2 and HSP60 proteins, the latter used as loading control.

E: Western Blot analysis of fibroblasts from Patient 2 (P2) and control fibroblasts (Ct) using antibodies against TARS2 and HSP60 proteins, the latter used as loading control.

Yeast studies

To test the possible deleterious effect of the mutations found in humans, we used a *Saccharomyces cerevisiae* yeast model. However, this investigation was possible only for the *VARS2* but not for the *TARS2* gene. Yeast has two genes encoding threonyl-tRNA synthetases: *THS1*, encoding a cytoplasmatic isoform and a putative mitochondrial isoform which aminoacylates only the canonical $tRNA^{Thr(UGU)}$, and *MST1*, encoding the mitochondrial isoform which aminoacylates

both mt-tRNA$^{Thr(UGU)}$ and the non canonical mt-tRNAThr$^{(UAG)}$, whose corresponding codons CUN encode, in yeast but not in humans, threonine instead of leucine (Su et al., 2011; Ling et al., 2012). The human Pro282 is conserved in Ths1, but not in Mst1, which lacks the N-terminal region containing this aminoacid. Introduction of the corresponding mutation in *THS1* would not have effect on mtDNA-dependent protein synthesis, since the mitochondrial enzyme Mst1 is present and can aminoacylate all the mt-tRNA^{Thr}, whereas introduction of mutant (or wt) *THS1* in a *MST1Δ* strain would not allow the mtDNA-dependent protein synthesis since the mt-tRNA$^{Thr(UAG)}$ would not be aminoacylated, and CUN codons would not be translated.

Contrariwise, the mutated Thr367 residue in human *VARS2* is conserved in the yeast

ortholog *VAS1* (Thr380) and can be investigated. However, *VAS1* codes for both the cytosolic and mitochondrial valyl tRNA-synthetases. We disrupted *VAS1* by homologous recombination, and re-expressed the wild-type cytosolic isoform, thus generating a viable but OXPHOS incompetent strain (*cytvas1*), which lacked the mitochondrial isoform. In this strain, we expressed either the wild-type *VAS1* gene (*VAS1*), or a Thr380Ile mutant allele (*vas1*^{T380I}), equivalent to the human Thr367Ile mutation (Supp. Figure S2).

The strain expressing *vas1*^{T380I} showed a division time in ethanol higher than the strain expressing *VAS1* (Figure 3), suggesting an OXPHOS-dependent growth defect. Accordingly, the respiration rate in *vas1*^{T380I} strain was slightly but significantly lower than

in *VAS1* strain (Supp. Figure S4). No obvious alterations were observed in the in-vivo mitochondrial protein synthesis assay (Supp. Figure S4). Interestingly, the supplementation of valine (40ug/ml) in the culture medium led to normalization of the division time (Figure 3) and recovery of respiration for *vas1*^{T380I} strain (Supp. Figure S4).

Complementation studies in fibroblasts

To validate the pathogenic role of the identified variants, we looked for a biochemical defects in mutant fibroblasts by evaluating oxygen consumption rate in cells grown in a galactose-rich, glucose-free medium, a condition that forces cells to depend on mitochondrial respiration rather than glycolysis for ATP production. We first immortalized P1 and P2 fibroblasts to avoid

discrepancies in measuring respiration due to culture passages, as is frequently observed in primary fibroblasts. We found a clear reduction of the maximal respiration rate (MRR) in both P1 and P2 immortalized cells compared to immortalized control fibroblasts, which increased to normal values after transduction with a recombinant lentiviral construct expressing the wt cDNA of either *VARS2* (for P1, and the corresponding control) or *TARS2* (for P2, and the corresponding control) (Figure 4A-B). Western Blot on P1 and P2 fibroblasts after transduction showed an increase in amount of VARS2 and TARS2 proteins respectively (Figure 4 C-D).

These results confirm the causative role for *VARS2* and *TARS2* variants in defective mitochondrial respiration of P1 and P2 cells, respectively.

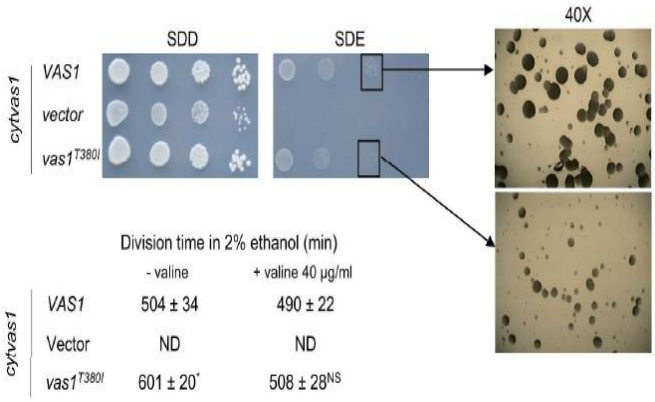
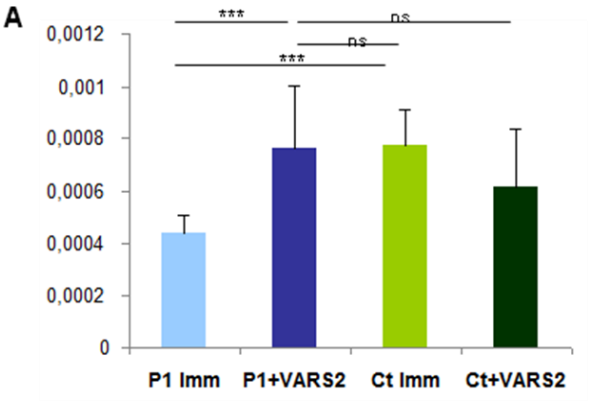
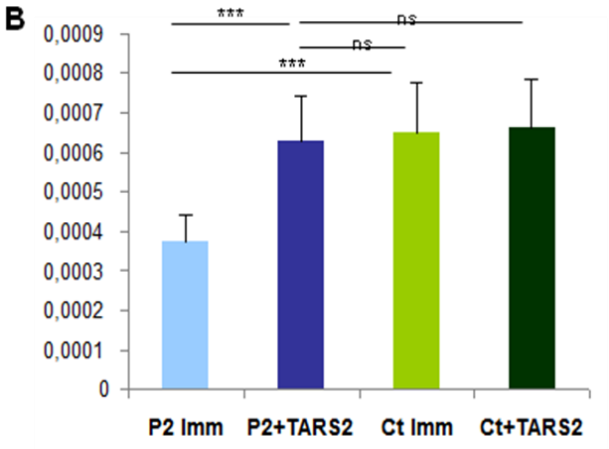


Figure 3: Yeast growth studies

Growth of *cytvas1* strain transformed with wt *VAS1*, *vas1*^{T380I} mutant allele or empty plasmid on SD medium supplemented with 2% glucose (SDD, left panel) or 2% ethanol (SDE, right panel). Cells were pregrown on SDD and plated after serial dilutions to obtain spots of $5x10^4$, $5x10^3$, $5x10^2$ and $5x10^1$ cells/spot. Pictures were taken after 2 days of growth on SDD and 3 days on SDE. Colonies spotted on the third spot of SDE were magnified with a Leica LM 2000 microscope. Division time was calculated by growing cells on liquid SDE medium and measuring the optical density at 600 nm every two hours when cells were in log phase of growth.

Values are mean of three independent experiments. *=p<0.05 by using a two-tailed, paired t-test.





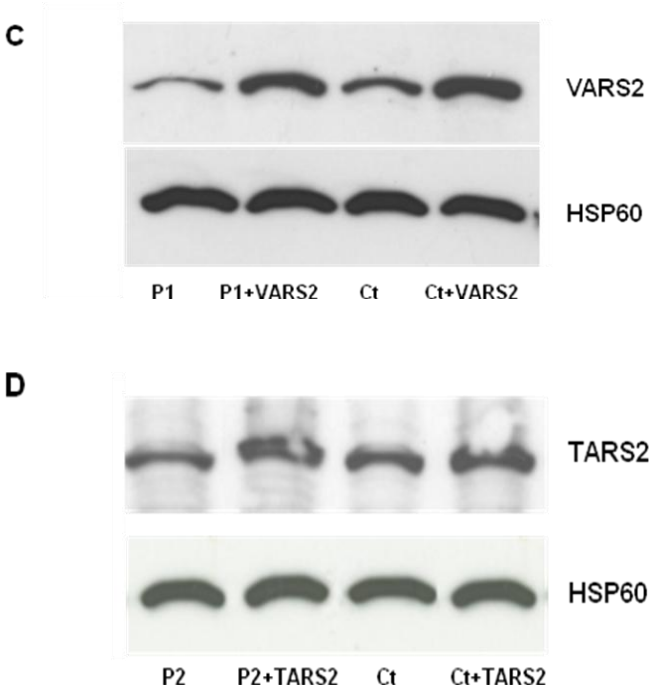


Figure 4: Complementation studies in mutant fibroblasts

A: Oxygraphy performed in immortalized P1 and control fibroblasts cultured in galactose medium in naïve condition and after infection with wt cDNA of *VARS2* (+VARS2). Y-axis values correspond to the

maximal respiration rate, expressed as $pMolesO_2$/min/cells. Data are represented as mean ± SD. Two-tail, paired t test was applied for statistical significance. ***: $p<0.001$; ns: non-significant ($p > 0.01$).

B: Oxygraphy performed in immortalized P2 and control fibroblasts cultured in galactose medium in naïve condition and after infection with wt cDNA of *TARS2* (+TARS2). Y-axis values correspond to the maximal respiration rate, expressed as $pMolesO_2$/min/cells. Data are represented as mean ± SD. Two-tail, paired t test was applied for statistical significance. ***: $p<0.001$; ns: non-significant ($p > 0.01$).

C: Western Blot analysis of samples reported in figure 4A using antibodies against VARS2 and HSP60, the latter used as loading control.

D: Western Blot analysis of samples reported in figure 4B using antibodies against TARS2 and HSP60, the latter used as loading control.

Discussion

Mutations in genes coding for mitochondrial aminoacyl-tRNA synthetases have been associated with diverse clinical presentations, usually inherited as early-onset autosomal recessive traits (Konovalova and Tyynismaa, 2013). We and others have reported a strict genotype-phenotype correlation for most of these conditions (Scheper et al., 2007; Edvardson et al. 2007; Steenweg et al., 2012, Sasarman et al. 2012). Since these genetic defects involve ubiquitously expressed enzymes all engaged in the very same enzymatic step of mitochondrial translation, the mechanisms leading to such different cellular and tissue specific phenotypes remain unexplained. Different hypothesis have been proposed to clarify the clinical variability associated with different *aaRS2* mutations, but

none seems to explain all aspects of this phenomenon that is probably the result of several different mechanisms altered in *aaRS2* mutant patients (Konovalova and Tyynismaa, 2013).

However, and similar to our *VARS2* and *TARS2* mutations, most mutations in *aaRS2* genes have been reported in single, or in just a few, cases, which hampers the establishment of definitive genotype to phenotype correlation. For instance, *AARS2* mutations were initially described as associated with hypertrophic cardiomyopathy in two families, but were later found also in one patient with leukoencephalopathy and no heart involvement (Diodato et al., 2013). Nevertheless, encephalopathy is the most common presentation in *aaRS2* mutations, as is in several other OXPHOS disorders,

probably because of the high energy request by the central nervous system. Interestingly, a specific MRI pattern seems to be associated to some *aaRS2* (i.e. *DARS2*, *EARS2*, *RARS2*) mutant patients. In our cases P1 MRI showed hyperintense lesions involving mainly the insula and fronto-temporal right cortex, whereas P2 MRI was reported to show thinning of corpus callosum (a neuroradiological finding in several neurodegenerative disorders) and bilateral lesions of the globi pallidi. However, the MRI of P3, brother of P2, was normal, although this can be due to the acquisition of the MRI at a very early age. Additional *VARS2* and *TARS2* cases will clarify whether these MRI patterns are specific or not.

Mutations in *aaRS2*, impairing the translation of the 13 mtDNA-encoded MRC subunits,

should in principle be associated with a biochemical deficiency of all MRC complexes, except cII. This biochemical profile was indeed found in muscle samples from the two *TARS2* mutant subjects (P2 and P3), whereas P1 muscle showed isolated cI deficiency. Complex I seems to be particularly prone to damage in mtDNA translation defects, as already reported for *MARS2* patients cells and in the corresponding mutant fly model (Bayat et al., 2012) or for mutations in other proteins involved in mitochondrial protein synthesis, such as mitochondrial methionyl-tRNA-formyltransferase (Haack et al., 2012). This propensity can partly be explained by the fact that 7 of the 13 mtDNA encoded proteins are subunits of cI, and by the complexity of its functional and structural interactions. In patients' fibroblasts, biochemical assays of

individual MRC complexes showed inconsistent alterations, indicating partial preservation of functional proficiency of the mutant enzyme or reflecting poor OXPHOS dependency. The same biochemical discrepancy has been also reported for mutations in other mt-aaRSs (Gotz et al, 2011; Sasarman et al, 2012; Bayat et al, 2012). However, we observed a consistent biochemical phenotype in both naïve and immortalized mutant fibroblasts by measuring whole mitochondrial respiration, especially in culturing conditions which force cells to exploit OXPHOS to produce energy. As already reported for *EARS2* mutant fibroblasts, reduced oxygen consumption rate is likely to depend on the cumulative impairment of the entire set of MRC complexes (Steenweg et al., 2012). We proved the pathogenic role of the

mutations identified in *VARS2* and *TARS2* by complementing the biochemical defects in patients' fibroblasts expressing the corresponding wt cDNAs.

In conclusion, we identified novel mutations in two mitochondrial aminoacyl-tRNA synthetase (VARS2 and TARS2), not yet reported as causing mitochondrial disease, thus expanding the list of *aaRS2*-associated diseases. We also confirmed the value of WES for the identification of disease-causing genes even in single patients presenting heterogeneous clinical syndromes, such as mitochondrial disorders.

Acknowledgements

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The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION ONLINE

VARS2 and *TARS2* mutations in patients with mitochondrial encephalomyopathies

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Detailed yeast methods with supplemental references, 4 supplemental Figures, 3 supplemental Tables

Supplemental Methods: Yeast strains, plasmids and media

The W303-1B genotype is *Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100*. Strains were grown in SD synthetic defined medium (0.69% Yeast Nitrogen base without amino acids ForMedium, Norfolk, UK) supplemented with amino acids and bases necessary for complementing the W303-1B strain auxotrophies, or SC synthetic complete medium (0.69% YNB without amino acids ForMedium, Norfolk, UK,, 1gr/L dropout mix (Kaiser et al., 1994) without uracil, tryptophan and, in case, valine). Media were supplemented with various carbon sources (w/v) (Carlo Erba Reagents, Milan, Italy), in liquid phase or after solidification with 20g/L agar (ForMedium, Norfolk, UK).

VAS1 was cloned under its natural promoter by PCR-amplification with oligonucleotides VAS1CFw and VAS1CRv (Supp. Table S2) and cloning of the *Sac*I-*Pst*I-digested *VAS1* fragment in pFL36 (Bonneaud et al., 1991). The plasmid was inserted in W303-1B through the Li-Ac method (Gietz and Schiestl, 2007). Since disruption of *VAS1* is lethal, we performed disruption of the gene in strain W303-1B harboring pFL36*VAS1*. Disruption was performed through one-step gene disruption by PCR-amplification of KanMX4 cassette (Wach et al, 1994) with primers VAS1DFw and VAS1DRv (Supp. Table S2) and transformation of the former strain, thus obtaining W303-1B *vas1Δ*/pFL36VAS1.

VAS1 fragment was subcloned from pFL36 to pFL38 and pFL39 (Bonneaud et al., 1991). All the mutations were introduced through the

overlap method (Ho et al., 1989) with appropriate primers (Supp. Table S2). pFL38*VAS1* was mutagenized to obtain an isoform of *VAS1* encoding the cytoplasmic but not the mitochondrial Vas1 isoform, called *cytvas1*, through mutagenesis of codon 1, which is the initiator codon for the mitochondrial isoform, which was changed to the codon for alanine GCG, as previously performed by others (Wang et al., 2003), thus obtaining pFL38*cytvas1*,. pFL39*VAS1* was mutagenized with appropriate primers (Supp. Table S2) to obtain mutant allele pFL39$vas1^{T380I}$.

W303-1B *vas1Δ* pFL36*VAS1* was co-transformed with pFL38*cytvas1*, and pFL39*VAS1* wt or pFL39$vas1^{T380I}$, and then pFL36*VAS1* was lost through plasmid-shuffling, thus obtaining strains encoding a

cytoplasmic wt Vas1 isoform and a wt or mutant Vas1 isoform.

Supplemental References

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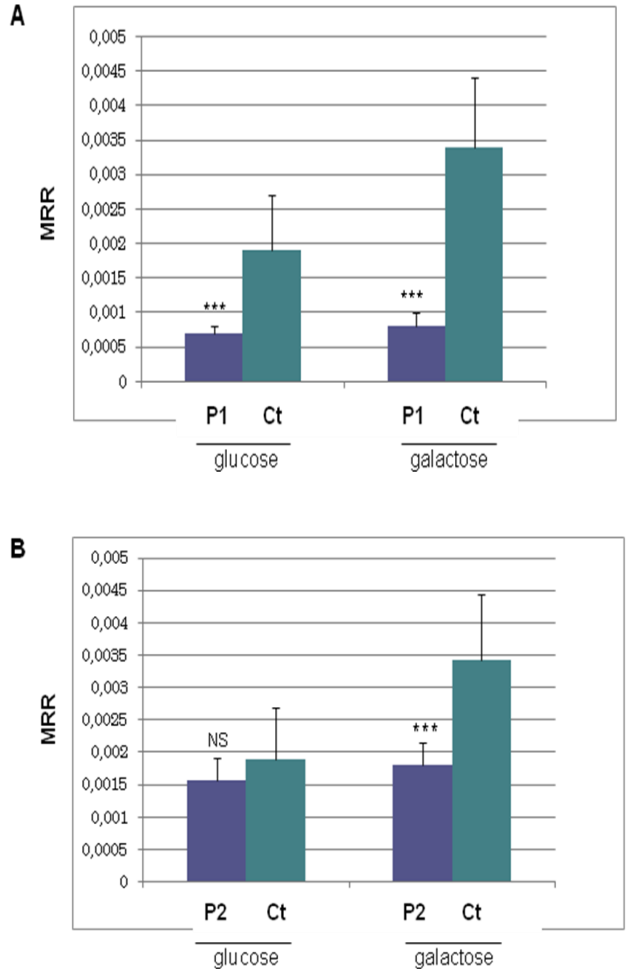
Supplemental Figure S1



Respiration in fibroblasts

Maximal respiration rate (MRR), measured in fibroblasts from patient 1 (P1, panel A), patient 2 (P2, panel B) and corresponding control subjects (Ct). MRR values are expressed as $pMolesO_2$/min/cells. Data are represented as mean ± SD. Two-tail, unpaired Student's t test was applied for statistical significance. ***: $p < 0.001$; NS: non-significant ($p > 0.01$).

Supplemental Figure S2

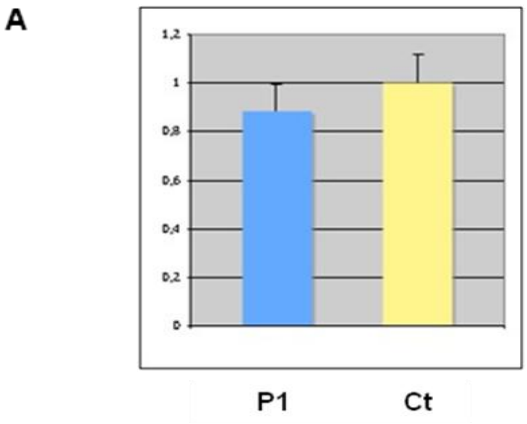
Phylogenetic conservation of VARS2 and TARS2

A. Alignment of VARS2 orthologs. The aminoacid residues corresponding to threonine

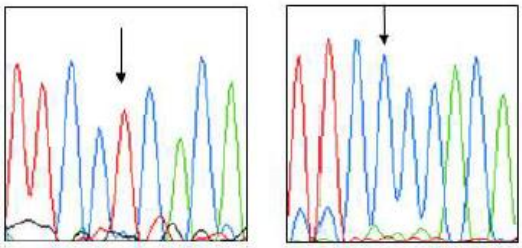
in position 367 of the human protein, mutated in patient 1, are in red bold. For *Saccharomices cerevisiae* the protein coded by *VAS1* was reported.

B. Alignment of TARS2 orthologs. The aminoacid residues corresponding to proline in position 282 of the human protein, mutated in patients 2 and 3, are in red bold. For *Saccharomices cerevisiae* the protein coded by *THS1* was reported.

Supplemental Figure S3

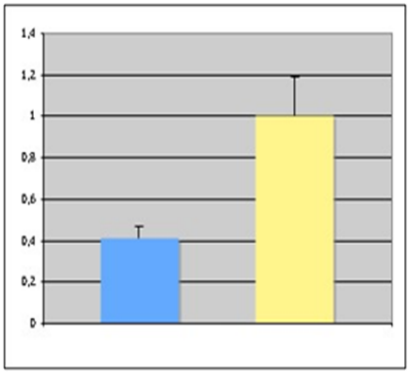


B



P2: c.845T Ct: c.845C

C



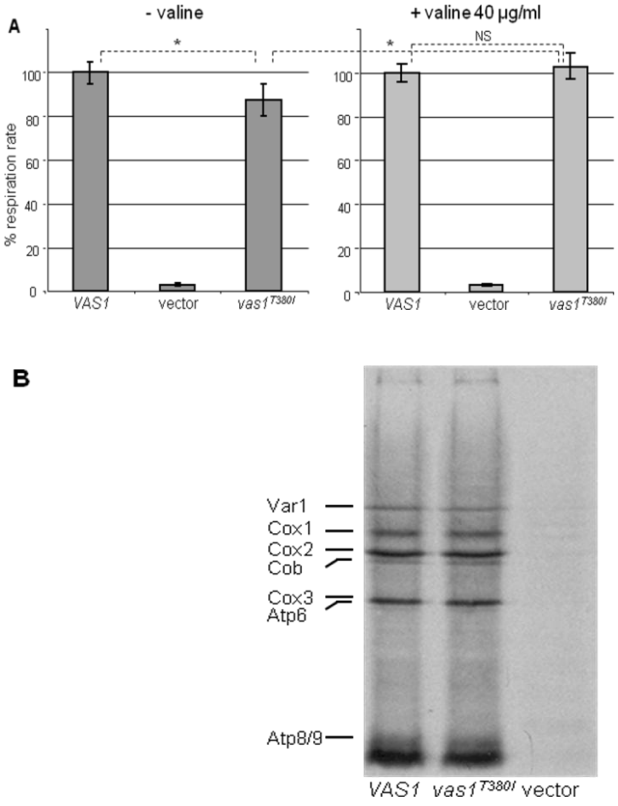
Analysis of *VARS2* and *TARS2* transcripts

A. Levels of *VARS2* transcript, normalized to that of the endogenous *GAPDH* cDNA, in fibroblasts from patient 1 (P1) and a control (Ct), obtained from 2 independent experiments, performed in triplicate.

B. Electropherograms of *TARS2* cDNA portion containing the c.845C>T variant, in patient 2 (P2) and a control subject (Ct).

C. Levels of *TARS2* transcript, normalized to that of the endogenous *GAPDH* cDNA, in fibroblasts from patient 2 (P2) and a control (Ct), obtained from 2 independent experiments, perfomed in triplicate.

Supplemental Figure S4



Yeast studies

A. Respiratory activity of wild type and mutant *vas1* yeast strains. Respiratory activity was measured after 18-hour growth in SD medium supplemented with 0.6% glucose, without valine (left panel) or with the addition of 40 µg/ml valine (right panel). Values are means of four independent experiments. *=$p<0.05$ in a two-tailed, unpaired t test.

B. *In vivo* mtDNA-dependent protein synthesis in yeast strains. Synthesis was performed after growth in SC medium supplemented with 2% galactose and 0.2% glucose when $OD_{600} \cong 1.5$

Supp. Table S1: Primers used for *VARS2* and *TARS2* PCR amplification

Assay	Gene symbol	Forward primer	Reverse primer	Exon
PCR (genomic DNA)	*VARS2*	TTTCCAGTTCTACTGCCTTTAGC	GCCACCATTTAAAACCCAAG	10
	TARS2	TGGCTCTTCCAGGACATCTT	CCATTTCTTTTTCTGGAGCA	6
	TARS2	TCATTTGAGTCTTGAAAAAGGTG	GCTCCCAATTTCATAATCAGG	8
Assay	**Gene symbol**	**Forward primer**	**Reverse primer**	
qPCR (cDNA)	*VARS2*	ACAGCCCCCGATATGTTGAG	GGCCTGATATTCTGGTTTGAAGA	
	VARS2	GACCTTGCTCGTTTCTACCC	ATGAAGAAGCACCTTGCTGA	
	TARS2	AGGTGGCGGTGTCTCCG	ACTGCCGTGTGTAGCCTGC	
	TARS2	GGCCGAACAGGTCCTTAAACA	CAGAGTTGAGGTCCCAGGGTT	
	GAPDH	CTCTGCTCCTCCTGTTCGAC	ACGACCAAATCCGTTGACTC	

Supp. Table S2: Oligonucleotides used for the yeast manipulation

	Primer forward	Primer Reverse
Cloning	cgggggagctcgacctatgacatttctctcacag	ccgccctgcagcacctcgtctagttgtatagc
Disruption	gttgttgtatgtccttcagatttatggaattacttagatggt gcttcgtacgctgcaggtcgacg	tattacattaatttttatctaccttcaatctacaatttcaaaga tatcatcgatgaattcgagc
Mutagenesis Cytvas1 [a]	ggaattacttagatggtGCgaataagtggttaaacaca ttatctaag	cttagataatgtgtttaaccacttattcGCaccatctaagt aattcc
Mutagenesis vas1^{T380I} [a]	cgatgaaaaactgatcattgctacaaTtagacctgaaa ctatatttgg	ccaaatatagtttcaggtctaAttgtagcaatgatcagtttt tcatcg

[a] In upper case the bases which have been changed to introduce the desired mutations

Supp. Table S3: In silico prediction of pathogenicity for the identified *VARS2* and *TARS2* mutations

	Mutation taster	**Polyphen2[a]**	**PMUT[a]**	**Panther[a]**
VARS2 c.1100C>T, p.Thr367Ile	Disease causing	Damaging (0.998)	Pathological (0.606)	Intermediate (0.45)
TARS2 c.845C>T, p.Pro282Leu	Disease causing	Damaging (0.974)	Neutral (0.254)	Probably deleterious (0.83)
TARS2 c.695+3A>G	Disease causing	/	/	/

[a] In parentheses the scores of pathogenicity (ranging from 0=benign to 1=highly damaging) obtained with the different softwares.
Mutation taster: http://www.mutationtaster.org;
Polyphen2: http://genetics.bwh.harvard.edu/pph2/index.shtml;
PMUT: http://mmb.pcb.ub.es/PMut;
Panther: http://www.pantherdb.org/tools/csnpScoreForm.jsp

CHAPTER 5

Summary, conclusions and future perspectives

SUMMARY

The aim of my PhD project was to identify new mitochondrial DNA and nuclear gene variants responsible for mitochondrial disorders, validating their deleterious effect and trying to understand their pathogenic role.

In order to achieve my aim, I started my project studying single probands or small families with a clinical and biochemical profile of mitochondrial disorders where the sequencing of mitochondrial DNA revealed the presence of new nucleotide variants, absent or with a frequency <1% in SNP databases. After the identification of a change in the mtDNA sequence and in order to demonstrate its pathogenic role, I followed a step-wise approach:

1) I evaluated if the identified variant caused a substitution in a highly conserved aminoacid residue and then I scored the pathogenicity of this change by *in silico* analysis using different bioinformatic tools.
2) I analyzed the mutation heteroplasmy in different tissues of the proband and her relatives through Restriction Lenght Polymorphism Analysis (RFLP) in order to correlate the percentage of mutation with the clinical phenotype.
3) I created a cybrid cell model harbouring variable percentage of the mutation and then used biochemical and molecular approaches to detect any pathological phenotype.

As part of my project I also tried to verify the co-pathogenicity of a mt-DNA variant (in *MTCO1*) and a nuclear gene mutation (in

AARS2) coexisting in one patient. I therefore created cybrid cell models harbouring variable amount of mutated mt-DNA; then wt and mutated *MTCO1* cybrid clones were treated with siRNA against the human *AARS2* gene, creating cell models with reduced amount of the AARS2 protein and different heteroplasmy for the *MTCO1* mutation. Finally, I performed oxygraphy in cybrid cells to verify a possible double genetic effect on the biochemical phenotype.

The second part of my PhD project was focused on the identification of nuclear genes responsible for mitochondrial disorders using the Whole Exome Sequencing (WES) approach.
Patients with a clinical and biochemical phenotype suggestive of mitochondrial

disorder were selected to perform WES on DNA samples. WES data were analyzed and the identified variants filtered out prioritizing homozygous or compound heterozygous nucleotide changes in genes coding proteins with known mitochondrial function. Using bioinformatic tools I searched if a nucleotide variant was absent in the general population (or had a frequency less than 0.2%) and if it is predicted to cause a pathogenic aminoacidic substitution; then I checked if the identified variant segregated within the family, that means if it was present in the homozygous state in the affected subject, in the heterozygous state in the parents, and in heterozygous state or absent in the healthy siblings.

To characterize the effect of the identified mutation I performed quantitative real-time

PCR to assess the level of gene transcripts; then I performed western blot on fibroblasts lysate to assess if the mutation caused instability of the protein structure and therefore a reduction of its amount.

To validate the pathogenic role of the nuclear gene variant I used cellular models (skin fibroblasts) from patients, presenting a biochemical phenotype, usually reduced oxygen consumption. I cloned the wt cDNA of the gene of interest into a lentiviral vector. Patients' fibroblasts were infected with viral supernatant. Then I performed oxigraphy on fibroblasts to confirm the rescue of the biochemical phenotype.

A better understanding of the genetic background of mitochondrial disorders is essential to make a proper and early diagnosis,

and to allow genetic counseling. This is important also to investigate and define the pathogenic mechanisms of these diseases in order to hypothesized and test new therapeutical approaches.

MTCOI variant

A 35-year-old woman presented with a phenotype characterized by a stroke-like episode, severe hearing loss, migraines, proximal myopathy, and secondary amenorrhea with low gonadotropin levels. A muscle biopsy showed ragged-red, cytochrome c oxidase-negative fibers; an isolated defect of cytochrome c oxidase activity was present in muscle mitochondria.

Sequence analysis of muscle mtDNA revealed a previously unreported heteroplasmic

m.6597C>A substitution in the *MTCOI* gene, encoding for the subunit I of cytochrome c oxidase, corresponding to p.Q232K aminoacid change. The Q residue in position 232 of MTCOI is highly conserved through phylogenesis. I performed the quantification by allele-specific RFLP-PCR analysis and found that the mutation was 95% heteroplasmic in muscle, 70% in urinary-sediment cells, 40% in cultured skin fibroblasts, and 30% in lymphocytes of the patient. The mutation was not detectable in urinary-sediment cells of the patient's mother, of one brother, and of her three healthy children.

I created transmitochondrial cybrids harbouring different percentage of mtDNA mutation load and, since I consistently failed to obtain homoplasmic mutant clones, I

selected two 80% heteroplasmic mutant ones. Then I compared the activities of individual MRC complexes with those of several wild-type cybrid clones derived from the same patient and found a severe decrease in complex IV activity. I also performed Western-blot immunodetection and densitometric measurement on one-dimension blue-native gel electrophoresis (BNGE) from 80% mutant cybrid lysates and mutant muscle showing a partial reduction of the holo-COX content compared to the wild-type samples, suggesting impaired assembly or stability, whereas the content of other MRC complexes was normal.

My results demonstrate that an *MTCOI* mutation can cause a MELAS phenotype with isolated reduction of COX activity in muscle; I also showed that the Q232K change can

severely impair the activity and just partly destabilize the structure of COX, in agreement with biochemical data and BNGE-based results.

AARS2

I was involved in a study focused on leukoencephalopathies of unknown cause with the aim to define a novel phenotype based on clinical and MRI findings and to identify the common causal genetic defect, shared by patients with this phenotype.

Our and another group carried out independent next generation exome sequencing studies in two unrelated patients with a leukoencephalopathy; WES revealed compound heterozygous mutations in *AARS2*,

encoding mitochondrial alanine-tRNA synthetase, in both patients. The MRI pattern in these patients was compared to available MRIs in a database of unclassified leukoencephalopathies and eleven patients with similar MRI abnormalities were selected; between them other 4 patients were found compound heterozygous for mutations in the *AARS2* gene.

The patient who came to our attention is a 32 years female with a clinical phenotype chracterized by cerebellar ataxia, dementia, psychotic features and primary ovarian failure. Biochemical assay showed severe isolated cytochrome c oxidase deficiency (15% of residual activity) in muscle homogenate, whereas the activities of all respiratory chain complexes, including cytochrome c oxidase, were normal in cultured skin fibroblasts. The

sequence of mitochondrial DNA in our patient revealed a heteroplasmic variant m.5979G>A (p.Ala26Thr) in *MTCOI*, reported as rare single nucleotide polymorphism (<0.1% in Mitomap database). I performed RFLP analysis in the patient and her family members and I found low levels of mutation loads in their tissues. I created transmitochondrial cybrids harbouring the *MTCOI* mutation and performed biochemical analyses that ruled out a primary causative role for this mitochondrial DNA variant.

WES identified a missense (c.149T>G, p.Phe50Cys) and a nonsense (c.1561C>T, p.Arg521*) heterozygous variant in our patient DNA sample.

Functional studies in yeast were performed and confirmed the pathogenicity of *AARS2* mutations in our patient.

The other five patients with *AARS2* mutations had childhood to adulthood onset neurological signs consisting of ataxia, spasticity and cognitive decline with features of frontal lobe dysfunction. MRIs showed a leukoencephalopathy with peculiar involvement of left-right connections and ascending and descending tracts, and cerebellar atrophy. All female patients had ovarian failure. None of the patients had cardiac abnormalities.

Mutations in *AARS2* have been found in a severe form of infantile cardiomyopathy in two families. This work presents six patients with a new phenotype caused by *AARS2* mutations, characterized by leukoencephalopathy and, in females, primary ovarian failure.

VARS2 and TARS2

Whole-exome analysis was performed in two subjects with a clinical and biochemical profile typical for a mitochondrial disorder.

The first patient had a clinical picture characterized by psychomotor delay, microcephaly and epilepsy; brain MRI displayed hyperintense lesions in the insula and fronto-temporal right cortex. Biochemical analyses revealed complex I deficiency in muscle homogenate whereas no defect was detected in fibroblasts. After the filtration strategy prioritizing homozygous or compound heterozygous mutations, as expected for recessive transmission suggested by the structure of our pedigree, WES revealed the presence of an homozygous missense mutation (c.1100C>T, p.Thr367Ile) in *VARS2*, the gene encoding the mitochondrial valyl tRNA-

synthetase. Sequence anlysis I performed on parents samples demonstrated that they were heterozygous for the mutant allele.

I performed oxygen consumption analysis in patient's normal and immortalized fibroblasts, compared to control ones, and I found a defective respiration in both basal and stressful conditions. Then I carried out quantitative Real-Time PCR, that revealed normal amount of *VARS2* transcripts, and western blotting on fibroblast lysates that showed instead a decreased amount of VARS2 protein, indicating an early degradation due to the instability of the mutant protein.

WES was also carried out in two siblings, male and female, presenting with axial hypotonia and severe psychomotor delay associated with multiple MRC defects in muscle but normal biochemistry in fibroblasts.

WES identified in both patients two variants in *TARS2*, encoding the mitochondrial threonyl tRNA-synthetase: a missense mutation (c.845C>T, p.Pro282Leu) and a nucleotide change in position +3 of intron 6 (c.695+3A>G). I confirmed that these mutations segregate within the family. The oxigraphy I performed in normal and immortalized fibroblasts of the sister showed a defective respiration rate only in stressful conditions. The qRT-PCR analysis revealed a decrease amount of *TARS2* transcript. In order to evaluate the effect of the intronic variant on *TARS2* transcript, I amplified cDNAs extracted from patient's and control fibroblasts. In the patient I did not detect any aberrant species but the band corresponding to the full-length transcript was found to contain only the mutated nucleotide T in position c.845,

suggesting that the intronic mutation in the splice site determines an highly unstable *TARS2* transcript. I then performed western blot analyses on female patient fibroblasts pellet that showed a clear reduction in the amount of protein compared to the control.

I cloned the wt cDNAs from *VARS2* or *TARS2* into a lentiviral vector and then patients' fibroblasts were infected with viral supernatant and selected upon exposure to 2μg/ml blasticidin. I performed oxigraphy on these fibroblasts expressing the wt copy of *VARS2* and *TARS2* genes respectively and I found the rescue of the biochemical phenotype.

These findings clearly demonstrate that the identified mutations in *VARS2* and *TARS2* are responsible for the mitochondrial phenotype of our patients.

My study reports the first mutations in *VARS2* and *TARS2*, encoding for two mitochondrial aminoacyl-tRNA synthetases, as cause of early-onset mitochondrial encephalopathy.

DISCUSSION and CONCLUSIONS

Patients with mitochondrial diseases present with a wide spectrum of phenotypes that can affect any organ, at any age. This clinical variability and the underlying genetic heterogeneity makes the diagnosis of these disorders very challenging.

However, with a high level of clinical alertness and an organized diagnostic approach, most mitochondrial diseases can be confirmed early in their course; moreover some of them can

benefit from precise molecular diagnosis, for instance making possible genetic counseling or allowing appropriate supportive treatment.

Currently, the biochemical diagnosis relies upon the enzymatic assay of respiratory chain complexes in different tissues, mostly in muscle biopsy. Unfortunately, clinical laboratories have considerable differences in their biochemical assay protocols and methods of interpretation, and this can lead to inaccurate diagnoses. It has been proved that first level screening based on microscale oxygraphy is more sensitive, cheaper and rapid than spectrophotometry for the biochemical evaluation of cells from patients with suspected mitochondrial disorders (Invernizzi et al, 2012).

Molecular diagnosis of mitochondrial diseases must take into account their dual genetic background and hence in the past years has been based on the compelte sequence of mtDNA and on direct sequencing of selected nuclear genes known to be associated with specific biochemical/clinical presentations.

Most mtDNA mutations function "recessively", this means that 60–90% mutation load is needed before a biochemical defect is noted. The prevalence of mtDNA mutations is difficult to establish with accuracy, especially because of the high asymptomatic carrier rate. Moreover, mtDNA mutations are associated with a very wide range of clinical expression. However, studies have shown a prevalence for specific mutations of 0.14–0.20%.

On the other hand, over 200 nuclear genes

have been identified that cause mitochondrial diseases, and the list continues to grow as over 1400 genes have been identified controlling mitochondrial structure and function.

The recent technological advances in the field of next-generation sequencing, allow now to perform cost-effective analysis in index cases, thus bypassing the laborious procedure of systematic Sanger sequence analysis of a great number of candidate genes. In particular the exome, i.e. the entire set of exons, constitutes less than 1% of the whole genome, but it is estimated to contain about 85% of the disease-causing mutations of monogenic disorders.

In addition, for the identification of new disease-genes, WES does not require large pedigrees and can also be applied to nuclear families or even singleton patients.

***MTCOI* variant**

Mutations in *MTCOI* are very rare, usually sporadic, and associated with a spectrum of conditions including sideroblastic anemia (Gattermann et al, 1997), myopathy with myoglobinuria (Karadimas et al, 2000), and stroke-like episodes, leading to severe, drug-refractory epilepsy (Tam et al, 2008). MELAS syndrome (MIM 540000) is associated in 90% of the cases, with the classic mutation m.3243G>A, in the mt-tRNA$^{Leu(UUR)}$ gene, or, less frequently, with mutations in other mt-tRNA genes or mitochondrial ND, resulting in CI deficiency in muscle. In most of these cases, including the m.3243G>A mutation, the muscle biopsy displays ragged red, COX positive, rather than negative, fibres.

Our patient presented with acute, reversible stroke, for which obvious vascular and cardiac

causes had been ruled out, and has a MELAS-like phenotype. Interestingly, the patient had also secondary amenorrhea, clearly caused by hypophyseal insufficiency. She showed profound and isolated decrease of COX activity in muscle histological sections and homogenate, suggesting a mutation in complex IV. Sequencing of mtDNA revealed the presence of a missense mutation in the *MTCOI* gene.

I found that the heteroplasmic load of the m.6597C>A change did segregate within the family and correlate with the clinical picture: both the mother of the patient, and her brother, who are asymptomatic, showed no mutation, at least in the biological samples that could be examined, i.e. urinary sediment and blood. Interestingly, the three sons of the patient are healthy and harbour no mutation, and this led

us to hypothesise a bottleneck-based selection of mutation-free oocytes in their mutant mother.

Accordingly to the pathological nature of the mutation, I consistently failed to obtain homoplasmic mutant cybrids, and this could be explained by the fact that this condition is possibly incompatible with cell survival or that the mutation exerts a toxic role rather than simply reducing OXPHOS proficiency. Other *MTCOI* mutations might be active with a similar mechanism, thus explaining both their rarity and non-transmission.

In silico analysis of the Q232K change based on the 3D crystal structure of COX suggested that, since the lateral chain of the Q232 is surrounded by negatively charged and hydrophobic residues belonging to both MTCOI and MTCOII subunits, the

substitution with a positively charged K could electrostatically perturb the region and affect the electron transfer from the CuA center in MTCOII to the heme a of MTCOI, impairing the interaction between the two CIV subunits. Moreover Q232 is next to the proton exit pathway and the K232 mutant residue could also disturb the efficiency of proton pumping.

Accordingly to in *silico* data, the biochemical analyses in 80% mutant cybrid clones revealed a profound decrease of CIV activity and BNGE studies showed a partial reduction in complex IV amount. Taken togheter, these results indicate that the Q232K MTCOI mutation mainly impairs the activity and partly destabilizes the structure of COX.

AARS2

AARS2 mutations have been reported in three subjects causing infantile hypertrophic cardiomyopathy, lactic acidosis, with early fatal outcome (Gotz et al, 2010). One patient harboured a homozygous missense mutation (c.1774C>T [p.Arg592Trp]), while the other was compound heterozygous for the p.Arg592Trp mutation and a missense mutation in exon 3, c.464T>G (p.Leu155Arg). Patients described in our work show a very different clinical picture, consisting of signs of neurological dysfunction and a leukoencephalopathy on MRI. Strikingly all female patients show primary ovarian failure and none has cardiac abnormalities. Ovarian failure is often present in mitochondrial disorders caused by mutations in different genes i.e. amino acyl tRNA synthetase

HARS2 (MIM*600783) and *LARS2* (MIM*604544), causing Perrault syndrome, a disease characterized by ovarian dysgenesis and sensorineural hearing loss and *POLG*, the gene encoding the mitochondrial DNA polymerase gamma (Luoma et al, 2004; Bekheirnia et al, 2012). MRI features described in our work consist in a leukoencephalopathy with a peculiar involvement of the frontal, parietal periventricular and deep white matter in a tract-like fashion, with involvement of the corpus callosum and ascending and descending connections; cerebellar atrophy was variable

The patient who has come to my attention had an early onset at two years of age with impaired balance and frequent falls but she remained nearly stable until the age of 15,

when she started to present severe cerebellar features, cognitive deterioration and psychosis. At 18 years of age she had secondary amenorrhea due to ovarian failure.

In this patient we detected a severe, isolated defect of complex IV activity in skeletal muscle, rather a multiple respiratory chain defect, which is expected in case of impaired mitochondrial translation. I excluded the possible additive effect of a mtDNA *MTCOI* variant creating transmitochondrial cybrids and performing biochemical assay in mutant and wild-type clones. The pathogenicity of the two variants found in our patient was instead experimentally proven by a recombinant yeast model. Interestingly, it was noted that the yeast p.Phe22Cys mutation, equivalent to p.Phe50Cys found in our patient, affects complex IV activity more than complex I+III

activity; on the contrary, the p.Leu125Arg mutation, equivalent to human p.Leu155Arg and described in the cardiomyopathy patients (Gotz et al, 2010), decreases both complex IV and complex I+III activities. The isolated complex IV defect found in our patients could be related to specific missense mutations, possibly influencing the main target tissue (brain versus heart). Götz *et al.* suggested that the p.Arg592Trp mutation could impair the editing activity of *AARS2*, thus determining an increased mistranslation of the alanine codon by serine or glycine. According to this hypothesis mistranslation would lead to cardiomyopathy, while a defective translation, caused by other amino acyl tRNA synthetase mutations, is usually not associated with heart damage (Konovalova et al, 2013).

Our patient has a milder clinical course

compared to the others and her neurological picture remains pretty stable. The yeast mutation equivalent to human p.Phe50Cys found in our patient has been proven to be deleterious only in stress conditions, and this could explain the milder clinical phenotype. The oxigraphy I performed in patient's fibroblast failed to detect a decrease in the respiration rate both in basal and stressful conditions; nevertheless the lack of a biochemical defect in fibroblasts of patients with mutations in mitochondrial aminoacyl tRNA-synthetases has been reported several times (Gotz et al 2010; Shahni et al, 2013).

Interestingly, the association of white matter involvement and ovarian abnormalities has been reported in vanishing white matter disease (MIM 603896), caused by mutations in any of the five genes (*EIF2B1* to *EIF2B5*)

encoding subunits of the translation initiation factor eIF2B.

VARS2 and TARS2

Mitochondrial aminoacyl-tRNA synthetases are ubiquitous enzymes involved in the same step of mitochondrial translation. The reason why mutations in *aaRS2* genes can cause such different and tissue-specific phenotypes remains unexplained (Konovalova and Tyynismaa, 2013).

In my work I describe the first patients with mutations in two mitochondrial aminoacyl tRNA synthetases, VARS2 and TARS2 encoding respectively for the valil- and the threonyl-tRNA synthetases.

These patients had a clinical picture characterized by an early onset encephalomyopathy and, for *TARS2* patients, a

fatal outcome in the first months of life. Encephalopathy is the most common presentation in *aaRS2* mutations, as is in other mitochondrial disorders, probably because of the high energy request by the central nervous system. Moreover, a specific MRI pattern seems to be associated to some *aaRS2* (i.e. *DARS2*, *EARS2*, *RARS2*) mutant patients. MRI of the *VARS2* mutant patient showed hyperintense lesions in the insula and fronto-temporal right cortex, whereas one sibling compound heterozygous for *TARS2* mutations had an MRI charcterized by thinning of corpus callosum (a neuroradiological finding in several neurodegenerative disorders) and bilateral lesions of the globi pallidi. Additional *VARS2* and *TARS2* cases will clarify whether these MRI patterns are specific or not.

Spectrophotometric assay of respiratory chain

complexes (MRC) revealed a complex I defect in muscle of the *VARS2* patient, whereas a multiple MRC defect in muscle of *TARS2* patients. Defect of mitochondrial translation, involving the 13 mtDNA-encoded MRC subunits, are expected to cause a combined respiratory chain defect, but complex I seems to be particularly sensible to damage, as already reported for *MARS2* patients cells and in the corresponding mutant fly model (Bayat et al., 2012) or for mutations in other proteins involved in mitochondrial protein synthesis, such as mitochondrial methionyl-tRNA-formyltransferase (Haack et al., 2012).

Biochemical analyses of the MRC activities in fibroblasts of *VARS2* and *TARS2* patients did not show any defect. This is often described in aaRSs mutations (Gotz et al, 2011; Bayat et al, 2012), probably indicating residual enzyme

activity or reflecting poor OXPHOS dependency.

In order to validate the pathogenicity of *VARS2* and *TARS2* mutations I immortalized patient's fibroblasts to obtain cell lines that can be propagated indefinitely. This allows an analysis to be repeated many times on genetically identical cells which is desirable for repeatable scientific experiments. Then I investigated for a biochemical phenotype measuring whole mitochondrial respiration, that is a more sensitive method to evaluate respiratory chain defects, both in basal and stress culturing conditions, forcing cells to rely on OXPHOS to produce energy. Once I confirmed a defective respiration rate in patients' fibroblasts compared to control, I carried out complementation experiments by cloning the wt *VARS2* and *TARS2* cDNA and

infecting immortalized cells, and I obtained the rescue of biochemical phenotype. Moreover, the homozygous *VARS2* mutation was validated in a yeast model, whereas this was not possible for *TARS2*, because of the redundancy of orthologs in yeast.

My results clearly indicate that the identified mutations in *VARS2* and *TARS2* are responsible for the severe mitochondrial phenotype in these patients.

FUTURE PERSPECTIVES

Mitochondrial disorders are characterized by an extreme clinical and genetic variability. Their prevalence is considered to be 1 to 5000 births. The majority of mitochondrial disease patients are still genetically undefined, even

after extensive mutational screening of known disease genes; this diagnostic limitation is partly due to the huge size of the mitochondrial proteome potentially involved in mitochondrial dysfunction, which is estimated to contain >1400 entries.

The mtDNA has an highly polymorphic nature and assessing the pathological nature of an mtDNA variant can be difficult. Traditionally, mtDNA variants have been defined as mutations if: absent in control populations, determining the substitution of a conserved aminoacid, segregating with the disease phenotype and with a relevant biochemical defect, and usually present as heteroplasmic changes. Exception to these rules is the mutation causing LHON that is typically homoplasmic in affected individuals. Evidence also suggests that the nuclear background of a

mtDNA mutation can influence its biochemical expression.

Considering that the complete sequencing of mitochondrial DNA is performed almost in every specialized laboratory, it's reasonable thinking that the major part of undiagnosed mitochondrial conditions is due to mutations in nuclear genes. Therefore, there is compelling need to boost the search for new genes associated with mitochondrial phenotypes, using large cohorts of biochemically well-defined patients and families, and the implementation of state-of-art technologies, for instance NGS, to complete the molecular dissection of mitochondrial disease.

NGS has considerably improved the diagnosis of mitochondrial disorders in the last years. This technology is providing insights into the genetic basis of diseases that often remain

undiagnosed even after extensive clinical workup.

Mitochondrial disorders are multisystemic and often dramatic conditions for which obtaining a precise molecular diagnosis is extremely important. First of all the identification of the genetic defect underlying these conditions allows clinician to give families a genetic counseling and then to assess the right clinical and therapeutical management.

Translational consequences of discovering new genes responsible for mitochondrial disorders are the possibility to extend the molecular screening in subjects that share common clinical and biochemical features, and to establish a precise genotype-phenotype correlation, and then acquiring insights into the molecular functions of the new indentified proteins. This knowledge will improve the

development of rational, evidence-based strategies for the identification of therapeutic targets that can be tackled by pharmacological, cellular or genetic interventions.

A recent Cochrane systematic review has confirmed that there is no effective treatment for mitochondrial disorders, and current therapeutic management is focused on treating complications. The therapeutical approach is mainly based on antioxidant compunds and vitamin supplementation.

There is therefore a clear need to develop new effective therapies. Anyway, new therapeutic approaches are emerging in the treatment of mitochondrial disorders. In case of mtDNA mutations the strategy is based mainly on altering the balance between wild-type and mutated mtDNA (Koene et al, 2009); recently it has been demonstrated that the transient

overexpression of *IARS2* gene, encoding for the mitochondrial isoleucyl tRNA synthetase, ameliorates viability of transmitochondrial cybrids derived from fibroblasts of a patient with an homoplasmic mutation in the mitochondrial tRNAIle (*MTTI*) gene associated with hypertrophic cardiomyopathy.

In the Unit of Molecular Neurogenetics (Institute of Neurology "Besta"), we are trying to obtain transmitochondrial cybrids harbouring different mtDNA mutations and to create a collection of these cell lines. This biobank will give us for instance the chance to try different approaches to increase/restore levels of wt mtDNA in mutant cybrids.

I would like to test the new therapeutic approaches in cybrids harbouring the *MTCOI* missense mutation, m.6597C>A (p.Q232K),

using TALENs (Moraes et al, 2013) or restriction endonucleases (Tanaka et al, 2002) to reduce levels of mutant mtDNA and then verify the possible rescue of the biochemical phenotype.

Regarding the AARS2 patients, I would like to extent the molecular screening eventually to patients in our institute with a similar clinical phenotype and with the same MRI features.

On the other hand, I'm setting up the conditions to perform in organello aminoacylation and protein synthesis assay in mitochondria extracted from our patient's immortalized fibroblasts; this will give me information about the molecular consequences of AARS2 mutations to the enzyme activity, possibly highlighting differences amongst mutations associated with either cardiomyopathy or leukodystrophy. Anyway

this is a risky project, in fact these experiments failed to show a molecular phenotype in cells with defective activity in other aminoacyl tRNA synthetases (Elo et al, 2012).

I would like to perform these assays also in mitochondria extracted from immortalized fibroblasts of VARS2 and TARS2 patients. It would be very interesting, if possible, to collect more patients with mutations in these two aminoacyl tRNA sytnhetases to verify if they share a common clinical and biochemical phenotype and then, if it is possible to recognize a specific MRI pattern, as in other aaRS2 diseases.
We are currently treating fibroblasts of VARS2 and TARS2 patients with culture medium supplemented with a mixture of aminoacids or, specifically, with valine or

threonine, trying to obtain an improvement of the biochemical phenotype observed by oxigraphy experiments.

Few mouse models of aaRS2 mutations are currently under study. The group of Aleksandra Trifunovic is currently dealing with DARS2 mouse models and they recently reported that constitutive Knock-Out (KO) mice had embryonic lethality, whereas conditional cardiac and skeletal muscle KO mice showed severly shortened life span and a gradual increase in heart/body weight ratio, with marked reduction of MRC complexes, except Complex II, in both heart and skeletal muscle.

My group and I would like to create a conditional AARS2 mutant mice model that will give us the chance to eventually study the clinical phenotype and the variable

biochemical effects in different tissues, and also to evaluate the possible variable amount of alanine between them. Mice model could be created also for VARS2 and TARS2 mutations and, if brain alterations are present, we could study why some brain areas are more affected than others.

The production of conditional tissue-specific mouse models for different aaRSs2 could provide an insight into the pathological mechanisms and the selective tissue-involvement caused by mutations in these ubiquitously expressed enzymes and might be a valid tool to study therapeutical approaches.

Nowadays we still miss an efficient treatment for mitochondrial disorders. Therapeutic management of these disorders relies mainly of antioxidant compounds and vitamin

supplementation. The knowledge of the pathogenic mechanism causing mitochondrial disorders is important to develop specific therapeutic strategies and this is the principle aim of my future researches.

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List of abbreviations

aaRS2: mitochondrial aminoacyl tRNA synthetases

AARS2: mitochondrial alanine-tRNA synthetase

ADP: Adenosine diphosphate

AIF: Apoptosis inducing factor

ATP: Adenosine triphosphate

CNS: central nervous system

CYB: Cytochrome B

COI: cytochrome oxidase subunit I

COX: Cytochrome c oxidase

CSF: Cerebro-spinal fluid

DNA: Deoxyribonucleic acid

FAD: flavin adenine dinucleotide

$FADH_2$: reduced flavin adenine dinucleotide

FASTKD2: Fas activated serine-threonine kinase domain 2

KSS: Kearns-Sayre syndrome

IM: Inner membrane

IMS: Inter membrane space

LHON: Leber hereditary optic neuropathy

MDS: Mitochondrial depletion syndrome

MR-1: Myofibrillogenesis regulator 1

MRC: Mitochondrial respiratory chain

mtDNA: Mitochondrial DNA

MTS: Mitochondrial targeting signal

NAD^+: oxidized nicotinamide adenine dinucleotide

NADH: reduced nicotinamide adenine dinucleotide

OM: Outer membrane

OMIM: Online Mendelian Inheritance in Man

OXPHOS: Oxydative Phosphorylation System

PEO: Progressive external ophtalmoplegia

PDHc: Pyruvate dehydrogenase complex

PNKD: Paroxysmal non kinesigenic Dyskinesia

ROS: Reactive oxygen species

RRF: Ragged red fibers

SDH: Succinate dehydrogenase

TCA: Tricarboxylic acid

TARS2: mitochondrial threonyl-tRNA synthetase

VARS2: mitochondrial valyl-tRNA synthetase

WEB RESOURCES

- ClustalW, http://www.ebi.ac.uk/Tools/clustalw/index.html
- Ensembl, http://www.ensembl.org
- GeneCards, http://www.genecards.org/index.shtml
- Mitoprot, http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html

- NCBI database, http://www.ncbi.nlm.nih.gov
- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
- Predotar, http://urgi.versailles.inra.fr/predotar/predotar.html
- Psort II, http://psort.ims.u-tokyo.ac.jp/form2.html
- TargetP, http://www.cbs.dtu.dk/services/TargetP
- TMHMM, http://www.cbs.dtu.dk/services/TMHMM/
- TMpred, http://www.ch.embnet.org/software/TMPRED_form.html
- Wolf-PSORT, http://www.wolfpsort.org

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