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Cardiolipin content in mitochondria from cultured skin fibroblasts harboring mutations in the mitochondrial *ATP6* gene

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Abstract The role of phospholipids in normal assembly and organization of the membrane proteins has been well documented. Cardiolipin, a unique tetra-acyl phospholipid localized in the inner mitochondrial membrane, is implicated in the stability of many inner-membrane protein complexes. Loss of cardiolipin content, alterations in its acyl chain composition and/or cardiolipin peroxidation have been associated with dysfunction in multiple tissues in a variety of pathological conditions. The aim of this study was to analyze the phospholipid composition of the mitochondrial membrane in the four most frequent mutations in the *ATP6* gene: L156R, L217R, L156P and L217P but, more importantly, to investigate the possible changes in the cardiolipin profile. Mitochondrial membranes from fibroblasts with mutations at codon 217 of the *ATP6* gene, showed a different cardiolipin content compared to controls. Conversely, results similar to controls were obtained for mutations at codon 156. These findings may be attributed to differences in the biosynthesis and remodeling of cardiolipin at the level of the inner mitochondrial transmembrane related to some mutations of the *ATP6* gene.

Keywords Cardiolipin · *ATP6* mutations · Mitochondria · Leigh syndrome

Abbreviations

CL	Cardiolipin
PG	Phosphatidylglycerol
MLCL	Monolysocardiolipin
PGP	Phophatidylglycerolphosphate
PA	Phosphatidic acid

Introduction

Most of the ATP in human cells is produced by the F1F0-ATP synthase located in the inner mitochondrial membrane (Saraste 1999) and serves for the many cellular functions of organs and tissues. Faulty ATP production occurs in severe encephalomyopathies, especially in infancy, a

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critical period for neural/brain development, which requests sufficient energy supply (Celotto et al. 2006)

The syndromes of neuropathy, ataxia and retinitis pigmentosa (NARP, MIM 551500) and maternally-inherited Leigh syndrome (MILS, MIM 516060) have been associated with a number of mutations in the mtDNA *ATP6* (*MTATP6*) gene encoding subunit 6 of the ATP synthase. This subunit is a transmembrane protein containing most of the residues involved in the proton translocating activity of the F0 domain of the ATP synthase (Fillingame et al. 2003). The number of disease-associated variants is growing at a considerable speed, as well as the attention for their functional characterization. Accordingly, we observed in vitro that, the L156R and L217R ("R mutations") display a more severe phenotype than the L156P and L217P variants ("P mutations") (Vázquez-Memije et al. 2009), and that there is a threshold effect for the "R mutations". We also noticed that the L156R mutation produces an F1 portion that is loosely bound to the membrane, considering the low oligomycin-sensitive ATPase activity, whereas the L217R mutant induces low activity of complex V, possibly reducing the rate of proton flow through the A6 channel. Conversely, "P mutations" retain partial ATP production and growth rate of fibroblasts.

The role of phospholipids in normal assembly and organization of the membrane proteins, multimeric protein complexes, and higher order supercomplexes, have been well documented (for a review, see Bogdanov et al. 2008). Cardiolipin (CL) a unique tetra-acyl phospholipid initially isolated from beef heart in the early '40s (Pangborn 1942) is an anionic phospholipid predominantly localized in the inner mitochondrial membrane (IMM), and is responsible for the modulation of activities of various enzymes involved in oxidative phosphorylation (OXPHOS). This includes cytochrome *c* oxidase (Robinson 1993), the ATP/ADP exchange protein (Horvath et al. 1990), and the F1F0-ATP synthase (Eble et al. 1990). The latter appears to have four high affinity sites for CL which may play a role in its optimal functioning (Eble et al. 1990). Cardiolipin is particularly susceptible to oxidation because it contains unsaturated side chains that are easily oxidized (O'Brien 1969). Peroxidation of CL is involved in the mechanism by which cytochrome *c* is released into the mitochondrial intermembrane space, whereas CL low content prevents adequate assembly of productive CL/cytochrome *c* complexes and peroxidation, thus increasing resistance to apoptosis (Huang et al. 2008). Depletion of CL or alteration of its acyl chain composition, and/or CL peroxidation have been associated with human diseases such as Barth syndrome (BTHS, MIM 302060), an X-linked disorder characterized by cardiac and skeletal myopathy, neutropenia and abnormal shape of mitochondria (Barth et al. 1983). Patients with BTHS show a deficiency of CL, especially its tetralinoleoyl form ((C18:2)4CL) in a variety of tissues (Vreken et al. 2000; Schlame et al. 2002).

Considering the key role of CL for proper functioning of OXPHOS (Hauff and Hatch 2006), we analyzed the membrane phospholipid composition of cultured skin fibroblasts carrying common mutations in *MTATP6* to investigate possible changes in CL profile, its relationship to levels of ATP production and the type of mutation. We observed that mitochondrial membranes with mutations at codon 217, but not at residue 156, showed a different content of CL compared to controls, suggesting that the position of the mutation influences different biosynthesis and cardiolipin remodeling.

Materials and methods

Chloroform, methanol, hexane, isopropyl alcohol and water of the highest purity (HPLC grade), were purchased from Sigma (St Louis, MO). The 1,1′,2,2′-tetramyristoyl-cardiolipin (sodium salt) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

Primary cell cultures

Human fibroblasts were obtained for diagnostic purposes from punch skin biopsies of a MILS patient (still unreported) harboring the mtDNA L156R mutation, a Leigh-like infant (patient 3 in Vilarinho et al. 2001) carrying the L156P, from the index patients reported in Dionisi-Vici et al. 1998 for the L217P mutation, and the Leigh patient reported in Carrozzo et al. 2001 for the L217R mutation. All the mutations were virtually homoplasmic (100% mutant mtDNA genomes) in primary cells. As controls, we used 5 cell lines obtained for diagnostic purposes from skin biopsies of age-matched children who were reportedly free of any metabolic or neuromuscular disorder. All cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 4.5 g/L glucose and 50 μg/ml uridine.

Isolation of mitochondria and extraction of lipids

Mitochondria were isolated by a differential centrifugation method, scraping monolayer cultures from six culture dishes (containing each about 7×10^6 cells) in 5 ml of an isolation buffer consisting of 250 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin (BSA), 10 mM Hepes and 10 mM Tris–HCl pH 7.43. Lipids were extracted from fibroblast isolated mitochondria (1 mg protein) using chloroform-methanol 2:1 (v/v) in the presence of $(C14{:}0)_4$CL as an internal standard according to the modified Folch extraction technique (Folch et al. 1957).

The total lipid extract was dried under a gentle stream of nitrogen and it was stored at −20 °C during 1 week. Lipid extracts were resuspended in 500 µl of chloroform-methanol (1/1, v/v) for their analyses.

Chromatographic conditions

A Varian 1200 series HPLC system (Varian, Cal, USA) was used with a normal-phase column (Nova-Pak silica, 150× 2 mm i.d. and 5 µm, particle size, Waters, Milford, MA). A gradient HPLC system was used to separate CL and the internal standard from other phospholipids. Chloroform/methanol, 90:10 containing 5 mM ammonium formate was used as a mobile phase A and methanol/water (90/10) with 5 mM ammonium formate was used as mobile phase B. The separation was achieved by using a gradient elution starting at 100% A, and ending at 100% B during 15 min. After that, the mobile phase B was maintained at 100% during 10 additional min. Finally, the solvent B was decreased to 0% and the column was again equilibrated during 5 min before the next run. The flow rate was 0.2 ml/min and the temperature of the column was 40 °C. A post column splitter was used to obtain 50 µl/min before introducing the sample to the electrospray ionization-mass spectrometry system (ESI-MS). The HPLC system was coupled on-line with a mass spectrometry Varian 1200 series, equipped with an ESI interface. The analyses eluted from HPLC were introduced into MS through a steel ESI needle operated at 3 kV in negative mode simultaneously. The nitrogen drying gas was approximately 8 L/min at 200 °C. The ion source and the ion voltage parameters were optimized with respect to the negative and positive ions of the standard phospholipids. The MS data were collected under full scan mode in the range of 600–1500 m/z.

Statistical analysis

Data are expressed as mean ± SE and assessed by using one-way ANOVA. Significance was set at $p<0.05$. Statistical analyses were performed with SigmaPlot 9.0 (Jandel Scientific Software Corporation, 2004, CA, USA).

Results

As preliminary settings, both natural CL from lipid extract of control mitochondria and (C14:0)4CL used as internal standard (IS), were clearly separated from other phospholipids by HPLC-MS in negative mode by using a scan mode in the ion range corresponding to each phospholipid analyzed (Fig. 1). In order to optimize the integration of peaks area corresponding to each phospholipid analyzed, scans from m/z:1350 to m/z:1500 to isolate CL, from m/z:1100 to m/z:1300 to isolate MLCL and from m/z:700 to m/z:900 to isolate PG were applied (Fig. 1). The area of both peaks, phospholipids and IS, were used to calculate the quantity expressed by mg of mitochondrial protein. The retention time of each phospholipid analyzed was compared to that of its corresponding standard.

Figure 2a shows CL content in fibroblast mitochondria harboring different mutations in *MTATP6.* Whilst mitochondria from cells carrying mutations at codon 156 did not significantly differ from controls. Mitochondrial membranes from L217R showed a 50% (48±5.2) increased content of CL whereas a 70% (71±2.7) reduction was observed in L217P cells. Figure 2b shows a considerable increase of the content of a precursor of CL, phosphatidylglycerol (PG), in mitochondrial membranes from all *MTATP6* cells which was more noticeable for mutations at codon 217 (9–11 times) than residue 156 (4–5 times). A significant increase (75%) of monolyso-cardiolipin (MLCL), an additional precursor of CL in L156P cells is observed in Fig. 2c, whereas the remaining mutations displayed a slightly decreased amount, if any, when compared to the MLCL content observed in controls. Since MLCL is considered an index of remodeling of CL undergoing deacylation of unsaturated fatty acid, the accumulation seen in L156P suggests low remodeling of CL. Figure 3 illustrates the spectra of different CL species in negative simply charged ion $[M\text{-}H]^-$ together with the corresponding ion to the IS (m/z:1242.4) in control and mutant mitochondria. In all spectra, the ion in m/z:1401.5, 1431, 1450.2 and 1472.7 corresponds to CL species that contain (C68)CL, (C70)CL, (C72)CL and (C74)CL, respectively. The cluster (C74)CL at m/z:1472.7 and 1474.3 corresponds to different CL species such as (C18:2)3(C20:4)CL, (C18:2)2(C18:1)(C20:4)CL which are enriched in linoleic (C18:2) and arachidonic (C20:4) acids. The cluster (C72)CL (m/z:1450.2) corresponds to different CL species rich in linoleic acid, for instance (C18:2)4CL, (C18:2)3(C18:1)CL or (C18:2)3(C18:0)CL. Whilst the (C70)CL cluster (m/z:1430) corresponds to palmitic [(C18:2)3(C16:1)CL] and palmitoleic acids [(C18:2)3(C16:0)CL] into CL species, the (C68)CL cluster contains double palmitic acid (18:2)2(C16:1)2CL, (18:2)2(C16:1)(C16:1)CL or (C18:2)2(C18:0)2CL. Table 1 summarizes the proportion of major molecular species of CL detected in all mitochondria analyzed. In mitochondria from L217R, L156P and L217P cells there is a general decrease or no change in the proportion of m/z:1401.5, 1403.4 and 1405 which correspond to the cluster of (C68)CL. However, some of these species are increased three times (m/z:1401.5) in the L156R mutation. The ion at m/z:1431 [cluster (C70)CL] is amplified in cells having the "R mutations" and reduced in the "P mutations". The clusters (C68)CL and (C70)CL showed a 198% and

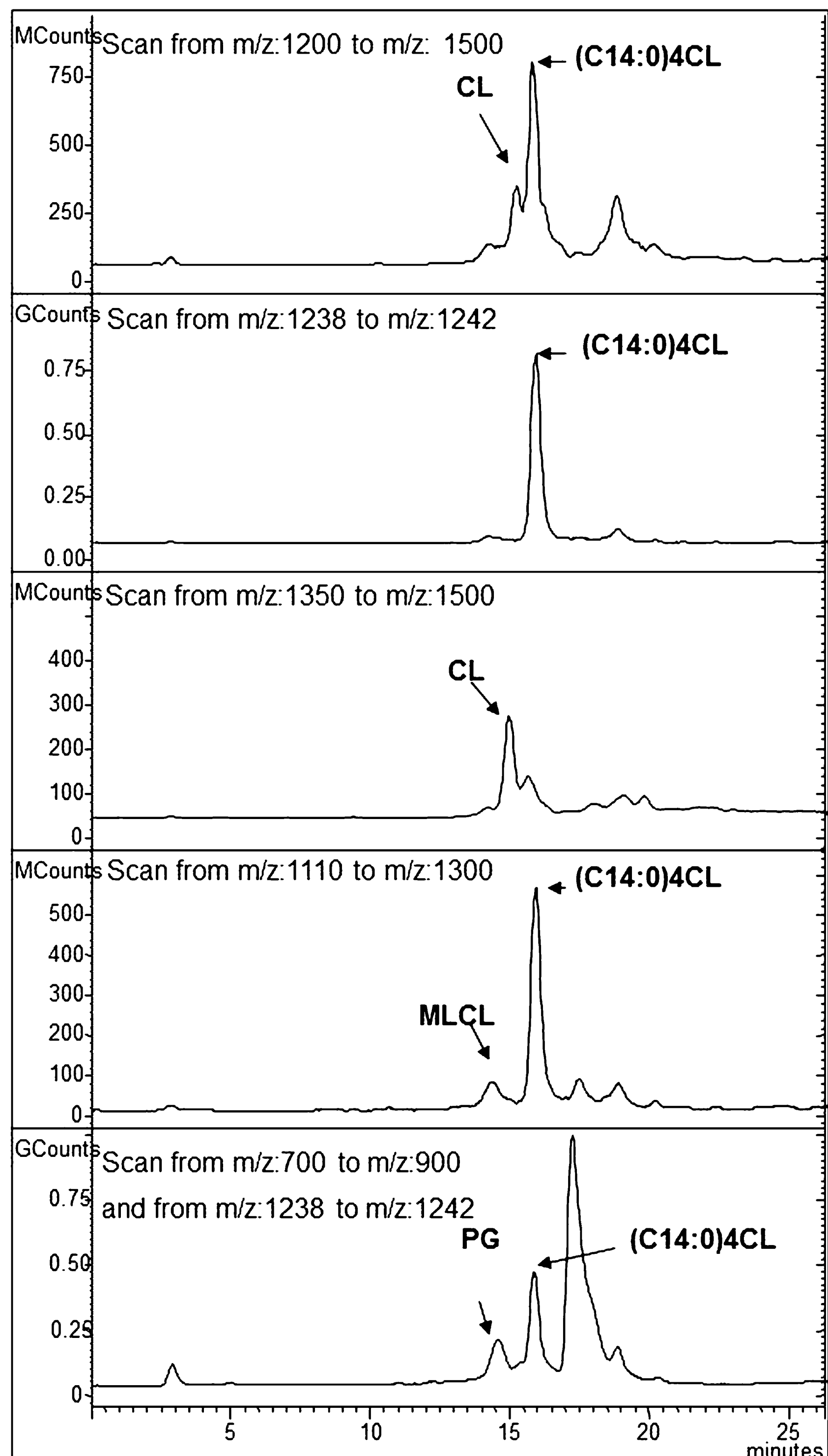

Fig. 1 Chromatograms of CL and internal standard (C14:0)CL, as well as MLCL and PG from lipid extract of the mitochondrial membrane. The HPLC conditions that allow separation and quantification of the different lipid classes are described in the Methods section. In brief, chloroform/methanol (90/10,v/v) containing 5 mM ammonium formate was used as a mobile phase A and methanol/water (90/10) with 5 mM ammonium formate was used as mobile phase B. The separation was achieved by using a gradient elution starting at 100% A, increasing to 100% B in 15 min. After that, the mobile phase B was maintained at 100% during 10 additional min. The MS data were collected under a scan in negative mode in the range of m/z 1200–1500 for CL and (C14)4CL. The scan applied for the detection of the different compounds were: CL, from m/z:1200 to m/z:1500; (C14)4CL, from m/z:1238 to m/z:1242.; PG, m/z:700 to m/z:900; and for MLCL the scan was performed from m/z:1110 to m/z:1300

47% increase in L156R cells compared to control mitochondria. The proportion of the ions at m/z:1450.2 corresponding to (C18)4CL species and containing exclusively linoleic acid were decreased in the L156R fibroblasts and increased in the other mutations with a highest amount in L217P mitochondria. The ion at m/z:1452.1 was found significantly increased in L156P; whereas the ion at m/z:1458.1, corresponding to the same cluster, namely (C72)CL was decreased in all mitochondria.

In the Clusters (C74), the ion at m/z:1472.7 corresponding to CL species enriched in linoleic (C18:2) and arachidonic acids (C20:4) such as (C18:2)3(C20:4)CL increased in all mutated mitochondria as compared to control. The ion at m/z:1474.3 corresponding to the CL

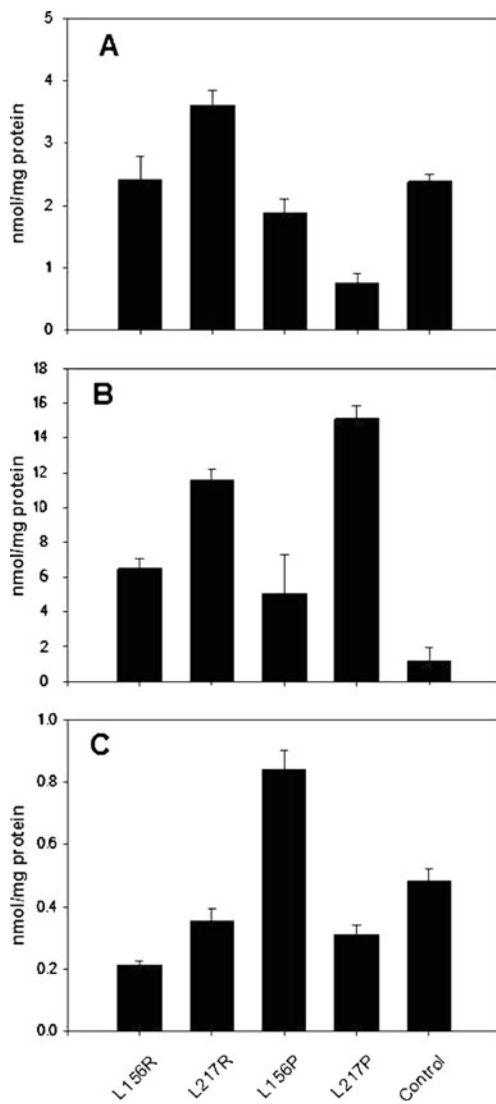

Fig. 2 CL (**a**), PG (**b**) and MLCL (**c**) levels in mitochondria from fibroblasts harboring different mutations in the *MTATP6* gene: L156R, L217R, L156P and L217P. Control corresponds to normal fibroblasts. Total phospholipids were extracted, separated and analyzed as described in Materials and Methods section. (C14)4CL was used as an internal standard and was analyzed by HPLC-ESI-MS

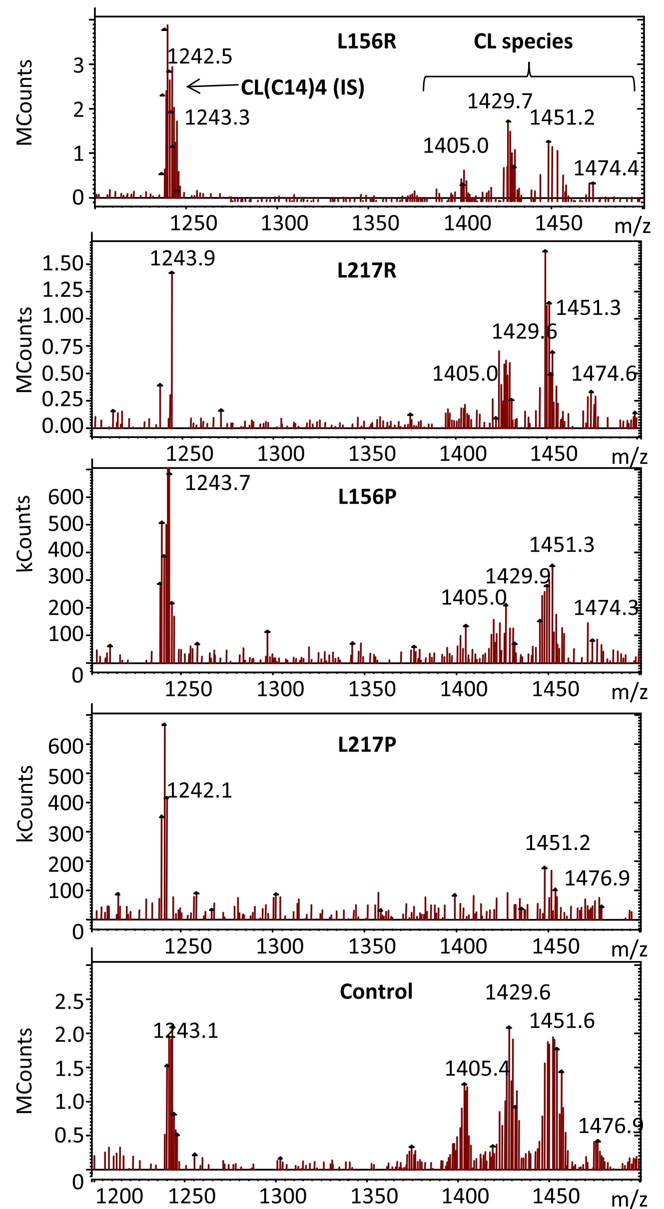

Fig. 3 Mass spectra of CL molecule species of mitochondria from fibroblasts harboring different mutations on the *MTATP6* gene. A scan between m/z:1200 to 1500 was performed to identify the different CL molecule species. The cluster of ions from m/z:1241 to m/z:1243 corresponds to the internal standard (C14)4CL. In all spectra the cluster of ions at m/z:1405, 1429 and 1451 match to CL species that contains (C68), (C70) and (C72) respectively, as total number of carbon related to four fatty acyl of CL. The cluster (C72)CL (m/z:1451) corresponds to different CL species containing four linoleic acids (C18:2)4CL. The cluster in (C70)CL (m/z:1429) corresponds to (C18:2)3(C16:1)CL where linoleic acid is substituted by one palmitoleic acid and the cluster (C68)CL corresponds to the replacement of two linoleic acids by two palmitoleic acids (18:2)2(C16:1)2CL

species containing oleic, linoleic and arachidonic acids was found increased in all mutations except in the L156P.

Discussion

In this work we investigated whether the metabolic consequences on mutated F1F0-ATP synthase might also include alterations in associated membrane lipids. For the function of the complex V and its coupling with the electron transport chain, it is necessary to have acidic lipids and especially cardiolipin which plays a key role in the process of oxidative phosphorylation (Kocherginsky 2009). Cardiolipin is able to form stable ion pairs with positively charged amino acids like lysine and arginine, leading to the selective binding of cardiolipin to complex V. Investigating the content of CL in cells with mutations in *MTATP6*, we

Table 1 Cardiolipin molecular species in fibroblast mitochondria harboring different mutations on the *MTATP6* gene

CL cluster	m/z	Control	L156R	L217R	L156P	L217P
(C68)CL	1401.5	4.1±0.3	12.2±0.9	4.7±0.6	2.3±0.2	4.8±0.2
	1403.4	6.6±0.5	7.5±0.6	3.2±0.2	4.1±0.3	4.6±0.2
	1405.0	7.6±1.0	7.8±0.4	6.2±0.6	4.6±0.6	3.2±0.2
(C70)CL	1431.0	13.9±1.1	20.4±1.3	16.5±1.1	9.8±0.6	6.9±0.5
	1433.7	11.8±0.7	9.7±0.7	3.1±0.4	7.0±0.2	Nd
(C72)CL	1450.2	22.5±1.4	17.3±1.3	36.6±1.4	25.6±1.0	38.6±2.4
	1452.1	21.5±1.9	16.9±1.2	15.3±0.8	30.7±1.0	14.0±0.8
	1458.1	10.5±1.2	7.4±0.6	3.1±0.3	8.2±0.4	6.5±0.5
(C74)CL	1472.7	2.3±0.4	3.8±0.3	8.3±0.9	10.2±0.8	7.4±0.9
	1474.3	4.8±0.4	5.6±0.5	9.0±0.7	2.4±0.1	15.4±0.5

Values correspond to the percentage ± ES from 3 different experiments. The percentage of each specie was calculated from the abundance of counts of each specie related to the total abundance of all species identified

(C68)CL contains two C16 carbon chains and two C18 carbon chains; (C70)CL contains one C16:0 carbon chain and three C18 carbon chains; (C72)CL contains four carbon chains; (C74)CL contains one C20 carbon chain and three C18 carbon chains. (C16 could be either plamitic or palmitoleic acid; C18 corresponds to oleic or linoleic acid and C20 is consistent with arachidonic acid

observed that mitochondrial membranes from fibroblasts harboring the L217R mutation had a higher content of CL compared to control cells. The opposite results seen in L217P mutated cells associated with a high content of the PG (CL precursor) suggest a decreased biosynthesis of CL. Mammalian CL is synthesized *de novo* via the CDP-DG (cytidine-5-diphosphate- 1,2-diacylglycerol) pathway (Hatch 1994). In the first step, PA (phosphatidic acid) and CTP are converted into CDP-DG by CTP:PA cytidylyltransferase or CDS (CDP-DG synthetase) (Kiyasu et al. 1963). Pulse–chase heart perfusion studies have indicated that one of the rate-limiting steps of CL biosynthesis in the heart is the conversion of PA into CDP-DG (Cheng and Hatch 1994). In the second and third steps of the pathway, CDP-DG condenses with *sn*-glycerol-3-phosphate to form phophatidylglycerolphosphate (PGP) and then phosphatidylglycerol (PG), catalyzed by PGP synthase and PGP phosphatase, respectively. PGP does not accumulate in tissues. In the last step of the CL biosynthetic pathway, PG is converted into CL by condensation with CDP-DG via CL synthase (Hostetler et al. 1971). Accumulation of PG suggests that the biosynthesis *de novo* of CL is affected with a possible block at the level of CL synthase. On the other hand, an increased level of PG and CL in L217R cells might indicate a high rate of biosynthesis or accelerated turnover. The results obtained in mitochondrial membranes from cells having a mutation at codon 156 are more difficult to be interpreted. In fact, mutations cells with at residue 156 had PG levels higher than control mitochondria whereas CL content remained wholly unaltered. These findings are in agreement with others observations (Schlame et al., 1999) who described only a slight increase of CL content in skeletal muscle biopsies from some patients with a mitochondrial disease; including one case carrying the L156R mutation (Schlame et al. 1999). Moreover, when determined in cultured skin fibroblasts, total CL and CL subclasses were found to be similar to controls in cultured skin fibroblasts from a child with MILS due to the L156P variant (Valianpour et al. 2002).

The significance of the findings reported in the present work might have multiple interpretations. One might argue that mutations replacing arginine for leucine increase the positive charge of the protein which is oriented with its positively charged arginine towards the negatively charged side of the membrane (Palsdottir and Hunte 2004). The acidic lipids in the membrane are oriented in the same direction probably because of the driving force in the membrane created by the protein asymmetry. The number of occurrences of acidic lipids as ligands with different amino acids changes are in the following order Arg>Lys>Tyr>His>Trp>Ser>Asp (Palsdottir and Hunte 2004). In addition, codon 217 is closer than residue 156 to the negatively charged side of the membrane (Schon et al. 2001; Angevine et al. 2003; Moore et al. 2008) and could induce enhanced biosynthesis of acidic CL which could ultimately interact with the positively charged arginine, as in the case of the L217R mutation. Membrane phospholipids biosynthesis is also thought to be regulated by the mitochondrial trans-membrane pH gradient and cation concentration in yeast (Cerbón and Calderon 1991). Therefore, the pathway for CL biosynthesis is regulated by the transmembrane pH component of the proton-motive force generated by the mitochondrial respiratory chain. For instance, in yeast, the uncoupler CCCP inhibited CL synthesis and oligomycin treatment resulted in a small increase of CL synthesis (Gohil et al. 2004).

A decrease in the mitochondrial content of CL is the most frequently reported pathological alteration of the CL profile. Low CL content associated with mitochondrial dysfunction has been observed in a range of disease conditions, including hypothyroidism, ischemia-reperfusion, heart failure, ageing, oxidative stress (Chicco and Sparagna 2007) as well as in fibroblasts harboring the L217P mutation (this study). However, reduced CL does not affect the activity of the ATPase since the rate of ATP synthesis in L217P cells is only reduced by 15–20% (Thyagarajan, et al. 1995; Kucharczyk et al. 2010), and complex V displays high affinity for CL, binding four CL molecules per each ATPase molecule (Eble et al. 1990). Thus, little amount of CL is sufficient to maintain the activity of complex V.

It is generally accepted that peroxidized cardiolipin is unable to preserve the activity of mitochondrial respiratory enzymes (Paradies et al. 1997, 1998, 2001, 2004; Musatov 2006). Peroxidation may lead to an overall loss of detectable CL content, by either preferential hydrolysis of peroxidized acyl chains by phospholipase A_2 (McLean et al. 1993), or direct decomposition of lipid peroxides (O'Brien 1969), or even by formation of CL-protein complexes that would no longer be detected as phospholipids (Toleikis et al. 1979). Moreover, evidence of high superoxide anion production and overproduction of ROS in L156P mitochondria (Baracca et al. 2007) might in part indicate the high MLCL levels as a manifestation of low membrane remodeling consequential to excess of peroxidized CL. The precise functional importance and significance of CL remodeling as the result of a decreased bioavailability of (C18:2)4CL fatty acid pattern in mammalian cells or alterations in the activity of acyl-specificity of cardiolipin remodeling enzymes remain relatively unexplored. Decreased levels of tetralineoyl-cardiolipin ((C18:2) 4CL) or a decreased content of cardiolipin (C18:2) have been reported in aged (Lee et al. 2006) and failing rat heart (Sparagna et al. 2005) even when enough C18:2 is provided in the diet. It has been proposed that the (C18:2) acyl chain configuration is a key structural requirement for the high affinity of CL to the inner membrane proteins in mammalian mitochondria (Schlame et al. 1990).

Data observed in the present study showed an increased cluster at m/z:1431 with lower m/z 1450 in L156R cells and reduced MLCL level, suggesting a higher than expected membrane remodeling, in the face of a stable CL biosynthesis. *De novo* CL synthesis was stimulated in L217R cells, despite that remodeling was similar to control mitochondria. Differences might relate to the specific slackly state of the L156R mutation (Vázquez-Memije et al. 2009).

The gross CL composition of mitochondria from control fibroblasts observed in this work, was in the range of the values reported by others (Valianpour et al. 2002; van Werkhoven et al. 2006) whereas *MTATP6* cells had fluctuations, which might reflect alterations in the development of mitochondrial membranes under a wide variety of genetic controls. To the best of our knowledge, this work represents the first indication of an abnormal turnover of the phospholipid composition in cells harboring a mtDNA point mutation. The different mutations seem to modulate the phospholipid composition of the membrane and previous reports support this argument. Tretyachenko-Ladokhina et al. (1993) have demonstrated that small changes in amino acid sequence cause dramatic changes in the hydrophobicity of the membrane-binding domain of cytochrome b_5, and that the complex lipid composition seen in membranes is the result, at least in part, of the protein complexity. The data obtained in the *MTATP6* mutations investigated herewith indicate a different biosynthesis and cardiolipin remodeling, dependent on the position of the mutation in the membrane. It is tempting to speculate that the mutated residues add a degree of "complexity" to the structure of subunit *6* of ATP synthase, which might partially influence disease severity as result of excessive oxidative stress or else in terms of ATP production.

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