

Review

Mitochondrial membrane lipid remodeling in pathophysiology: A new target for diet and therapeutic interventions

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ABSTRACT

Mitochondria are arbiters in the fragile balance between cell life and death. These organelles present an intricate membrane system, with a peculiar lipid composition and displaying transverse as well as lateral asymmetry. Some lipids are synthesized inside mitochondria, while others have to be imported or acquired in the form of precursors. Here, we review different processes, including external interventions (e.g., diet) and a range of biological events (apoptosis, disease and aging), which may result in alterations of mitochondrial membrane lipid content. Cardiolipin, the mitochondria lipid trademark, whose biosynthetic pathway is highly regulated, will deserve special attention in this review. The modulation of mitochondrial membrane lipid composition, especially by diet, as a therapeutic strategy for the treatment of some pathologies will be also addressed.

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Abbreviations: AIF, apoptosis-inducing factor; ATP, adenosine triphosphate; BTHS, Barth syndrome; CL, cardiolipin; cyt c, cytochrome c; DNA, deoxyribonucleic acid; DOX, doxorubicin; ER, endoplasmic reticulum; GD3, ganglioside GD3; IMM, inner mitochondrial membrane; iPLA2 γ , calcium-independent phospholipase A2 γ ; MAM, mitochondria-associated membrane; MLCL, monolysocardiolipin; MPT, mitochondrial permeability transition; mtDNA, mitochondrial DNA; OMM, outer mitochondrial membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; PTP, mitochondrial permeability transition pore; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP binding protein with low Pi; UCP, uncoupling protein.

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1. Mitochondria: evolution, physiology and the role of their membrane lipids

Mitochondria are membrane-enclosed cellular structures, which are classically defined as energy-producing organelles, although their influence extends to many other cell functions. In the classic, although controversial at the time, endosymbiotic theory proposed by Lynn Margulis in the late sixties [1], an amitochondriate anaerobic eukaryote engulfed an oxygen consuming proteobacterium. In one of those chance events that most likely allowed for the expansion of life on Earth, the engulfed bacterium was not destroyed, but rather lived in symbiosis with its host. The emergent biological system became equipped with skills to adapt to different types of environment, especially oxygen-rich niches. In fact, this new aerobic organism, an ancestor of eukaryotic cells, earned the advantage of using a wider variety of substrates to generate even higher amounts of energy, which provided it with a replicative superiority in relation to competition [2]. By its turn, the proteobacterium gained a better protection from the harshness of the environment and a steady flux of substrates. Over millions of years, the proteobacterium evolved into what we now recognize as the mitochondrion. Further evolution steps resulted in a remodeling of mitochondria to suit the needs of fungi, animals or plants. Thus, despite being mostly similar to their mammalian counterparts, plant mitochondria have interesting differentiating features [3], including inner membrane alternative oxidases (AOX) [4] and type II NAD(P)H dehydrogenases [5], which result in high metabolic plasticity and make plants endowed with a remarkable potential for adaptation. Interestingly, taking into account the widespread taxonomic distribution of AOX, which is also present in prokaryotes and, among eukaryotes, in fungi, plants and animals, it has been proposed that the proteobacterial ancestor from which mitochondria evolved were provided with AOX and this had been lost over evolutionary time by vertebrates and arthropods [6].

Mitochondrial bioenergetics depends largely on the physiology of the inner mitochondrial membrane (IMM), which hosts the redox complexes of the respiratory system and phosphorylation apparatus that performs the highly efficient energy-generating process known as oxidative phosphorylation. In this process, the energy made available from the oxidation of nutrients is used to drive ATP synthesis. Electrons from specific substrates, funneled to nicotinamide nucleotides (NAD⁺ or NADP⁺) or flavin nucleotides (FMN or FAD), enter the mitochondrial electron transport chain, through which they are transferred to oxygen [7]. As electrons flow through a sequence of membrane-bound protein and non-protein carriers, protons are pumped from the mitochondrial matrix to the intermembrane space. Therefore, according to the chemiosmotic theory introduced by Peter Mitchell [8], the free energy of substrate oxidation is conserved as a transmembrane proton electrochemical potential (Δp), part of which composed by the electric component ($\Delta\Psi$) which is used to drive ATP synthesis by the ATP synthase complex, through a rotational catalysis mechanism [9]. Mitochondrial oxidative phosphorylation accounts for about 90% of cellular oxygen consumption and provides more than 80% of the energy demands for cellular life metabolism [10]. However, besides cellular energy production, mitochondria have other functions in the cell, including the modulation of calcium signaling, regulation of cell death, the maintenance of cellular redox balance and the housing of important biosynthetic pathways [11]. Therefore, it is fair to say that mitochondria function

as gatekeepers of cell life and cell death. In the latter case, mitochondria have been associated with both apoptotic and necrotic cell death [11], discussed in detail in the next section.

Mitochondrial membrane lipids are involved in a number of processes as diverse as protein biogenesis, energy production, membrane fusion and apoptosis [12]. Moreover, spatially defined lipid distribution (Fig. 1A) may also affect mitochondrial processes as fusion and fission or the topology of proteins in the membrane plane [12]. In the particular case of energy production, evidence that membrane lipids modulate mitochondrial respiration has been generated by dietary manipulations of the lipid content in mitochondrial membranes. These approaches have shown that dietary interventions that are able to influence mitochondrial membrane lipid composition, hence modifying its physical properties [13–20], alter respiration [20–29] as well as other mitochondrial processes involving generation of reactive oxygen species (ROS) [30] and Ca²⁺-induced MPT [20,31]. The role of membrane lipids in mitochondria function is best exemplified by cardiolipin (CL), the signature phospholipid of those organelles. CL has been linked to a number of important mitochondrial processes, including oxidative phosphorylation [32], apoptosis [33] and the assembly and function of mitochondrial membrane proteins [34].

The phospholipid composition of mitochondrial membranes is thought to be under genetic control, and varies markedly between different tissues and organs within an individual animal, representing a specific feature of each membrane [35].

Under physiologic conditions, phospholipid composition of mitochondria relies on the conjugated expression and activity of a number of proteins involved in lipid synthesis and interconversion. Mitochondrial regulation of phospholipid levels is not fully understood, but it depends on the action of mitochondrial proteins, as reported for CL and PE [36]. On the other hand, studies on genetic manipulation of mitochondria membrane lipid content [37–39] point to the need to maintain a “physiologic” lipid composition of mitochondria membranes. We envision that environmental factors, including diet, can impact the genetic control of lipid synthesis, remodeling and incorporation in membranes, thus inducing alterations in membrane lipid composition. Despite diet-induced changes in the expression/genetic regulation of mitochondrial proteins have been reported [40,41], the mechanisms involved in the regulation of mitochondrial lipid content and their susceptibility to the interference of external stressors at the level of gene/protein expression were not yet fully clarified. An alternative way for external interventions to alter mitochondrial membrane lipid composition may be related with the supply of different lipid precursors, which may be in excess or not available for lipid synthesis.

In the following sections, we will address issues regarding the modulation of mitochondrial membrane lipid composition in the context of apoptosis, cancer or other diseases and aging (Section 2), as well as the possibility to explore diet-induced alterations in mitochondrial membrane lipids as a strategy for therapeutic purposes (Section 3).

2. Remodeling of mitochondrial membrane lipids in pathophysiology: an early event or a consequence?

A great deal of data has been accumulated regarding alterations of cell and organellar lipids in health and disease conditions. An

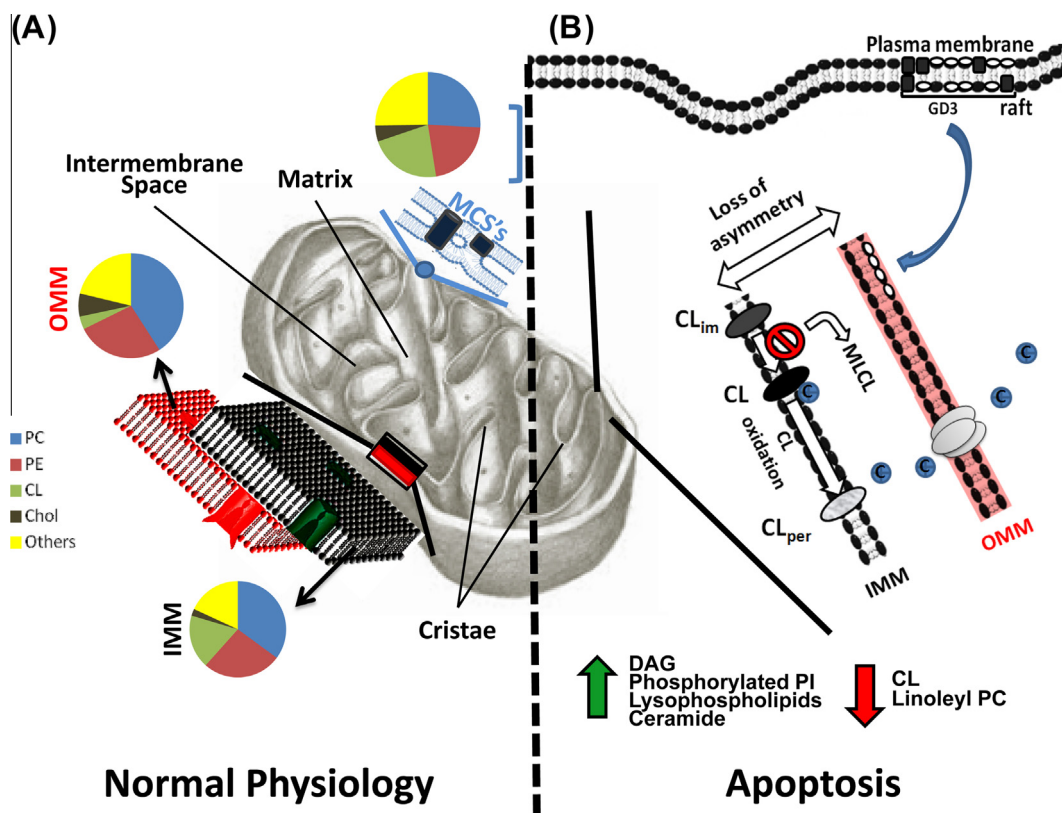


Fig. 1. Panel A (left) shows the general structure of mitochondria and most relevant differences in terms of membrane lipid composition between the inner mitochondrial membrane (IMM), the outer mitochondrial membrane (OMM) and mitochondrial contact sites (MCS's). The IMM displays invaginations (cristae) and defines the mitochondrial matrix. The inter-membrane space is delimited by IMM and OMM. The general lipid composition (in percentage of the total lipid) of the IMM, the OMM and MCS's reports to mouse liver mitochondria, according to Ardail et al. [256]. Panel B (right) shows some of the membrane lipid alterations described to occur during apoptosis, namely GD3 translocation from plasma membrane lipid rafts to mitochondria, an increase in ceramide, a decrease in CL content, and impairment of its acyl chain remodeling (indicated in the figure by a prohibition sign), resulting in MLCL accumulation. CL peroxidation, followed by cyt c ("c") release through pro-apoptotic pores in the OMM, loss of CL asymmetric distribution between the IMM and OMM, increase in DAG, phosphorylated PI and lysophospholipids (including, as described, MLCL), as well as a decrease in linoleyl PC were also reported as taking place during apoptosis. CL_{im}: immature CL; MLCL: monolysocardiolipin; CL_{per}: peroxidized CL.

important question is why membrane lipids are altered. This is an issue to be solved by functional lipidomics, which focuses on unraveling the molecular mechanisms that trigger (upstream events) and are triggered by (downstream events) membrane lipid alterations. Therefore, the major challenge for lipidomic research is to find out how membrane lipid changes are associated to known or unknown cell signaling pathways. In this section, alterations of mitochondria membrane lipids related to apoptosis, cancer or other diseases and aging are reviewed, and a mechanistic perspective is provided whenever sufficient data are available to support it.

2.1. Mitochondrial membrane lipids and apoptosis

2.1.1. Mitochondrial pathway for apoptosis

As discussed briefly above, mitochondria have an important role in the modulation of cell death. The "intrinsic" or mitochondrial pathway to cell death becomes activated in response to a variety of apoptogenic stimuli, such as growth factor deprivation, hypoxia, cell detachment, oxidative stress and DNA damage [42,43]. This process results in increased mitochondrial membrane permeability and ultimately in the release of pro-apoptotic factors such as cytochrome *c* (cyt *c*), Smac/DIABLO, Omi/Htr2A, and AIF (apoptosis-inducing factor) from mitochondria, leading to the activation of a highly complex and regulated cell death program. Several mechanisms have been proposed to occur during the permeabilization of OMM, as will be described in the following section. Another pathway leading to apoptosis involves mitochondrial calcium overload, which triggers the opening of the mitochondrial permeability tran-

sition pore (PTP) through a phenomenon termed mitochondrial permeability transition (MPT). *In vitro*, the MPT causes disruption of oxidative phosphorylation and mitochondrial swelling due to influx of water, leading to the rupture of the mitochondrial outer membrane (OMM) and subsequent release of pro-apoptotic factors [44]. Indeed, more than an *in vitro*-only effect, the MPT is a common phenomenon in tissue injury [45].

When cellular ATP levels become abnormally low, which may result from mitochondrial dysfunction, classical apoptotic triggers typically cause necrotic demise, showing that sustained ATP production is required for the cells to engage the apoptotic program [46]. Necrosis results in the swelling of the cell and the disruption of the plasma membrane, with consequent release of cellular content to the extracellular space. As opposed to apoptosis, uncontrolled necrosis triggers an inflammatory response that can cause further damage to surrounding cells [47].

2.1.2. Mitochondrial membrane remodeling and signaling during apoptosis

Several alterations in mitochondrial lipid content and distribution during apoptosis have been reported (Fig. 1B). An increase in diacylglycerol (DAG), monolysocardiolipin (MLCL) and phosphorylated phosphatidylinositol (PI) was described in mouse liver mitochondria. On the other hand, CL and linoleic acid-containing phosphatidylcholine (PC) contents were reported to be decreased, with the same trend observed in mitochondria from other origins [48]. These lipid changes appear to reflect a deficiency in the biosynthesis of PC, impacting downstream the remodeling of CL,

since this process requires PC species as acyl donors. Thus, an increase in MLCL is often concomitantly found with the initial loss of CL [49]. The content in other lysolipids (C16:0 and C18:1 lysoPC) also increases during the early stages of the apoptotic process [50] and may play a crucial, although not fully clarified, role in assisting the pro-apoptotic proteins Bax and Bak in the process of OMM permeabilization [48].

During apoptosis, OMM CL content has been reported to increase from very low to up to 40 mol% of its total content [51]. On the other hand, CL distribution between the two leaflets of the IMM [52] was shown to change from 60:40 mol% between the inner leaflet (matrix side) and the outer leaflet (intermembrane space side) to about 30:70 mol%, during the early phase of apoptosis, with CL translocation to the outer leaflet of the IMM resulting in increased association with cyt *c*. Moreover, CL peroxidation was suggested to have a key role in mitochondrial apoptotic signaling. Thus, upon an apoptotic stimulus, a tight complex between cyt *c* and CL is formed and cyt *c* is converted from a loosely attached electron carrier protein to a peroxidizing molecule [53,54]. Then, a catalytic peroxidase activity selectively targeted towards CL is initiated. This oxidation process leads to a decrease of cyt *c* affinity for CL and apparently mediates the release of pro-apoptotic factors from mitochondria into the cytosol, including cyt *c* itself, by mechanisms not yet fully understood. [51]. Interestingly, CL as well as phosphatidylethanolamine (PE), predominates in IMM-OMM contact sites. These phospholipids are both non-bilayer lipids, adopting an inverted hexagonal phase (H_{II}) in the absence of membrane constraints. Although somehow speculative, it has been proposed that non-bilayer lipids could be involved in membrane fusion at the contact sites, fostering CL access to the cytosolic face of OMM [55], where it would directly interact with tBid, offering a docking site for this active form of the pro-apoptotic protein Bid. This would be the first step for triggering the action of Bax and Bak (other pro-apoptotic Bcl-2 proteins), thus leading to OMM permeabilization. A more recent view of the involvement of CL-enriched contact sites in apoptosis-associated events was presented by Sorice et al. [56]. In this work, a hypothetical model was presented proposing that contact sites act as activating platforms (raft-like microdomains) at which caspase-8 is anchored, cleaving its substrate (Bid). The resulting product, tBid, is then recruited to promote Bak/Bax oligomerization. The segregation of these domains from the rest of the OMM would create membrane defects, fostering apoptotic factor leakage.

Sphingolipids also play a crucial role in the early stages of apoptosis. Mitochondria are provided with enzymes that catalyze the synthesis and the hydrolysis of ceramide, a lipid that occupies a central position in sphingolipid metabolism [57]. Thus, ceramide can be generated in the endoplasmic reticulum as well as in mitochondria [58] by *de novo* synthesis, which can involve the acylation of sphinganine to form dihydroceramide in a reaction catalyzed by ceramide synthase, or the reverse reaction of ceramide hydrolysis catalyzed by ceramidase, using as substrates sphingosine and fatty acids.

It is well known that ceramide levels in mitochondria undergo a significant increase *prior* to the onset of apoptosis. On the other hand, ceramide-induced adverse effects on mitochondria have been extensively reported, namely increased generation of ROS, alteration of calcium homeostasis, collapse of the inner mitochondrial $\Delta\Psi$ and release of proteins from the intermembrane space [59]. However, the mechanisms underlying ceramide-induced apoptosis at the mitochondrial level are not yet fully clarified. According to some authors [59–61], this event involves a direct action of ceramide on mitochondria, although the immediate consequences of this interaction and the role on apoptotic alterations are still under debate. Siskind et al. demonstrated that ceramide forms protein-permeable channels in isolated mitochondria, thus raising the permeability of the OMM not only to cyt *c*, but also to all small

proteins (up to 60 kDa in size) at physiologically relevant ceramide concentrations [60]. Interestingly, these channels, formed by transmembrane columns of ceramide molecules held together by intermolecular hydrogen bonds [59], were not formed within the plasma membrane of erythrocytes [60], revealing a certain specificity for the lipid environment of the OMM. Other authors alternatively proposed that the direct interaction of ceramide with the OMM results in its permeabilization due to the formation of ceramide-rich macrodomains which recruit Bax molecules, regulating their insertion and subsequent oligomerization to form pores [61]. Therefore, ceramide was proposed as a membrane-based stress calibrator, acting through a reorganization of the OMM structure. This effect is compatible with ceramide propensity to promote lateral phase separation in a fluid phospholipid membrane inducing the formation of extremely ordered and rigid ceramide-enriched platforms [62]. This has been proposed to serve clustering of receptor molecules, reorganization of signaling molecules and to constitute, at the plasma membrane level, sites to promote and amplify signaling processes [63]. As an alternative to a direct interaction of ceramide with mitochondria, the activation of the pro-apoptotic proteins BAX/BAK mediated by the sphingolipid metabolites sphingosine-1-phosphate (Sph1P) and hexadecenal was proposed as the mechanism underlying OMM permeabilization [64]. According to this proposal, ceramide, constitutively generated from sphingomyelin by a neutral sphingomyelinase in a mitochondrial-associated compartment, is transferred to mitochondria and produces Sph1P and hexadecenal, which directly regulate BAK and BAX, respectively, by stabilizing the active conformation of these effector proteins. Hexadecenal, a product of Sph1P degradation, would promote BAX monomer conformational alterations that favored oligomerization, leading hence to permeabilization of OMM and apoptosis.

GD3, a ceramide-based glycolipid, has been reported to accumulate during apoptosis in mitochondria, where it is supposed to trigger specific events involved in the apoptogenic program by interacting with mitochondrial raft-like microdomains [65]. In fact, GD3-enriched raft-like domains are formed during Fas/CD95-mediated apoptotic signals, consisting of well-ordered structures connected to the internal mitochondrial cristae in mitochondria [66]. After a pro-apoptotic stimulus, a number of mitochondrial proteins, such as ATP synthase, VDAC-1, VDAC-2 [67] and hFis [68], converge to those raft-like mitochondrial microdomains, which contribute to apoptosis-associated modifications and late apoptogenic events, as previously mentioned [69]. GD3 was considered as an actual structural component involved in the formation of the PTP, and contributing to the formation of a multimolecular complex that includes VDAC-1, Bcl-2 family and fission proteins, such as hFis [68]. Following CD95/Fas crosslinking, Bax, Bak, caspase-8 and tBid are also recruited to these CL- and GD3-enriched mitochondrial raft-like microdomains, as mentioned above [66,56]. Therefore, GD3 has been proposed as playing a crucial role in the context of MPT induction and cyt *c* release and subsequent cell death promotion [66].

2.2. Remodeling mitochondrial membrane lipids to survive: the case of cancer cells

The classical metabolic phenotype of cancer cells consists of an increase in glycolytic fluxes under aerobic conditions, which was first described by Otto Warburg almost a century ago [70]. The Warburg effect involves a much more complex metabolic picture, involving the orchestration of multiple factors [71], and is reflected in pronounced alterations in bioenergetic and apoptotic-related cellular signaling. These can account for oncosuppression, oncogenesis, tumor progression and chemotherapy resistance shown by cancer cells, which are remarkably more resistant to death induction than their normal analogs [72]. Several specific

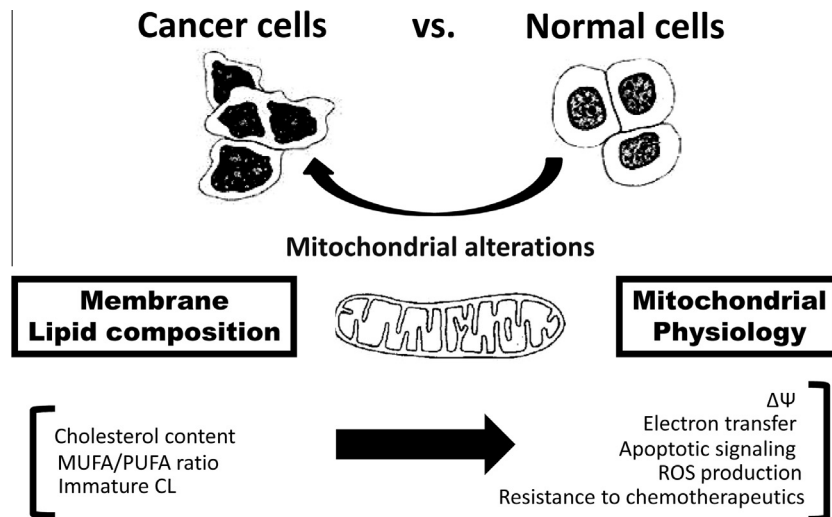


Fig. 2. Some features of mitochondrial membrane lipid composition (left) and mitochondria bioenergetics (right), which are altered in cancer cells as compared to normal cells. Bioenergetic remodeling is known to occur in the majority of cancers, involving multiple partners, including transcription factors and metabolites, often resulting in increased glycolytic fluxes with decreased oxidative phosphorylation. Increased mitochondrial electric transmembrane potential is a trademark characteristic of many tumors, which is susceptible to be targeted by positively-charged molecules. Mitochondrial membrane lipid alterations, including changes in cardiolipin remodeling and in cholesterol content, can contribute to modify the activity of that organelle, including through inhibition of the respiratory chain. At the same time, membrane lipid alterations, especially concerning the fatty acyl chains that compose cardiolipin, can increase the resistance to chemotherapeutics through altering the peroxidation index of that phospholipid (MUFA/PUFA ratio).

alterations associated with mitochondrial function have been observed in cancer cells, with the activities of certain enzymes involved in oxidative phosphorylation being decreased in mitochondria from these cells. Increased gluconeogenesis and glutaminolysis, a decrease in pyruvate oxidation, increased lactate production accompanied by decreased fatty acid beta-oxidation have been reported in mitochondria from different types of tumors [73].

In the whole metabolic remodeling framework in cancer, the mitochondrial membrane composition was described to undergo alterations (Fig. 2). Analysis of the inner membrane lipid composition of various tumor mitochondria indicated a higher cholesterol content [74–76], along with changes in the relative amount of individual phospholipids [77]. Alterations in lipid acyl components have also been observed, the phospholipids of mitochondria from tumour cells showing generally shorter and less unsaturated acyl chains than those from normal cells [78,79].

Lipid composition alterations in mitochondrial membranes are expected to impact the mitochondrial phenotype, which may contribute to the Warburg effect and to the decreased susceptibility to apoptotic stimuli. Altered mitochondrial membrane composition and dynamics in cancer cells may decrease the activity of the respiratory chain, a relationship that has already been described for CL [77]. On the other hand, a remodeling in mitochondrial membrane lipid composition may also contribute to the increased mitochondrial $\Delta\Psi$ observed in many tumors [80,81]. Increased mitochondrial $\Delta\Psi$ may have as a consequence an increased generation of ROS by the respiratory chain, which may be important in the context of cancer development, including by regulating Kras-mediated tumorigenicity [82], and stabilizing the hypoxia-inducible factor-1 α [83]. The mechanisms underlying these phenomena appear to involve both protein and membrane lipid alterations. One possibility is that altered ATP synthase protein content/activity can decrease proton conductance through the F_o subunit, increase mitochondrial $\Delta\Psi$ and decrease ATP generation thus contributing to the Warburg effect [84]. Uncoupling proteins (UCP) would also be an attractive candidate to explain those alterations, since a decreased expression of this class of proteins in tumors would in theory contribute to increase mitochondrial $\Delta\Psi$. Nevertheless,

the still incomplete knowledge of the functional meaning of the different UCP isoforms justifies that the results obtained so far regarding the function of this class of proteins in different tumors is still under discussion [85,86]. In fact, an increase in UCP2 is identified in breast and in many other tumors, including leukemia, ovarian, bladder, esophagus and lung [87]. Ectopic expression of UCP2, surprisingly, was able to increase the tumorigenic properties of a breast cancer cell line. The authors proposed that increased expression of UCP2 results from an adaptive response to the hyperpolarization of mitochondrial membranes [87], showing that modulation of UCP2 content is a consequence and not a cause of hyperpolarized $\Delta\Psi$. In terms of membrane lipids, the cholesterol content has been shown to increase in the inner mitochondrial membrane of Ehrlich tumor mitochondria, an effect that was suggested to increase membrane order and decrease proton passive permeability through that membrane, thus increasing $\Delta\Psi$ [74]. Other outcomes of mitochondrial membrane cholesterol enrichment include reduced activity of the respiratory chain [88], promoting a phenotype characteristic of the Warburg effect, and increased resistance to chemotherapy [75]. The role of cholesterol in chemotherapy resistance has been related to its capacity to increase membrane lipid packing, thus decreasing mitochondrial membrane permeabilization and impairing Bax-driven release of cytochrome c to the cytosol [75]. Some studies have suggested that the increase in cholesterol in mitochondrial membranes may be directly related with caveolin-1 deficiency [89], which has been described to underlie carcinogenesis in different models including colorectal and breast cancer [90,91]. Interestingly, loss of tumor stromal caveolin-1 leads to increased oxidative stress and induced the so-called “reverse Warburg effect”, in which tumor-associated fibroblasts with deficient mitochondrial function and increased oxidative stress and autophagic fluxes feed tumor cells with substrates for the respiratory chain [91,92]. Other studies have suggested that the outer membrane protein voltage-dependent anion channel (VDAC) is involved in cholesterol distribution in mitochondrial membranes in tumors [88].

CL has also attracted a lot of interest in a cancer context. A shotgun lipidomics study aiming at determining CL content and composition in mitochondria from subcutaneously grown brain

tumors including astrocytoma and ependymoblastoma, a stem cell tumor, and two microgliomas, showed evident alterations in CL content and composition in all tumors [77]. These abnormalities included an abundance of immature molecular species along with alteration of mature molecular species, probably related to defects in CL synthesis and remodeling. Data regarding this mitochondrial phospholipid in terms of cancer are very diverse, ranging from the appearance of immature molecular species and decreased CL content in brain tumors [77], decreased CL content in rhabdomyosarcoma cells [93], increased CL content and fatty acid remodeling in a rat model of peritoneal carcinosis [94] to unaltered total CL amount in a mouse strain with high propensity to form spontaneous gliomas [95]. This last study involved two strains of mice, one of which (VM/Dk) with a much higher propensity to develop gliomas than the other, taken as the control strain (C57BL/6j). The VM/Dk strain not only contained higher amounts of ethanolamine glycerophospholipids, phosphatidylserine (PS) and ceramide, but also displayed a lower number of CL species than the control strain [95]. Interestingly, although the activity of complexes I–III was decreased in the VM/Dk strain, complex IV activity was higher [95]. This disparity of impact on respiratory chain activity may have resulted from the differences in CL species, which should have influenced the rate of ROS production and ATP generation, although sadly these parameters of mitochondrial function have not been measured in this work.

Regarding the drug resistance phenotype, studies on MCF-7 human breast cancer cells showed an increase of CL content (expressed in percentage of cell total phospholipids) in doxorubicin-resistant and cisplatin-resistant cancer cells, as compared to drug-sensitive parental cells [96]. However, other lipid alterations were observed in those drug resistant cells, namely an increase in cholesterol and sphingomyelin content, which the authors suggest to be involved in lipid raft dynamics, thus affecting protein–protein and protein–lipid interactions. The characterization of CL species in drug-sensitive vs. drug-resistant cells was not performed in this study. Evidence has been obtained that CL remodeling is important in determining CL peroxidation catalyzed by *cyt c* and consequent *cyt c* dissociation from the inner mitochondrial membrane [53,54]. Therefore, the decreased resistance to apoptosis in tumor cells may be predictably related with less oxidizable CL species. In terms of CL content and its relationship with cell resistance to apoptosis, a very comprehensive study has been carried out by Huang et al. [37] showing that CL deficiency counteracts rather than promotes, as stated by other authors [97], cytochrome *c* release and apoptosis induced by several stimuli. Huang and collaborators remarked that discrepant results obtained by Choi et al. might be due to the methodology used for CL quantification: a radioactive method based on the integration of [³H] palmitic acid into phospholipids instead of evaluating the content of CL with respect to the total phospholipid phosphorus, as performed by Huang et al. These authors argued that, since palmitate has shown to be a poor substrate for CL synthase [98], a predictable low incorporation of the radiolabel in CL should have occurred, thus disabling an accurate determination of CL content changes relative to other lipids.

Facing these different results, it is not easy to establish a model for the effects of CL on cancer etiology. As a general consequence, we may anticipate that alterations in CL:protein ratio or in CL species may alter the activity of different respiratory chain proteins, thus inhibiting electron flux and the efficiency of oxidative phosphorylation [94], contributing to the Warburg phenotype. But the story is certainly far more complex. The balance between proapoptotic and anti-apoptotic proteins present in the OMM, critical in the context of apoptosis, is altered in a variety of tumors. In fact, the mitochondrial apoptotic pathway is compromised by a readjustment between anti-apoptotic and proapoptotic promoters, in favor of the former [99].

Targeting mitochondrial alterations has become a promising strategy for the induction of apoptosis in tumor cells. The aforementioned proliferative and bioenergetic differences between normal and tumor cells provide an opportunity to selectively target cancer cell mitochondria or even use them as biomarkers for early detection of cancer [71,73]. As an example, the above mentioned differences in $\Delta\Psi$ have been used as part of a therapeutic strategy, in which delocalized lipophilic cations are selectively accumulated in tumor cells. Some of these compounds have in fact exhibited efficiency in the demise of cancer cells either *in vitro* or *in vivo* [100–108]. However, efficient mitochondrially-targeted therapies must be based on the coordinated targeting of as much cancer features as possible, namely cellular energy metabolism, mitochondrial stability and mechanisms responsible for apoptosis resistance [109].

In summary, this section highlighted a particular case of mitochondrial membrane remodeling towards cell survival and fast proliferation, with different examples pointing to lipid membrane and protein alterations in tumors, which can contribute to the Warburg phenotype and to escape from basal or chemotherapy-induced cell death. Cardiolipin appears to be particularly involved in this process, a phenomenon that is observed in the context of other human pathologies (see the following section).

2.3. Cholesterol and cardiolipin alterations in human disease: beyond cancer

Several pathologic conditions have been proposed to promote changes in mitochondrial lipid composition, which may be implicated in alterations of mitochondrial function and in disease progression. Two lipids will be specially focused in this section: CL and cholesterol. Alterations associated with CL content and fatty acid composition have shown to play an important role in a disease context. Reduced CL levels (the most frequently reported pathological alteration regarding this lipid) [110], CL oxidation and/or alteration of its molecular composition have been associated with mitochondrial dysfunction in multiple tissues and in several physiopathological conditions, including ischemia/reperfusion, different thyroid states, diabetes, heart failure and Barth syndrome [111–115]. An important note of caution is that in most cases there is no definitive evidence whether the alteration in CL amount/species is located upstream or downstream of the disease process and the impact of that specific defect on the overall mitochondrial phenotype.

2.3.1. The Barth Syndrome

Barth Syndrome (BTHS) is a paradigmatic example of a disease in which a clear association between the pathological phenotype and a defect on a mitochondrial lipid has been established. BTHS is an X-linked disease [116], often fatal in childhood and resulting in congestive heart failure in infants [117]. This multisystem inherited disorder is characterized by cardiomyopathy, skeletal myopathy, growth retardation, neutropenia and organic aciduria [118–120]. A broad spectrum of clinical manifestations, with different levels of severity, has been observed in BTHS patients, which complicates the diagnosis of the disorder, due to the difficulty in differentiating it from other cardiomyopathy syndromes in infants, based solely on clinical findings.

Studies of familial cardiomyopathies have shown that this disease is linked to the TAZ or G4.5 gene (previously termed tafazzin), located at Xq28 [116], which is highly expressed in the cardiac and skeletal muscle. The primary transcript of this gene can produce different mRNAs by alternative splicing, producing proteins that differ at the N-terminus and at the central region [116]. The detection of a significant sequence homology between the TAZ gene and genes coding for phospholipid acyltransferases led to the hypothesis that impairment of lipid metabolism should be involved in

BTHS [121]. Abnormalities of the respiratory chain, not confined to a specific respiratory complex, were detected in mitochondria from different cells of BTHS patients [122,123], strengthening the hypothesis that the cause of this disease resided on membrane lipid composition alterations with consequent perturbation of lipid–protein interactions. The impairment of mitochondria function, in turn, could explain the occurrence of characteristic clinical manifestations of BTHS, such as cardiomyopathy and muscle weakness. Since CL plays a crucial role in mitochondria complex assembling, stability and function [124,32], it would not be surprising that TAZ mutations impacted on CL homeostasis. In fact, a decrease of CL content [117] and alterations in CL remodeling/maturation, reflected in impaired incorporation of linoleic acid into CL [125] and a specific decrease of tetralinoleoyl-CL [117,126], were reported in a variety of tissues from BTHS patients. An extensive study involving children with genetically proven BTHS and children exhibiting BTHS-like disorders (lacking TAZ mutations) showed that CL analysis in platelets can provide a valuable diagnostic tool to identify cardiomyopathies caused by TAZ mutations, since only in this case CL abnormalities were detected [117]. However, besides CL, other phospholipids, namely phosphatidylcholine and phosphatidylethanolamine (PE), exhibited a non-characteristic fatty acid composition, resulting in a deficiency of palmitoyl-arachidonoyl-glycerol species in several tissues, with potential impact on eicosanoid-dependent signal pathways [117].

The synthesis of characteristic CL species in mitochondria involves two steps: (a) transfer of an activated phosphatidyl group from phosphatidyl-cytidine monophosphate to phosphatidylglycerol (PG), catalyzed by cardiolin synthase and (b) CL molecule remodeling by tafazzin-catalyzed transacylation, in order to produce cell and tissue-specific CL species, namely tetralinoleoyl-CL, a dominant molecular species in animal and plant tissues [127]. Therefore, it is conceivable that alterations of the characteristic CL acyl-composition detected in BTHS result from impaired tafazzin activity. On the other hand, accumulation of MLCL molecules has been associated with BTHS [128]. Malhotra et al. [129] stated that this effect was due to the action of a mitochondrial calcium-independent phospholipase A₂ (iPLA₂-VIA), which promoted CL deacylation. Using a tafazzin-deficient *Drosophila* model of BTHS, which exhibited poor motor function in parallel with abnormal mitochondria, low CL content and CL species with abnormal fatty acid composition, the authors demonstrated that the inactivation of mitochondrial calcium-independent iPLA₂-VIA could be a strategy to revert manifestations associated with tafazzin deficiency [129]. Also, the chemical inhibition of calcium-independent phospholipase A₂ from Barth patient lymphoblasts restored the MLCL/CL ratios to the values detected in normal cells. Since an important consequence of TAZ mutations that contribute to BTHS is related to MLCL accumulation, the inhibition of the deacylation step should be envisaged as a partial solution of the problem. On the other hand, this therapeutic intervention should spare healthy cells and target specifically the tissues affected by the disorder.

Another interesting issue concerns the specificity of CL acyl patterns, resulting *a priori* from fostering specific transacylations in normal mitochondria. Recent studies showed that tafazzin itself does not display a high specificity for their lipid substrates, reacting with a great variety of phospholipids and lysophospholipid species [130–132], although its activity is greatly influenced by the physical properties of the matrix in which lipid substrates reside [130], being lower for lipids highly packed in bilayer phases. Moreover, Schlame et al. [130] showed that the characteristic CL species of normal mitochondria (tetralinoleoyl-CL) could be formed *in vitro* in the presence of purified tafazzin, under conditions that favor inverted hexagonal lipid arrangements. It was thus proposed that tafazzin acts in specific membrane lipid domains, explaining why its activity does not equilibrate the fatty acid

composition of all mitochondrial phospholipids [130,133]. On the other hand, tafazzin-induced formation of tetralinoleoyl-CL (a non-bilayer lipid) in highly curved lipid domains is expected to have a great impact on fostering mitochondria fission and fusion phenomena, which require transient destabilization of lipid bilayer, or on favoring lipid arrangements in membrane regions with very high curvature, such as those in contact sites between the inner and outer mitochondrial membranes. Notably, these regions are enriched in CL and seem to play a crucial role in clustering and organization of apoptotic signals on mitochondria [56], as previously reported in this work.

2.3.2. Cardiac pathology

A loss of heart mitochondria CL following ischemia has been described in the early 1980s [134,135], and has since been well documented in a variety of tissues. CL depletion accompanied by loss of cytochrome *c* during the ischemia phase was shown to lead to increased generation of hydrogen peroxide by the respiratory chain [136]. On the other hand, chronic cardiac overload leading to experimental heart failure, results in loss of tetralinoleoyl-CL with consequent decrease in the activity of cytochrome *c* oxidase [115]. A similar effect was observed in rats suffering from rapid pressure-overload hypertrophy and failure by chronic aortic banding. In this case, a reduction in the linoleoyl content in cardiac mitochondria CL was reported [137,138]. A reduction of heart CL content was also reported during the progression of heart failure in cardiomyopathic hamsters. The authors of this report ascribed this event to a reduction of the diacylglycerol content in heart and impaired phospholipid biosynthesis [139]. CL loss during cardiac pathologies may result from deficient biosynthesis/remodeling or increased degradation (including oxidation) and certainly affect mitochondrial energy generation [140].

2.3.3. Diabetes and other metabolic diseases

Recent reports indicate a reduction of mitochondrial CL levels and alterations of CL acyl chain remodeling, resulting in increased incorporation of the highly unsaturated docosahexaenoic acid, in the hearts of streptozotocin (STZ)-treated diabetic mice [141]. Whether these specific CL alterations, occurring in an early stage of diabetes, influence the development or progression of diabetic cardiomyopathy remains unknown. A recent study demonstrated that transgenic expression of CL synthase decreases the incidence of CL remodeling processes that ultimately cause degeneration of mitochondrial function during STZ-induced diabetes [142]. Not only mitochondrial dysfunction was mitigated by cardiolipin synthase expression in the diabetic myocardium, but also a marked increase in protective eicosanoids was observed [142]. Although this interesting study allows foreseeing a therapeutic role for this enzyme within the scope of diabetes-induced alterations of mitochondria bioenergetics and signaling, the truth is that we are far from understanding the mechanisms underlying the benefic effects resulting from the manipulation of cardiolipin synthase.

In another context, an increase in CL synthase activity and CL content in rat heart [143–145] and liver mitochondria [146] was found in hyperthyroidic rats, which resulted in an increase in the activity of multiple CL-dependent mitochondrial proteins and processes. Conversely, drug-induced hypothyroidism resulted in a marked loss of CL content and CL synthase activity in heart mitochondria [147]. This study also showed that rat heart monolysocardiolipin acyltransferase activity is regulated in parallel and in a concerted manner with the biosynthesis and content of cardiac CL.

A significant decrease in CL mitochondrial content and a relevant increase in the level of peroxidized CL were reported in non-alcoholic fatty liver disease. These changes in CL content were related to the decrease in complex I activity found in liver mitochondria from rats to which this disease was diet-induced, since

the activity of complex I was completely restored to the level of control livers by exogenously added CL. Moreover, the effect of exogenous CL was not reproduced by other phospholipids nor by peroxidized CL [148].

2.3.4. Parkinson's disease and oncogenic-associated cachexia

Intriguing evidence for a potential role of CL alterations in Parkinson's disease has been provided [149]. Mice lacking α -synuclein exhibited a 22% reduction in CL mass in the brain, a 25% reduction in the CL content in n-6 polyunsaturated fatty acids (including C18:2), and a 51% increase in the CL content in saturated fatty acids. A 23% reduction in PG (a CL precursor) was also detected, without any changes in the content of other brain phospholipids or mitochondrial density, suggesting that the CL biosynthesis pathway should have been selectively impaired.

Cachexia is a serious consequence of a carcinogenic process. Inflammation and disruption of metabolic and energy balance result in a decrease of body mass, mostly originating from a depletion of muscle tissue [150]. Using animal models of peritoneal carcinosis, a decreased efficiency of liver mitochondrial function was observed, concomitantly with an increase in energy wasting and with a lower n-6/n-3 PUFA ratio in the carcinosis group, as compared to the control group [94]. Interestingly, very large (12-fold) increase in ROS generation by hepatic mitochondria was observed in the same group [94]. Although this alteration was not investigated in other tissues (namely skeletal muscle), this investigation makes way for therapies aimed at improving mitochondrial efficiency to prevent cachexia derived from different pathologies, including cancer. Moreover, a recent review [151] provided clues suggesting that alterations in CL composition may be behind a decrease in mitochondrial efficiency in cancer cachexia.

2.3.5. Drug-induced mitochondrial toxicity

Cardiolipin has also been involved in the mechanisms of toxicity of several drugs. One particular example is Doxorubicin (DOX), an anti-cancer agent whose clinical usage is associated with a cumulative and dose-dependent cardiotoxicity, in which mitochondria appear to play an important role [152–154]. DOX inhibits the activity of several mitochondrial proteins in the heart [155–157], which result from a specific drug interaction with CL, excluding this phospholipid from the lipid matrix surrounding the protein complexes indirectly affected by DOX [158–160]. Recent data demonstrated that tacrine, an acetylcholinesterase inhibitor used in the treatment of Alzheimer's disease, promotes a significant disturbance in the mitochondrial phospholipid content in the brain of treated rats, including a decrease in CL and an increase in oxidized aminophospholipids [161].

On the other hand, a great diversity of mitochondrial-active molecules, such as the anti-inflammatory nimesulide [162], the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazine (FCCP) [163] and the precursor of vitamin K menadione (2-methyl-1,4-naphthoquinone or vitamin K3) [164] disturb lipid order and organization in mitochondrial membrane-mimicking models, which may contribute to the adverse mitochondrial effects exerted by those compounds. Non-lamellar CL-containing structures induced by FCCP and menadione were suggested to have a key role in the insertion of those molecules in IMM lipid milieu and influence lipid and protein topology, namely in CL-enriched regions such as the contact-sites, with predictable impact on mitochondrial functioning.

2.3.6. Mitochondrial cholesterol alterations and disease

Accumulation of cholesterol in mitochondria seems to be a key step in the progression of some pathologic conditions, including steatohepatitis, carcinogenesis and Alzheimer disease [165].

Interestingly, Alzheimer disease has been considered as a disorder of mitochondria-endoplasmic reticulum (ER) crosstalk, which occurs through mitochondria-associated ER membrane (MAM) [166]. This ER sub-domain, which is physically connected to the OMM, constitutes a crossroad between signal transduction and metabolic pathways, with impact on mitochondria function and, hence, on cell fate [167]. In recent years, this membrane compartment has deserved special attention in a lipidomic perspective, since it has been recognized as an intracellular lipid raft-like structure, enriched in cholesterol and sphingolipids organized in a liquid-ordered phase, thus promoting an increased thickness of the bilayer as compared to the surrounding ER membrane [166]. Such structural characteristics make MAM able to recruit certain proteins, excluding others. Among others, proteins whose transmembrane domains favor a “hydrophobic matching” with MAM bilayer will be preferentially selected to reside in that region. According to the “MAM hypothesis” for AD [166], the key catalytic component of the γ -secretase complex (presenilin), its substrate (APP, from amyloid- β precursor protein) and A β (the small peptide that results from the processing of APP) are enriched in MAM. On the other hand, Winkler and collaborators [168], carrying out a systematic study on the activity of γ -secretase reconstituted in membrane model systems differing in the thickness of the lipid bilayer showed that the generation of the long toxic A β with 42/43 amino acids is significantly higher in thinner membranes, probably as a consequence of changes in presenilin I conformation or tilting. Therefore, the authors proposed the regulation of the thickness of γ -secretase host membrane as a new potential target for AD therapy.

Cholesterol also regulates the association between mitochondrial and endoplasmic reticulum (ER) membranes in MAM [169], which means that disturbances in cellular cholesterol content alters the association between the two organelles, predictably with important consequences for the cell. In fact, a tighter membrane association (apparently favored by cholesterol depletion [169]) has been found to promote apoptosis resulting from Ca²⁺ mitochondrial overloading.

Another important aspect related to cholesterol regards the role of caveolin-1, an important component of caveolae, which are invaginations of the plasma membrane, rich in cholesterol and with important roles in membrane trafficking and cell signaling [170]. Caveolin-1, however, is also hosted in MAM and induces cholesterol efflux from the ER to the plasma membrane, reducing MAM cholesterol content and its entry into mitochondria [89]. Thus, caveolin-1 deficiency causes free cholesterol accumulation in mitochondrial membranes, increasing membrane order, decreasing electron flux through the respiratory chain and increasing ROS generation and apoptotic signaling [89,171], which can be related to the progression of several diseases. The consequences of caveolin-1 depletion in cancer were previously described in Section 2.2.

2.4. Mitochondrial membrane lipid composition changes in aging

The free radical theory of aging [172] stated that normal aging is the result of the oxidative damage to tissues mediated by free radicals produced as a byproduct of oxidative metabolism. Subsequently, mitochondria have been seen as the main source and target of free radicals in the context of aging [173]. Progressive loss of mitochondrial function has been associated with aging and several experimental evidence have supported this interdependence [174], including: (i) the occurrence of mitochondrial DNA (mtDNA) deletions, punctual mutations or depletion; (ii) age-dependent decline in the activity of mitochondrial electron transport complexes; (iii) increase in the production of oxygen free radicals; (iv) changes in the morphology of mitochondria and (v) decrease or collapse of

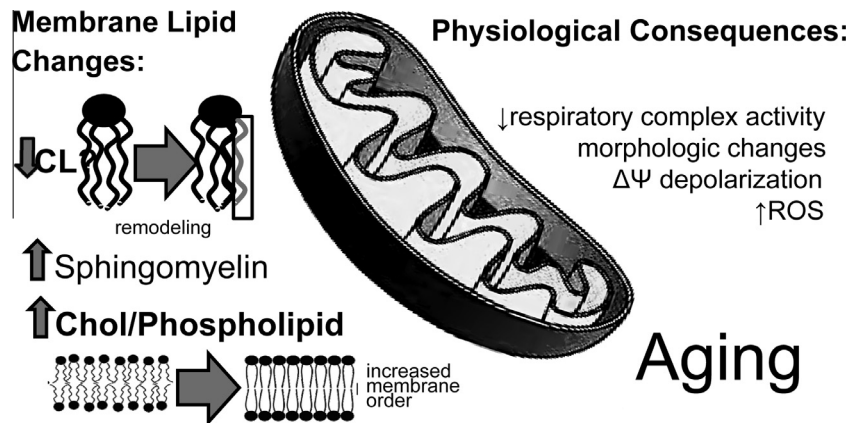


Fig. 3. Aging-promoted changes in mitochondrial membrane lipid composition. Besides morphological and physiological alterations, aging has been reported as holding consequences in mitochondrial membrane lipid content. A decrease in CL content and alterations in CL remodeling have been proposed to occur with aging, although some controversy still remains in this subject. An increase in sphingomyelin content and in the cholesterol (Chol) to phospholipid ratio was also suggested, which should be correlated with the reported increase in membrane order with aging. Overall, membrane alterations can have bioenergetic costs, with a decrease in mitochondrial activity and ATP production.

transmembrane $\Delta\Psi$. Loss of mitochondrial apoptosis regulation has also been reported in aged cells [175].

The membrane theory for aging conceives life span as being inversely related to the content in unsaturated membrane phospholipids [176]. A positive impact of calorie restriction has been suggested in this context, leading to a decrease in mitochondrial ROS production in multiple tissues [177] and extending mean and maximum lifespan in a variety of species [178]. Since lipid peroxidation is closely related to ROS-induced toxicity, the beneficial effect of calorie restriction on the aging process is attributed to a decrease in membrane fatty acid unsaturation, thus diminishing lipid peroxidation [179]. Concordantly, calorie restriction has been reported to decrease the content in PUFAs in membranes from a number of tissues [180–182].

Changes in mitochondrial membrane fatty acid composition can also influence oxidative stress by promoting changes in membrane-mediated processes such as proton leakage across the IMM [183]. Other aging-induced alterations, such as decreased membrane fluidity resulting from lipid peroxidation, as well as decreased CL content and altered activity of the respiratory chain have been reported (Fig. 3). Due to the influence of CL in the activity of several anionic carrier systems [184–186] and some of the electron transport complexes [187–191], including their supramolecular arrangements [192,193], alterations in CL content may account for the reported age-dependent decline in mitochondrial performance [183]. In fact, this has been supported by data obtained in rat heart [194,195], brain [196] and liver mitochondria [197], as well as in human epidermal cells [198]. Paradies and co-workers reported a consistent age-dependent loss of cardiac mitochondrial CL, correlated with a decreased activity of a number of proteins including the mitochondrial phosphate transporter [199], cytochrome c oxidase [200], adenine nucleotide transporter [200], carnitine-acylcarnitine translocase [201] and the pyruvate carrier [202]. It was demonstrated that acetyl-L-carnitine restored the function of all those CL-dependent proteins by restoring the levels of CL, although the molecular mechanism underlying this effect had not been clarified. The authors hypothesized an eventual influence of acetyl-L-carnitine in the activity of CL synthase [202]. However, a selective reduction of CL content with aging is still controversial, since other studies reported no alteration [203] or even an increase [204] in CL content in rat heart mitochondria, although in the former the alteration was detected only for inter-fibrillar mitochondria in Fischer 344 rats. CL remodeling is likely

to be affected during the aging process. Several studies suggest that CL remodeling may be altered during aging, including an increase in the content of highly unsaturated fatty acids, namely arachidonic and docosahexaenoic acids, and a decrease in linoleic acid content in aged rat heart mitochondria [205], whereas another study showed an increase in CL long chain saturated fatty acids (arachidic and lignoceric acids) [204]. Similar changes were not observed in other phospholipid classes, namely in PE [204–206], or plasma unesterified fatty acids [205], suggesting the specificity of the alterations towards CL.

With regard to changes in the amount of other membrane lipids, a decrease in total phospholipid with a parallel increase in cholesterol, resulting in a progressive aging-dependent increase in the molar ratio of cholesterol/phospholipid, have been reported in hepatic mitochondria from aged animals [207]. Paradies et al. also reported an increase in cholesterol (43%) and a decrease in the phospholipid content (15%) in heart mitochondria, with CL being the principal responsible for phospholipid loss (28% decrease in aged rats) [195]. Another study reported higher cholesterol content in heart mitochondria, along with an increased percentage of sphingomyelin [204].

Decreased mitochondrial membrane fluidity, as previously mentioned, also seems to be a characteristic of aging. Vorbeck and colleagues [207] reported a significant aging-dependent increase in the lipid structural order as measured by steady-state fluorescence polarization using the probe 1,6-diphenyl-1,3,5-hexatriene, which might be correlated with the increased cholesterol/phospholipid molar ratio discussed before. A similar effect was reported for brain mitochondria [208].

Altogether, the abovementioned data suggest that the aging process is associated with a progressive remodeling of mitochondrial membranes, which impacts the capacity of mitochondria to generate ATP and to control apoptotic signaling (Fig. 3). One of the key players appears to be CL, which has a relevant and critical role in both aspects. Therefore, it is expectable that strategies able to delay the loss of CL or avoid CL detrimental alterations should also delay the loss of mitochondrial capacity and ROS generation, possibly preventing in this manner some of the cellular phenotypes associated with aging. An interesting open question in this regard is whether the possible CL alterations occurring during aging, often resulting in mitochondrial dysfunction, can be reversed by diet or exercise. And, in this case, it would be challenging to identify the mechanisms involved, in order to improve potential strategies to counteract the aging process.

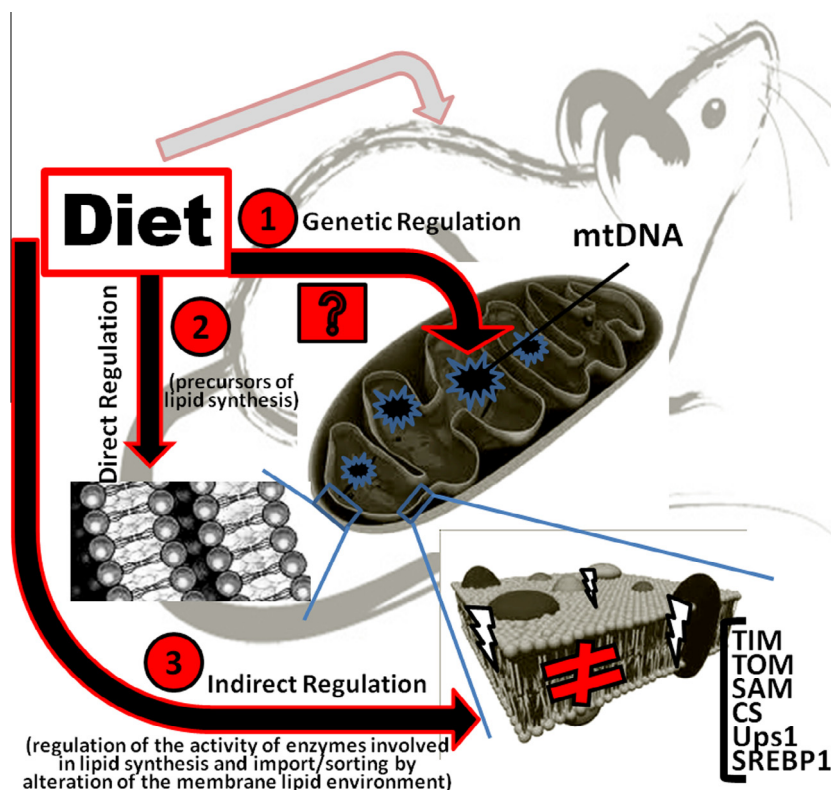


Fig. 4. Proposed pathways for diet-induced alterations in mitochondrial membrane lipid composition. External factors, such as diet, can create an environmental stress that impacts the physiologic genetic control (with changes in the expression/genetic regulation of mitochondrial proteins), possibly resulting in an imbalance in the expression of proteins involved in lipid synthesis or remodeling. The mechanisms involved in the regulation of mitochondrial lipid content through the interaction of external stressors with genetics/protein expression are currently not known. An alternative mechanism for external dietary interventions to alter mitochondrial membrane lipid composition is based on a switch in the availability of precursors for lipid synthesis, which can be provided by the diet or further metabolic processing. Another model is based on the indirect regulation of lipid composition through the modulation of the activity of key-enzymes of lipid metabolism by the membrane lipid environment, which might be modified (\neq) by the diet in terms of lipid composition or in terms of biophysical membrane properties. Some proteins that can undergo this type of modulation include TIM (translocase of the inner mitochondrial membrane), TOM (translocase of the outer mitochondrial membrane), SAM (sorting and assembly machinery), CS (cardiolipin synthase), Ups1 and SREBP1 (sterol regulatory element binding protein 1).

3. Mitochondrial membrane lipids as a target for diet interventions

As previously mentioned, mitochondrial lipid composition, although obeying a characteristic pattern, displays a highly dynamic behavior, depending on the physiological or pathological conditions. This plasticity in lipid composition, sensible to external modulation may substantiate the emergence of therapeutic applications. In fact, by modulating the lipid composition of mitochondrial membranes and, hence, interfering with mitochondrial function, it is predictably possible to regulate and improve mitochondrial function in the context of injury and disease (Fig. 4). In this context, the investigation of the mitochondrial alterations induced by diets with different fat composition can be instrumental to influence cell fate. Studies establishing a correlation between diet, mitochondria membrane lipid composition and mitochondria/cell physiology will open new trends to identify the etiology of diseases and to develop novel therapeutic strategies. One possibility is to establish dietary interventions that counteract or prevent alterations of membrane lipid properties associated to the occurrence of several diseases. In fact, dietary benefits have been revealed for some diseases, namely cardiovascular. Increasing evidence regarding a correlation between the prevalence of cardiovascular diseases in mammals and the type of lipids included in the diet has been found [209–211]. Benefits provided by tuna fish and sunflower seed oils were observed in cases of reduced ventricular fibrillation in occlusion, ventricular tachycardia and risk of

sudden cardiac death [212]. Fish oil also proved to be advantageous against atherogenesis, mortality from coronary heart disease, intimal hyperplasia and atherosclerosis [213–217]. Preventive effects and cardioprotective properties have also been reported for rapeseed oil (optimized oils) [218], including reduction of mortality rate and infarct size [219]. Thus, this oil has been suggested to be used as a functional supplement, aiding in stroke prevention and protection. Dietary benefits towards some kinds of cancer have also been reported, with an inverse correlation between fish oil consumption and the occurrence of male and female colorectal cancer [220] and breast carcinoma [220,221]. Advantages of a dietary control have also been proposed for type 2 diabetes [222]. Some mitochondrial fatty acid oxidation disorders may be targeted by dietary intervention, as well [223].

The importance of diet as a valid therapeutic approach is illustrated by the manageable modulation of the acyl chain unsaturation in CLs. In fact, CL was suggested to be more susceptible to undergo acyl chain changes upon dietary manipulation as compared to other phospholipid classes, such as PC and PE [224]. On the other hand, some studies revealed that tetraoleoyl-CL did not undergo oxidation when cyt *c*/tetraoleoyl-CL complexes were incubated in the presence of H_2O_2 [51]. In this context, the enrichment of mitochondrial membranes with non-oxidizable molecular species of CL was proposed as a potential mechanism for protecting cells against apoptosis [51]. Conversely, an increase in PUFAs, resulting in increased generation of highly oxidizable CL species, would make the targeted cells more susceptible to oxidative modification and, ultimately, to

apoptotic cell death. This approach may represent an interesting perspective when attempting to develop antitumor strategies to overcome the increased resistance of tumor cells to pro-apoptotic drugs [51].

We have also demonstrated recently that rats fed a rapeseed oil-rich diet for 11, 22 and 33 days undergo alterations of mitochondrial membrane lipid composition and properties, as well as a decrease in state 3 respiration [20]. This study demonstrated that the modified diet resulted in a higher incorporation of oleic acid in CL species [20], as well as in a decrease in hydroperoxide production by the respiratory chain [20]. One particular learning from these experiments was that lipid content of mitochondrial membranes was altered after only 11 days of modified, rapeseed oil-based diet, suggesting that diet can have an immediate effect on the composition (and properties) of mitochondrial membranes, modulating the bioenergetics of that organelle.

In another approach, recent data demonstrated that the dietary content in fatty acids modulated the type of CL species found in the liver of rats with non-alcoholic fatty liver disease [225]. Interestingly, different dietary fatty acid patterns (lard-rich vs. fish oil-rich diet) showed to modulate differently CL synthase gene expression and CL remodeling. Concomitantly, the activity of mitochondrial proteins was differently affected. While ATP synthase activity showed a positive correlation with increased CL content, the opposite was observed for complex II + III activity [225]. The differential effects of dietary fatty acid patterns on CL species impact mitochondrial capacity, which, in its turn, may interfere with beta-oxidation of fatty acids, thus resulting in progression of the disease.

Taking all previous data into account, it seems fair to emphasize the relevance that dietary approaches may hold in a therapeutic context. The synergistic effects of diet and pharmacological drugs, namely mitochondria-active compounds, could also represent a valid path to explore.

Some evidence have been accumulated underlining the influence of dietary lipids in the action of certain membrane-active drugs (e.g., anesthetics and alcohols) [226]. A correlation between diet-induced changes in membrane lipid composition and the pharmacological effects of volatile anesthetics, such as halothane, isoflurane, methoxyflurane and cyclopropane, has been established [227]. The authors demonstrated that the increased anesthetic action in rats fed a fat-free diet was specifically reversed by linoleic acid supplementation. The action of the local anesthetic tetracaine was also proposed to be dependent on lipid membrane composition [228]. Curcumin, a natural spice used for generations in traditional medicines, was proposed to indirectly regulate the action of membrane proteins by changing the physical properties of the membrane rather than acting by direct binding to proteins [229]. Similarly, many other molecules, including drugs and environmental contaminants, particularly those whose lipophilicity implies a strong incorporation into the lipid bilayer, have shown a biological activity mediated or highly influenced by direct interactions with membrane lipids [230–239]. Therefore, it is fair to assume that biological effects of such compounds should be modulated by an appropriate diet.

Focusing specifically on mitochondria, it was reported that the ATPase of heart mitochondria from animals fed diets with a high content in long chain-fatty acids exhibited increased oligomycin sensitivity and decreased 2,4-dinitrophenol-induced stimulation [240]. The authors suggested that *in vivo* changes in the thickness of the lipid bilayer might alter mitochondrial ATPase activity.

Studies carried out in our laboratory with a great diversity of compounds, including drugs [233,234], insecticides [241], herbicides [242] and other synthetic compounds [243,244], which exhibit high toxicity towards mitochondrial functioning, have suggested that mitochondriotoxicity is often mediated by effects exerted by the compounds on mitochondrial membrane lipids.

Thus, data gathered from biophysical studies using different technical approaches and a variety of membrane model systems showed that membrane physical properties, such as fluidity, lateral heterogeneity and curvature stress, were affected by those compounds in such a manner that adverse repercussions would be expected in mitochondrial functioning [233,234,237,243–247].

4. Open perspectives and words of caution

Therapies addressed to mitochondrial lipids may be successfully used in the context of mitochondrial disease and dysfunction, due to the strict association of lipids such as CL with mitochondria membrane proteins and their involvement in mitochondrial function. Recent evidence suggests membrane-lipid therapies as potentially useful by themselves or in combination with other therapies, to treat common pathologies, such as cancer, cardiovascular diseases, neurodegenerative processes, obesity, metabolic disorders, inflammation, and infectious and autoimmune diseases, but only future studies will enable determining the full potential of this novel therapeutic approach [248]. Most drugs used with pharmacological purposes have been designed to target specific membrane proteins, at relatively low concentrations. However, some of these drugs hold side pharmacological effects at higher concentrations [249]. These less specific effects towards membrane-associated proteins may arise from the interaction of lipophilic drugs with the lipid bilayer, causing changes in their structural and elastic properties, and resulting in alterations of protein distribution in the plane of the membrane and/or in alterations of protein functional conformation [249–252]. The intricate interplay between lipids and membrane protein structure and function makes it reasonable to infer that lipid bilayers may influence drug action towards membrane protein targets also at low concentrations. The disruption of membrane lipid rafts and alterations of protein distribution between raft and non-raft domains have been proposed as the means by which drugs may influence membrane protein activity with a relative specificity [253].

Considering all data mentioned above, the use of specific diets to modulate drug membrane-activity should seriously be taken into account in situations of either managing membrane-localized toxicity or potentiating treatment in a disease context. Diet manipulation may then represent a way to reduce the toxic outcomes brought by the use of some drugs or to assist their pharmacological action. Further work is necessary in order to fully appraise the potential of dietary interventions in improving the chemotherapeutic activity of pharmacological agents. In this context, membrane lipid-mediated therapeutic strategies have been proposed [248], in which the molecular targets are the lipids and the structures they form (membrane-lipid therapy). The final goal of this approach is to develop drugs able to control the activity of membrane-associated proteins by regulating membrane lipid composition and/or structure.

Finally, it is clear that CL has a very special role in the modulation of mitochondrial function at health and disease conditions, determining also the triggering of the mitochondrial pathway for apoptosis. Since it has been demonstrated that CL synthase has the unexpected role of modulating the physiology of mitochondrial membranes, increasing their resistance to metabolic stress [142], one can aim at targeting this enzyme to improve a mitochondrial disease-related phenotype. How this can be achieved or which pathologies can be targeted by this approach are questions that require further investigation. Finally, a word of caution: despite many mechanisms have been proposed implicating CL content alterations in different pathologies, this may also result from variations in the overall mitochondrial mass. This is often an overlooked aspect in many studies, in which proper internal standards (e.g., CL content/citrate synthase activity) should be used.

Nevertheless, there is strong and clear evidence that in Barth syndrome, among other conditions, disruption of CL metabolism results in a decrease of CL mass without loss of mitochondrial content [254,110]. Other works, however, overlooked the determination of CL content/mitochondrial units [136,255]. Therefore, caution should be taken prior to infer that different conditions result in a decrease in CL content without ensuring that mitochondrial copy number in cells remained constant.

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