Biochimica et Biophysica Acta 1838 (2014) 1657–1679

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Lipid Replacement Therapy: A natural medicine approach to replacing damaged lipids in cellular membranes and organelles and restoring function☆,☆☆

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ARTICLE INFO

Article history:
Received 18 August 2013
Received in revised form 30 October 2013
Accepted 9 November 2013
Available online 21 November 2013

Keywords:
Membrane phospholipids
Mitochondrial function
Inflammasome
Fatigue
Degenerative illnesses
Fatigue
Lipid oxidation

ABSTRACT

Lipid Replacement Therapy, the use of functional oral supplements containing cell membrane phospholipids and antioxidants, has been used to replace damaged, usually oxidized, membrane glycerophospholipids that accumulate during aging and in various clinical conditions in order to restore cellular function. This approach differs from other dietary and intravenous phospholipid interventions in the composition of phospholipids and their defense against oxidation during storage, ingestion, digestion and uptake as well as the use of protective molecules that noncovalently complex with phospholipid micelles and prevent their enzymatic and bile disruption. Once the phospholipids have been taken in by transport processes, they are protected by several natural mechanisms involving lipid receptors, transport and carrier molecules and circulating cells and lipoproteins until their delivery to tissues and cells where they can again be transferred to intracellular membranes by specific and nonspecific transport systems. Once delivered to membrane sites, they naturally replace and stimulate removal of damaged membrane lipids. Various chronic clinical conditions are characterized by membrane damage, mainly oxidative but also enzymatic, resulting in loss of cellular function. This is readily apparent in mitochondrial inner membranes where oxidative damage to phospholipids like cardiolipin and other molecules results in loss of transmembrane potential, electron transport function and generation of high-energy molecules. Recent clinical trials have shown the benefits of Lipid Replacement Therapy in restoring mitochondrial function and reducing fatigue in aged subjects and patients with a variety of clinical diagnoses that are characterized by loss of mitochondrial function and include fatigue as a major symptom. This Article is Part of a Special Issue Entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

Contents

Abbreviations: ABR, auditory brainstem responses; AD, Alzheimers disease; ADP, adenosinediphosphate; AGEs, advanced glycation end products; ATP, adenosinetriphosphate; CAPD, chronic ambulatory peritoneal dialysis; CDP-DAG, cytidinediphosphate-diacylglycerol; CFS, chronic fatigue syndrome; CL, cardiolipin; CR, caloric restriction; CVD, cardiovascular disease; DAG, diacylglycerol; DAMPs, damage associated molecular patterns; DHA, docosahexaenoic acid; eNOS, endothelial nitric oxide synthase; EPA, eicosapentaenoic acid; EPL, essential phospholipids; ETC, electron transport chain; FA, fatty acid; FDA, US Federal Drug Administration; F-MMM, Fluid—Mosaic Membrane Model; GRAS, generally recognized as safe; HDL, high density lipoproteins; HNE, 4-hydroxynonenal; IL, interleukin; LDL, low density lipoproteins; LRT, lipid replacement therapy; MAM, mitochondria-associated membrane; MAPK, mitogen activated protein kinase; MDA, malondialdehyde; ME, myalgic encephalomyelitis; MetSyn, metabolic syndrome; MIM, mitochondrial inner membrane; MOMP, mitochondrial outer membrane permeabilisation; MPTP, mitochondrial permeability transition pores; mRNA, messenger RNA; mtDNA, mitochondrial DNA; NAFLD, non alchoholic fatty liver disease; NASH, Nonalcoholic Steatohepatitis; NCD, non communicable diseases; NF-κB, nuclear factor kappa B; NLRP3, nucleotide-binding oligomerization domain (NOD)-like receptor protein 3; PAMPs, pathogen-associated molecular patterns; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; RC, respiratory chain; RNS, reactive nitrogen species; ROS, reactive oxygen species; TCA, tricarboxcylic cycle; TLR, toll like receptor; TNFα, tumor necrosis factor alpha; tRNA, transfer RNA; UCP, uncoupling protein

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http://dx.doi.org/10.1016/j.bbamem.2013.11.010

1. General introduction

The use of dietary membrane lipids and oral and intravenous lipid supplements to modify cellular and intracellular membranes in order to improve health or treat specific medical conditions has a rich history [1,2]. Membrane lipids are known to be essential to cellular membrane function and cell viability [3,4], and thus their modification and restoration by exogenous membrane lipids remains a useful approach for maintaining and restoring cellular membrane function [5,6]. Cell membranes control a variety of cellular processes, including whether cells live or die as well as the maintenance of a structural and ionic barriers, intercellular communication networks, transport, secretion, recognition, adhesion and other important cell functions [4,7–9]

Membrane lipids provide at least four major requirements for cellular health [9,10]. They are used as: (*i*) an important energy storage reservoir; (*ii*) the matrix for all cellular membranes, enabling separation of enzymatic and chemical reactions into discrete cellular compartments; (*iii*) bioactive molecules in certain signal transduction and molecular recognition pathways; and (*iv*) important functional molecules that undergo interactions with other cellular constituents, such as proteins and glycoproteins. This latter characteristic is an absolute requirement for the formation, structure and activities of biological membranes [3,4,7–9].

2. Introduction to membrane lipids

The most common membrane lipids are the glycerophospholipids [9,10]. These are essential for membrane structure and are found in the membranes of all lower and higher living species, but other phospholipid forms, such as the substitution of sphingosin for glycerol (sphingomyelins or ceramide-1-phosphorylcholines), are also commonly found in cell membranes, mainly on their exterior surfaces [9,10]. Another common membrane constituent is cholesterol, the only sterol found in abundance in membranes [4,6,9,10]. In addition, there are also acylglyerols, fatty acids (FAs) and many other minor lipid constituents of cellular membranes of largely unknown function [9,10].

The membrane glycerophospholipids have attached FA chains that are ester-linked to the glycerol group. The nature and saturation of the attached FA chains of the phospholipids generate dramatic effects on membrane packing and fluidity [10–12]. Unsaturated FAs, such as oleic acid and linoleic acid, confer a high degree of conformational flexibility of the unsaturated hydrocarbon chains within membranes due to their occupying a slightly wedge-shaped space. This generally results in looser packing and a more fluid membrane [3,11,13]. In contrast, saturated FA, such as stearic acid and palmitic acid, confer rigidity that results in a less fluid or more organized membrane [12].

There are lipid compositional differences between different membranes of the cell [9,10,12,14]. The concentrations of sterols (cholesterol and cholesterol esters) and sphingolipids (sphingomyelin, ceramide and gangliosides) increase from the endoplasmic reticulum to the cell surface [9,10,14]. For example, cholesterol/phospholipid ratios increase from 0.1 in the endoplasmic reticulum membranes to 1.0 in the plasma membrane [9]. In addition, sphingolipids such as gangliosides are quite asymmetrically distributed on the outer surface leaflets of cell membranes [15]. Similarly, other neutral lipids, such as phosphatidylcholine (PC), reside preferentially on the outer leaflet or surface of the cell membrane, whereas anionic phospholipids, such as phosphatidylserine (PS) and phosphatidylinositol (PI) tend to reside on the inner leaflet of the cell membrane. The asymmetric distributions of lipids between inner and outer membrane leaflets as well as in the plane of the membrane are important in determining key membrane physical properties (deformation, curvature, compression, expansion) and functional interactions within membranes [11,15–17,20–22].

There are also important differences in the lateral organization of lipids in membranes [18,20,21]. Lipid cooperative behavior ensures that lipids organize laterally in the plane of the membrane in a non-random, non-uniform fashion [18,20,21].

The matrix of cellular membranes is largely formed by glycerophospholipids, especially PC and phosphatidylethanolamine (PE), the most abundant phospholipids along with sphingomyelins in cell membranes [10,12,14,16,17]. Under physiological conditions membrane phospholipids are present in various fluid, semi-solid and solid phases that are organized into domains characterized by different lipid spatial arrangements and rates of rotational and lateral movements [9,10,18,20,21]. The different lipid phases (domains) in membranes have profound consequences for membrane properties, organization and activities [14–22].

3. Cell membrane structure and membrane models

The most important observation on membranes over the last 100 years was that of Gorter and Grendel, who proposed that membrane lipids must be present in a bilayer configuration [23]. Indeed, an asymmetric lipid bilayer forms the matrix of all biological membranes [4,9,11,15–17,19–24]. This hypothesis was used by Danielli and Davson [25] and later by Robertson [26,27] as the basis for tri-layer models of membrane structure. The tri-layer models, such as the Unit Membrane Model [28], possessed unfolded membrane proteins bound to the head groups of phospholipids on each side of the lipid bilayer by electrostatic and other forces [26,27].

The current accepted model for cellular membranes, at least at the sub-micrometer scale, is the Fluid-Mosaic Membrane Model (F-MMM) [29]. At the time the F-MMM was introduced, the accepted model for cellular membrane structure was still the tri-layer membrane model

with most proteins present in extended forms (beta configurations) bound to the lipid bilayer by electrostatic and other forces [26,27]. However, this model could not explain various data on membrane structure and did not take into account the ability of many components in membranes, especially most phospholipids, to rapidly diffuse in the membrane plane [29]. As first proposed, the F-MMM depicted biological membranes as a matrix made up of a fluid bilayer of phospholipids with mobile globular integral membrane proteins and glycoproteins intercalated into the bilayer [29]. It did not take into account specialized lipid domains or regions of low lipid lateral mobility, data that was largely unavailable at the time the model was proposed.

Over decades of membrane research overwhelming support has accumulated for the F-MMM, but with some specific modifications [30,31]. The proposal that intrinsic or integral membrane proteins are globular structures inserted into a fluid (fluid-disordered) or a fluid-ordered (fluid-crystalline) lipid matrix, has remained unchanged [29,30]. However, the revised F-MMM presents membranes more as heterogeneous, with domains of fluid and structured lipids and integral membrane proteins, peripheral membrane proteins and membrane-associated complexes (cytoskeletal and extracellular matrix complexes) above and below the membrane [30,31].

4. Phospholipids and their fatty acid chains

As mentioned in Section 2 the major structural lipids in eukaryotic cell membranes are the glycerophospholipids, such as PC, PE, PS, PI, and phosphatidylglycerol (PG). These glycerophospholipids contain hydrophobic diacylglycerol (DAG) tails that constitute the main hydrophobic matrix of biomembranes [3,9–11]. The DAG tails of glycerophospholipids contain saturated or *cis*-unsaturated fatty acyl chains of varying lengths. In mammalian cell membranes most PC molecules have at least one *cis*-unsaturated fatty acyl chain, which renders them fluid at room temperatures, although some membrane regions may not be in a fluid state [9–11,18–20]. PC usually accounts for greater than 50% of the phospholipids in eukaryotic cellular membranes, but there are also significant percentages of PE, PI, PS and PG [9,10,12]. In addition, the sphingolipids with their hydrophobic ceramide backbones constitute another major class of membrane lipids, and this class of lipids is mainly found on the exteriors of cell membranes where some of these molecules display oligosaccharide chains [9,10].

Due to their different polar head groups the glycerophospholipids occupy different geometric spaces in the membrane plane. For example, since PE has a relatively small polar headgroup size, it assumes a more conical geometry compared to PC. Addition of PE to PC bilayers imposes lateral curvature stress [18,21]. This is important in some forms that membranes take, such as membrane budding, fission and fusion. Thus changes in local membrane composition can determine important properties, such as membrane blebbing and budding.

Although lipid head groups are important in membrane protein–lipid interactions, another major interaction is through hydrophobic structural matching, for example in glycerophospholipids mediated mainly through protein-DAG acyl chain interactions [32]. Such hydrophobic matching can be disrupted by oxidative modification of the DAG acyl chains. For example, enzyme activation can occur when acyl chains are disordered by oxidization [11,33]. This is thought to change acyl chain packing and disrupt hydrophobic interactions. In general, hydrophobic structural matching is facilitated by the appropriate conformational states of the lipid molecules or by selection of appropriate lipid species that provide the best hydrophobic structural match [21,22,34].

FAs occur in mammalian cells in a variety of chain lengths and unsaturation states (some FAs found in mammalian membranes are listed in Table 1). Common FAs in dietary replacement studies are: oleic acid (9-octadecenoic acid; 18:1Δ9 or 18:1[n-9]), linoleic acid (9,12-octadecadienoic acid; 18:2Δ9,12 or 18:2[n-6]), α-linolenic acid (9,12,15-octadecatrienoic acid;18:3Δ9,12,15 or 18:3[n-3]), and arachidonic acid (5,8,11,14-eicosatetraenoic acid; 20:4Δ5,8,11,14 or 20:4[n-6]) [35]. This latter FA is a precursor for prostaglandins. Mammalian cells are unable to synthesize FAs with double bonds at specific positions, such as the Δ9 position, and therefore certain unsaturated FAs, for example linoleic and linolenic acids, are essential dietary FAs, especially for the synthesis of arachidonic and docosahexaenoic (DHA, 22:6Δ4,7,10,13,16,19 or 22:6[n-3]) acids. The FA *cis*-double bonds have dramatic effects on lowering the melting points of phospholipids and increasing their motional properties [36]. This can lead to lipid lateral phase separation, domain formation and differences in membrane fluidity [35].

Glycerophospholipids are synthesized mainly in the endoplasmic reticulum in four steps: (*i*) synthesis of the backbone glycerol-3-phosphate molecule, (*ii*) using FA acyl coenzyme A (CoA) attachment of FAs to this backbone to produce phosphatidic acids, (*iii*) dephosphorylation to 1,2-DAG, and (*iv*) addition of a hydrophilic head group, such as phosphocholine to make PC, phosphoserine to make PS, phosphoethanolamine to make PE, or phosphoglycerol to make PG. PI is formed directly from phosphatidic groups by addition of inositol. In addition, some glycerophospholipids are made by alterations of existing molecules, such as methylation of the ethanolamine group to form choline, or exchange of head groups. The assembly of the various glycerophospholipids can also take place in the inner mitochondrial membrane [37,38].

5. Mitochondrial structure and function

Subsumed and adapted by eukaryotic cells between 1 and 3 billion years ago [39], the sacrificial and symbiotic alpha-proteobacterium that forms the genetic basis of mammalian mitochondria has a dual membrane reminiscent of those present in bacteria [40,41]. This intricate dynamic membrane system, with a peculiar lipid composition,

Table 1
Fatty acid nomenclature for some common mammalian unsaturated FA*.

Systematic name	Common name	Abbreviations
9-hexadecenoic acid	Palmitoleic acid	16:1A9
9-octadecenoic acid	Oleic acid	18:1A9 or 18:1 (n-9)
9-eicosenoic acid	Gadoleic acid	20: 1A9 or 20:1
9,12-octadecadienoic acid	Linoleic acid	18:2A9,12 or 18:2 (n-6)
9,12,15-octadecatrienoic acid	a-Linolenic acid	18:3A9,12,15 or 18:3 (n-3)
6,9,12-octadecatrienoic acid	y-Linolenic acid	18:3A6,9,12 or 18:3 (n-6)
8,11,14-eicosatrienoic acid	Dihomo-y-linolenic acid	20:3A8,11,14 or 20:3 (n-6)
5,8,11-eicosatrienoic acid	Mead acid	20:3A5,8,11 or 20:3 (n-9)
5,8,11,14-eicosatetraenoic acid	Arachidonic acid	20:4A5,8,11,14 or 20:4 (n-6)
4,7,10,13,16-docosapentaenoic acid	Docosapentaenoic acid	22:5A4,7,10,13,16 or 22:5 (n-6)
7,10,13,16-docosatetraenoic acid	Adrenic acid	22:4A7,10,13,16 or 22:4 (n-6)
7,10,13,16,19-docosapentaenoic acid	Clupanodonic acid	22:5A7,10,13,16,19 or 22:5 (n-3)

* Modified from Stubbs and Smith [35].

displays transverse as well as lateral asymmetry with some lipids being synthesized inside mitochondria, while others are imported or acquired in the form of precursors [42,43].

The membranes of mitochondria form a distinct dual framework which forms an intermembrane space and matrix compartment. The matrix contains a complex mixture of enzymes important for the synthesis of ATP molecules, in addition special mitochondrial ribosomes, tRNAs, mRNAs and the maternally dominant mitochondrial DNA (mtDNA) [44,45].

The outer membrane of the mitochondrion is a relatively simple membrane containing a phospholipid bilayer and protein structures called porins which render it permeable to molecules of about 10 k Daltons or less (ions, nutrient molecules, ATP, ADP, and other relatively small molecules) and other membrane components. The inner mitochondrial membrane (MIM) is a highly complex structure freely permeable only to oxygen, carbon dioxide, and water [46–49]. Embedded in the MIM are the four respiratory chain (RC) complexes, ATP-synthase (complex V), ubiquinone, carnitine-palmitoyl-transferase II, which make up the electron transport chain (ETC), and carriers for anions, cations and redox equivalents.

Mitochondria use oxidative phosphorylation via the tricarboxcylic (TCA) cycle and the ETC to produce energy. ETC oxidative phosphorylation accounts for about 90% of cellular oxygen consumption and provides more than 80% of the cellular energy demands [50].

In addition to cellular energy production, mitochondria provide other critical functions in the cell, including the modulation of calcium signaling, regulation of cell death, the maintenance of cellular redox balance, innate immune signaling [51] and the housing of important biosynthetic pathways, especially for certain lipids [52]. Therefore, it is reasonable to claim that mitochondria function as gatekeepers of cell life and cell death [53].

Mitochondrial membrane phospholipids are comprised of mainly PE and PC. Uniquely they also contain the tetra-acyl phospholipid cardiolipin (CL). CL constitutes some 15–20% of the mass of total mitochondrial phospholipid [54]. Both PE and CL are non–bilayer-forming phospholipids, a feature best explained by their conical shapes. This shape allows the formation of hexagonal phases that can be observed for isolated lipids, depending on the pH and ionic strength [55]. PE is an abundant phospholipid present in all cellular membranes and essential for cell survival, whereas CL is exclusively found in MIM where it is required for oxidative phosphorylation, ATP synthesis, and mitochondrial bioenergetics. CL is essential for MIM structure and function as well as maintaining MIM transmembrane potential [56].

CL is biosynthesized from PG and cytidinediphosphate-diacylglycerol (CDP-DAG) by the enzyme CL synthase on the inner face of the MIM. CL is highly sensitive to damage of its double bonds by oxidative mechanisms due to its rich content of linoleic acid (with the exception of brain) and being located adjacent to the site of reactive oxygen species (ROS) production in the MIM, which places it at greater risk of oxidative insult. CL is of significant functional importance due to its unique ability to interact with respiratory chain proteins and its role in maintaining MIM fluidity and osmotic stability [57].

Perhaps the most important property of CL is its central role in supporting the activity and organization of the mitochondrial respiratory chain. It binds to the cytochrome bc1 complex (complex III) and cytochrome c oxidase (complex IV), complexes that form high molecular weight super-complexes that in effect supersize the mitochondrial respiratory chain, and in doing so develop a mechanism that allows for greater nutrient availability to ensure mitochondrial electron chain function remains viable even in periods of nutrient depletion and stress [58,59].

Mitochondria are also able to respond quickly to changes in transmembrane potential as the penalty for failure to adapt to changes in MIM potential promoting mitochondrial collapse (mitophagy) and associated cellular autophagy. The electron and proton transfers of chemiosmotic energy coupling generate a remarkable transmembrane potential of 150–200 mV across the MIM yielding an equivalent field strength of about 30 million volt/m, matching that discharged by a bolt of lightning [60]. Failure to maintain the mitochondrial inner membrane potential results in the collapse of available cellular energy, blocking active transport across the cell membrane, and increasing free-radical leakage.

Various stress conditions, including increased metabolic rates, hypoxia, or membrane damage all markedly induce mitochondrial ROS production [61]. ROS, as well as a range of 'danger signals,' including pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, peptidoglycans, bacterial nucleic acids and sterile, host derived, damage-induced molecules called damage associated molecular patterns (DAMPs) are released in the face of cellular and mitochondrial damage. These include: extracellular ATP, K^+ efflux and uric-acid crystal's [62], which induce the assembly of intracellular multiprotein inflammatory complexes called the inflammasome [63].

Inflammasomes are key intracelleular signaling platforms that detect pathogenic microorganisms and sterile stressors, as well as some environmental triggers, such as crystalline silica, alum and asbestos [64,65]. Of the known inflammasomes the best characterized, and one that is perceived to sense sterile injury is formed by a pattern recognition receptor called NOD-like receptor pyrin domain containing three (NLRP3). The nucleotide-binding oligomerization domain-like receptor (NLR) proteins are a group of multimeric protein complexes that consist of an inflammasome sensor molecule, the adaptor protein ASC and caspase 1. Once these protein complexes have formed, the inflammasomes activate caspase 1, which proteolytically activates the pro-inflammatory cytokines interleukin-1 (IL-1) and IL-18 and may result in a unique programmed cell death known as pyroptosis and inflammation [66]. The NLRP3 inflammasome also triggers innate immune defenses through the maturation of these pro-inflammatory cytokines. In particular, the NLRP3 inflammasomes are activated by the ROS released by damaged mitochondria, suggesting that mitochondria are also essential for an inflammatory immune response [64].

6. Oxidative damage to cellular membranes

Oxidative stress occurs when the production of ROS, such as superoxide anion radicals, hydroxyl radicals and hydrogen peroxide, and reactive nitrogen species (RNS), such as peroxynitrite anion, are in excess of the cell's ability to destroy these molecules using natural antioxidants [67–70]. Cellular targets of ROS/RNS include nucleic acids, proteins and lipids [69–73], and mitochondrial structures are especially sensitive to oxidative damage [70,73]. ROS and RNS are produced by multiple cellular oxidative pathways, including xanthine oxidase, NAD(P)H oxidases, monoamine oxidases, cyclooxygenases, lipoxygenases, and as described in Section 5, the mitochondrial ETC [68,73,74].

The MIM is one of the most abundant sources of ROS, and it is produced as a consequence of the uncoupling of the electron transport chain from ATP generation [67,73,74]. Usually the levels of ROS/RNS are low in cells, and any damage that is caused is constantly repaired [67,69]. Low levels of ROS are used in cell signaling and may be important in the aging process by the induction of mitochondrial *hormesis*, the cellular response to low levels of toxins [75]. However, at higher concentrations ROS/RNS become toxic to cells, especially their membranes [67,75]. To counteract this, mitochondria are equipped with enzymatic and non-enzymatic systems to control ROS/RNS production and prevent their dissemination within and out of this organelle [70]. As discussed in the previous section, excess ROS and RNS damage in mitochondria can result in triggering of mitophagy and apoptosis.

In addition to oxidative damage to unsaturated FA, CL and other lipid molecules [67,68,74], ROS/RNS can damage DNA and proteins [69–71]. For example, ROS can stimulate opening of L-type voltage-sensitive calcium channels, resulting in increased intracellular calcium concentrations, as seen in neurodegeneration and stroke patients [74–76]. Once

released, the ROS/RNS can penetrate mitochondrial and cell membranes and diffuse outside cells to cause widespread tissue damage [77].

The reaction of ROS/RNS with cellular membranes is particular damaging, causing oxidation of double bonds in phospholipid unsaturated FA to aldehyde products, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), 4-oxo-2-nonenal and acrolein [67,78]. These reactive products covalently bind to protein thiol groups and other cellular materials, altering their function [67]. They are also markers of neurodegeneration, inflammation, diabetes, atherogenesis and other pathogenic processes linked to oxidative stress and lipid peroxidation [67,77–80].

In mitochondria, ROS free radicals are also capable of causing oxidation of CL. Consequently, CL remodeling has been implicated in the etiology of mitochondrial dysfunction and is associated with a host of pathophysiological conditions, including diabetes, obesity, heart failure, hyperthyroidism, neurodegeneration, and aging, all of which are characterized by increased levels of oxidative stress, CL deficiency, and enrichment of docosahexaenoic acid (DHA) content in CL [81].

The phospholipids of mitochondria are sensitive to ROS/RNS damage because of their high content of certain unsaturated FA, such as DHA (22:6, n-3) and its sister molecule eicosapentaenoic acid (EPA, 20:5, n-3) [82,83]. These FAs, which are found at high concentrations in fish oils, constitute important components in phospholipids of mitochondria by providing fluidizing properties to the MIM and supporting proton transport processes [83]. For example, there is a direct relationship between the contents of unsaturated FA in mitochondria, such as DHA and EPA, and the ability to maintain a proton gradient across the MIM [84]. They also have the property of decreasing the contents of cholesterol in the plasma membranes of aortic endothelial cells and making their membranes more fluid and controlling membrane permeability [82,83,85].

The mitochondrial unsaturated FA oxidized products are also very important in inducing apoptosis by reaction with mitochondrial permeability transition pores (MPTP) [86,87]. MPTP are voltage-dependent channels that function during calcium-dependent apoptosis. Increases in mitochondrial ROS production alters MPTP and initiates Ca^{2+} release, modifying Ca^{2+} cell signaling and causing mitochondrial calcium

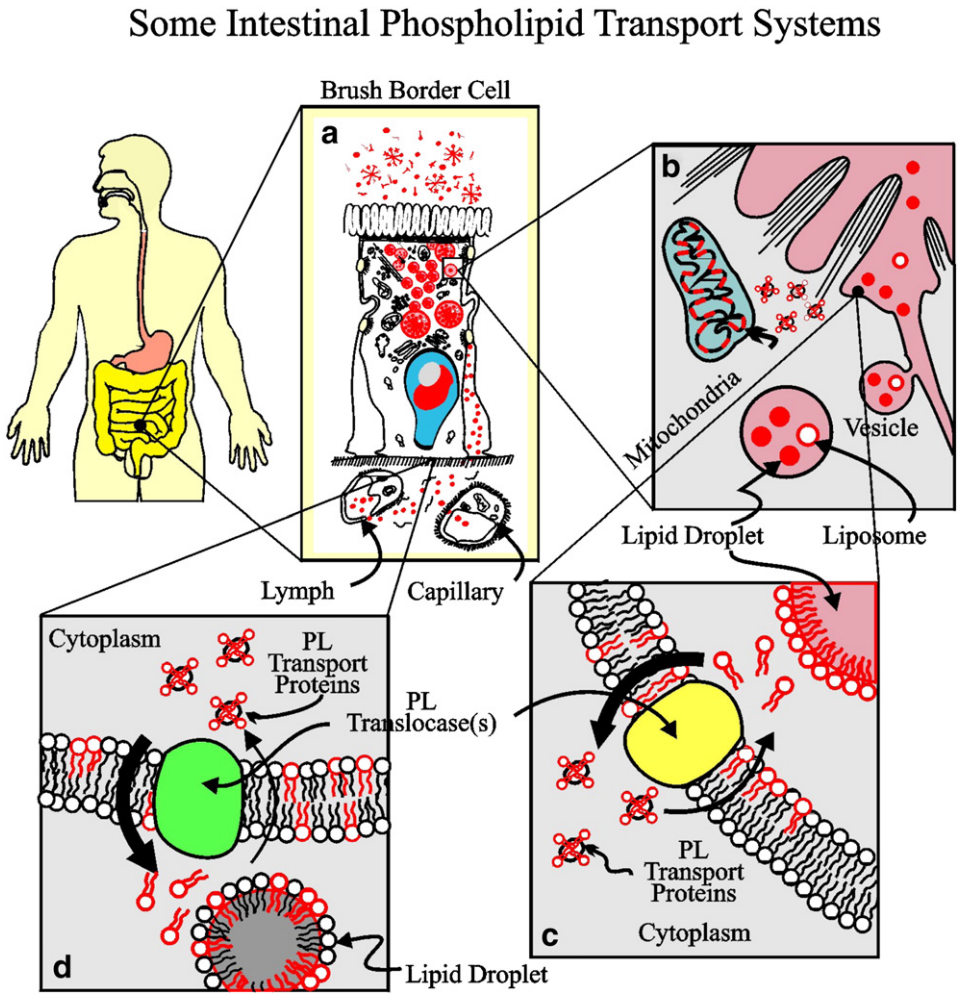

Fig. 1. Some phospholipid transport systems. Most orally ingested phospholipids are absorbed and transported in the upper small intestines by brush border epithelial cells after their dispersion and enzymatic digestion. (a) Lipid Replacement Therapy (LRT) phospholipids are usually protected from complete disruption and enzymatic degradation by bound oligosaccharides, permitting transportation into cells as small lipid droplets and vesicles by endocytotic processes. At the distal or basolateral regions of the brush border cells excess lipid vesicles and droplets can be extruded by a reverse exocytotic process and eventually transported to lymph and blood vessels. (b) The endocytotic transport of lipid droplets and vesicles into brush border epithelial cells is shown in more detail. In addition, some of the phospholipids are absorbed by the epithelial cell plasma membrane and transported as simple phospholipids or their degradation products to organelle membranes, including mitochondria, by phospholipid transport systems. (c) After absorption by the brush border epithelial plasma membrane, phospholipids are flipped to the inner plasma membrane surface by phospholipid translocases. At the inner plasma membrane surface the phospholipids can be absorbed by phospholipid transport and carrier proteins and moved to other membranes. (d) At the brush border cell basolateral surface excess phospholipids are delivered by transport proteins to the inner surface of the plasma membrane. There they are flipped to the outer surface by phospholipid translocases where they can be absorbed by lipid droplets and further transported. Not shown in the figure are lipid transport by direct membrane-to-membrane contact and lipid droplet- and vescile-to-membrane contact and temporary fusion.

loading, which further increases ROS production, repeating the process until mitochondrial begin swelling, eventually followed by cell death [88]. Modifying mitochondrial unsaturated FA composition by dietary supplementation with DHA can alter mitochondrial Ca^{2+} homeostasis and function by delaying MPTP opening and Ca^{2+} induced apoptosis [89,90].

7. Lipid metabolism and transport

Oral phospholipids are dispersed, partially or completely degraded and absorbed in the gastrointestinal tract [91,92]. Although some hydrolysis takes place in the stomach (10–30%), most fat hydrolysis and absorption occurs in the upper small intestine [93,94]. With increased load, however, fat is also absorbed in the distal small intestine [95]. The process is quite efficient; in clinical experiments more than 90% of radiolabeled phospholipids were absorbed within hours, and the blood concentration indicated that about 20% of an administered phospholipid dose was transported into the blood within 6 h [96,97].

Since phospholipids can be oxidized and degraded during their ingestion, digestion and eventual adsorption by the intestinal lumen, oral Lipid Replacement Therapy (LRT) phospholipids are usually protected from the acidic environment of the gut, disruption by bile salts and hydrolysis by phospholipases and other enzymes from the pancreas and gut microflora [98]. This is accomplished by complexing LRT phospholipid micelles and liposomes with certain fructooligosaccharides, which bind to and protect the phospholipids from phospholipases and bile salts [99,100]. They are also better protected from oxidation in this form [100].

Once phospholipid micelles and liposomes are protected by their binding of specific protective oligosaccharides, they can be taken in largely intact by the gastrointestinal brush border cells (Fig. 1) [94,101]. Although some hydrolysis of phospholipids occurs during this process, most micellar phospholipids are absorbed intact by mucosal cells as unoxidized, undegraded phospholipids [95]. When dietary phospholipids are protected from the action of bile salts and pancreatic lipases by oligosaccharides, however, such as in LRT, their overall absorption is reduced but with less degraded products [102]. On the other hand, when uncomplexed dietary membrane phospholipids are directly absorbed by mucosal cells, most of these molecules are partially degraded by pancreatic phospholipases, usually by removal of one or even both of the acyl FA chains and further degradation [103].

Morphological studies have revealed that undigested dietary lipids and phospholipids are present in the small intestine mainly as small lipid droplets and micelles (50-1,000 Å in size) that are taken in largely intact into intestinal cells (Fig. 1) [104]. Although electron microscopic methods cannot distinguish the lipid compositions of lipid droplets and micelles, these materials are absent in fasting controls [104]. The intestinal absorptive cells also transport individual phospholipids and their degradation products, such as FAs, using specific membrane transport systems, but the small droplets (called chylomicrons once inside cells) and micelles are transported via a pinocytosis process [104]. There they accumulate inside intestinal brush border cells as larger droplets or chylomicrons [105], especially in the most differentiated cells at villous tips [104,105].

In addition to the pinocytosis transport system, intestinal bush border plasma membranes can also directly absorb free phospholipids (and other lipids) and phospholipids bound in micelles and lipid droplets into their outer plasma membrane leaflets. Dermer observed that the microvillous plasma membranes became thicker on their outer surfaces during phospholipid and FA absorption, and this was attributed to the direct insertion of lipid molecules into the outer surface or outer leaflets of the microvilli membranes [106]. Once phospholipids like PC are enriched in the plasma membranes of the colonic mucosa, they help protect this structure from pathogenic processes like ulcerative colitis and other chronic inflammatory conditions. It was proposed that they do this by providing cell surface hydrophobicity and modulating the signaling state of the mucosa, a regulatory component of the inflammatory signaling pathway [107].

After phospholipids, DAG and FAs are incorporated into the outer leaflet of a cellular membrane, there are several different transmembrane lipid-translocase or flipping molecules (flipases, flopases and scramblases) that can transfer the phospholipids and FAs to the other membrane surface [108–110]. In the case of lipid molecules that have been transferred from the outer to the inner surface of the plasma membrane, they can then be picked up by intracellular lipid carrier, transporter and transfer molecules and transported to intracellular membranes, or they can simply diffuse to recipient cellular membranes and partition into the membrane [111,112]. Alternatively they can be stored inside cells as vesicles or in some cells as lipid droplets [113]. The flipping of phospholipids to the inner surface of the plasma membrane may promote formation of transport vesicles by inducing membrane curvature and blebbing [109]. A possible reason for the existence of multiple mechanisms for the transfer of lipid molecules from the intestinal lumen of brush border cells into these cells and beyond may be to provide redundancy for this critical process.

Once inside intestinal cells phospholipids and other lipids are usually delivered to various membranes and organelles via carrier, transfer or transport proteins [111,112], or as mentioned above, they can be stored as chylomicrons that are found only during fat absorption [104,114]. Lipid chylomicrons are mostly phospholipids (70–75%), but they also contain cholesterol (5–10%), triglycerides (13–25%), FAs and other lipids [114]. Not only do they store phospholipids in mucosal cells, but they are also used to transfer lipids to the endoplasmic reticulum, Golgi and other organelles, and also to other adjacent cells [104,105].

Vesicles released from Golgi membranes of mucosal cells can contain small lipid droplets or larger chylomicrons, and these lipid-loaded vesicles have been observed to be transported to the basolateral surface for release by a process called reverse pinocytosis. Eventually they find their way to the cells lining the lymph or circulatory systems [104]. There pinocytosis and transport processes can be repeated until the lipid droplets or chylomicrons are eventually released into the lymph or blood.

In addition to lipid droplets [113], the usual method of lipid transfer inside cells makes use of a wide variety of intracellular lipid carriers or transfer proteins, each specific for a given type of lipid or lipid class [111,112]. These lipid transport systems usually function on a mass action basis where membranes that contain high concentrations of certain membrane lipids deliver their excess lipids to membranes with lower lipid concentrations.

When intracellular membrane phospholipid-binding or transfer proteins were isolated and studied in vitro, investigators found a transfer preference for polyunsaturated phospholipids but not for phospholipids with different acyl chain lengths. This indicated that intracellular phospholipid transfer proteins can distinguish unsaturated acyl chains of varying lengths in membrane phospholipids [115]. A slightly different result was obtained by other investigators with a membrane phospholipid transfer protein from bovine liver. They found that this membrane phospholipid transfer protein preferentially extracted and transferred PC long chain unsaturated FA (fluid phase PC) [116]. Of course, once they arrive at their destination, membrane phospholipids are also modified enzymatically, and their FAs and head groups can be replaced to reflect the usual composition of the membranes at their final destination [117]. This system can also be reversed to remove oxidized or damaged lipids from membranes and eventually degrade them or export them [118].

There is also an additional system for transferring lipids inside cells. Membranes and organelles can transfer lipids within cells by direct contact and diffusion. For example, endoplasmic reticulum and mitochondria can transfer membrane lipids by direct contact transfer through specific junctions called the mitochondria-associated membrane (MAM) [119,120]. In addition, certain cellular organelles have their own specific lipid transport systems to move phospholipids inside

these structures. For example, mitochondria possess lipid transport proteins that shuttle membrane phospholipids between inner and outer membranes, probably to insure maximal exchange of damaged membrane phospholipids with undamaged phospholipids during fusion [121,122].

Once in the circulation, lipids, such as membrane phospholipids, steroids, FAs and other lipids, can be bound by plasma carrier molecules, absorbed by lipoproteins, such as high- and low-density lipoproteins (HDL and LDL), or bound to blood cells, such as erythrocytes. The blood lipoproteins are an important transport system for membrane phospholipids in the circulation. They also protect phospholipids and other lipids from oxidation and enzymatic digestion during transport. In man, the amounts of membrane phospholipids exchanged and preferentially transported by HDL lipoproteins are more than 20-times the amounts transported by red blood cells [123]. In addition, membrane phospholipids can displace and help remove cholesterol from the circulation by displacing it from erythrocytes and circulating lipoproteins [123,124].

The half-life of high concentrations of membrane phospholipids injected intravenously into the circulation indicates that after a rapid decrease in concentration due to liver clearance (approximately 80% reduction within 15 min), residual phospholipids may be present for some time before being deposited into tissue stores. In one study in man the concentrations of intravenously injected radiolabeled-phospholipids fell rapidly in the blood (almost 100% after 10 h), but the residual phospholipids slowly declined with a half-life of 59 days [125,126]. This may not be a good model for dietary or oral membrane phospholipid turnover in the blood circulation, however, because the concentrations of phospholipids in the blood are many times higher after intravenous injection than those transported from intestinal sources [126]. Le Kim and Betzing studied polyunsaturated PC absorption in rats and found that the disappearance from the gastrointestinal tract was relatively rapid in the first 6–8 h after instillation but became considerably slower thereafter [127]. Importantly, more than 90% was absorbed within 24 h, and essentially all of the membrane phospholipids absorbed after oral administration were eventually incorporated into tissues [96,127]. This indicates that the gastrointestinal transport and utilization of membrane phospholipids is a very efficient process.

After their transport in the blood to organs and tissues, membrane phospholipids and other lipids are transferred to the plasma membranes of endothelial cells. This process repeats almost in reverse the transfer of membrane phospholipids from gut endothelium to the blood. From endothelial cells at sites distant from the gut, membrane phospholipids are then transferred to tissue and organ cells and eventually to intracellular membranes. The entire process follows a mass action concentration gradient from the gut to tissues (and back again for damaged/oxidized phospholipids). As mentioned above, there is also a natural process to reduce cholesterol in membranes and tissues, because the lipoproteins with high phospholipid unsaturated FA have a fluidizing effect on lipoproteins and membranes, effectuating cholesterol removal from cellular stores in tissues and cells [128,129]. Once the transferred membrane phospholipids are present at their destination sites, they can also be modified enzymatically to other phospholipids, or they can have one or two of their FAs exchanged with unsaturated FA or modified in situ. The end result is to transform cellular membranes so that enzymes, receptors and other components of membranes are more active, and membranes are less permeable, less deformable and more functional [130–136].

8. Lipid replacement methods

Membrane lipids can be replaced (LRT) using dietary sources, oral supplements or intravenous introduction of membrane phospholipids (so-called "essential" phospholipids). There are advantages and disadvantages to each of these methods. Plant sources of dietary polyunsaturated membrane phospholipids, such as legumes or cabbage, are a good starting point for dietary supplementation [6,137]. Although the normal average dietary uptake of diet-derived membrane phospholipids is not known to any accuracy, it is considered in the range of 2–8 g per day [6]. However, the amounts of raw material, such as soy beans, required to obtain a daily dose of approximately 1.8 g of membrane phospholipids is approximately 15 kg of beans [137]. This makes dietary sources of enough membrane phospholipids unappealing and impractical. In addition, dietary sources of membrane phospholipids are not protected from oxidation, disruption and digestion before and during intestinal delivery. Oral supplements, on the other hand, can deliver therapeutic doses of membrane phospholipids as part of a daily supplement regimen, and oral membrane phospholipid supplements can be protected from oxidation, bile disruption and enzymatic digestion using protective fructooligosaccharides [5,99,100].

Most oral supplements for membrane repair and replacement utilize sources that contain mixtures of glycerophospholipids, n-3 and n-6 unsaturated FA and other lipid components derived from legumes, milk, liver, fish, krill, and other sources [6,90,138–141]. Many of these have also been tested in laboratory animals. For example, animals supplemented with n-3 unsaturated FAs showed changes in mitochondrial membrane phospholipid FA composition, improved mitochondrial function and altered Ca^{2+}-induced mitochondrial permeability transition pore opening [142]. This was accomplished by modification of the MIM, or more specifically by replacing CL FAs with specific unsaturated FA to improve inner membrane fluidity and CL-ETC interactions. For example, feeding rats for 10 weeks with a DHA and EPA unsaturated FA supplement resulted in modifying their cardiac mitochondrial CL

Table 2
Some clinical effects of dietary LRT supplement NTFactor on fatigue scores.[a]

Subjects/patients	n	Av age	Time on LRT	Fatigue Scale reduction (%)[b]	Reference
Chronic fatigue[c]	34	50.3	8 week	40.5**	Ellithorpe et al. [354]
Aging, chronic fatigue[d]	22	68.9	12 week	35.5*	Agadjanyan et al. [166]
Chronic fatigue syndrome[d]	15	44.8	8 week	43.1*	Nicolson & Ellithorpe [144]
Obesity, fatigue[d]	35	42	8 week	24*	Ellithrope et al. [251]
Aging, chronic fatigue[e]	67	57.3	1 week	36.8**	Nicolson et al. [230]
Lyme disease, fatigue[f]	17	52.4	8 week	26*	Nicolson et al. [231]
Gulf War Illness, fatigue[g]	16	42.2	8 week	34.6*	Nicolson et al. [147]

[a] Modified from Nicolson and Settineri [147].
[b] Piper Fatigue Scale [147].
[c] Propax™ with NT Factor.
[d] NT Factor®.
[e] Healthy Curb™ with NT Factor®.
[f] Advanced Physician's Formula™ with NT Factor®.
[g] ATP Fuel® with NT Factor®.
** $P < 0.0001$.
* $P < 0.001$ compared to without NT Factor.

FAs and delaying Ca^{2+}-induced mitochondrial permeability transition pore opening [143].

In terms of daily use for humans, the most convenient, efficient, safe and cost effective method of membrane phospholipid administration has been the use of daily oral supplements [6]. Of the oral supplements available, most are crude soy, egg yolk or marine lecithin preparations that lack oxidation, bile and phosphatase protection. In addition, most of these preparations have not been carefully analyzed for phospholipid composition. However, there are oral membrane phospholipid supplements, such as NTFactor®, that fulfill the requirements for efficacy, oxidation and degradation protection, safety and convenience [5,144,145]. The NTFactor supplement, its use and clinical results, will be discussed in more detail in Section 11. NTFactor comes in many oral forms, but almost all contain from 1.8 to over 2 g of phospholipids (>30% PC plus PI, PE, PS and other phospholipids with mostly unsaturated FA) [144,145]. Some NTFactor-containing supplements also contain probiotic bacteria, various vitamins and minerals and other ingredients. Some of these oral supplements can be compositionally quite complex. For example, a specific supplement for mitochondrial support, ATP Fuel®, contains 2 g NTFactor and also NADH, Coenzyme-Q10, vitamin E, α-ketoglutaric acid, L-carnitine, and other ingredients [147]. All of these oral supplements contain some antioxidant, such as low concentrations of vitamin E, to protect the phospholipids and unsaturated FA from oxidation during storage and ingestion.

Other specific oral phospholipid preparations, such as PS supplements, usually made from bovine brain or soy, have been used to treat memory loss in aged subjects or in Alzheimer's disease (AD). In the case of AD patients supplementation with 300 mg per day of bovine PS for 6 months provided cognitive improvement relative to placebo controls [148]; however, this was not seen in another study on elderly subjects with age-associated memory impairment that received 300-600 mg soy PS daily for 12 weeks [149]. Although administration of PS alone has health benefits, the use of more complex mixtures of membrane phospholipids containing PC, PS, PE, PI, etc. are considered more useful [5,144,146].

Intravenous administration of membrane phospholipids ("essential" phospholipids or EPL) can deliver high phospholipid concentrations without the need for inhibiting intestinal disruption, but they are still susceptible to enzymatic and oxidative damage. In addition, daily intravenous delivery comes with some risk for adverse events (infection, blood vessel damage, thrombosis, pruritus, dyspnoea, urticaria, etc.), and it is much more expensive and ~~its~~ administration must be professionally supervised. Nonetheless, there are many published reports on the clinical usefulness of intravenous membrane phospholipids [1,2]. EPL intravenous preparations, such as Essentiale®, contain 1 g phospholipids, mainly PC (>75% PC, with some PE, PI and other phospholipids, ethanol, tocopherol, ethylvanilllin, vitamins B6, B12, nicotinamide, and sodium D-pantothenate) [2,6]. Other membrane phospholipid products are listed in Table 2 of Küllenberg et al. [6].

9. Pre-clinical and clinical safety studies

Some of the most important preclinical studies on LRT are analyses of acute and chronic toxicity, including dose effects, perinatal and postnatal toxicity and mutagenic and carcinogenic potentials. Importantly, none of these studies, which were mostly conducted in laboratory animals (mice, rats and rabbits), demonstrated any acute or chronic toxic effects of membrane phospholipids given by oral, subcutaneous or intravenous administration. From multiple studies toxic or lethal doses could not be established in laboratory animals. Nor could any doses of membrane phospholipids be established that caused any mutagenic or carcinogenic events. In mice, rats, rabbits and dogs daily oral doses up to 3.75 g/Kg body weight produced no effects (reviewed in [2]). Thus using single or repeated dose administration no toxicity could be established in young, adult, pregnant or fetal laboratory animals. For example, no toxicity was found in pregnant rats or rabbits or with their fetal offspring at doses up to and above 1 g/Kg [2].

When the effects of membrane phospholipids were examined in laboratory animals receiving carcinogens, simultaneous administration of membrane phospholipids inhibited the formation of tumors (reviewed in [2]). For example, supplementation of pure PC in rats reduced preneoplastic liver nodule formation [150]. These and other studies resulted in the U.S. Federal Drug Administration (FDA) classifying membrane phospholipids used in LRT in the category 'Generally Recognized as Safe' (GRAS) [151].

The long-term pharmacological effect of membrane phospholipids on rodents was examined by Wagener et al. [152]. The membrane phospholipids were given in chow or water daily at doses ranging from 0.01 to 5 g/Kg body weight. No effects were found in the central or peripheral nervous systems, including reflexes, analgetic, spasmoltyic or spasm-influencing functions, renal function, heart and vascular function, or other measures of pharmacological toxic effects [152]. Long-term administration of membrane phospholipids in the chow of laboratory rodents has proven to be beneficial not harmful. For example, Seidman et al. [153] examined the protective effect of feeding rodents membrane phospholipids on age-related hearing loss and DNA deletions associated with aging. Rats aged 18–20 months were fed membrane phospholipids (NTFactor) or placebo for 6 months and their auditory brainstem responses (ABR) and MIM potentials and mitochondrial DNA deletions were examined every two months. ABR were measured by measuring hearing thresholds and sensitivities, mitochondrial inner membrane potentials were assessed by using blood lymphocytes labeled with rhodomine-123 and monitoring fluorescence with a flow cytometer, and DNA deletions were determined by extracting DNA from various brain regions and amplification of mitochondrial mtDNA sequences (ND1-16srRNA and other sequences).

There were significant differences found between the experimental and placebo groups in ABR, MIM potential and the presence of mtDNA deletions [153]. By 4-months administration of NTFactor there was significant preservation of hearing threshold at all frequencies tested in the experimental group. In addition, NTFactor prevented age-related decline in mitochondrial inner membrane potential and reduced the incidence of common mtDNA deletions found in aging rats. The effects were attributed to the ability of NTFactor to repair mitochondrial and other membranes and to the ability of the phospholipid unsaturated FA to reduce the effects of ROS damage on mtDNA [153].

High doses of membrane phospholipids have also been given to humans with no apparent toxicity. For example, patients with hepatic encephalopathy due to decompensated liver cirrhosis were administered 2 g per day of EPL intravenously for several weeks with no apparent adverse events. Patients receiving EPL showed significant improvements in their liver disease and had significantly prolonged survivals compared to a control group that did not receive the membrane phospholipids [154]. In phase I/II clinical trials on patients with cardiovascular disease, PI was given at doses over 5 g per day. This phosphatidyl lipid was able to increase plasma HDL and apolipoprotein A-1 levels and reduce triglyceride levels with no evidence of any toxicity [155].

Long-term administration of relatively high doses of LRT has actually improved cardiovascular blood markers. In addition to the studies above, in 35 older (average age 60.7) subjects receiving over 2 g per day oral NTFactor for over 6 months showed no evidence of adverse events. In fact, their cardiovascular blood marker levels, such as homocysteine, improved during the trial [155]. Similarly, 58 patients with fatiguing illnesses received doses of 2 g per day oral NTFactor for 2 months without incident [147]. A follow-up on these patients found that most had continued using the LRT supplement for over a year with no adverse events. Cohn et al. [157] have reviewed experimental and clinical studies on the use of membrane phospholipids in the treatment of cardiovascular diseases and have concluded that there is no evidence of toxicity.

In a variety of clinical studies membrane phospholipids have been shown to be safe and effective and have a positive impact in human disease (reviewed in [2,6,157]). Most clinical studies have used oral membrane phospholipids in the range of 1.5-3 g per day or intravenous administration of 0.5-2 g per day [2,6,144–146]. Membrane phospholipids from soy, egg yolk, milk and marine sources have been used in doses over 3 g per day orally or intravenously with no adverse effects. In a few cases does up to 45 g of membrane phospholipids were given orally without adverse effects [158]. In fact, the administered phospholipids actually reduced the side effects of drugs and other treatments [2,6,145]. Thus the use of purified membrane phospholipids at high daily doses has no apparent toxic effects in animals or man.

10. Aging and energy requirements

Aging is a degenerative process associated with progressive accumulation of deleterious changes with time, reduction of physiological function, including increased fatigue and increased chances of disease and death. The causes of normal aging are multi-factorial with no single mechanism able to explain all aspects. An understanding of some of the molecular mechanisms driving the aging process may provide new insights into the pathophysiology of diseases associated with aging and potential new interventions to limit the rate of aging [159–162].

López-Otín et al. [159] have proposed that there are 9 hall marks related to aging: (*i*) genomic instability, (*ii*) telomere attrition, (*iii*) mitochondrial dysfunction, (*iv*) cellular senescence, (*v*) epigenetic alterations, (*vi*) loss of proteostasis, (*vii*) deregulated nutrient sensing, (*viii*) stem cell exhaustion and (*ix*) altered intercellular communication. One of the most significant hallmarks was however, not mentioned: inflammation ("Inflamm-aging") [160] and its effect on aging. Inflamm-aging describes the close relationship between low-grade chronic innate immune driven inflammation and aging that has been linked to a wide spectrum of age-related disorders in various organs [161].

The inflamm-aging process is associated with a decline in autophagic and mitophagic capacity that impairs cellular and mitochondrial housekeeping, leading to protein aggregation and accumulation of dysfunctional mitochondria. This can result in diminished oxidative phosphorylation, reduced MIM trans-membrane potential and increased permeabilization of the outer mitochondrial membrane [162]. In turn, this provokes ROS/RNS production and oxidative stress, resulting in other changes, such as loss in membrane fluidity due to lipid peroxidation and decreased CL content [163,164]. These changes probably account for the reported age-related declines in mitochondrial function and other hallmarks of aging [165].

Importantly, LRT can reverse these age related changes. For example, NTFactor use in aged subjects has been shown to improve mitochondrial function, reduce fatigue and increase cognition, suggesting that the phospholipid lipid membranes of the mitochondria were functionally improved through oral LRT supplementation [166]. More impressively, the mitochondrial function of the aged group was restored to the same level of function as that displayed by a healthy 29 year-old control [166].

Overall, age-related membrane alterations can have significant bioenergetic costs, such as a decrease in mitochondrial activity and ATP production. In addition, the essential transport of phospholipids across leaflets of the membrane bilayer is highly dependent on the presence of ATP. As discussed in Section 7, membrane translocases are generally ATP-dependent [109,110] and as ATP production declines so does the utilization of existing membrane-and-serum derived lipids for the purpose of achieving asymmetrical membrane management and composition [167].

Since mitochondrial production of ATP is directly linked to maintenance of MIM trans-membrane chemical/electrical potential [168], providing lipid substrates via oral supplements or diet manipulation represent attractive options for managing age-related decline. A group of mitochondrial FA oxidation disorders have been successfully targeted by dietary intervention, indicating that dietary composition and supplementation have valid roles in phospholipid replacement strategies [169].

The mitochondrial membrane permeability event MOMP is a decisive event in the functional decline and eventual execution of apoptosis or programmed cell death. It is also causally linked to a decline in bioenergetic function via different mechanisms, not merely due to cytochrome c dispersion. This includes at higher levels the generation of fatigue, and the increased production of DAMPs or 'alarmins' [170], further provoking risk for MIM electrochemical potential decline, resulting in additional reduction in ATP production [171].

Other processes contributing to aging and mitochondrial dysfunction are also linked to alterations in the phospholipids of the MIM. Several groups have reported pre-apoptosis-associated changes in CL content, including oxidation [172], "reorganization" [173], and even relocation of CL from the MIM to other membrane compartments [174].

One of the most contemporary examples includes selective peroxidation of MIM CL in cells undergoing apoptosis. CL peroxidation products are required for the mitochondrial membrane permeabilization, release of pro-apoptotic factors and completion of the cell death program. Therefore, the search for effective inhibitors of CL peroxidation may be critical to discovery and development of anti-apoptotic supplements.

Recent studies have found that autophagy (the programmed destruction of cells) and mitophagy (the programmed destruction of mitochondria) function as major sensors of redox signalling at the interface between cell stress adaptation and cell death. Autophagic activities are mediated by complex molecular machinery, including membrane phospholipids. Dysfunction of autophagy may result in abnormal mitophagy, loss of mitochondrial function and oxidative stress, which are some of the molecular hallmarks of aging [175]. The age-related accumulation of dysfunctional mitochondrial likely results from the combination of impaired clearance of damaged organelles by autophagy and inadequate replenishment of the cellular mitochondria by mitochondriogenesis as well as optimal membrane lipid availability for maintenance of MIM potential [176].

Mitophagy is a selective type of autophagy, whereby damaged or superfluous mitochondria are eliminated to maintain proper mitochondrial numbers and quality. While mitophagy shares key regulatory factors with the general macroautophagy pathway, it also involves distinct steps that are specific for mitochondrial elimination [177]. The subsequent release of DAMPs from damaged membranes is recognised by intracellular danger-sensing multiprotein platforms called *inflammasomes* [178–180]. Recent studies have clearly indicated that ROS, such as superoxide ($O(2)(-)$) and hydrogen peroxide ($H(2)O(2)$) production induced by damaged mitochondria, can stimulate inflammasome formation as a consequence of their role as sterile inflammation or para-inflammation promoters [181–183]. Damaged but functional mitochondria can release up to tenfold more hydrogen peroxide, potentially triggering more sterile inflammatory responses [184]. However, other studies have shown that nitric oxide may actually inhibit the triggering of the keystone NLRP3 inflammasome; suggesting a co-dependant oxidation relationship for the purpose of maintaining innate immune activation and subsequent adverse age related changes in membrane tissues [185].

Although ROS and RNS have been classically known for their damaging effects, increasing evidence of their importance in regulating and maintaining normal homeostatic processes in living organisms has been accumulating. Therefore, the term 'redox regulation' seems to better describe the redox status of mitochondria and its consequences. One of the areas where redox balance is most comprehensively required is in the MIM.

NLRP3 is a key immune related receptor with high affinity for numerous compounds is activated by many danger signals, such as ROS/RNS, soluble ATP, mtDNA, cathepsin B (released from destabilized

lysosomes) and aggregated proteins, all of which evoke cellular stress and are involved in the aging process [186]. NLRP3 activation is also enhanced in many age-related diseases, such as atherosclerosis, obesity and type 2 diabetes [187]. NLRP3-induced cytokines provoke inflammatory responses and accelerate the aging process by inhibiting appropriate autophagy and mitophagy [188]. To avoid cellular damage, ROS-generating mitochondria are constantly removed by mitophagy [189]. Inhibition of autophagic and mitophagic capacity with aging generates the inflammaging condition via the activation of inflammasomes [190].

Exploratory studies using FAs to manipulate the expression of NLRP3 inflammasomes indicate that omega-3 (n − 3) unsaturated FA, such as DHA and EPA, exhibit anti-inflammatory properties via their inhibition of the inflammasome [191,192]. This is likely achieved through various mechanisms, including the manipulation of cell membrane lipids as well as inhibition of primary and secondary triggers, in particular through the compression of NFκB [193]. This provides an early stage mechanism for the possible use of dietary lipids to inhibit inflammation and for LRT utilizing cell membrane-specific phospholipids to compress the rate of mitochondrial-induced DAMP production through reduction of membrane permeabilization and thus limit age-related innate immune inflammation.

For almost a century two primary hypotheses have dominated the concepts of aging. The first appreciated that an organism's intrinsic metabolic rate is an important determinant of life span and was referred to as the "rate of living hypothesis" [194]. This was merged with the "free radical theory of aging" proposed initially by Harman [195], and expanded on since by many authors [196–198]. Harman suggested that aging might be mediated by macromolecular damage through reactions involving ROS [195]. Today, a version of the free radical theory of aging, focusing on mitochondria as source as well as target of ROS, is considered a valid theory to explain aging [199].

Recognition of the important role of mitochondria in aging has led to the mitochondrial free radical theory of aging, which considers mtDNA mutations to be the initiating, primary event in the aging process, because it has been observed that mutated mtDNA molecules accumulate with age [200,201]. The age-dependent accumulation of mutations in mtDNA can, in principle, be explained by six mechanisms that result from replication errors and unrepaired damage through increased production of ROS and an imbalance in the expression of antioxidant enzymes [202,203].

Studies in several species have revealed a wide spectrum of alterations in mitochondria and mitochondrial mtDNA with aging, including: (*i*) Increased disorganization of mitochondrial structure, (*ii*) decline in mitochondrial oxidative phosphorylation (oxphos) function, (*iii*) accumulation of mtDNA mutations, (*iv*) increased mitochondrial production of ROS and (*v*) increased extent of oxidative damage to DNA, proteins, and lipids and (*vi*) insufficient antioxidative enzymes [177,195–205].

LRT provides protection for ROS-related damage to mtDNA, proteins and lipids, decline in MIM potential, and provides substrates for CL regeneration and membrane repair [2,5,86,87]. Thus LRT is capable of decreasing or preventing age-related mitochondrial stress-induced adverse effects and may have significant potential in the reduction of age-related disorders [153,166].

Mitochondria cannot be synthesized de novo, and thus mitochondrial mass, fusion, and fission are important factors in coping with impaired function. Effective control of mitochondrial biogenesis and turnover, therefore, becomes critical for the maintenance of energy production, the prevention of endogenous oxidative stress and the promotion of healthy aging. Fusion of mitochondria helps mitigate cellular stress by mixing the contents of partially damaged mitochondria with undamaged mitochondria as a form of complementation in which undamaged phospholipids and co-factor nutrients are utilized or re-used to maintain their viability. Mitochondrial fusion remains a largely unknown process albeit some steps have now been exposed. Legros et al. used a green and a red fluorescent protein targeted to the mitochondrial matrix to demonstrate that mitochondrial fusion occurs in human cells, is efficient and achieves complete mixing of matrix contents within as little as 12 h [204]. This showed that fusion requires mitochondria to be viable and is mediated by mitofusins. These mitofusions activate the process in which mitochondria of healthy cells undergo fusion.

The course of action occurs in three steps: (*i*) The mitochondria align themselves, end to end, (*ii*) the outer membranes of the two organelles fuse with each other, (*iii*) the inner membranes fuse with each other, thus forming a larger intact mitochondrion [205]. Mitochondrial fusion therefore, represents a rescue mechanism for impaired mitochondria by the mixing of contents (proteins, lipids and mitochondrial DNA) and the unification of the mitochondrial compartment, permitting it to maintain its functionality and allow it to continue to play roles in cellular development, healthy aging and energy dissipation [206,207].

Fission of mitochondria is needed to create new mitochondria, but it also contributes to quality control by enabling removal of damaged mitochondria and can facilitate apoptosis (or mitophagy) during high levels of cellular stress. Mitochondrial fusion, fission and membrane repair also provide suitable quenching molecules for ROS/RNS free-radicals and thus diminish inappropriate mitochondrial collapse and mitophagy. These processes occur throughout the lifecycle of the cell and the functional outcomes of the use of LRT to support them may be reflected in increased ATP synthesis, decreased membrane permeabilization, increased MIM potential, diminished DAMP production and eventually decreased levels of fatigue and improvements in various organ functions, such as cognition and mood [166,153].

Additional improvements in mitochondrial repair have also been noted in diets that are limited in caloric intake without malnutrition, or they involve periods of reduced nutritional intake followed by periods of modest surplus and an adequate intake of minerals, utilization of antioxidants such as CoQ_{10}, vitamin E, curcumin, resveratrol and rotterlin and other associated or complementing nutrients [208–212]. This indicates that supplemental sources of mitochondrial-related nutrients and LRT may provide redox regulation and membrane functional benefits with a subsequent reduction in age-related mitochondrial disorders.

11. Fatiguing illnesses

Fatigue is considered a complex, multidimensional sensation that is poorly understood but perceived to be a loss of overall energy and feeling of exhaustion and an inability to perform even simple tasks without exertion [213–215]. At the cellular level moderate to severe fatigue are related to loss of mitochondrial function and diminished production or leakage of ATP [216,166]. Intractable fatigue lasting more than 6 months that is not reversed by sleep (chronic fatigue) is the most common complaint of patients seeking general medical care [213,217].

During aging and chronic diseases oxidative damage to mitochondrial membranes impairs mitochondrial function [214,218–220]. For example, chronic fatigue syndrome patients present with evidence of oxidative damage to DNA and lipids [218,219], such as oxidized blood markers and oxidized membrane lipids that are indicative of excess oxidative stress [220,221]. These patients also have sustained, elevated levels of peroxynitrite due to excess nitric oxide, which can also result in lipid peroxidation and loss of mitochondrial function as well as changes in cytokine levels that exert a positive feedback on nitric oxide production [222].

In addition to fatigue in chronic illness patients, fatigue is also one of the most common symptoms in cancer [145,146]. It occurs in cancer patients from the earliest forms of cancer to the most progressed forms of metastatic disease [223,224]. Along with pain and nausea, cancer-associated fatigue is one of the most common and disabling symptoms in cancer [223,224], especially in advanced cancers [225,226]. Cancer-associated fatigue is not well understood, but it is thought to be a combination of the effects of the cancer itself plus the effects of cancer treatments [227].

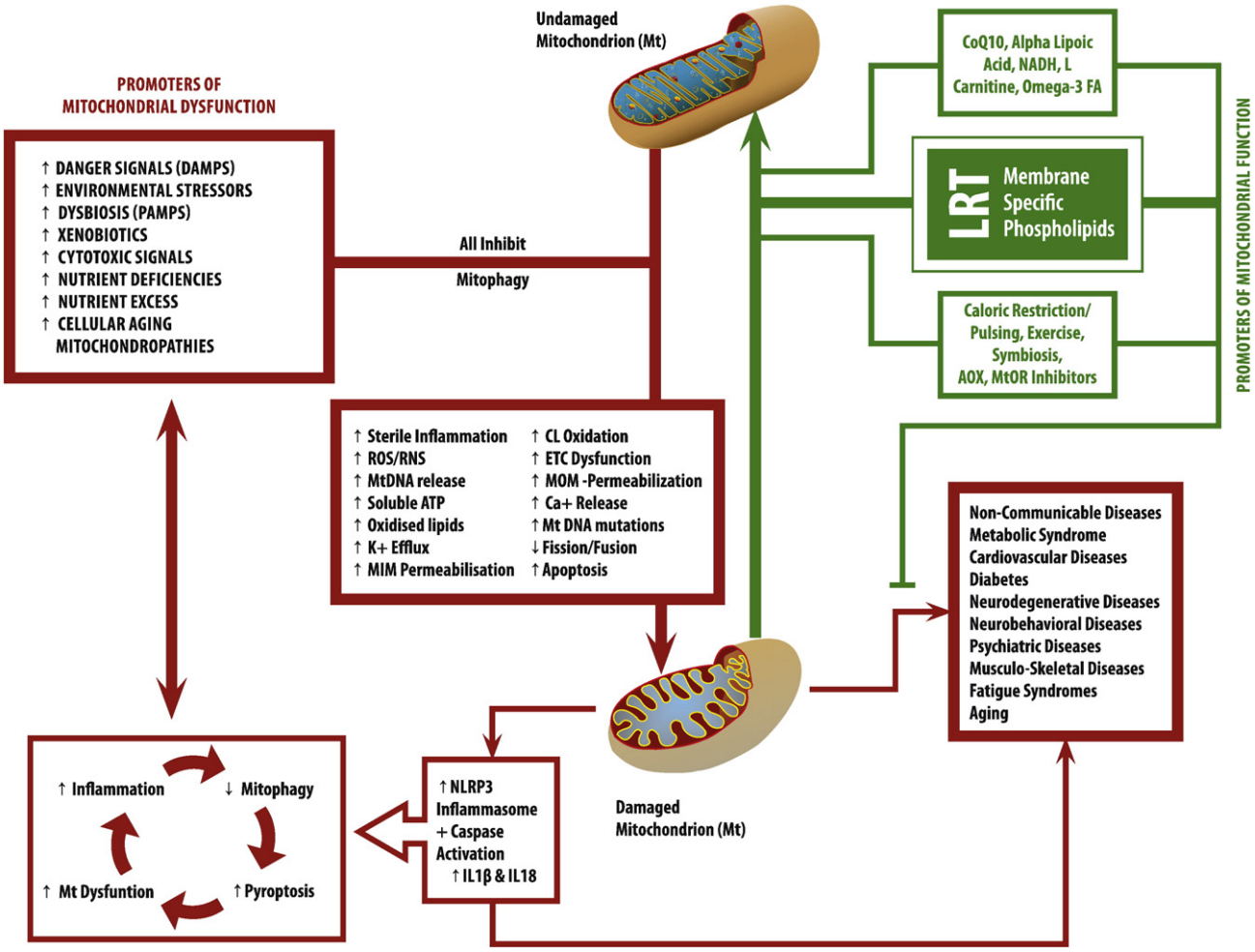

Fig. 2. Mitochondria contribute to a wide variety of cellular, molecular and immune interactions. Oral phospholipids (LRT) have demonstrated direct and beneficial effects on the functional health of mitochondria, contributing to a reduction in loss of functionality. In addition, lifestyle and nutrition are also recognized to play a role in mitochondrial fitness and function. More recently mitochondria are understood to be a significant source of signalling molecules for the promotion of an intracellular protein complex; the inflammasome. Mitochondria and inflammasomes feature at the centre stage of several complex chronic diseases and functional disorders via the production of cellular energy, and promotion of inflammatory enzymes and cytokines. LRT, associated cofactor nutrients and lifestyle changes are safe beneficial enhancers of mitochondrial function and present opportunities for disease symptom management via inhibition of mitochondrial dysfunction. *Abbreviations*: AOX, antioxidants; CoQ10, coenzyme Q10; CL, Cardiolipin; ETC, electron transport chain; IL-1β, interleukin 1-beta; IL-18, interleukin-18; MIM, Mitochondrial inner membrane; MOM, mitochondrial outer membrane; Mt, mitochondria, MtDNA, mitochondrial DNA; MtOr, mammalian target of rapomycin; NADH, nicotinamide adenine dinucleotide; NCDs, non-communicable diseases; NLRP3, Nod-like receptor family protein 3; ROS/RNS, reactive oxygen species/reactive nitrogen species.

Until recently cancer-associated fatigue was rarely treated and thought to be an unavoidable symptom [223] that was also associated with depression and anxiety [225]. Since fatigue or loss of energy is a core aspect of diagnosing depression, both fatigue and depression are often diagnosed together in cancer patients, usually by self-assessment, and they are considered to be part of a clinical symptom cluster or co-morbidity [226]. Cancer-associated fatigue can vary in degree from mild to severe, which is often seen during cancer therapy. Fatigue is often a significant reason why patients discontinue anti-cancer therapy [227]. Eighty to 96% of patients receiving chemotherapy and 60-93% receiving radiotherapy experienced moderate to severe fatigue, which continued for months or even years after the cancer therapy was completed [228].

LRT has been used to treat cancer-associated fatigue and the fatigue-effects of cancer therapy [145,146]. Using NTFactor cancer-associated fatigue was reduced approximately 30% within 8 weeks [145]. A vitamin-mineral mixture with NTFactor has been used in cancer patients to reduce common adverse effects of cancer therapy, such as chemotherapy-induced fatigue, nausea, vomiting, malaise, diarrhea, headaches and other side effects [229]. In advanced metastatic colon, pancreatic and rectal cancers LRT was used to reduce adverse chemotherapy effects. NTFactor supplementation resulted in significantly fewer episodes of fatigue, nausea, diarrhea, constipation, skin changes, insomnia and other effects. Eighty-one percent of patients on chemotherapy that used NTFactor experienced an overall improvement in quality of life parameters. In a double-blinded, cross-over, placebo-controlled trial on advanced cancers the patients on chemotherapy plus LRT (NTFactor) showed fewer adverse effects of chemotherapy, and LRT resulted in improvements in the incidence of fatigue, nausea, diarrhea, impaired taste, constipation, insomnia and other quality of life indicators [229].

LRT has been used in studies with severe chronic fatigued patients to reduce their fatigue [144,147,166,230] (Table 2). For example, the effects of NTFactor on fatigue in moderately fatigued subjects were also determined to see if mitochondrial function improved with administration of NT Factor [166]. In this clinical trial there was good correspondence between reductions in fatigue and gains in mitochondrial function. After 8 weeks of LRT with NTFactor, mitochondrial function was significantly improved, and after 12 weeks of NTFactor supplementation, mitochondrial function was found to be similar to that found in young healthy adults (26.8% increase, $p < 0.0001$) [166]. After 12 weeks of supplement use, subjects were placed on placebo without their knowledge for an additional 12 weeks, and their fatigue and mitochondrial function were again measured. After the 12-week placebo period, fatigue and

mitochondrial function were intermediate between the initial starting values and those found after eight or 12 weeks on the supplement, indicating that continued supplementation is required to show improvements in mitochondrial function and maintain lower fatigue scores [166]. Similar results on the effects of NTFactor on fatigue were found in patients with chronic fatigue syndrome (CFS/ME) and/or fibromyalgia syndrome, Gulf War Illness or chronic Lyme Disease [163] (Table 2).

NTFactor has also been used in combination LRT studies with other mitochondrial supplements to treat long-term chronic illness patients with moderate to severe fatigue [144,231]. These patients had been ill with intractable fatigue for an average of over 17 years, had been seen by many physicians (>15), and had taken an average of over 35 supplements and drugs with no effect on their fatigue [147]. On the combination LRT, however, they responded with significant reductions (30.7%, $p < 0.001$) in fatigue within 60 days. Regression analysis of the data indicated that reductions in fatigue were gradual, consistent, and occurred with a high degree of confidence ($R^2 = 0.960$) [147]. The combination supplement proved to be a safe and effective method to significantly reduce fatigue in long-term patients with intractable chronic fatigue [147,231].

12. Degenerative diseases, the metabolic state and mitochondrial function

Degenerative diseases are primarily the non-infectious or non-communicable prevalent diseases (NCD) whose incidences increase with aging and certain behaviors. The main human degenerative diseases are: cardiovascular diseases, neoplastic diseases and neurodegenerative diseases. These include hypertension, cardiopathies, including coronary disease and myocardial infarction, cerebrovascular incidents (including cardiovascular diseases or strokes), neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, among others. Other NCDs such as obesity, chronic respiratory diseases, diabetes, autoimmune diseases and arthritis are also important. Metabolic syndrome (MetSyn), type 2 diabetes, cardiovascular diseases, hypertension and other related diseases will be discussed in the next section. Nutrition and environmental stress exposures have been identified as major modifiable determinants of NCD [232–234].

As discussed in previous sections of this review, maintenance of functional mitochondria is essential in order to prevent degenerative processes leading to disease and aging. These outcomes share basic mechanisms—in particular, mitochondrial age and function within an individual [235].

Mitochondrial dynamics, especially fission and fusion, play a crucial role in ensuring mitochondrial function and quality, but the process may also generate and spread molecular damage through a population of mitochondria if adequate membrane lipids or other macro and micro nutrients are either unavailable or consumed in excess [236]. Computational simulations have indicated that this molecular dynamic is advantageous when mitochondria are undamaged or only marginally damaged. In contrast, at a higher degree of damage mitochondrial dynamics may be disadvantageous, implying that supporting favorable mitochondrial dynamics with suitable lipids (LTR) and other important nutrients along with appropriate caloric restriction will confer a health advantage [237] (Fig. 2).

Mitochondrial defects, systemic inflammation, and oxidative stress are at the root of most NCDs, and improved mitochondrial bioenergetics along with positive changes in lifestyle represents an opportunity for suitable intervention [238]. Potential therapeutic approaches currently available to slow down age-related functional declines that predispose to NCDs include insulin sensitizers [239], exercise to promote mitofusin [240] and antioxidant treatments to reduce ROS/RNS [241]; however, the effectiveness of existing antioxidants alone is suboptimal, because most antioxidants are not selective for mitochondria and fail to penetrate the MIM. In addition, unlike ubiquinone or tocopherols, most nutritional antioxidants do not attach covalently to lipophilic triphenylphosphonium cations to facilitate transport across the phospholipid bilayers into the mitochondria [242]. Other possible therapeutic approaches, such as caloric restriction or reduction in caloric intake without malnutrition (CR) or intermittent fasting, remain controversial and their benefits yet to be fully determined [243,244].

Experiments with a combination of phospholipids (LRT) and antioxidants have been successful in improving mitochondrial bioenergetics in animal and human studies [144,153,166,230,231,251] and have the added effect of resolving inflammation. Manipulating cellular bioenergetics represents a new way to treat NCDs and inflammatory and immune diseases via the reprogramming of the inflammatory and metabolic response. This includes lifestyle and behavioral changes related to food selection combined with LRT to provide important mitochondrial support. It is well understood that unmodified western diets lead to mitochondrial dysfunction and higher susceptibilities to inflammation, apoptosis, NCDs and aging [8].

One of the mechanisms involved in the management of mitochondrial functionality and NCDs is the mammalian target of rapamycin (mTOR), a well-conserved serine/threonine kinase that regulates cell growth in response to nutrient status [244]. Two distinct complexes have been identified: mTORC1, in which mTOR is bound to the protein partner raptor, and mTORC2, in which mTOR is bound to another protein partner called rictor [245]. Dysregulation of TORC1 and TORC2 activity is closely associated with various NCDs, including diabetes, cancer and neurodegenerative disorders as well as aging [246].

By sensing the abundance of various nutrients and regulating the activity of critical processes such as autophagy and translation, the TORC1 signaling pathway lies at the intersection between nutrient, environmental and innate mechanisms of aging and NCDs. TORC1 signaling regulates mitochondrial biogenesis, oxidative stress and turnover in mammals as well as in lower organisms. Also, defects in the clearance of damaged mitochondria by TORC1-regulated autophagy contribute to ROS/RNS accumulation [247]. CR without malnutrition and periodic bouts of short-term nutrient excess [248] and specific inhibitors of MTOR such as rapamycin [249] beneficially affect mitochondrial function, suggesting that mitochondria are highly responsive nutrient sensors and effectors, some of the implications of which are discussed in the next section. The natural sensitivity of mitochondria to energetic substrate levels and their recently discovered ability to dynamically undergo function-defining morphology transitions that influence the integrity of the mitochondrial genome constitute a novel potential mechanism to explain long-term modulations of health and disease [250].

Lifestyle changes plus LRT may present opportunities to enhance mitochondrial fusion, and reduce inflammation and oxidative stress and thus assist in the management, prevention or treatment of NCDs. An example of this is a small pilot trial in which LRT supplementation modified metabolism through body mass reduction and appetite restraint [251]. Thirty subjects (Mean Age = 56.8; 24 females and 6 males) used oral NTFactor (500 mg) and alpha-amylase inhibitor (500 mg) 30 min before each meal. Participants were told to eat and exercise normally and weight, waist and hip measurements were taken weekly. Appetite and sweet cravings were assessed weekly by standard methods. Fatigue was determined using the Piper Fatigue Scale [252], and blood samples were taken prior to and at the end of the trial for lipid and chemical analyses.

Sixty-three percent of the participants lost an average of 6.11 ± 0.28 lb (2.77 ± 0.12 Kg) ($p < 0.001$) along with average reductions of 2.51 ± 0.05 in. (6.4 ± 0.13 cm) ($p < 0.0001$) and 1.5 ± 0.04 in. (3.8 ± 0.10 cm) ($p < 0.0001$) from waist and hip circumferences, respectively. The entire group lost an average of 3.63 ± 0.13 lb (1.65 ± 0.11 Kg) ($p < 0.001$) with average reductions of 1.59 ± 0.03 in. (4.04 ± 0.06 cm) ($p < 0.0001$) and 1.13 ± 0.02 in. (2.87 ± 0.05 cm) ($p < 0.0001$) from waist and hip circumferences, respectively. Weight loss and body measurement decreases were gradual, consistent and significant, along with reductions in body mass index (BMI) and basal metabolic rate (BMR) measurements. Overall hunger was reduced

44.5% ($p < 0.001$), with reduced cravings for sweets and fats, and there was a 23.9% reduction in fatigue ($p < 0.009$). Along with fatigue reduction there was a 26.8% perceived improvement ($p < 0.004$) in cognition and ability to concentrate, remember and think clearly. Blood lipid profiles at the end of the trial suggested improved cardiovascular lipid profiles, and there were no adverse events from the product [251].

The weight loss effects of LRT in clinical studies may be due to the replacement of membrane lipids with glycerophospholipids containing particular unsaturated FA. In support of this Vögler et al. fed rats diets high in stearic, elaidic, oleic, linoleic or 2-hydroxyoleic acids or control vehicle for 7 days and found that in the test animals food intake was lower and the animals lost body weight, mainly through reduction of adipose fat mass. Only treatment with C-18 oleic acid or 2-hydroxyoleic acid induced body weight loss (3.3 and 11.4%, respectively). They attributed the effect to enhanced energy expenditure due to changes in UCP1 expression and phosphorylation state of the cyclic AMP-response element-binding protein CREB in adipose tissue [253].

Other uses of membrane phospholipids to modify NCD related functional decline include supplementation with oral PS to improve memory loss and cognition. Richter et al. [254] recruited 30 male and female subjects (age 50–90 years, average 74.6 years) with memory impairments unrelated to neurological disease, stroke, intracranial hemorrhage, brain lesions, diabetes, infections or inflammatory processes for a 12-week study to determine if 300 mg PS per day modified outcomes in 6 different tests of memory and cognition. At the end of the study participants showed significant improvements in memory recognition ($p = 0.004$) and recall ($p = 0.006$), total learning ($p = 0.013$), executive functions ($p = 0.004$), metal flexibility, and visual spatial learning. There were no adverse events during the trial, and interestingly both mean systolic and diastolic blood pressure values were reduced at 12 weeks in comparison to baseline values [254]. Similarly, Kato-Kataoka et al. [255] conducted a double-blind, randomized clinical trial on 78 subjects (50–69 years) to determine if 100–300 mg oral PS per day versus placebo affected memory scores. They found that PS supplementation significantly improved behavioral memory functions, especially short-term memory and cognitive function in low-scoring (delayed word recall) participants ($p < 0.01$). There were no adverse events and no changes in vital signs or laboratory tests [255].

13. Metabolic syndrome, diabetes and cardiovascular diseases

Metabolic syndrome (MetSyn), thought to be the precursor to type 2 diabetes and cardiovascular diseases (CVD), is made up of several interrelated disturbances of glucose and lipid homeostasis in obese individuals. This includes insulin resistance, changes in blood lipid profiles, abnormal glucose tolerance, hypertension and vascular inflammation, as well as a background of multiple genetic abnormalities [256,257]. There are a number of major risk factors for MetSyn: (*i*) abdominal obesity, (*ii*) elevated fasting plasma glucose, (*iii*) artherogenic dyslipidemia (increased triacylglyerols, increased LDL and reduced HDL), (*iv*) elevated blood pressure, and (*v*) the presence of prothrombotic and proinflammatory molecules [257,258]. MetSyn has also been called Syndrome X [259] or insulin-resistance syndrome [259], and it is estimated that in the age group over 60 in North America, over 40% have some symptoms of MetSyn [260]. The above risk factors are also found in CVD, type 2 diabetes, hypertension, and other diseases [256,261–263].

The interacting principles of MetSyn have been proposed by Grundy [258] as obesity plus genetic factors and endogenous metabolic susceptibility, such as manifested by insulin resistance and other characteristics [256,258]. Along with the multiple risk factors listed in the paragraph above and the laboratory test results listed below, a diagnosis of MetSyn can be made, although there is still some discussion as to the relative merits of using MetSyn as a diagnosis in clinical practice [264]. Insulin resistance, increased abdominal fat, genetic factors, physical inactivity, advancing age, inflammation and endocrine dysfunction also help establish the metabolic susceptibility of MetSyn, which when combined with additional laboratory risk factors, such as high LDL, low HDL, high triacylglyerols, elevated blood glucose, elevated plasminogen activator inhibitor-1 and c-reactive protein, among other tests, also increase dramatically the likelihood of life-threatening MetSyn-associated diseases later in life [257,265].

One of the initial signs of MetSyn is insulin resistance [261]. Insulin secreted by the pancreatic (beta) cells in response to increased circulating levels of glucose and amino acids is essential for development, growth, apoptosis and maintenance of glucose homeostasis by regulating gene expression and carbohydrate, lipid and protein metabolism [266]. Insulin regulates glucose homeostasis by reducing hepatic output and increasing the rate of glucose uptake in tissues as well as increasing lipid synthesis in liver and fat cells and reducing triglyceride breakdown in fat and muscle. When the circulating concentrations of insulin are insufficient to regulate the above processes, insulin resistance occurs. This, in turn, can lead to clinically diverse syndromes, such as type A syndrome, leprechaunism, Rabson-Mendenhall syndrome and often type 2 diabetes [267].

Insulin resistance is one of the primary events in the development of MetSyn, and it is thought to induce the biochemical, pathophysiological and clinical sequelae of MetSyn and eventually its associated diseases [257]. Houston and Egan [267] have listed several factors that are involved in insulin resistance and MetSyn: (*i*) multiple genes (thus it is a polygenetic disorder), (*ii*) epigenetic contributions (nutrition, low birth weight, etc.), (*iii*) visceral obesity, (*iv*) body-mass index, (*v*) caloric and carbohydrate intake, (*vi*) sedentary lifestyle, (*vii*) age, (*viii*) ethnicity, (*ix*) gender, (*x*) menopausal status, (*xi*) alcohol consumption (*xii*) inflammation and (xiii) dysbiosis. One of the emerging inflammation mechanisms involved in the generation of metabolic disorders is the activation of the NLRP3 inflammasome, via various triggers, including mitochondrial DAMPs [268], lysosomal membrane disruption [269] and high fat diets [270] through the generation of the cytokines IL-1β and IL-18. The activation of this inflammation complex contributes to the development of visceral adiposity and insulin resistance. Because of its wide distribution in different tissues and organs, the NLRP3 inflammasome protein complex may represent a crucial signaling pathway that facilitates organ crosstalk and local injury in tissues target of metabolic damage [271].

The significant role of inflammasome components in the generation of altered gut microbiota composition has recently demonstrated that NLRP3 inflammasome regulates both the gastrointestinal microbiome and is activated by pathobionts and associated dysbiosis, which affects host susceptibility to metabolic disease onset and progression beyond the gastrointestinal tract, including obesity and diabetes. In particular, the NLRP3 modulation of the intestinal microbiota through multiple inflammasome components has been demonstrated to be a significant determinant of NAFLD/NASH progression as well as many other aspects of MetSyn, including weight gain and glucose homeostasis [272].

In terms of cellular lipids, defects in the capacity to metabolize FAs and glucose are thought to play important roles in insulin resistance and MetSyn [273]. Accumulations of DAG, triacylglycerol and free FAs in non-adipose tissues correlate strongly with insulin resistance [274,275]. For example, increases in free fatty acids may play a role in blocking insulin signal transduction [265]. DAG, in particular, has been implicated in insulin resistance by activating distinct isoforms of protein kinase C, which in turn can directly modulate insulin signaling by phosphorylating and inhibiting the tyrosine kinase activity of the insulin receptor and activating genes responsible for FA-induced impairment of insulin action [275]. Gene expression modifications in adipose tissue are thought to be responsible for enhanced secretion of MetSyn-related factors, such as the pro-inflammatory cytokine TNFα and the tissue-specific protein adiponectin [276], and in muscle tissue decreased oxidative capacity and fat accumulation may also induce skeletal muscle insulin resistance and contribute to the development of type 2 diabetes [273].

Although no single lesion or gene can account for MetSyn and its associated diseases, various studies point to mitochondrial dysfunction as a major component, especially in the development of type 2 diabetes [273,277,278]. Gene expression studies have shown that there is a coordinate reduction in oxidative gene activities along with increased expression of several other genes in type 2 diabetes patients [279]. Using microarray assays to monitor gene expression clusters several oxidative genes were found to be down-regulated, supporting the notion that more generalized mitochondrial dysfunction occurs in type 2 diabetes [273]. This also correlated with reduced muscle ETC activity [277] and decreased whole body anaerobic capacity in type 2 diabetes patients [280]. In addition, in type 2 diabetics genetic polymorphisms have also been found that are involved in FA oxidation and in factors that control transcriptional activities (reviewed in [267]).

MetSyn and type 2 diabetes patients show reduced fat oxidative capacities [281]. In obese, pre-diabetic and diabetic patients free FA levels are increased together with decreases in fat oxidative capacity, and this can result in accumulations of FAs and acylglyerols in beta cells and other tissues, which have been shown to correlate strongly with insulin resistance [257,273,282].

Continuous production of superoxide and peroxide are necessary for normal cellular functions [283], but in MetSyn and associated diseases oxidants, mainly ROS/RNS, are over-produced [273,284]. Excess superoxide produced continually as a byproduct of normal mitochondrial respiration can directly damage iron sulfur center-containing enzymes, be converted to hydrogen peroxide (and ultimately to hydroxyl radical) and also react with nitrogen oxide to produce peroxynitrite, a very reactive RNS [285]. As discussed in previous sections, FAs are particularly sensitive to ROS/RNS oxidation, resulting in the formation of lipid peroxides, which are cytotoxic and lead to free-radical damage to other lipids, proteins and DNA, and this is especially important in MetSyn, type 2 diabetes and cardiovascular (CVD) and renal diseases [273,286]. Free FAs also accumulate, particularly in muscle cell mitochondria where ROS/RNS damage can occur, and there they are thus prone to peroxidative events that result in damage to mitochondrial membranes, proteins, DNA and the activation of the NLRP3 inflammasome [287]. Once mitochondrial membrane lipids have been modified, they are less likely to maintain the low levels of proton leakage and membrane fluidity required to maintain the proper MIM membrane potential.

Uncoupling proteins (UCPs), such as UCP2 and UCP3, are involved in regulating ETC activity, and UCP3 and other UCPs also prevent build-up of excessive concentrations of ROS/RNS by limiting oxidative phosphorylation [285]. Also, it has been suggested that UCP3 functions to remove FA anions formed by oxidative reactions that can build-up during excess FA partitioning into the mitochondria [284]. These FA anions can cause reactions with other lipids, proteins and DNA. Type 2 diabetes patients have been found to have about one-half the normal levels of UCP3 in their skeletal muscles [288].

Pancreatic beta cells contain mainly UCP2, and while activation of UCPs can reduce ROS/RNS production in peripheral tissues, it may disrupt glucose-stimulated insulin secretion in beta cells [289]. Thus the reduced levels of UCPs in MetSyn and type 2 diabetic patients could indicate a defective feedback mechanism between ROS-lipid peroxides and mitochondrial protection against fat accumulation, and thus could contribute to oxidative mitochondrial damage [273].

Type 2 diabetes is thought to occur as a consequence of persistent hyperglycemia which causes: (*i*) formation of advanced glycation end products (AGEs, the products of nonenzymatic glycation and oxidation), and their oxidation and interactions with cell receptors and cellular accumulation; (*ii*) activation of various isoforms of protein kinase C; (*iii*) induction of the polyol pathway; and (*iv*) increased hexosamine pathway flux [289–291]. Most of these pathways are associated with elevated oxidative stress and over-production of superoxide (and thus ROS/RNS) during hyperglycemia, but the link between hyperglycemia and increased mitochondrial superoxide production may not be mediated solely by the mitochondrial redox state [289]. Therefore, an increase in mitochondrial ROS/RNS in response to hyperglycemia is the defect that likely leads to the pathological consequences of hyperglycemia. Moreover, hyperlipidemia as a consequence of obesity results in increased FA oxidation products that stimulate insulin secretion, resulting in hyperinsulinemia. This, in turn, down-regulates insulin receptors, reducing insulin action and increasing blood glucose levels [292]. Mitochondrial excess oxidative stress likely contributes to progression to type 2 diabetes by disrupting the ability of beta cells to respond to elevated blood glucose [289]. Eventually, apoptosis is stimulated by excess ROS/RNS, resulting in loss of beta cells by apoptosis and hence reduced production of insulin [293].

Preventing damage to cellular and mitochondrial membranes is important in preventing loss of electron transport function and cellular energy seen in MetSyn and type 2 diabetes [292]. This can be accomplished, in part, by neutralizing excess ROS/RNS with various types of antioxidants or increasing free-radical scavenging systems [289,292,294]. In MetSyn and diseases caused or promoted by excess ROS/RNS dietary supplementation with low molecular weight antioxidants, plus some replacement of accessory molecules, such as the metal ion cofactors zinc, manganese, copper, vanadium, chromium and selenium necessary for antioxidant and other enzymes, and certain vitamins with some antioxidant properties (C, E, A, CoQ_{10}) can be used to maintain antioxidant levels and free-radical scavenging systems [267,289,292,295,296]. However, supplementation with low molecular weight antioxidants, enzyme and other cofactors and vitamins, may not be sufficient to maintain cellular components free of ROS/RNS damage, and antioxidants alone cannot replace damaged cellular components, especially the phospholipids in membranes [295,296]. In addition, once extensive damage and cell death has occurred over time, it may be impossible to reverse these changes by supplementation alone [296].

Patients with MetSyn and type 2 diabetes have been found to be deficient in certain antioxidant vitamins and minerals, correlating with oxidative excess in these patients [296–298]. However, despite the evidence for a connection between excess oxidative stress in MetSyn and associated diseases, a link between the intake of antioxidant nutrients, even in high concentrations, and the ability to prevent or delay MetSyn disease progression to type 2 diabetes has been difficult to prove [296–299]. Often randomized, controlled clinical trials failed to show any significant benefit of antioxidants, whereas initial cohort studies suggested otherwise [300].

Since MetSyn and associated diseases show excess ROS/RNS damage to membranes and other structures, LRT should be useful in replacing damaged membrane components and restoring unoxidized phospholipids in blood lipoproteins. In fact, administration of membrane phospholipids along with changes in diet can help remove oxidized phospholipids and cholesterol from HDL and LDL [301]. For example, treatment of type 2 diabetic patients with oral LRT resulted in decreased serum triglyceride levels (37% reduction over 12 months) and reduced lipid peroxidation products compared to placebo [302]. In terms of LRT producing reduced blood peroxidation products, one study showed a significant reduction in the levels of acyl-hydroperoxides, of Schiff's bases, diene/triene conjugates as well as MDA in patients who were taking membrane phospholipids [303]. In contrast, there have been variable results with fasting glucose levels in type 2 diabetics on oral EPL [257], but some studies have found a significant reduction in blood sugar levels in patients with type 2 diabetes given 1.2 g of oral phospholipids for 60 days compared to a control group with diet alone [304].

MetSyn also appears to be a precursor condition to hypertension and CVD, such as atherosclerosis, heart diseases and stroke [256,258,260,261,267]. Damage to the endothelium causes endothelial dysfunction and impaired release of nitric oxide and loss of its antiatherogenic and other properties. At the early stages of insulin-resistance, before the development of MetSyn in obese young adults, reductions in vascular smooth muscle nitric oxide vasodilatory capacity

have been seen using positron emission tomography [305]. Thus insulin resistance is linked to endothelial dysfunction through nitric oxide-mediated vasodilation. Vascular dysfunction may be one of the initial steps in the development of hypertension, MetSyn, type 2 diabetes and CVD. Nitric oxide formed in endothelial cells by the action of endothelial nitric oxide synthase (eNOS) can be inactivated by superoxide anion radical to form the NRS peroxynitrite anion, which can cause further oxidative damage and depress nitric oxide-related, endothelial-dependent, acetylcholine-induced arterial relaxation [305]. In MetSyn many of the altered blood components, such as excess free FAs and LDL, also decrease eNOS activity [306]. A decrease in eNOS and an excess of angiotensin-II synthesis or action causes vasoconstriction, growth promotion and pro-thrombotic, pro-inflammatory, pro-oxidant states [267].

Endothelial cell dysfunction can also be caused by inflammatory cytokines and infiltration by inflammatory macrophages. For example, adipose tissue plays a role in endothelial dysfunction by producing pro-inflammatory cytokines, such as IL-6 and TNFα as well as other factors. Molecules like TNFα activate the important NF-κB transcription factor and can indirectly induce serine phosphorylation of the insulin receptor, thereby interfering with insulin receptor signaling pathways [307,308]. Activation of the NF-κB transcription pathway also increases production of nitric oxide [309]. Adipose cells express receptors for inflammatory cytokines, and the infiltration of adipose tissue by inflammatory macrophages stimulates over-production of ROS, which is a common feature of obesity [310].

Conditions like hypertension are directly related to vascular dysfunction and MetSyn, which can be preceded by insulin resistance for decades before becoming apparent in most patients [311]. As indicated above, the primary abnormalities associated with hypertension include loss of eNOS and reduced nitric oxide availability, up-regulation of the MAPK pathway, inflammation of the vascular endothelium and accumulation of angiotension converting enzymes, collagen overproduction and other factors [312–314]. Hypertension is also linked to insulin resistance, excess oxidative stress, mediated mainly by ROS/RNS, and changes in endothelial and smooth muscle cells that eventually result in vascular inflammation. In endothelial cells excess ROS/RNS can affect a variety of cellular targets and can initiate apoptosis and modify gene expression [315].

Atherosclerosis involves chronic inflammatory damage to blood vessels due to lipid accumulation, inflammatory response, vessel cell death and thrombosis, which can eventually result in the occlusion of coronary vessels and heart disease. Atherosclerosis is characterized by a number of risk factors, including abnormalities in lipoprotein subclass distribution, increases in vascular acute phase response proteins, changes in vascular endothelial cell adhesion molecules and certain cytokines [316,317]. ROS/RNS play an essential physiological role in maintaining vascular integrity, but when they are in excess, they serve a pathological role in cardiovascular dysfunction. In addition to type 2 diabetes and hypertension, excess production of ROS/RNS is also associated with atherosclerosis, ischemic heart disease and congestive heart failure [282,316].

The sensitive immune-based, Toll-like receptors (TLRs) are also recognized as key orchestrators of atherosclerosis, which is driven by inflammation. The pattern-recognition receptor CD36 coordinates the intracellular conversion of oxidized LDL, amyloid-beta, and amylin peptides, into crystals or fibrils, which results in lysosomal disruption and activation of the NLRP3 inflammasome in atherosclerosis, Alzheimer's disease, and diabetes. CD36 has been found to be a central regulator of inflammasome activation in sterile inflammation associated with DAMPs. The ligation of CD36 may possibly be the common molecular event that links the recognition of sterile ligands with priming and activation of the NLRP3 inflammasome in atherosclerosis, Alzheimer's disease and type 2 diabetes [318].

Atherosclerosis is thought to begin with abnormalities in lipoprotein subclasses, such as triglyceride-rich lipoproteins, their remnants, and changes in HDL and LDL, hallmarks of MetSyn [319]. During the development of MetSyn these lipoproteins and their remnants are susceptible to oxidation [320], and the presence of the oxidized lipoprotein subclasses is significantly associated with an abundance of macrophages in atherosclerotic lesions [321]. When they interact with the blood vessel wall, the oxidized, proinflammatory lipoprotein subclasses can induce endothelial adhesion molecules, resulting in selective leukocyte recruitment, attachment to the endothelium and transmigration into the intima [322,323]. These leukocytes differentiate into inflammatory, ROS-producing macrophages that are abundant within thickened vessel walls. As this occurs, smooth muscle cell numbers decline and foam cells form that release growth factors, cytokines, metalloproteinases and more ROS/RNS, which perpetuates and amplifies vascular remodeling [324]. Unstable atherosclerotic plaques form slowly over time, and eventually some of the unstable plaques break off and form thrombi that can occlude blood vessels and interrupt blood flow. When this occurs in the heart, myocardial infarction, ischemia, heart failure and sudden death can occur.

Endothelial and adipose dysfunction and insulin resistance are thought to be among the most basic physiologic abnormalities that link MetSyn and CVD [305,325]. The exact mechanism of endothelial dysfunction and insulin resistance and the contribution of dyslipidemia remain only partially known. Hsueh and Quiñones [305] have argued that endothelial dysfunction occurs early in the pathogenesis of insulin resistance, MetSyn and the development of related diseases, suggesting that vascular damage associated with excess oxidation, inflammation and thrombosis is a primary event in the development of MetSyn, CVD and other diseases. Also, since macrophages are also recruited to adipose tissue, changes occur in adipose cells in parallel with changes in endothelial cells, such as induction of secreted adipokines (IL-1, IL-6, leptin, c-reactive protein, serum amyloid A, plasminogen activator-1, chemerin and others) [325].

Although it is doubtful that LRT can modify or reverse late-stage changes described above that result in type 2 diabetes, atherosclerosis or CVD, use of membrane phospholipids such as PC can change the composition and oxidation state of circulating lipoproteins. For example, intravenous administration of polyunsaturated PC to laboratory animals resulted in the rapid uptake of 50–80% of the PC into HDL and about 20% into LDL and very-low LDL, whereas oral PC administration resulted in about 60% of the PC being incorporated into HDL and less than 20% into LDL fractions within 36 h [326]. Interestingly, the administration of PC resulted in removal of cholesterol from serum lipoproteins and membranes. The transported cholesterol was deposited mainly in the liver [327]. In a long-term (10 year) experiment using seven rhesus monkeys fed high cholesterol diets (120 mg cholesterol per 100 g of diet), Wong et al. [328] found that seven weeks of oral lecithin in their diets significantly lowered total cholesterol, LDL cholesterol and triglyceride levels. Other animal studies have also shown that administration of LRT resulted in reductions in cholesterol, LDL-cholesterol and triglycerides (reviewed in [257]). In an interesting study using mini-pigs fed a cholesterol and coconut oil diet for 24 weeks Samochowiec et al. [329] found that the serum levels of triglycerides, cholesterol free FAs and beta-lipoprotens gradually increased with time. Using this model to study the effects of LRT EPL was administered (up to 280 mg/Kg body weight), and after 8 weeks of treatment Samochowiec et al. found a dose-related reduction in total lipids, cholesterol esters, free cholesterol and triglycerides [330]. At the highest doses used there was a reduction in atherosclerotic plaques observed in the aortas and heart valves.

In addition, increased lipid peroxidation is one of the first changes associated with the development of MetSyn, and it is thought to be important in hypertension, type 2 diabetes, atherosclerosis and CVD. According to this hypothesis oxidized lipoproteins, such as oxidized LDL, play an important role in promoting MetSyn-associated diseases, and agents that reduce lipoprotein lipid oxidation can inhibit or attenuate the pathogenesis of these diseases [331,332]. In fact, LRT has been shown to reduce lipid peroxidation in patients with ischemic heart

disease [333]. For example, Serkova administered oral LRT (1.8 g phospholipids) for 3 weeks to a group of patients with angina pectoris and at the end of treatment found that there was a significant reduction ($p < 0.01$) in oxidized serum lipids, an increase in HDL cholesterol and a reduction in erythrocyte hemolysis due to peroxidation [333].

Use of LRT in a controlled clinical setting has demonstrated that blood levels of cholesterol, LDL-cholesterol and triglycerides can be reduced. For example, a risk for long-term dialysis patients is ischemic cardiovascular complications, and these patients tend to have high lipid values. To study this two groups of ten patients who had hyperlipidemia (serum cholesterol greater than 260 mg/dl, LDL cholesterol greater than 180 mg/dl and triglyerides greater than 200 mg/dl) were given 2.7 g per day oral PC or placebo for 6 weeks [334]. In this double-blind, randomized study the 6 week treatment was followed by a two-week wash-out phase, and lipid parameters were determined at 2, 4 and 6 weeks of treatment. Two weeks after PC administration there was a reduction in LDL-cholesterol of 32 mg/dl compared to the stable placebo controls ($p < 0.01$). By four weeks triglycerides decreased by 58.2 mg/dl ($p < 0.001$) and by six weeks there was a reduction in triglycerides of 43.3 mg/dl compared to the placebo group (4 weeks, −5.7 mg/dl and 6 weeks, −11.4 mg/dl, $p < 0.01$). There were few side effects of the treatment, and they were similar in both test and placebo arms of the study [334]. Patients with hyperlipidemia were also tested with oral PC. In a double-blind study of patients with type II hyperlipidemia participants received either three doses of oral polyenylphosphatidylcholine (0.9 g per day) or placebo, and their blood lipid levels determined [335]. Total cholesterol and LDL-cholesterol were lowered significantly, and there was a downward trend in apoprotein B, triglycerides and VLDL-cholesterol and an upward trend in apoprotein A1 compared to the placebo group [335]. As discussed above, administration of membrane phospholipids along with changes in diet and related caloric restriction as well as suitable macro- and micro-nutrient concentrations can help replace oxidized phospholipids and cholesterol from HDL and LDL [301].

Thus there is the potential to reverse some of the lipid changes that are important in MetSyn development and possibly prevent other diseases with the use of LRT. It is interestingly that long-term use of LRT in the form of oral NTFactor and vitamins reduced significantly markers for CVD risk, such as homocyteine, erythrocyte sedimentation and fasting insulin levels [156]. In a group of patients with all of the above factors above the threshold for risk, LRT using oral NTFactor resulted in return of average test results to the normal ranges within 6 months [156]. Future studies should document whether LRT can impede or change the course of the development of MetSyn and its subsequent associated diseases.

14. Final comments and future directions

In this review we have concentrated on a few examples of how LRT can be used to repair and replace oxidatively damaged membrane glycerophospholipids and restore function. Using mitochondria as an example of a critical membrane-bound enzyme and electron transport system we have discussed how LRT can be used to restore MIM transmembrane potential and recover ETC function. Indeed, multiple clinical trials have proven the usefulness of LRT in reducing the symptoms associated with loss of mitochondrial function and improving quality of life in patients with a variety of diagnoses as well as providing important anti-aging effects on important cellular structures [336]. In addition to the uses of LRT described in this review, it has been used in laboratory animals and humans (mainly anecdotally) to treat toxic liver and kidney damage caused by carbontetrachloride, alcohols, glactosamine, acetaminophen, tetracycline, solvents, detergents, thioacetamide, indomethacin, anesthetics, ionizing radiation, immune-mediated hepatitis and others. LRT reduced the toxic effects of these agents and promoted organ regeneration after exposure [1]. In humans LRT has been used mainly in uncontrolled studies to treat damage caused by fat embolism, non-steroidal anti-inflammatory drugs, liver-damaging anti-microbial drugs, lethal hepatic toxins, fatty liver due to malnutrition and viral hepatitis (reviewed in [2]).

The treatment of viral hepatitis using membrane phospholipids has been extensively investigated in uncontrolled and controlled clinical trials [337,338]. For example, Wallnöfer et al. [337] found that hepatitis patients treated with intravenous EPL usually reported earlier improvements in dyspepsia, nausea, epigastric pain, fullness in the epigastrium and other symptoms as well as showing improvements in hepatomegaly and presence of ascites. Laboratory tests also improved, such as more normal blood liver enzyme levels, total protein, bilirubin, among other tests, and histological analysis of liver biopsies indicated earlier regeneration of hepatocytes [337]. In a blinded, controlled trial Kordac et al. [338] treated 20 patients with active, chronic hepatitis for one year with intravenous EPL. In the treatment group there was a significant reduction in hepatomegaly ($p < 0.01$), and liver enzymes, such as γ-glutamyltransferase, hepatic excretory capacity (bromsulphalein test) and γ-globulin levels ($p < 0.05$), and other parameters, such as serum albumin levels, also improved compared to controls. Although most results on treatments of patients with chronic hepatitis with intravenous EPL tended to be variable, in general, the patients with the more severe forms of hepatitis with lowest liver function and highest blood liver enzyme levels responded best with greater improvements over time [339,340].

Treatments with intravenous membrane phospholipids have also clinically improved cirrhosis of the liver and decreased time required for recovery. Pogromov et al. [341] treated 25 patients with advanced liver cirrhosis with oral membrane phospholipids. After 3 months, nearly all blood biochemical parameters improved and were found to be within the normal range. Kalab and Cervinka [342] also found improvements in clinical and blood biochemical tests in 30 patients treated with EPL for 6 months, and in particular IgA decreased to within normal range in these patients. In other studies, patients with moderately severe to severe cirrhosis caused by type B hepatitis virus were treated with intravenous EPL for 3 months [343]. Thirty-seven patients were given EPL and compared to 27 patients who received a vitamin preparation alone. In the control group there were no changes in liver function from pre-treatment values, but in the treatment group there was a significant improvement in liver function and in 63% of the test group there was an absence of hepatitis B antigen but this was seen in only 14% of the control group [343].

LRT has also been used in chronic ambulatory peritoneal dialysis (CAPD). Among the first to use administration of membrane phospholipids to CAPD patients, di Paolo et al. [344] found that CAPD patients not only released electrolytes, creatinine and urea into the ascites fluid, they also secreted phospholipids and other membrane-derived material which was removed by dialysis. The released membrane phospholipids were thought to improve peritoneal dialysis by lowering surface tension. Therefore, they supplemented CAPD dialysis with 250 mg of membrane phospholipids like PC intravenously or 400 mg orally each day and found significant increases in mean CAPD ultrafiltration within 72 h. There were also significant increases in creatinine and urea clearance in the treated patients receiving CAPD, and they were able to restore normal physiological conditions in patients with abnormal but not in patients with normal ultrafiltration rates [344].

During pregnancy gestosis or toxemia of pregnancy can occur where patients display hypertension, edema and proteinuria [345]. Gestosis is thought to be caused by chronic intravascular clotting and fibrin deposition in the uteroplacental bloodstream, which can affect uteroplacental perfusion and fetal development [345]. Risk factors include pre-existing vascular conditions, chronic nephropathies, liver dysfunction and diabetes mellitus. The more severe the clinical presentation the higher the levels of lipid peroxidation products found in the serum and erythrocyte membranes of these patients [346]. To treat these conditions patients were given intravenous or oral membrane phospholipids. For example, using 52 patients Bottiglioni and Tirelli [347] administered EPL twice daily at a dose of 500 mg per day in last

trimester of pregnancy and found that clinical symptoms disappeared. Edema subsided, and liver and kidney function tests were normalized.

There are a number of studies that show that membrane phospholipids given orally or intravenously improve a variety of neurological conditions. Examples include cerebral circulatory disorders, involutional dementias, Friedreich's ataxia, multiple sclerosis, encepthalomyelitis, neurotoxicosis and other conditions (reviewed in [2]). Although these studies are for the most part preliminary, they point to the potential use of membrane phospholipids to treat a variety of acute and chronic neurological conditions.

In future studies on the use of membrane phospholipids for LRT more attention must be directed at determining if the clinical and biochemical changes brought about by the use of LRT actually cause long-term or only temporary changes that are slowly reversed in time. This was apparent in previous cross-over studies on chronic fatigue patients where fatigue slowly returned and mitochondrial function slowly decayed after placebo was substituted for membrane phospholipids [166]. In clinical situations where an acute toxic insult is removed during or before treatment, such as in the use of LRT to reverse the toxic or poison exposures listed above, it seems reasonable that after the insult is removed and cellular damage repaired that LRT will no longer be necessary. However, in most chronic conditions the presumed insult(s) are not temporary and may continue for an unforeseeable period of time without dietary and behavior modifications. In these cases LRT may be a lifelong requirement to maintain health.

The role of LRT in maintaining mitochondrial function is considered particularly important in reducing the effects of aging and helping provide for a healthy lifestyle [336]. Typical 21st century sedentary life favors chronic metabolic oversupply [348], a physiological state that promotes mitochondrial fission and fragmentation and damages mitochondrial membranes through mitophagic inhibition. This can alter mitochondrial membrane function, mutate mtDNA and compromise cellular structure and function. In contrast, caloric restriction or pulsing between restriction and modest increases in caloric intake, including nutrient dense foods and LRT and exercise can induce a state of metabolic undersupply that triggers mitochondrial fusion and appropriate mitophagy. This establishes a life-sustaining intracellular state that promotes cell survival and maintains the integrity of mtDNA. A healthy balance of both sides of mitochondrial dynamics (fusion and fission) promotes healthy cellular adaptation [349].

In addition, other endogenous cellular components, enzymes, messengers and metabolites, among other important cellular molecules, are sensitive to oxidative damage, especially damage from excess generation of ROS/RNS. There is increasing recognition of the NLRP3 inflammasome pathway in triggering and responding to sterile inflammation. Such inflammation of non-infectious origin has put this innate immune sensor at the crossroads of metabolic disease and inflammation. The accumulation of mitochondrial DAMPs during chronic inflammatory diseases is increasingly hypothesized to contribute to low-grade inappropriate systemic inflammation and disease pathogenesis. LRT provides us with the an opportunity to beneficially modify mitochondria-promoted DAMPs and reduce endogenous activation of persistent low grade inflammation and in doing so reduce one of the most significant causal factors in metabolic and aging illnesses — inflamm-aging [350].

Maintenance of MIM trans-membrane potential and coordination of ETC and uncoupling reactions keeps ROS/RNS at normal physiological levels. LRT helps to repair and replace damaged mitochondrial membrane phospholipids to maintain "healthy" mitochondrial membranes [336]. It is likely that LRT may also be important in maintaining other membrane structures, such as endoplasmic reticulum, nuclear, Golgi and other membranes. It is also likely to be equally important in the maintenance and function of various plasma membranes, including the membranes of neural, immune, gastrointestinal, vascular, musculoskeletal and other cells. Future studies will undoubtedly focus on the use of LRT to modify and repair various tissue and cellular membrane systems.

Finally, although this review has focused on the use of LRT in restoring cellular membrane functions, it can also be used to have new, selective effects, such as modifications of membrane-drug and –messenger interactions [351]. For example, it is known that the membrane recruitment, localization and functions of heterotrimeric, dimeric and subunits of messenger G proteins ($G\alpha\beta\gamma$, $G\beta\gamma$ and $G\alpha$) are dependent on membrane phospholipids, and these interactions can be modified by modulation of the membrane phospholipid organization through substitution of particular glycerophospholipids and their FAs [352,353]. Thus designing specific LRT using particular phospholipids with certain FA acyl chains that have specific effects on cellular functions, such as drug and messenger effects, could be useful in developing new combination therapies for a variety of conditions and diseases [351].

Acknowledgment

We thank Claire Gardin for the expert assistance in preparing this manuscript and John Michael for the artwork assistance.

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