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Review

Glued in lipids: Lipointoxication in cystic fibrosis

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ABSTRACT

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutations in the CF transmembrane regulator (CFTR) gene, which encodes a chloride channel located at the apical surface of epithelial cells. Unsaturated Fatty Acid (UFA) deficiency has been a persistent observation in tissues from patients with CF. However, the impacts of such deficiencies on the etiology of the disease have been the object of intense debates. The aim of the present review is first to highlight the general consensus on fatty acid dysregulations that emerges from, sometimes apparently contradictory, studies. In a second step, a unifying mechanism for the potential impacts of these fatty acid dysregulations in CF cells, based on alterations of membrane biophysical properties (known as lipointoxication), is proposed. Finally, the contribution of lipointoxication to the progression of the CF disease and how it could affect the efficacy of current treatments is also discussed.

1. Introduction

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutations in the CF transmembrane regulator (CFTR) gene. CFTR is located at the apical surface of epithelial cells and CFTR dysfunction leads to altered chloride transport. Improper chloride transport results in mucus dehydration, which becomes thick and sticky, causing many of the symptoms associated with CF. More than 2000 mutations grouped into 5 to 7 classes have been described to date depending on their impacts on the CFTR protein [1]: Class I mutations directly impact protein production and mainly correspond to nonsense mutations; Class II mutations, which include the most prominent mutation F508del-CFTR, result in protein misfolding and affect the trafficking of the CFTR protein which is retained at the endoplasmic reticulum (ER) level by the ER quality control; Class III mutations are associated with altered gating of the CFTR channel; Class IV mutations result in a decrease in CFTR channel conductance of chloride and bicarbonate ions; Class V mutations lead to a strong decrease in the levels of the CFTR protein, mostly related to alternative splicing; Class VI mutations reduce CFTR stability at the apical surface; Class VII mutations, such as large gene deletions, are considered as unrescuable mutations, since they cannot be pharmacologically rescued *per se*.

Even if apparently difficult to directly connect to CFTR dysfunction, at least at first glance, disturbances in lipid metabolism have been a persistent observation in CF, and this since more than 50 years, long before the identification of the CFTR gene [2, 3]. More particularly, such disturbances are characterized by low levels of so-called Essential Fatty Acids (EFA), namely Linoleic Acid (LOE) and docosahexaenoic acid (DHA), in plasma and tissues from patients with CF [4]. However, if these aspects have been of interest for many researchers until then, apparent discrepancies have emerged among studies, probably related to the analysis methods used, the kind of lipid species analyzed (if the EFA can be found in the organism as free species, they can also be esterified into complex lipids), and intrinsic variations between tissues. Moreover, due to these apparent disparities, clear connections between these lipid dysregulations and the course of the disease have been difficult to establish, raising the reasonable question of such alterations being a second-side effect or, rather, an important parameter to consider, as a potent contributor to the severity of the disease [5].

In this context, a first goal of the present review will be to pinpoint a general consensus from previous studies on the nature and the potential origins of the fatty acid dysregulations observed in CF, taking into consideration the lipid class and the tissue studied. In a second step, based on the literature generated with other models displaying similar lipid dysregulations, we will highlight the common impacts generated by such disorders on cell function, and their relevance to the CF phenotype. Finally, we will propose a unifying mechanism based on the effects of EFA deficiency on the properties of cellular membranes and how it could explain, at least in part, the progression of the CF disease and the limits which are reached with current treatments.

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2. Fatty acid homeostasis: general principles

The main pathways of fatty acid homeostasis are summarized in Fig. 1.

Fatty acids vary one from the other on the basis of two criteria: the length of their acyl chains and the number of double-bonds, also referred to as unsaturations. The number of unsaturations is used to classify fatty acids as Saturated (Saturated Fatty Acids; SFA), Monounsaturated (Mono-Unsaturated Fatty Acids; MUFA) and Polyunsaturated (PolyUnsaturated Fatty Acids; PUFA). A Prime example of SFA is Palmitate, the main constituent of Palm oil, bearing 16 carbons in its acyl chain and no unsaturation (16:0). The most common MUFA is Oleate (18:1), which is exquisitely enriched in plant oils, such as Olive oil. If these species can be obtained from the diet, it has to be noted that mammalian cells display the machinery to build up their own SFA and MUFA. SFA are synthesized in the cytoplasm on a soluble fatty acid synthase (FAS) [6]. SFA are then converted to their MUFA counterparts by stearoyl-CoA desaturases (SCDs), a family of Δ-9 fatty acid desaturases, which are embedded in the ER membrane, and are strictly oxygen-dependent. SCD1 appears to be the predominant isoform and is expressed ubiquitously among tissues [7]. SCD1-preferred substrates are Palmitate and Stearate (18:0), which are converted to Palmitoleate (16:1) and Oleate (18:1), the major MUFA of membrane phospholipids and storage-neutral lipids (Triglycerides (TG) and Steryl Esters; see below) [8].

By contrast, the body cannot synthesize PUFA "from scratch" and must therefore obtain basic blocks from the diet, which are referred to as essential fatty acids (EFA; Fig. 1). There are two families of EFA, which are classified on the basis of the position of the first double-bond from the last carbon of the acyl chain (the omega (ω) carbon): ω-3 and ω-6. The $\omega-6$ building block is linoleic acid (LOE; 18:2) and the ω-3′s is α-linolenic acid (LON; 18:3). End-products are synthetized from these precursors by successive desaturation and elongation steps in the ER [5]. The main end-PUFA species found in Man are the ω-6 Arachidonic Acid (AA) and the ω-3 Docohexaenoic Acid (DHA). Noteworthily, both AA and DHA can also be directly obtained from the diet. For example, DHA is present in fish oils.

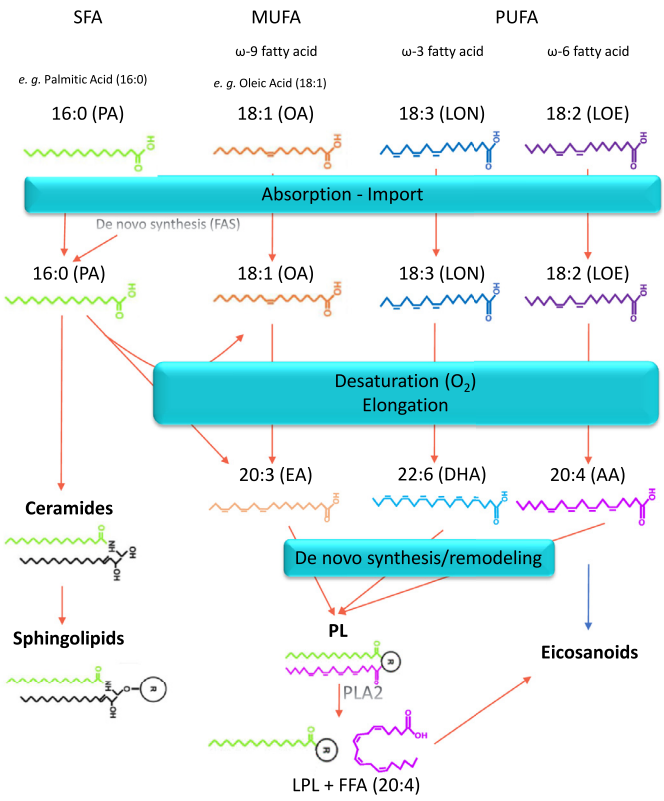

Fig. 1. An overview of cellular Fatty Acid homeostasis. The main pathways modulating the repertoire of fatty acids available to the cell and the major pathways leading to their distribution within complex lipids are displayed. See text for details. SFA: Saturated Fatty Acid; MUFA: MonoUnsaturated Fatty Acid; PUFA: PolyUnsaturated Fatty Acid; PA: Palmitic Acid; OA: Oleic Acid; LON: Linolenic Acid; LOE: Linoleic Acid; EA: Eicosatrienoic Acid; AA: Arachidonic Acid; DHA; DocosaHexaenoic Acid; PLA2: Phospholipase A2; LPL: LysoPhosphoLipid; FFA: Free Fatty Acid; SPT: Serine PalmitoylTransferase; FAS: Fatty Acid Synthase.

As a consequence, cells from our body therefore dispose of a repertoire of fatty acids, which may however greatly vary depending on the organ considered (see below), originating from both exogenous (diet) and endogenous (synthesis) sources. The various fatty acids may thereafter have different fates (Fig. 1).

Virtually all fatty acids can enter the composition of membrane Phospholipids (PLs). Fatty acids can be esterified into PLs through two alternative pathways: the *de novo* pathway (or Kennedy pathway), which corresponds to the complete synthesis of a PL from elementary building blocks, and the remodeling pathway (or Lands cycle), which consists of the substitution of one acyl chain in a preformed PL [9]. The acyltransferases involved in these pathways have been identified and their substrate specificity accounts for the acyl chain diversity of PLs. Systematic analysis of fatty acid distribution within the PLs of various tissues revealed that each organ displays a characteristic signature: in rats, organs can be classified as highly SFA-, MUFA- or PUFA-enriched PL [10]. Relevant to the present review, among all the organs studied, the lung appears as the organ displaying the lower proportion of PUFA within its PLs [10]. Therefore, sculpting the fatty acyl content of PLs is the result of the contribution i) of the repertoire of available fatty acids in a given cell from our body, which may compete one with the other to enter the PL synthesis/remodeling pathways, and ii) of the enzymatic machinery that is turned on in this specific cell type (*e. g.* specific acyltransferases).

Excess fatty acids can also be stored in form of their neutral esters, Triglycerides (TG) and Steryl esters (SE), in a process that occurs in the ER [11]. However, whereas levels of TG are high in cells exposed to MUFA, SFA generally poorly accumulate within TG [12]. Instead, SFA preferentially accumulate within other lipid species, including membrane PL, ceramides, or diacylglycerol (DAG), an intermediate in TG synthesis [12, 13].

The PUFA AA is also an important lipid since it is the precursor of inflammatory mediators known as eicosanoids, which include prostaglandins (PG), leukotrienes, and thromboxanes. ([5]; Fig. 1). Interestingly, if AA can be generated by *de novo* synthesis from LOE (see above), it can also be released from PLs by the selective action of Phospholipase A2 (PLA2), a family of enzymes that catalyze the hydrolysis of ester bonds at the sn-2 position of phospholipids, generating a free fatty acid and a lysophospholipid [5].

To summarize, maintaining the correct balance between the various fatty acids is extraordinary complex and greatly varies depending on the available building blocks and the most operational metabolic/catabolic pathways in the cell type/tissue considered.

3. Constant dysregulation of fatty acid profile in CF

The first evidences describing altered fatty acid profiles in cystic fibrosis have been reported more than 50 years ago [2, 14, 15], and since then, many consecutive studies have reported similar findings. However, many discrepancies still exist between several studies. This is likely due, at least partly, to the fact that different methods for fatty acid characterization/quantification and tissue samples have been used. To establish clear comparisons, it is important to consider whether the fatty acid profile that is provided in a given study corresponds to free fatty acids (NEFA; *i. e.* non-esterified within complex lipids, such as TG and PLs), total fatty acids (total FA; free and esterified in various (if not all) complex lipids) or fatty acids within a given

lipid species (TG or PL, for example). This is, of course, highly dependent on the methods used for lipid extraction, separation (if any) and analysis (mainly Gas Chromatography and Mass spectrometry). Moreover, if we focus on the specific case of PLs, it is well-established that the fatty acid signature greatly depends on the tissue/cell type considered, and that no extrapolation can easily be made between various tissues if the same specific determinations have not been driven in all the relevant organs with the same method (see above).

Concerning cellular and animal models of CF, important parameters should be considered to avoid inappropriate extrapolations to CF patients and misinterpretations related to lipid-associated phenotypes.

First, *in vitro*, one should keep in mind the culture conditions can greatly influence the fatty acid signature of the cells. For example, knowing that the main fatty acid desaturases are strictly oxygen-dependent (see above), oxygen scarcity, which is encountered in patient lungs suffering CF [16], results in an increase in SFA-enriched PLs in bronchial epithelial cells at the expense of MUFA-containing ones [17, 18]. Interestingly, most experiments performed on CF bronchial epithelial cells are generally performed under normoxia, therefore leading to higher MUFA/SFA ratios than encountered *in vivo*. The choice of serum for culture media supplementation is also an important point to consider. A very interesting example is the case of horse serum, which displays a fatty acid profile that matches at best the one encountered in the human plasma, particularly in terms of LOE concentrations [18, 19]. These observations suggest that great care should be taken before extrapolating the results obtained with cultured cells to *in vivo* situations, at least concerning the recapitulation of the fatty acid content.

Concerning animal models, one should consider that the fatty acid signature in organs greatly varies among species. For example, and relevant to the present review, PUFA constitute 16 and 26% of PC and PE in rat lungs, respectively [10], whereas, in human bronchial secretions, PUFA are found in very low levels in these PLs, corresponding to 0.8 and 9% of total fatty acyl chains in PC and PE, respectively [20]. Accordingly, the lower respiratory tract of the murine models appears to be poorly representative of the lower airways of CF individuals, therefore limiting their interest for the study of pulmonary disease progression in CF [21]. Finally, in animal models, the diet greatly influences the fatty acid signature in several organs, including the lungs, therefore generating possible discrepancies in lipidomic data and/or phenotype severity among various studies [10, 22].

Concerning this latter point, the exact same caution should be taken with CF patients, who may suffer metabolic diseases with various severities (pancreatic insufficiency, CF-related diabetes (CFRD), Cystic Fibrosis-related Liver Disease (CFLD)...) with well-known impacts on the circulating fatty acid distribution, and are exposed to uncontrolled diets or various diet supplements. Moreover, chronic infection with *Pseudomonas aeruginosa*, which is a recurrent situation encountered in CF, has been shown to influence the lipidomic signature in plasmas from CF patients [23]. Altogether, these parameters very likely influence the overall fatty acid balance, and render the identification of a homogenous definition of this balance very touchy among such an heterogenous patient population.

These elements being considered, some consensus however appear to emerge within CF individuals (Table 1).

First, decreased levels of the ω-3 series fatty acid DHA has been a consistent observation in various tissues/organs from CF patients. Decreased total DHA levels were observed in total FA in plasma [24-28], but also in nasal- and rectal-biopsy tissues [29] from CF patients. This decrease was also more specifically observed in the PL fractions in blood/plasma/serum [30-33] and cord serum samples [34].

Concerning the ω-6 series fatty acids, decreased LOE levels in the blood and tissues of CF patients have been a very consistent observation in the CF literature [5]. LOE decreased levels where observed in total FA extracted from plasma [24, 25, 27, 28], but also from Postmortem fat-, psoas muscle-, heart muscle-, liver- and lung-tissues and nasal-biopsies [25, 29]. As for DHA, this decrease was also more specifically observed in the PL fractions in blood/plasma/serum [25, 33, 35] and chord serum samples [34].

LOE being the precursor of AA (Fig. 1), one may expect to observe either an overall decrease of this long chain PUFA, due to limitations in precursor levels, or an increase, in case of an augmented flux through the ω-6 pathway. Indeed, both observations have been made (Table 1): AA levels have been reported to be either decreased, increased or unchanged in total FA extracted in Plasma from CF individuals [24-28], unchanged in Postmortem tissues [25] and rectal biopsies [29] and decreased in nasal biopsies [29]. AA levels were either unchanged or decreased in PL fractions from blood/serum/plasma [25, 30-33, 35], but were augmented in almost all PL species, except for PE, in bronchial secretions from CF patients [20].

To conclude this part, it appears that CF is associated with a global decrease in PUFA, and particularly in DHA and LOE. However, the intensity of this decrease highly depends on the lipid species considered, and, even when considering a specific species, depending on the organ/tissue on interest (Table 1).

Interestingly, parallel to this decrease in PUFA, increases in the levels of SFA, and more specifically Palmitate (16:0) and Stearate (18:0) have also been reported in CF individuals, particularly in circulating lipids, namely NEFA and Cholesteryl esters [36, 37] and in PL extracted from lung samples, *i. e.* bronchial secretions [20] and freshly dissociated bronchial epithelial cells [17, 18]. Altogether, these cumulative processes result in an overall FA imbalance towards more saturated species.

4. The potential origins of these dysregulations

The main pathways which could participate in fatty acid dysregulations are displayed in Fig. 2.

4.1. Malabsorption of precursors as an origin of these dysregulations

A common trait to most CF patients is exocrine pancreatic insufficiency, which results in altered fat absorption at the intestinal level and malnutrition. Such processes could account, at least in part and despite contradictory observations, in EFA deficiency [29, 38]. Alterations in chloride and water transport across duct cells result in a diminution in fluid flow by the gland, leading to duct obstruction [39]. On the long term, this leads to cellular damage and atrophy, resulting in a decrease in the secretion of digestive enzymes. However, even if supplementation with exogenous pancreatic enzyme displays beneficial effects, malabsorption cannot be fully circumvented in CF patients, as noted by persistent steatorrhea and malnutrition [40].

Another important determinant of EFA deficiency may be Cystic Fibrosis-associated Liver Disease (CFLD), which affects approximately 30% of patients and is now considered as the third cause of death in CF [41]. The CFTR protein is embedded in the apical membrane of the cholangiocyte. At this level, it regulates the amounts of electrolytes and water in the bile by affecting the transport of sodium and chloride ions and the secretion and alkalinisation of the bile. Bile alkalinisation regulates digestive functions by playing a central role in the solubility of its organic components. Since the bile breaks down complex fats into free fatty acids, which can be taken into the body by the digestive tract, bile alterations are likely an important contributor to EFA deficiency.

4.2. Increased flux through the ω-6 pathway

Another important observation is that the metabolic conversion of LOE to ω-6 metabolites, including AA, is upregulated in CF cells, indicating increased flux through the ω-6 pathway [42]. This likely occurs because loss of CFTR function, related to mutation, results in the

Table 1
Fatty Acid alterations in tissues from patients with Cystic Fibrosis.

	DHA decrease	LOE decrease	AA increase
Total Fatty Acids			
Plasma			
Witters *et al.*, 2013	Yes	Yes	No: decrease
Farrell *et al.*, 1985	Yes: influenced by the diet	Yes	No: decrease
Guilbault *et al.*, 2009	Yes	N.R.	Yes
Aldamiz-Echevarria *et al.*, 2009	Yes	Yes	No
Fat, Psoas Muscle, Heart			
Farrell *et al.*, 1985	N.R.	Yes	No
Nasal-Biopsy Tissues			
Freedman *et al.*, 2004	Yes; severity increases with P.I.	Yes.	Yes; severity may increase with P. I.
Rectal-Biopsy Tissues			
Freedman *et al.*, 2004	Yes	N.R	No
Phopholipids			
Blood			
Van Biervliet *et al.*, 2007	Yes	Yes	No
Plasma			
Guerrera *et al.*, 2009	Yes, especially in severe patients (decrease in DHA-containing PC (i.e. PC 38:6))	Probably (decrease in LA-containing PC (PC 36:2))	No: rather a decrease in AA-containing species (i. e. PC 36:4) in severe patients
Farrell *et al.*, 1985	N.D.	Yes	No
Grothe *et al.*, 2015	Yes: decrease in DHA-containing PC	No	No: decrease in AA-containing PC species
Serum			
Strandvik *et al.*, 2001	Yes	Yes	No
Maqbool *et al.*, 2008	N.R.	Yes	No: decrease in AA-containing PL species
Cord Serum			
Lloyd-Still *et al.*, 1991	Yes	Yes	Yes
Bronchial secretions			
Gilljam *et al.*, 1986	N.D.	Depends on the lipid species considered: decreased in PA and PE, increased in PC, unchanged in PI	Depends on the lipid species considered: increased in all PL species except for PE
Red Blood Cells			
Rogiers *et al.*, 1984 (b)	N.D.	Yes (PC analyzed)	Yes (PC analyzed)
Platelets			
Rogiers *et al.*, 1984 (b)	N.D.	Yes (PC analyzed)	No: (PC analyzed)
Rogiers *et al.*, 1984 (a)	N.D.	Yes (PC and PE analyzed)	No (PC and PE analyzed)
Triglycerides			
Plasma			
Farrell *et al.*, 1985	N.R.	No	No
Cholesteryl esters			
Plasma			
Farrell *et al.*, 1985	N.R.	No	No
Rogiers *et al.*, 1984 (b)	N.D.	Yes	No
Non-Esterified (Free) Fatty Acids			
Plasma			
Farrell *et al.*, 1985	N.R.	No	No
Rogiers *et al.*, 1984a	N.D.	Yes	N.D.

The most recurrent hypotheses that have been formulated concerning Fatty Acid dysregulations in CF tissues are decreases in DocosaHexaenoic Acid (DHA) and Linoleic Acid (LOE) levels and an increase in Arachidonic Acid (AA). Here, the data from various studies were separated depending on the class of the lipid analyzed and the tissue considered. The green color indicates that the data obtained fit the corresponding hypothesis, and the red color that contradictory results have been observed. N.R. not reported; N.D. not detected; P.I.: Pancreatic Insufficiency.

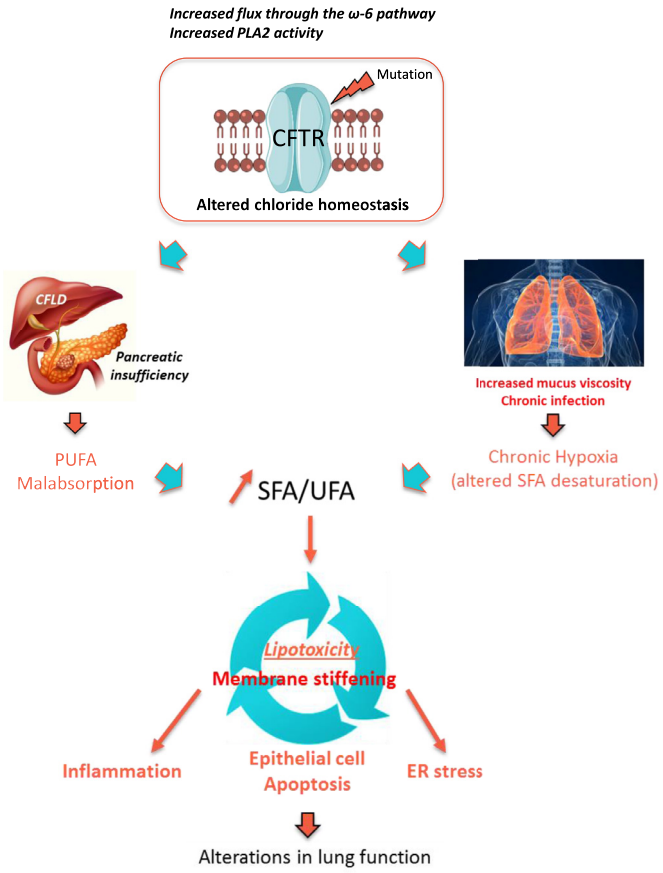

Fig. 2. The potential origins of lipointoxication in CF. Lipointoxication is defined as an imbalance among Saturated Fatty Acids (SFA) and Unsaturated Fatty Acids (UFA) within the main membrane constituents, namely Phospholipids (PL). Altered CFTR function results in several organ dysfunctions which could account for alterations in the repertoire of fatty acids available to the cells (and particularly bronchial epithelial cells), with an excess of SFA at the expense of UFA. These processes include Cystic Fibrosis-related Liver Disease (CFLD) and pancreatic insufficiency, leading to malabsorption of PolyUnsaturated Fatty Acids (PUFA), but also hypoxia, which precludes the desaturation steps required to form UFA from SFA (Fig. 1). Moreover, mutations in the CFTR genes are also associated to an increased flux through the ω−6 pathway, which may account for decreased levels of LOE and DHA, and increased activity of Phospholipase A2 (PLA2), leading to a decrease in the amount of PUFA-containing PL species (Fig. 1). See text for details.

activation of AMP-activated Protein Kinase (AMPK), which, in turn, stimulates the expression and consequently the activity of key fatty acid desaturases (namely Δ5 and Δ6 desaturases) leading to the synthesis of AA from LOE in CF cells ([43]; Fig. 1). Such a mechanism could account for the low levels of LOE in CF due to its increased metabolism to AA. Competitions between the ω-3 and ω-6 pathway could also account for the observed reduced levels of DHA in CF. Accordingly, medium supplementations with DHA or its precursor EPA (eicosapentaenoic acid) normalize the expression and activity of both Δ5 and Δ6 desaturases and result in a decreased production of AA in CF cells [44]. As a consequence to this mechanism, CF cells appear to display a deficit in both LOE and DHA within their PLs, which matches very nicely the observations made in humans ([42]; Table 1).

4.3. Release of AA from PL by phospholipase A2

As already mentioned, non-esterified AA is a precursor of very active inflammatory mediators known as eicosanoids, which include PG, leukotrienes, and thromboxanes. Even if increased levels of AA in CF are subject to caution (Table 1), high AA levels may contribute to inflammation in some patients *via* this pathway. Non-esterified AA can be released by the hydrolysis of selective PL species, *e. g.* PC (36:4), bearing a SFA (Palmitate) at its sn-1 position and a AA at the sn-2, by the action of phospholipase A2 (PLA2; see above; [45]). Since the release of AA by phospholipases is rate-limiting for the eicosanoid synthesis, an abnormal activity of PLA2 resulting in the liberation of AA from PLs could be a possible mechanism to explain the inflammatory state in certain patients with CF [4]. Relevantly, increased activity of PLA2 has been reported in plasma from patients with CF [4].

4.4. Hypoxia as an augmenting parameter

As already mentioned, mammalian cells display the machinery to build up their own MUFA from SFA, *via* the action of Δ-9 stearoyl-CoA desaturases (SCDs), and particularly SCD1. DHA and AA are synthesized from precursors, are metabolized to end products (including AA and DHA) by a series of alternating desaturation and elongation reactions. The desaturation steps are catalyzed by Δ-5 and Δ-6 desaturases [5]. All fatty acid desaturases have in common that they are exquisitely oxygen-dependent [46]. Hypoxia is a well-known situation encountered in CF [16, 47]. In good agreement with potential impacts of hypoxia on the FA balance, exposing bronchial epithelial cells to oxygen scarcity results in an increase of SFA at the expense of UFA, and this, independently of the functional status of the CFTR channel [18]. Moreover, it has been demonstrated that lung transplantation in cystic fibrosis normalizes EFA profiles, suggesting direct correlations between the respiratory capacity of patients and the fatty acid signatures [28].

Altogether, these data suggest that hypoxia may be an important contributor to EFA (and more generally UFA) deficiency in CF.

5. UFA deficiency and cellular membrane properties: the lipointoxication theory

Whatever the mechanism involved, which implication in the fatty acid signature may greatly vary depending on the patient considered, decrease in UFA within PLs appears to be a hallmark of CF. Moreover, a clear link has been established between the intensity of this UFA deficiency in circulating PLs and the severity of the disease [31].

PLs are the most abundant lipid classes whatever the organ considered [48]. A recent study performed on human bronchial tissue samples and dissociated bronchial epithelial cells showed that PLs constitute approximately 70% of all lipid species, with PC being the most abundant species, followed by ethanolamine-containing PLs, and, to lesser extents, PI and PS [49]. Of note, in mammalian cells, there is a gradual enrichment of SFA-containing PL species at the expense of unsaturated ones along the organelles of the secretory pathway (ER > Golgi > plasma membrane) ([50]; Fig. 3A). The fact that such a subcellular gradient exists suggests that the correct balance between saturated and unsaturated PL species in the various organelles has decisive functions [9].

In the studies displayed in Table 1, UFA deficiency in CF PL was paralleled by stable or increased levels of SFA, therefore resulting in an overall increase in the percentage of saturated- vs-unsaturated-chains within these lipid species. In bronchial epithelial freshly dissociated from CF patients, a 2-fold increase in disaturated PC species and a 3-fold increase in disaturated PE, at the expense of unsaturated species, were observed as compared to non-CF individuals [17].

The balance within PLs between SFA, MUFA and PUFA is known to regulate the biophysical properties of cellular membranes (Fig. 3B).

Numerous studies have demonstrated that maintaining the balance between SFA, MUFA and PUFA within PL is essential to guarantee optimal membrane biophysical properties, compatible with the selective processes taking place within cellular compartments, such as vesicular budding or the trafficking and function of membrane proteins (see below; [9]). Cumulative studies of biophysics and

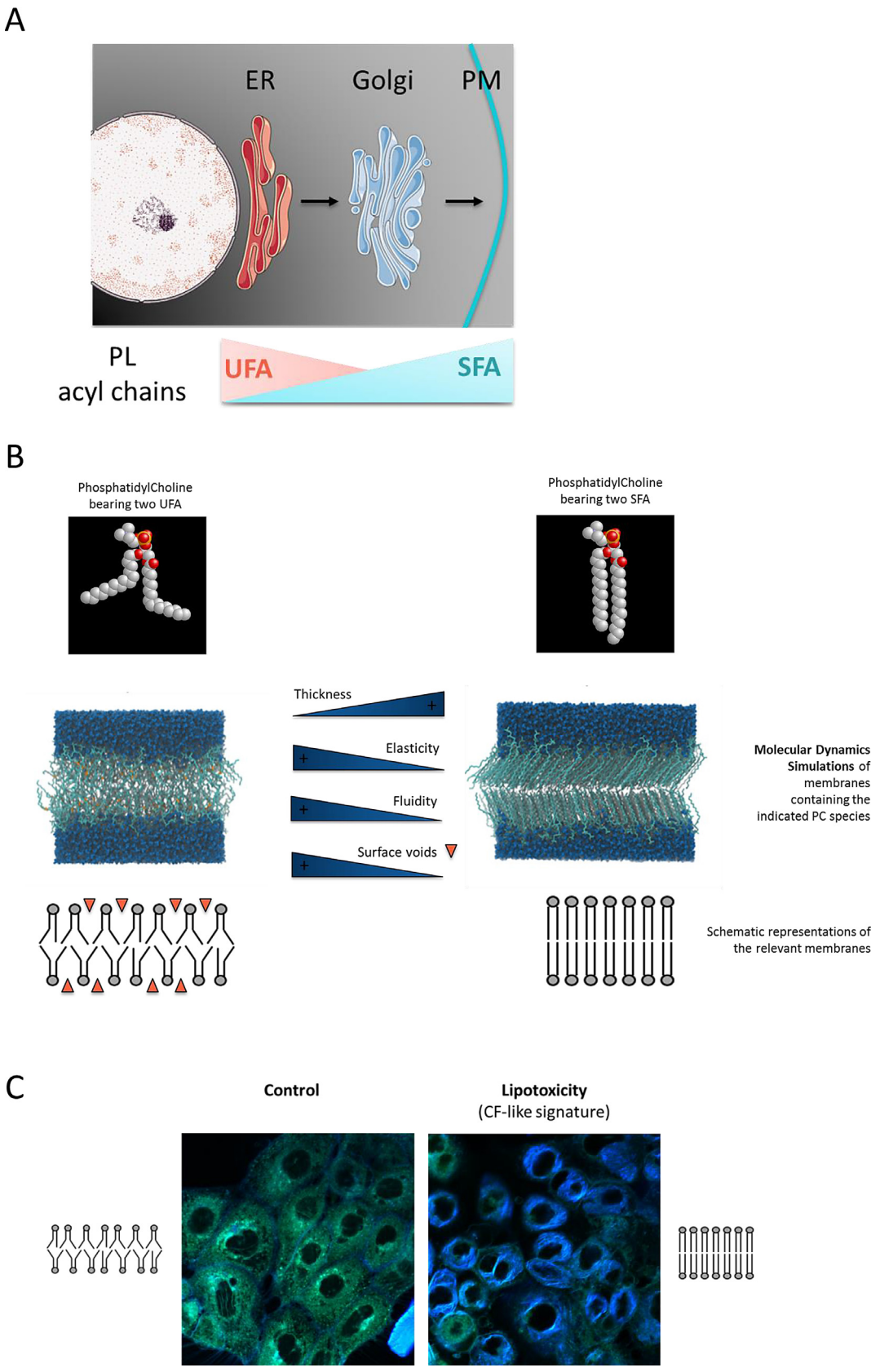

Fig. 3. The fatty acid composition of PL modulates membrane properties. (A) In mammalian cells, there is a gradual enrichment of Saturated Fatty Acid (SFA)-containing Phospholipid (PL) species at the expense of unsaturated ones (UFA) along the organelles of the secretory pathway (Endoplasmic Reticulum (ER) > Golgi > plasma membrane). (B) The ratio between SFA and UFA within PL (PhosphatidylCholine in the example displayed here) regulates several membrane properties including width, elasticity, fluidity and surface voids. Such impacts can be visualized using Molecular Dynamics Simulations. See text for details. (C) Bronchial epithelial cells bearing the F508del-CFTR mutation were cultivated either under normal conditions (Control) or under conditions recapitulating the SFA/UFA imbalance observed in freshly-dissociated bronchial epithelial cells obtained from CF patients (Lipointoxication). They were then incubated with the Laurdan probe which has the property of inserting into cellular membranes and displays different emission wavelengths depending on the overall organization of the lipid bilayer. A green color is characteristic of a fluid-membrane, and a blue color of a highly-ordered, rigid membrane. As shown, Lipointoxication results in a stiffening of the intracellular membranes, and particularly of the ER one. Adapted from Kadri et al. [17].

molecular dynamics simulations, have shown that different biophysical parameters are directly regulated by this ratio, such as the thickness of the membrane, its permeability, its elasticity, its plasticity or its surface roughness (surface voids) (Fig. 3B; for review, see [9]). For example, a membrane rich in PL containing SFA will be globally more organized due to the packing of the fatty acid chains, generating thick membranes, impermeable to water and to small hydrophilic molecules, but poorly elastic and malleable (Fig. 3B). In addition, it has been demonstrated that PUFA cannot simply considered as "super MUFA" but have the property of contorting to generate malleable and extremely deformable membranes [51]. In contrast, MUFA, due to their simple double bond, tend to form kinks within the membrane, consequently generating elastic, but poorly deformable membranes [9]. All of these results therefore demonstrate that the fatty acid content of PL, in a given membrane, has been scrupulously selected during evolution to meet specific physiological needs.

Considering the above, one may anticipate that perturbating the fatty acid balance within PLs cannot be without consequences on the trafficking and the structure/function of transmembrane proteins, including the CFTR channel ([52, 53]; Fig. 3B). Among many examples in the literature, and relevant to the present review, it has been demonstrated that modifying membrane thickness alters the folding on transmembrane domains. Indeed, the length of transmembrane helices appears to have evolved during evolution to match the precise thickness of the membrane the protein is naturally embedded in [54]. Perturbating this parameter drives transmembrane segments to adjust to these changes by either compressing/tilting (reduction in membrane thickness) or stretching (increase in membrane thickness) to avoid hydrophobic mismatch (which results from the exposition of their hydrophobic residues to the aqueous phase), with major impacts on their conformation, and, as a matter of fact, on their trafficking and function [55]. Another crucial parameter is the formation of surface voids, which correspond to cavities at the membrane surface, and which formation is intimately related to the fatty acyl chain composition of PLs ([56]; Fig. 3B). Surface voids are strictly required for the attachment/recruitment of the COPI (Coat protein complex I) and COPII machineries, which are responsible for bidirectional membrane trafficking between the ER and the Golgi. Not surprisingly, affecting the SFA/UFA ratio within PLs in these organelles alters the recruitment of these machineries, with global impacts on the secretory pathway and on the trafficking of plasma membrane proteins, from their site of synthesis (the ER) to their final destination [57, 58].

As a corollary to these observations, and due to the general nature and the conservation of the mechanisms involved, altered equilibria within SFA and UFA have been demonstrated to lead to dramatic dysfunctions in virtually all cell types tested so far (Fig. 4; for review, see [13]), including bronchial epithelial cells (see below). We refer to these fatty acid balance issues within PL under the term of lipointoxication.

6. The impacts of lipointoxication on the physiology of CF

The main impacts of lipointoxication on various cellular processes are summarized in Fig. 4.

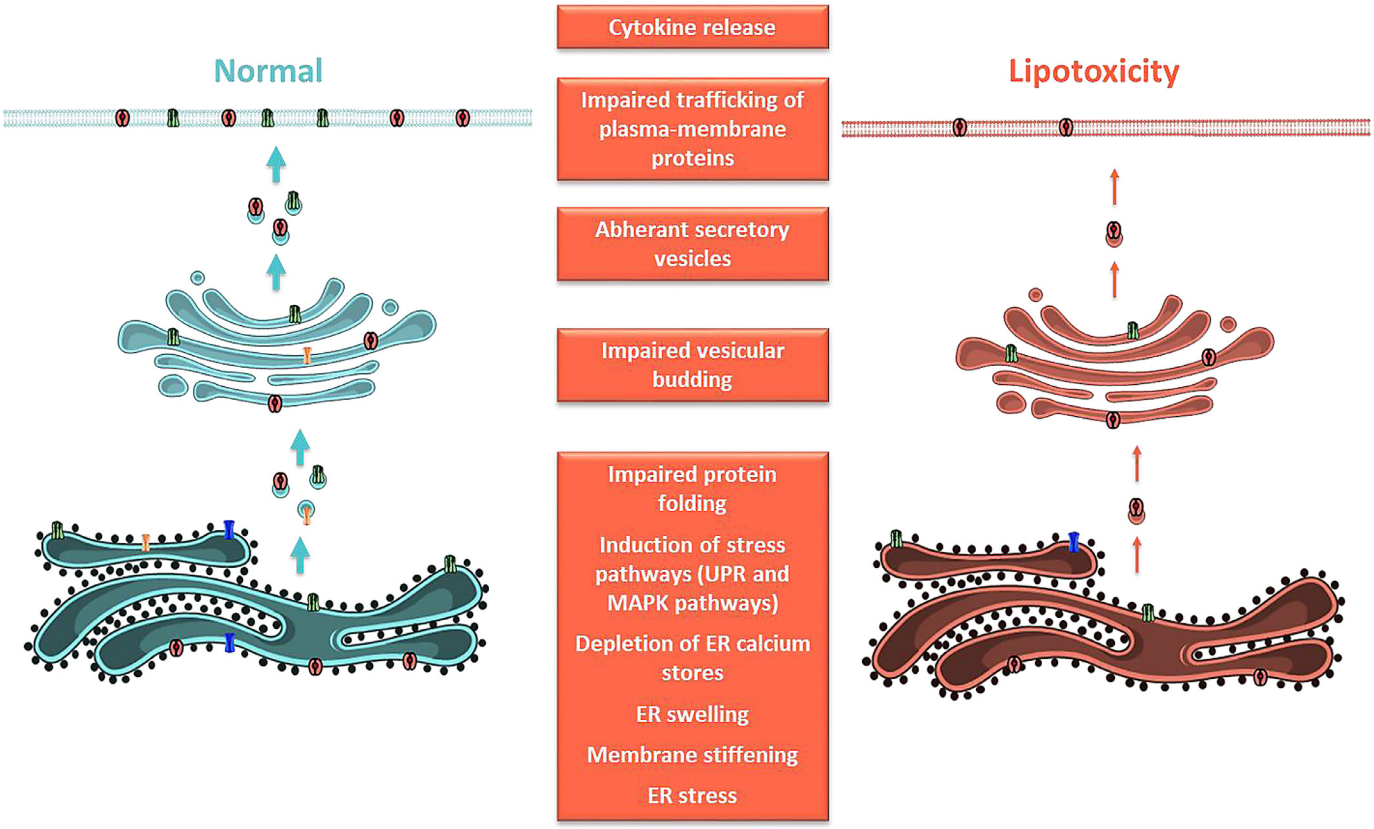

Fig. 4. Lipointoxication impacts crucial cellular processes. The impacts of lipointoxication on several crucial cellular processes have been demonstrated (and are conserved) in many cell types, including bronchial epithelial cells. By perturbating the overall membrane organization (Fig. 3), an SFA/UFA imbalance within PL results in the induction of stress pathways (Unfolded Protein Response (UPR) and MAPK pathway), altered protein folding, depletion of calcium stores and impaired vesicular budding in the ER. Later in the secretory pathway, lipointoxication is also associated with reduced vesicle formation at the Golgi level. Overall, these alterations result in a global disruption of the secretory and of the trafficking of plasma-membrane protein to their final destination. If sustained, lipotoxicty ultimately leads to cell death (apoptosis). Perturbed SFA/UFA balance has also been associated to an increased release of cytokines, including the pro-inflammatory chemokine IL8. See text for details.

6.1. Lipointoxication and the ER function: the ER stress

The ER is the organelle where the biosynthesis and the folding of transmembrane and luminal proteins occurs. To maintain the correct balance between ER protein load and folding capacity, cells have developed a pathway known as the unfolded protein response (UPR) [59]. The UPR is under the control of integral protein sensors, including the inositol-requiring enzyme 1 (IRE1) and protein kinase-like ER kinase (PERK) [59], which are maintained under an inactive monomeric form by the interaction of their luminal domain with the chaperone BiP. When unfolded proteins accumulate within the ER, these repressive complexes are disrupted, leading to the oligomerization of IRE1 and PERK, and the activation of the UPR cascade. Induction of the UPR limits protein overload *via* a general decrease in translation initiation and coordinated upregulation of genes encoding proteins involved in folding (*e.g.* chaperones) and ER-associated degradation (ERAD) by the proteasome.

UPR induction has been a recurrent observation in CF tissues (for recent review, see [60]).

Since UPR activation is intimately linked to misfolded-protein overload, it may appear logical that specific mutations, *i. e*. Class II mutations, which result in CFTR misfolding, activate the UPR *per se*. However, UPR induction occurs when unfolded proteins are expressed at high levels and accumulate in the ER [61]. In contrast, F508del-CFTR, the main Class II mutation encountered in patients, is expressed at very low levels and quickly degraded by the proteasome [62]. Furthermore, UPR induction is not CFTR-mutation specific, since such an induction can be observed in all CF patients, independently of the mutation. Altogether, these data suggest that the tissue environment resulting from CFTR dysfunction rather than CFTR mutations *per se* are responsible for UPR induction [60].

Many studies have demonstrated that the UPR is activated upon SFA overload, in virtually all cell types, including, among others, pancreatic β-cells [63-65], hepatocytes [66], muscle cells [67], adipocytes [68], yeast [69], and, relevant to the present review, bronchial epithelial cells [17, 18].

Because exogenously supplied fatty acids are efficiently incorporated into PLs, and that the ER is naturally enriched in UFA-containing PL species (Fig. 3A), saturated PLs are likely the direct cause of ER-stress as a result of their impact on membrane properties. Membrane perturbations *via* excessive saturated PLs include modification of calcium transport, alterations in protein folding, and changes in the oligomerization status of IRE1 and PERK. For example, lipointoxication promotes depletion of ER calcium stores in various cell types [63, 70], including bronchial epithelial cells [18], in a process that could be related to direct effects of saturated PLs on sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) pump activity [63]. Saturated PLs also induce the accumulation of misfolded proteins. Treating SFA-intoxicated cells with pharmacological chaperones that stabilize protein conformation alleviates SFA-induced UPR [69, 71, 72]. Various causes could account for misfolded protein accumulation under SFA treatment. First, the translocon, which catalyzes the translocation of newly translated proteins towards the lumen, is sensitive to membrane organization [73]. Second, many ER chaperones are dependent on calcium [74], the homeostasis of which is altered under SFA accumulation. Intriguingly, recent studies suggest that increased stiffness of the ER membrane due to saturated-PL accumulation can also induce UPR by favoring the direct clustering of IRE1 and PERK [75].

Of course, lipointoxication-related induction of the UPR could have great impacts on CFTR function and trafficking. Indeed, reciprocally, it has been shown that induction of the UPR decreases wild-type CFTR expression at the transcriptional, translational, and maturational levels [76].

When sustained, lipointoxication induces a global disorganization of the ER morphology. Indeed, under persistent lipointoxication, the ER tends to swell, and loses its ability to form a diffuse network through the cytoplasm to rather appear as stacks [17, 65, 69]. Under these circumstances, the UPR switches from anti- to pro-apoptotic leading to cell death in virtually all cell types (for review see [13]). Importantly, apoptosis as an important mechanism for airway epithelial cell loss in CF airways is recognized [77, 78].

Relevant to CF, reconstitution of the SFA/UFA ratio observed in bronchial epithelial cells from CF patients in cell lines resulted in the induction of the UPR, ER swelling/stack formation and, when most dramatic signatures were recapitulated, apoptosis [17, 18]. Relevant with the lipointoxication concept, increased stiffness of the ER membrane could be directly assessed *in cellulo* (Fig. 3C) and molecular dynamics simulations performed using CF and non-CF PL compositions revealed that SFA overload in CF PL impacts crucial cellular properties, including membrane thickness, area per lipid and lipid diffusion, all being *bona fide* indicators of membrane stiffening [17].

The role of a membrane origin in such disorders was strengthened by the fact that restoring a correct SFA/UFA balance in PL by co-supplementation of the medium with UFA, or the use of a lipophilic molecules, namely Mannide Monoleate, which displays the property of inserting within the membrane and of acting as a membrane reshaper to restore central membrane parameters, both relieved the ER stress, restored normal properties of the ER membrane and countered SFA-induced apoptosis [17, 18].

6.2. Lipointoxication and inflammation

Another important characteristic of CF is excessive and sustained lung inflammation [79]. If the roles of bacterial infection and induction of proinflammatory pathways *via* the production of AA-derived eicosanoids are undeniable [79], direct augmenting impacts of lipointoxication on these processes should be considered. Indeed, SFA-related inflammation has been demonstrated in many studies in different cellular models, including adipocytes, cardiac fibroblast, hepatocytes, skeletal muscle cells, and pancreatic β-cells, in processes that can be fully reversed by the restoration of the SFA/UFA balance (for review, see [13]). This effect is manifested by SFA-activation of proinflammatory pathways, with the increased secretion of proinflammatory cytokines and chemokine, among which are tumor necrosis factor a (TNFα), Interleukin-6 (IL6), and IL8. High levels of IL8 can be detected in the bronchial lavage (BAL) fluid from patients with CF [80]. To some extent, IL8 appears to derive from bronchial epithelial cells themselves, since airway epithelium plays a crucial role in initiating and augmenting host defense mechanisms. Relevantly, increased IL8 secretion was also demonstrated in bronchial epithelial cells under a situation which recapitulated the SFA/UFA imbalance observed in patients, a process which was relieved by UFA addition or treatment with a membrane reshaper [17]. IL8 secretion has been shown to be under the dependence of p38 MAPK (mitogen-activated protein kinase) signaling pathway in several cell types, including bronchial epithelial cells [81, 82]. Accordingly, UFA and membrane reshapers both relieved SFA-induced induction of the MAPK-pathway [17].

6.3. Lipointoxication and CFTR trafficking

The altered ER-function observed under lipointoxication has important impacts on protein folding (see above) but also on the trafficking of membrane-anchored proteins. Protein trafficking between the various compartments relies on the ability of the organelle membranes to bend in order to form transport vesicles, a property that is intimately related to the membrane properties and requires sufficient amounts of UFA-containing PLs [9]. Relevantly, it has been demonstrated that ER-to-Golgi protein trafficking is precluded in SFA-treated mammalian β-cells [83]. These observations fit *in vitro* experiments showing that liposomes formed of saturated PL display a lower capacity to recruit proteins of the Coat Protein complex (COP

II) machinery, which are essential for vesicular budding [84]. This process is likely to contribute to the retention of misfolded proteins in the ER, therefore reinforcing the protein overload in this compartment. Later in the secretory pathway, saturated-PL overload in the Golgi apparatus also impacts the recruitment of proteins of the COP I machinery, therefore leading to a reduction in the number, and an altered morphology of secretory vesicles "en route" to the plasma-membrane [57, 58]. Therefore, lipointoxication results in global alterations of the overall secretory pathway. The human genome is thought to code for approximately 5000 membrane proteins, most of them being synthesized and folded in the ER and further transported to their final destination along the secretory pathway, among which the CFTR and its functional partners. Therefore, lipointoxication is susceptible to impact the trafficking of hundreds of membrane proteins in the various cells of our body.

If the specific impacts of SFA-overload on CFTR trafficking in epithelial cells have not been studied in details so far, the fact that correctors of the F508del-CFTR appear to be less efficient under lipointoxication is in good agreement with the hypothesis of potent effects of lipointoxication on this process [18].

6.4. Lipointoxication and CFTR function

Finally, the activity of membrane proteins is highly dependent on the properties of the bilayer they are embedded in, and changing such properties has highly deleterious impacts on their function and/or stability at their site of action [52, 53]. An exhaustively-studied example is the light receptor rhodopsin, which function is highly connected to the presence of PUFA within its surrounding PL [9]. If the specific effects of membrane lipids on the activity of the CFTR channel have not been studied in great details so far, a pioneering study demonstrated that selective PL species are important for CFTR trafficking and contribute to the pathology of CF [85]. More recently, it has been shown that selective lipid interactions with purified CFTR enhance its catalytic activity [86]. Moreover, it has been proposed that the CFTR potentiator VX-770 and its derivatives destabilize the pharmacologically-rescued F508del-CFTR channel, by their potential non-specific effects on the lipid bilayer [87]. Altogether, and even if additional studies will be undoutfully required, these data demonstrate that CFTR activity/stability is highly connected to its surrounding lipids and that modulating the membrane properties clearly impacts its function.

7. Outstanding questions

If the mechanisms leading to fatty-acid remodeling within PL in various CF tissues are complex and likely result from the alteration of multiple mechanisms, which amplitude may vary from one patient to the other, *bona fide* modifications of the SFA/UFA balance have been repeatedly reported in CF organs, including the lung. Considering the known impacts of such an imbalance on membrane properties and the crucial effects of membrane homeostasis perturbations on the induction of stress pathways, organelle integrity, and membrane-protein trafficking and function, it is likely that lipointoxication plays a role in the etiology of the disease. An important question that still remains however is the contribution of this process to the course of the disease. Since the intensity of the lipointoxication signature nicely correlates with the severity of the disease [23, 31], and that lipotoxicty can account, by itself, for several traits associated to CF (including ER stress, stress pathway induction, inflammation and epithelial cell apoptosis), it is reasonable to consider lipointoxication as a potent contributor to the severity of the disease. Moreover, lipointoxication may also be a limiting parameter to the efficiency of existing treatments for CF. Even if additional studies will be required, the fact that F508del-CFTR correctors appear to be less efficient under lipointoxication reinforces this hypothesis [18]. Considering these observations, reevaluating the efficiency of existing treatment in cellular models recapitulating at best the lipid signature encountered *in vivo* will likely help at understanding the discrepancies observed between the data obtained *in cellulo*, in non-lipointoxicated cellular models, and the results obtained *in vivo*, which appear most often quite deceptive as compared to the expected results from *in vitro* experiments [88, 89].

8. Search strategy and selection criteria

Data for this review were obtained from PubMed searches and references from relevant articles using combinations of the following criteria: "Cystic Fibrosis", "Lipidomics", "Lipids", "Fatty Acids", "Phospholipids", "Essential Fatty Acid Deficiency", "ER stress", "Membrane Biophysics", "Structure/Function of Membrane Proteins", "Secretory Pathway" and "Membrane Protein Trafficking". Articles published in English from 1962 to 2020 were included with particular emphasis on studies from 2010 to 2020.

Contributors

Drs Vandebrouck and Ferreira contributed equally to this work. Both authors read and approved the final version of the manuscript.

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