Biochimica et Biophysica Acta 1822 (2012) 690–713

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Review

Oxidative stress and antioxidant therapy in cystic fibrosis ☆,☆☆

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ARTICLE INFO

Article history:
Received 20 October 2011
Received in revised form 16 December 2011
Accepted 17 December 2011
Available online 28 December 2011

Keywords:
Cystic fibrosis
Antioxidant
Oxidative stress
Reactive oxygen species
Inflammation
Glutathione

ABSTRACT

Cystic fibrosis is a lethal autosomal recessive condition caused by a defect of the transmembrane conductance regulator gene that has a key role in cell homeostasis. A dysfunctional cystic fibrosis transmembrane conductance regulator impairs the efflux of cell anions such as chloride and bicarbonate, and also that of other solutes such as reduced glutathione. This defect produces an increased viscosity of secretions together with other metabolic defects of epithelia that ultimately promote the obstruction and fibrosis of organs. Recurrent pulmonary infections and respiratory dysfunction are main clinical consequences of these pathogenetic events, followed by pancreatic and liver insufficiency, diabetes, protein-energy malnutrition, etc. This complex comorbidity is associated with the extensive injury of different biomolecular targets by reactive oxygen species, which is the biochemical hallmark of oxidative stress. These biological lesions are particularly pronounced in the lung, in which the extent of oxidative markers parallels that of inflammatory markers between chronic events and acute exacerbations along the progression of the disease. Herein, an abnormal flux of reactive oxygen species is present by the sustained activation of neutrophils and other cystic fibrosis-derived defects in the homeostatic processes of pulmonary epithelia and lining fluids. A sub-optimal antioxidant protection is believed to represent a main contributor to oxidative stress and to the poor control of immuno-inflammatory pathways in these patients. Observed defects include an impaired reduced glutathione metabolism and lowered intake and absorption of fat-soluble antioxidants (vitamin E, carotenoids, coenzyme Q-10, some polyunsaturated fatty acids, etc.) and oligoelements (such as Se, Cu and Zn) that are involved in reactive oxygen species detoxification by means of enzymatic defenses. Oral supplements and aerosolized formulations of thiols have been used in the antioxidant therapy of this inherited disease with the main aim of reducing the extent of oxidative lesions and the rate of lung deterioration. Despite positive effects on laboratory end points, poor evidence was obtained on the side of clinical outcome so far. These aspects examined in this critical review of the literature clearly suggest that further and more rigorous trials are needed together with new generations of pharmacological tools to a more effective antioxidant and anti-inflammatory therapy of cystic fibrosis patients. This article is part of a Special Issue entitled: Antioxidants and Antioxidant Treatment in Disease.

1. Introduction

Cystic fibrosis (CF) is a lethal autosomal recessive disorder caused by a single gene defect. This was identified in 1989 to map on the chromosome 7 and to correspond to the gene coding for the transmembrane conductance regulator (CFTR) that is mainly expressed in the apical membrane of epithelial cells that line mucous membranes and submucosal glands [1]. Several mutations have been identified to cause this gene defect with the Phe508del, or ΔF508, as one of the most common mutations in Caucasians. The prevalence at birth varies in the different regions according with ethnic background, from roughly 1 in 3000 white Americans and northern

☆ This article is part of a Special Issue entitled: Antioxidants and Antioxidant Treatment in Disease.
☆☆ For the "Working Group on Inflammation in Cystic Fibrosis" of the Italian Society for Cystic Fibrosis and the Italian Cystic Fibrosis Research Foundation.

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doi:10.1016/j.bbadis.2011.12.012

Europeans to 1 in 350,000 in Japan. Mutations are grouped in 6 classes based on the type of defect caused on CFTR protein metabolism and function. Several physiological processes affected by these mutations are related to the role of CFTR as anion channel. This mainly regulates chloride efflux, but other and larger anions such as reduced glutathione cross the plasmalemma throughout this transmembrane protein widely expressed in diverse epithelial tissues. Other ion transport systems are under its influence, such as bicarbonate anion and sodium channels, so that a defective CFTR can impair several processes such as cell volume and pH regulation, transepithelial transport, membrane conductance, and the GSH-related antioxidant and detoxification activity in the extracellular milieu [2,3]. CFTR dysfunction is associated with an altered fluid and electrolyte composition of secretions, their increased viscosity and progressive obstruction and fibrosis of organs [4]. The severity of these CF symptoms varies independently of the type and number of mutations diagnosed, suggesting that CFTR gene and its mutations interact with other genes at the transcriptional and post-translational level to influence a wide series of physiological processes. Lung, pancreas and liver are severely affected by these events, and recurrent infections of the airways together with pancreatic insufficiency and diabetes are most common conditions secondary to CF [1].

The presence of a defective CFTR appears to produce a redox imbalance in epithelial cells and extracellular fluids and to cause an abnormal generation of reactive oxygen species (ROS). A constitutive defect of GSH metabolism together with a lowered intake and absorption of fat-soluble antioxidant vitamins (vitamin E and carotenoids) could contribute to a defective antioxidant protection, which is believed to exacerbate oxidative stress indices along with the progression of clinical status [5–7]. The development of inflammatory and degenerative lesions in target tissues such as lung, pancreas and liver further exacerbate the shift from normal to abnormal flux of ROS in several organs, thereby leading to develop systemic oxidative stress. This is a chronic-degenerative trait common to other and severe inflammatory diseases such as chronic kidney disease and some auto-immune syndromes (reviewed in [8,9]), which may conspire with further mechanisms to worsen the prognosis of this inherited disorder (recently reviewed in [10]).

In view of these aspects, the CF patient is assumed to have a higher antioxidant demand. This has provided the rationale for the systematic investigation of antioxidant levels in blood and targeted tissues of CF patients, mainly the epithelium and lining fluids of the airways, and to plan for antioxidant interventions that might rescue specific defects of these patients. These also include the use of anti-inflammatory agents and nutritional formulations which can produce an "antioxidant effect", i.e. the lowering of oxidative stress indices, as a result of their direct or indirect action. Despite a number of promising in vitro and pre-clinical observations, antioxidants used as oral supplements or directly administered in the CF airways have failed to provide convincing evidence at the clinical level. Future efforts are required to identify more advanced agents and therapeutic strategies that may enhance secondary prevention and chemotherapy of airway inflammation and oxidative stress in CF patients. Advances in the approaches capable of improving nutritional status and antimicrobial therapy are of main relevance to further ameliorate quality of life and survival rates in CF.

These aspects will be discussed in this manuscript with the aim of providing an updated review of the literature as well as of strategies and future directions of antioxidant therapies in CF patients.

2. Inflammatory pathways and oxidative stress in CF

2.1. Progressive inflammatory damage in CF lungs and the contribution of oxidants

Evidence supporting the occurrence of oxidative stress in CF is by now established and extensive [6,10–12]. As introduced above, CF-related defects of the pulmonary epithelium and a sustained PMN activation by recurrent infections create the conditions for an abnormal flux of reactive oxygen species (ROS) in the CF lung (Fig. 1) between events of acute and chronic inflammation. Abnormalities of markers of ROS activity and inflammation are most evident during acute respiratory exacerbations and show improvement with the

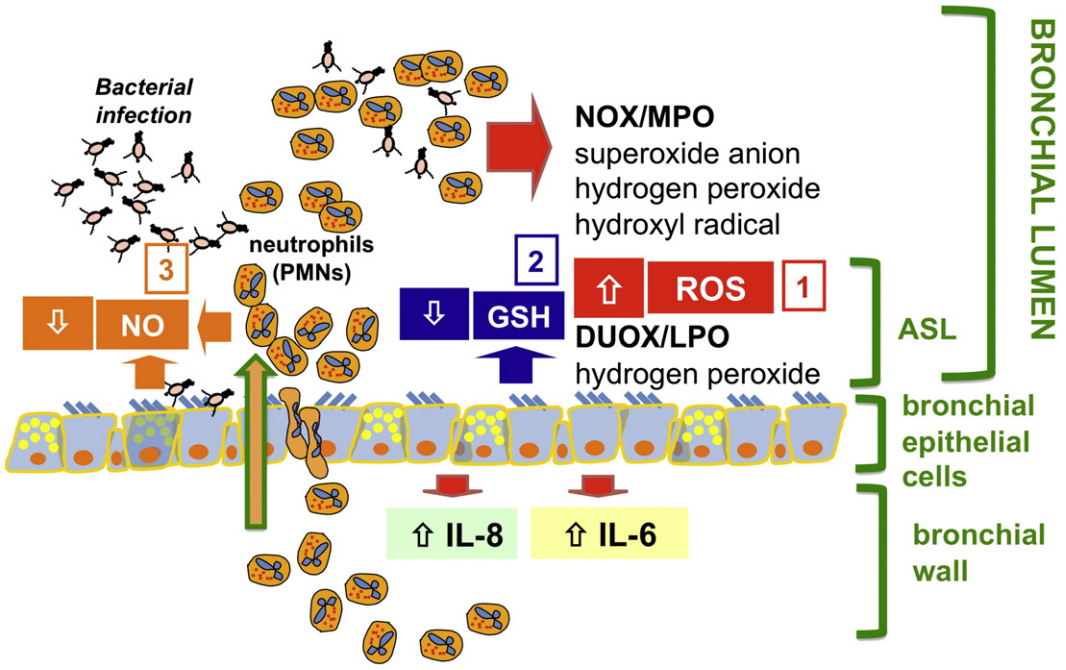

Fig. 1. Oxidative unbalance in conductive airways of patients affected by cystic fibrosis. airway surface liquid (ASL) in CF bronchi is characterized by 1. increased concentration of reactive oxygen species (ROS), 2. lowered levels of glutathione (GSH) and 3. reduced nitric oxide (NO). The net increase of pro-oxidative species in ASL, as a result of derangements of both neutrophils and bronchial epithelial cells, contributes to the progressive lung tissue damage and to the amplification of the inflammatory response in CF airways. This is characterized by the release of chemokines and cytokines (e.g. IL-8 and IL-6, respectively). NOX, NADPH oxidase; MPO, mieloperoxidase; DUOX, dual oxidase; LPO, lactoperoxidase, IL, interleukin.

intensive treatment of the infection [13–15]. The fact that relapses or successful therapy of infection do not normalize these markers demonstrates the presence of a chronic inflammatory syndrome that is intrinsic to the CF defect. In this context, ROS may lose their physiological role in the killing of pathogens, to turn into toxic effectors responsible for the damage of the pulmonary epithelium as well as of other components of the lung parenchyma and lining fluids. Importantly, ROS can modify the thiol homeostasis of extracellular fluids and epithelia [16] and promote the activation of MAPK signaling pathways [17], which regulate both the NFkβ-dependent and -independent transcription of pro-inflammatory genes and other molecular effects associated with the immuno-inflammatory imbalance observed in the CF lung.

Hallmark of the chronic inflammatory lung disease in CF is the release of chemokines, mainly interleukin (IL)-8 [18,19], leading to the neutrophil recruitment in the bronchial lumen (see [20] for a review). Whether CF lung inflammation arises independently and before bacterial infection remains to be fully established, although IL-8 and pro-inflammatory cytokines have been found in bronchoalveolar lavage fluids of CF infants even before the onset of an overt bacterial infection [21]. Although directed against infective agents, the chronic inflammation in CF lungs is largely recognized as mainly responsible for the progressive tissue damage leading to respiratory insufficiency. Dissection of the pathophysiology of CF chronic lung inflammation should take into account the bronchial epithelial cells expressing the mutated CFTR protein, the polymorphonuclear neutrophils recruited into the bronchial lumens and the bacterial infection itself, with special regards to *Pseudomonas aeruginosa*, the most common gram negative microorganism which colonizes CF airways [22]. Thus, novel anti-inflammatory therapies against the progressive damage of the CF respiratory tissue should be mainly aimed i) to reduce the excessive recruitment of neutrophils, by intervening on the transmembrane signaling pivoting the excessive expression of IL-8 [23–26], ii) to inactivate proteases released by the neutrophils continuously activated by bacterial products [27] and iii) to circumvent the effect of the unbalanced production of oxidants, deriving from both phagocytes and bronchial epithelial cells [22,28–30]. Identification of oxidants produced in the CF airway tract is of high importance, in order to identify novel molecular targets for specific pharmacological intervention.

2.2. Respiratory epithelial cells and neutrophils as sources of oxidants in the CF lung

It is largely accepted that neutrophils migrating inside the CF bronchial lumina release large amounts of reactive oxygen species (ROS), including the superoxide anion ($O_2^{-}\bullet$), hydrogen peroxide (H_2O_2) and the hydroxyl free radical (OH), mainly by the activation of the NADPH oxidase (NOX). To such exaggerated ROS production contribute both the continuous interaction of neutrophils with bacteria and bacterial degradation products and the inability to engulf bacteria in biofilms, leading to a condition of "frustrated phagocytosis". Neutrophils are therefore recognized as a major source of ROS in the airway surface liquid (ASL) of young children with CF [14,31]. However, bronchial ciliated and alveolar type II epithelial cells by themselves are able to produce significant amounts of ROS, through the two isoforms of NADPH oxidase expressed in the apical membrane of these epithelial cells, namely DUOX1 and DUOX2 [32]. A major proposed function of DUOXs is to support lactoperoxidase (LPO), which is in turn released by goblet and submucosal gland airway cells, to generate bactericidal hypothiocyanite ($OSCN^-$) starting from thiocyanate (SCN^-) and H_2O_2. Thus the DUOX/LPO coupled system of the respiratory epithelial cells parallels the NOX/mieloperoxidase (MPO) system of phagocytes in releasing and processing oxidants in the ASL. While the NOX/MPO system of phagocytes is mainly activated in the infection-induced respiratory burst, the release of ROS from epithelia is continuous and even independent of the presence of overt bacterial infection [33]. Interestingly, it was proposed that the efficiency of the DUOX/LPO defense system is dependent on the ion transport function of the CFTR Cl^- channel, which could also intervene both in the conductive secretion of SCN^- (for LPO function) and of HCO_3^- (for pH adjustment) into the ASL [34]. However, more recent ex vivo observations, while confirming a positive role of DUOX/LPO system in producing $OSCN^-$ as a general defense mechanism of the airways, do not directly relate SCN^- concentrations in ASL with CFTR function [35]. The LPO-mediated scavenging of H_2O_2 has suggested a role of SCN^- as physiological antioxidant of ASL [36], which may be defective in CF. Besides this role of LPO and SCN^-, an abnormal flux of H_2O_2 in the CF airways may also depend on other factors that are associated with an altered metabolism of ROS. For instance, lowered levels of Lactoferrin (LF) have been described in CF secretions [37]. This iron-chelating homologue of transferrin contained in the granules of neutrophils is also secreted by several mucosal tissues in biological fluids to contribute antimicrobial effects by a variety of mechanisms including the chelating activity of iron as Fenton chemistry catalyst [38].

As regards the progression of CF lung disease, in the early phases, before the onset of chronic bacterial colonization, epithelial DUOX continuously releases H_2O_2, thus being a predominant source in respect to the NADPH oxidase from phagocytes. The latter is mainly active when the respiratory burst is "triggered on demand" by infectious components. On the other side, in advanced phases of chronic infection of CF lungs, neutrophil-derived ROS are predominant [32], due to neutrophil activation as well as to the decreasing number of H_2O_2-producing ciliated cells, which are reduced by extensive apoptosis and tissue remodeling. A further reduction of epithelial DUOX activity has been observed as a result of infection with *P. aeruginosa* in conductive airways. *P. aeruginosa*-derived toxin pyocyanin in fact inhibits DUOX-dependent H_2O_2 production by consuming intracellular NADPH, which represents an interesting adaptive mechanism to downregulate innate anti-bacterial defenses [39].

2.3. Redox disturbances of CF airways: the role of GSH, NO and H_2O_2

Intracellular impairment of redox balance between oxidants and anti-oxidants has been proposed to occur in CF bronchial epithelial cells, although a significant difference in respect to normal CFTR-expressing cells is still controversial and debated [40]. Three major issues have been investigated concerning the intracellular redox balance in CF bronchial cells, namely i) a defect in GSH homeostasis [41,42], ii) an alteration of nitric oxide (NO) metabolism and iii) an imbalance of intracellular H_2O_2 production.

As far as GSH is concerned, the defective CFTR channel function has been proposed to lead to a lowered cell content of this tripeptide, which is crucial to control the flux of H_2O_2 in both the CF bronchial epithelial cells and lining fluids. This aspect – discussed in detail below in this review – is associated with characteristic defects of GSH-related enzymes and may represent a key underlying factor in the oxidative stress of CF airways.

Altered NO concentration has been found in chronic respiratory diseases such as bronchial asthma and chronic obstructive pulmonary disease [43], and reduced NO concentrations have been observed in the bronchial airways of patients affected by CF, which directly correlated with worsening of lung function [44]. Thus NO concentration in CF lung exhalate has been subsequently tested as a possible marker of pulmonary exacerbations and/or the inflammatory/infective status and its fluctuations over time [45,46].

This opened the way to further analysis of the mechanisms of this derangement, and it has been proposed that an excessive production of asymmetric dimethylarginine, an inhibitor of endogenous NO Synthase (NOS), could be involved in the reduced concentration of NO in CF airways [47–49]. Possible corrective therapies, such as the inhalation of L-arginine, have provided preliminary evidence of

correction of the defective NO concentration and improvement of lung function in CF patients [49–51].

Although exhaled NO is decreased in CF, increased immunohistochemical staining for nitrotyrosine was demonstrated in lung tissues from CF patients [52]. Therefore, a decreased production or accelerated metabolism of NO could be present in association with an abnormal reactivity of this radical and its derived species (NOx) toward biomolecular components of the CF airways. Peroxynitrite is one of the most relevant mediators of the biological activity of NO with toxic properties and damaging activity on several biomolecules [53].

A third emerging issue takes into consideration the expression of different enzymatic systems affecting the redox balance in CF bronchial epithelial cells. For instance, intracellular concentration of H_2O_2 has been found abnormally elevated in both immortalized bronchial and primary nasal epithelial cells derived from CF patients, both in the presence and the absence of proinflammatory cytokines [54]. This has been accompanied by a marked decrease of expression of proteins regulating H_2O_2 levels, such as thioredoxin 1 (TRX-1), glutathione-S-transferase pi (GST-pi), peroxyredoxin (PRDX) 6, TRX-dependent peroxide reductase (PRDX-1), catalase and, conversely, a significant increase of Mn superoxide dismutase (SOD2) [54]. Interestingly, to link these modifications with CF specific characteristics, dysfunctional CFTR channel was found associated with reduced activity of the transcription factor Nrf-2 (nuclear factor-erythroid 2 p45 subunit-related factor 2), which could at least in part explain the differential expression of the enzymatic systems resulting in the elevated intracellular steady-state concentration of H_2O_2 found in CF nasal and bronchial epithelial cells [54]. In synthesis, the ASL of CF patients during advanced stages of the lung disease contains elevated concentrations of ROS, mainly derived from neutrophils migrated into the airway lumen, and a reduced concentration of NO, which can strongly contribute to respiratory tissue injury together with the proteases released by the activated neutrophils. The homeostatic role of the GSH-related defenses appears constitutively impaired by the dysfunctional CFTR, thus increasing susceptibility to develop oxidative stress and lung tissue degeneration (Fig. 1), as described in detail in the sections below.

2.4. Conductive airway epithelium as target of ROS

Oxidants can target different biomolecules to damage epithelial cells and extracellular fluids of the airways. Lipid peroxidation and post-translational modifications of proteins on both cell membranes and extracellular targets are common biomarkers of this damage, which can occur by the direct reaction between ROS and biomolecules or through the formation of second-generation reactive byproducts [9,55]. All levels of this interaction between ROS and biological components can produce toxic and bioactive intermediates. Oxidants are known to activate second messengers through phospholipases A2, C and D, and to induce the production of cytokines and mucins, a series of molecular events that contribute to progressive obstructive disease and reduction of lung function [56]. Besides the direct oxidative damage to cellular structures of the bronchial epithelial cells, the excessive concentration of oxidants in CF, both in the ASL lining the apical membranes and inside the bronchial cells, has been studied in respect to the regulation of the inflammatory response.

ROS are often considered a sort of second messengers in activating the Nuclear Factor (NF)-κB, which is in turn involved in the activation of transcription of several proinflammatory cytokines and chemokines [57]. For instance, it has been shown that H_2O_2 partly controls NF-κB activation by IL-1beta, by facilitating the activation of NIK and subsequent phosphorylation of IKKβ [58]. In this respect, a direct link has been proposed between the excessive production of intracellular H_2O_2 and the elevated expression of IL-6 and IL-8, the most abundant pro-inflammatory cytokine and neutrophilic chemokine found in CF airways [54], which has been further confirmed [59]. Moreover, oxidants could be synergic in the induction of mucins, as promoted by neutrophil elastase, which further impairs ASL fluidity in CF [60]. Finally, bacterial infection with *P. aeruginosa* strains releasing the toxin pyocyanin (PCN) has been shown to reduce ion transport through the CFTR channel, thus potentially counteracting the therapeutic effects of correctors and potentiators of mutated CFTR protein [61,62]. In summary, excessive oxidants in CF conductive airways have different negative effects in the amplification of the already excessive lung inflammation and secretion of mucin, together with a direct deleterious effect on CFTR channel function.

2.5. Oxidative stress and surfactant

Oxidative stress and inflammation in cystic fibrosis can affect surfactant biophysical activity thus leading to early alterations of lung function in patients with CF [63]. Altered phospholipid-to-protein ratios and phospholipid subclasses, a modified fatty acid profiles, and decreased association of proteins such as SP-A with lipid components of isolated surfactant, indicate that components of this fluid are considerably altered and dysfunctional in lower respiratory tract secretions of CF patients [64].

Oxidative damage of surfactant may involve both lipid and protein components. Alteration of lipid components can in turn generate toxic lipid species with cytotoxic activity towards nearby epithelial cells [65]. Altered protein components have been shown in cystic fibrosis [66]. Notably, surfactant protein D, which is an important innate host defense molecule, becomes unable to agglutinate bacteria when it is modified by oxidation, which facilitates pathogen colonization in the lung [67]. In a cross-sectional analysis of CF patients with mild lung disease, reduced surfactant activity was correlated to increased neutrophilic airway inflammation, but not to lung function [68]. So far, longitudinal measurements of surfactant function in CF patients are lacking and it remains unclear how these alterations relate to progression of airway inflammation as well as to the rate of decline of pulmonary function [69].

2.6. Laboratory indices of oxidative stress in CF

Appropriate biochemical and clinical tools are of importance for the monitoring of antioxidant therapies in CF, and a crucial aspect is the selection of proper biomarkers and protocols to assess biological pathways of oxidation.

Pancreatic insufficiency and a diminished bile acid pool cause malabsorption of important essential nutrients and other dietary components in CF. Of particular significance is the malabsorption of fat-soluble antioxidants such as carotenoids, tocopherols and coenzyme Q-10 (CoQ-10), which act as chain breakers in the peroxidation reactions of polyunsaturated lipids. Accordingly, lipid peroxidation is one of the main signs encountered in the CF plasma, buccal mucosal cells, breath condensate and BALF as measured by the non-enzymatic oxidation product of arachidonic acid 8-iso prostaglandin F2α [5,70–73]. Elevations of this and other eicosanoids in human body fluids and tissues have been found in a diverse array of human disorders, including atherosclerosis, diabetes, obesity, cigarette smoking, neurodegenerative diseases, and many others [74]. Further, treatments for some of these conditions, including antioxidant supplementation, have been shown to decrease the levels of this class of biomarkers. In CF patients, respiratory exacerbations increase plasma levels of 8-iso-F2α [70], the levels of which in the breath condensate negatively correlate with respiratory function data [71]. At the same time, successful in vivo antioxidant therapy by GSH inhalation has been shown to decreased PGE levels in BALF, in association with changes in the number and activity of leukocyte subpopulations responsible for lung inflammation [73].

Oxysterols, a biomarker of cholesterol oxidation, were found to increase in CF plasma as a further proof of the abnormal lipid

metabolism and increased susceptibility to oxidation of lipoprotein lipids in CF patients [7]. Importantly, an increased oxidative burden of lung and blood lipids may produce bioactive lipid products that further sustain CF symptoms. Besides to 8-iso-F2α and other analogues with some bioactivity [74], arachidonic acid oxidation may contribute to persistent platelet activation and pulmonary dysfunction in CF via generation of bioactive isoeicosanoids [75], which provides sufficient rationale for a prevention therapy with fat soluble antioxidants such as vitamin E. Evidence has recently accumulated on the systemic effects of oxysterols on various tissues and organs [76]. The role of this lipid oxidation product alone or in combination with other factors could be further investigated in the context of mechanisms and clinical progression of multi-organ failure of CF patients.

The impaired pancreatic and liver functions of CF patients represent the underlying factor for a defective lipoprotein metabolism and hypocholesterolemia, which exponentially increase the burden of damage by cholesterol and other lipids due to reduced blood transport of fat-soluble antioxidants with nascent VLDL particles. Besides oxysterol accumulation, plasma fatty acid composition is also affected [7], and an increased ratio between unsaturated and saturated fatty acid species may contribute to lower cellular antioxidant defenses.

Altogether, these findings suggest that lipid oxidation biomarkers can provide a reliable measure of systemic and lung-specific oxidative stress in CF.

Markers of protein damage are also detectable in the airways of children with CF and their levels are observed to parallel the extent of neutrophilic markers and lung dysfunction [14,31,66]. Bronchoalveolar lavage proteins undergo halogenation of Tyr residues, a radical-mediated process presumably depending on MPO enzyme activity and assessed through the analysis of 3-chlorotyrosine and 3-bromotyrosine. Thiocyanate and protein carbonyls are also useful biomarkers to assess the inflammation-related injury of BALF proteins in CF [14,31].

3. Antioxidants in CF

3.1. Glutathione and its related defense system

3.1.1. Defects in GSH homeostasis

Other studies have pointed to alterations in the levels of extracellular antioxidants in respiratory tract lining fluids [3]. In particular, the analyses of broncheoalveolar lavages (BAL) have revealed the presence of decreased levels of GSH in the alveolar epithelial lining fluid of CF patients. The normal level of extracellular GSH in the lung is 140 times that present in blood plasma, and the lung is a net importer of circulating GSH [77]. As a result, GSH concentration in ELF is close to 400 μM, whereas significantly reduced GSH levels are present in adult CF patients [41]. Low levels of GSH have been observed in plasma and blood neutrophils [41,78] suggesting systemic GSH dyshomeostasis in CF. Moreover, some studies have shown that at the cellular level, the CFTR mutation causes mitochondrial depletion of GSH [79,80]. The consequences of this defect are still difficult to be understood due to our poor knowledge about the exact functions of GSH in the lung, but there are reasons to believe that the decrease of GSH in the ASL contributes to lung infection and inflammation [42,81]. At the same time, it must be underlined that a recent study has shown that the GSH content in sputum samples is higher in CF patients than in healthy people, indicating that GSH deficiency in CF is restricted to the lower respiratory tract [82]. Several hypotheses have been proposed to explain such a local increase in GSH, including the possibility that it may derive from apoptotic neutrophils. The release of high levels of GSH may be part of a compensatory mechanism aimed at controlling disulfide bonds-mediated aggregation of mucins. Secretions of the upper respiratory tract contain abundant levels of these glycosylated and cysteine-rich proteins, likely playing a role in the airway antioxidant defense. Indeed, these proteins are potent in vitro ROS scavenges [83] and their synthesis is upregulated upon oxidative stress via epidermal growth factor receptor (EGFR) transactivation [84].

GSH, a major component of cellular antioxidant defenses, exerts important functions related to its electron-donating capacity, including protection from the damaging effects of ROS and regulation of a plethora of cellular events, such as gene expression, proliferation and differentiation, apoptosis and immune response [85]. GSH is synthesized by two sequential ATP-dependent reactions catalyzed by γ-glutamylcysteine synthetase (recently, renamed glutamate-cysteine ligase) and GSH synthetase. The reaction catalyzed by γ-glutamylcysteine synthetase, i.e. the formation of γ-glutamylcysteine from glutamate and cysteine, is the rate-limiting step in GSH synthesis and is feedback regulated by GSH itself. In fact, this enzyme represents an interesting case of redox-regulation of catalytic activity that is mediated by the reversible formation of disulfide bonds [86]. Oxidizing conditions causing GSH depletion promote the formation of a disulfide bond between the catalytic and the regulatory subunits of the enzyme, leading to a conformational change which favors the binding of glutamate. In contrast, physiological levels of GSH reduce this disulfide bond, thus explaining GSH feedback inhibition.

Either in intracellular or extracellular compartments, GSH is predominantly found in the reduced form, although small amounts of the oxidized disulfide forms (GSSG or GSSR, where a GSH molecule is linked to a free or a protein thiol) can always be detected. GSSG is produced by the catalysis of glutathione peroxidase, during the detoxification from hydrogen peroxide and other peroxides, or by the direct reactions of GSH with electrophilic compounds, such as radical species. Despite the bulk of GSH synthesis occurring in the cytoplasm, GSH is distributed in intracellular organelles, including the endoplasmic reticulum, mitochondria and nucleus. Under physiological conditions, the GSH to GSSG ratio in these compartments is high, frequently > 100/1, but this may change under conditions of oxidative stress [87]. A major exception is represented by the endoplasmic reticulum, where GSSG is present at much higher levels to favor disulfide bond formation [88]. Interestingly, the compartmentalization of GSH in separate pools within organelles allows localized alterations in the balance between GSH and GSSG that may have considerable functional and pathological significance [89]. This could be particularly important in CF, in view of the above mentioned studies showing that cultured CF cells have an apparent normal GSH/GSSG content, but are characterized by a marked decrease in mitochondrial GSH in association to elevated mitochondrial ROS [79,80].

Different roles of high levels of GSH in the ELF may be hypothesized, including: a) preventing inflammation and tissue damage by scavenging the ROS spontaneously generated in this highly oxidizing environment, actively produced by neutrophils during inflammation or originating from lipid peroxidation, b) regulating the redox status of membrane proteins involved in the transduction of signals leading to changes in the expression of genes involved in the immune response, c) controlling mucus viscosity by breaking disulphide bonds, d) modulating the response to bacterial infections. This last possibility is suggested by the observation that GSH significantly increases in the ASL of wild type mice following *P. aeruginosa* infection, whereas this response is not observed in CFTR mutant mice [90]. Interestingly, some authors have suggested the ability of GSH to control *P. aeruginosa* growth and resistance to antibiotics, although these studies should be considered with some cautions due to the likely use of unbuffered GSH [91].

Although the decreased levels of GSH in ELF could be due to increased consumption during inflammation-related oxidative stress [41,92], GSH deficiency in CF ELF likely derives from CFTR channel dysfunction. In fact, comparable alterations in GSH extracellular content characterize the lung of CFTR knockout mice [93], indicating that this defect is correlated to mutations in CFTR.

In particular, ELF and lung tissue from CFTR knockout (*Cftr* KO, B6.129P2-Cftr^{tm1Unc}) and wild-type mice were compared for GSH content and the activities of glutathione-related enzymes [93]. In the ELF, the concentration of GSH was significantly decreased in the *Cftr* KO mice compared to WT, whereas tissue concentrations of GSH were similar [92]. In the *Cftr* KO lung, the activities of glutathione reductase and glutathione peroxidase were increased, whereas the activity of γ-glutamyltransferase was unchanged. Two indicators of oxidative stress, thiobarbituric acid reactive substances (TBARS) and 8-hydroxy-2-deoxyguanosine (8-OHdG), were also increased in the *Cftr* KO lung tissue [90]. These data support the hypothesis that a mutation in the CFTR gene can affect the antioxidant defenses in the lung and may contribute to the exaggerated inflammatory response observed in CF. Thereby, CFTR could be considered as an important actor of ELF antioxidant homeostasis and thus an intrinsic cause of oxidative imbalance in CF airways of human patients as well as *Cftr* KO mice.

Moreover, CFTR belongs to the MRP/ABC family of proteins which includes several GSH transporters and some in vitro studies have indicated that CFTR may mediate GSH export across membranes [2,3,94]. Despite these evidences, there is still debate about the physiological implication of CFTR in GSH transport outside the cells as other studies have raised the possibility that CFTR may not actually conduct GSH, but regulate its transport indirectly through chloride transport [85].

3.1.2. In vitro studies suggesting a protective role of GSH in CF model systems

Possible protective roles of extracellular GSH in the CF lung have been long proposed and recent in vitro studies have provided further and more robust support to this clue. For example, it has been suggested that GSH may control the levels of chlorinated compounds formed by the activity of myeloperoxidase, a neutrophil-released protein abundantly present in CF patients secretions [95,96] and prevent NK-κB activation [96]. Other studies have suggested that CFTR mutant cells produce higher levels of proinflammatory cytokines in response to *P. aeruginosa* diffusible material with respect to wild type cells, through a mechanism involving the activation of NADPH oxidase. This effect may be significantly reversed by the addition of extracellular GSH [97].

GSH could also play an important role in protecting the lung epithelia from the toxic effects of pyocyanin (PCN), a redox-active exotoxin released by *P. aeruginosa* which is supposed to cause a variety of deleterious effects on the airway physiology [98]. PCN levels as high as 130 μM have been measured in pulmonary secretions of patients with CF and individuals with chronic bronchiectasis [99]. At concentrations within the range measured in the sputum from CF patients, PCN induces a drastic reduction of intracellular GSH [100,101], promotes death of cultured cells [100] and causes pathophysiological alterations in the lung of wild type mice that are consistent with the changes observed in CF patients [102]. PCN toxicity likely derives from its ability to accept electrons from cellular reductants and then react with oxygen to generate superoxide and other ROS [103]. Therefore, GSH deficiency is explained either by the reaction of GSH with such oxidants or through the direct reaction of PCN with GSH that leads to the formation of a PCN radical [103]. However, the electron transfer from GSH to PCN does not occur at neutral pH and recent observations suggest that extracellular GSH provides significant protection against the toxic effects of PCN [104]. Moreover, GSH can react with PCN to form a stable adduct which is likely redox inactive [105]. Although the relationships between extracellular GSH and PCN are still contradictory, it is worth mentioning that extracellular GSH increases to millimolar levels in the ELF of wild type mice infected with *P. aeruginosa*, indicating that GSH may be useful to resist to bacterial colonization [90]. Interestingly, in vitro studies have revealed that extracellular GSH inhibits the ability of *Burkholderia cenocepacia* to enter epithelial respiratory cells and reduces bacterial induced expression of proinflammatory cytokines (D'Orazio, Pacello and Battistoni 2011, unpublished results).

3.2. GSH-based antioxidant therapies

3.2.1. Preclinical studies in animal models of CF

Mice genetically modified for the *Cftr* gene, along with acute and chronic infection induced by CF-related pathogens, are a key asset in CF research. Although much has been learned through these CF mouse models, limitations in the ability of this species to recapitulate spontaneous lung disease and several other organ abnormalities seen in CF humans, including few airway mucous glands, have created a need for additional species on which to study CF [106]. To this end, pig and ferret CF models have been generated and are currently being characterized [107,108]. These new larger animal models have phenotypes that appear to closely resemble human CF disease seen in newborns, and efforts to characterize their adult phenotypes are ongoing. However, mice have been the dominant species by which to study CF disease processes in vivo and develop therapies for the past two decades including GSH-based antioxidant treatment.

Despite limitations and significant species differences between mice and humans, these models proved to be useful tools to mimic the initial and progressive bronchopulmonary infection typical of CF patients [109]. In particular, the model of chronic infection, which challenge bacterial cells with agar as an immobilizing agent, has been extensively characterized, and induce the long-term persistence of the bacterial infection and lung pathology, including airway inflammation [110,111]. Lung pathology associated with chronic experimental infection resembled some aspects of the advanced chronic pulmonary disease at autopsy in CF patients [112–114]. Both naturally occurring and experimental infections frequently manifest bronchopneumonia, bronchiectasis, mucus plugging, epithelial metaplasia, fibrosis, and alveolar exudates with inflammatory cells. Lymphoid hyperplasia, which was prominent in the infected animals, was also a common finding in the lungs of CF patients. In addition, significant differences with regard to weight loss, BAL neutrophil counts and cytokine concentrations have been detected. Infected mice had a rapid though transient rise in absolute neutrophil counts, TNF-α, IL-1β, IL-6, MIP-2, and KC in bronchoalveolar lavage (BAL) [110,115]. In addition, the generation of CF mice has allowed the possibility of in vivo testing of novel therapies before entering in clinical trial. These include the pre-clinical evaluation of antibiotics and biotechnological drugs, as well as of natural and synthetic anti-inflammatory agents that reduce the excessive recruitment of neutrophils and the progressive damage of the respiratory tissue by the unbalanced production of oxidants.

Regarding antioxidants, GSH and its pro-drug N-acetylcysteine (NAC) remain the so far most investigated antioxidant agents in CF and several strategies have been proposed to improve systemic and lung GSH status of CF patients based on pre-clinical studies, including the administration of nebulized GSH by inhalation or the oral supplementation of GSH or NAC. Oral GSH administration can raise serum and lung tissue GSH levels in rodents [116,117]. In these studies GSH was administered to animals dissolved in saline or PBS. The pharmacokinetic profile of an oral bolus dose of GSH (300 mg/kg) was determined in mice in other studies [118]. Plasma, ELF, BAL cells, and lung tissue were analyzed for GSH content. There was a rapid elevation in the GSH levels that peaked at 30 min in the plasma and 60 min in the lung, ELF, and BAL cells after oral GSH dosing. Oral GSH treatment produced a selective increase in the reduced and active form of GSH in all lung compartments examined. Oral GSSG treatment (300 mg/kg) resulted in a smaller increase of GSH levels. To evaluate the role of CFTR in this process, *Cftr* KO mice and gut-

Table 1
Intervention trials on antioxidant therapy in CF patients found at ClinicalTrials.gov database[a].

Rank	Title	PI	Location	Recruitment	Interventions	Age groups	Phases; number enrolled	Study designs[b]	Outcome measures	Start and completion date	NCT ID
1	Efficacy and safety study of inhaled GSH in CF patients	Griese M.	Germany	Completed	Drug: • reduced GSH sodium salt • 0.9% saline (control)	C|A|S	II 138	1. R|E 2. Safety/eff 3. PA 4. DB (Sub, Inv) 5. Treatment	• Differences between inhaled GSH and inhaled normal saline with respect to the area under the curve of FEV1% predicted within the period from baseline to week 24; • Treatment changes with respect to the variables:spirometry, peak flow, quality-of-life, weight/height, percentage of neutrophils/other cell types (induced sputum), induced sputum levels of GSH/inflammatory mediators, pulmonary exacerbation	Jul-07 May-10	00506688
2	A phase I study of inhaled sodium pyruvate for the treatment of CF	Billings M.C.E.	United States	Terminated	Drug: • Inhaled sodium pyruvate	A|S	I 70	1. R|E 2. Safety/eff 3. PA 4. DB 5. Treatment	• Assessment of safety of inhaled sodium pyruvate in Subs with CF. Subs will be evaluated for the presence of symptoms and safety laboratory measurements. • Determination of improvement in lungs of CF Subs as determined by measurement of FEV1 and measurement of inflammatory markers in induced sputum.	Feb-06 n.a.	00332215
3	Inhaled GSH versus placebo in CF	Marsico S.	Italy	Recruiting	Drug: • Inhaled reduced GSH • Physiological solution	C|A	III 150	1. R|E 2. Eff 3. PA 4. SB (Sub) 5. Treatment	• FEV1% • Small airway function; exercise capacity; BMI; dyspnoea; cough; quality of life; pulmonary exacerbations; markers of oxidative stress (H_2O_2)in serum and in EBC; epithelial inflammatory markers on BNEC	Jun-10 Dec-12	01450267
4	Safety and efficacy of an antioxidant-rich multivitamin supplement in CF	Sagel S.D.	United States	Completed	Dietary supplement: • AquADEKs	C|A	II–II 17	1. NR|E 2. Safety/eff 3. SGA 4. OL 5. Treatment	• Plasma levels of β-carotene • Plasma levels of coenzyme Q-10, retinol (Vitamin A), 25-hydroxy vitamin D, α- and γ-tocopherols (Vitamin E), PIVKA-II	Aug-07 Nov-09	01018303
5	Efficacy and safety of epigallocatechin gallate (EGCG)/tocotrienol in 18 patients with splicing-mutation-mediated CF	Kerem E.	Israel	Not yet recruiting	Dietary supplement: • EGCG • Tocotrienol • EGCG + tocotrienol	A|S	n.a. 18	2. Safety/eff 3. CA 4. OL 5. Treatment	• Changes in nasal chloride secretion as assessed by TEPD, with assessment of mean changes in TEPD by drug compared to baseline and the proportion of patients with a chloride secretion response by drug compared to baseline • Pulmonary function testing: FEV1, FVC, MEF25-75	Sep-09 Jun-11	00889434
6	The effect of inhaled NAC compared to normal saline on sputum rheology and lung function	Van Daele S.	Belgium	Terminated	Drug: • Acetyl-Cys • Normal saline	C|A	IV 19	1. NR|E 2. Eff 3. CA 4. OL 5. Treatment	• Changes in visco-elasticity and lung function	Jan-10 Dec-10	00996424

7	Safety of orally administered curcuminoids in adult subjects with CF	Goss C.	United States	Completed	Drug: • Standardized turmeric root extract	A	I 11	1. NR|E 2. Safety/eff 3. SGA 4. OL 5. Treatment	• Safety and tolerability of 14 days of treatment with orally administered curcuminoids as assessed by adverse events, laboratory parameters, and spirometry. • Pharmacokinetics of repeated doses of orally administered curcuminoids; change in NPD measurements. change in sweat chloride measurements.	Apr-05 Jan-06	00219882
8	Nasal potential studies utilizing CF transmembrane regulator (CFTR) modulators	Rowe S.	United States	Recruiting	Other: quercetin	C|A	II 46	2. Eff 3. SGA 4. OL 5. BS	• NPD(NPD) Biomarker • Residual CFTR activity	Mar-10 Nov-11	01348204
9	Safety and tolerability of inhaled nitric oxide in patients with CF	Sagel S.	United States	Completed	Drug: • Nitric oxide for inhalation • Nitrogen	C|A|S	II–II 18	1. R|E 2. Saf 3. PA 4. DB (Sub, Care, Inv, OA) 5. Treatment	• Safety and tolerability of drug, assessed by change in methemoglobin levels, oxygen saturation, FEV1 • Assess the difference in sputum bacterial density before and after NO inhalation, and the difference in lower airway inflammatory measures before and after NO inhalation	Jul-04 Dec-08	00570349
10	NAC Phase IIB: a multi-center, phase IIB, randomized, placebo-controlled, DB study of the effects of NAC on redox changes and lung inflammation in CF patients	Conrad C.	United States	Completed	Drug: • NAC	C|A|S	II 80	1. R|E 2. Eff 3. SGA 4. DB (Sub, Care, Inv, OA) 5. Treatment	• Change in the logarithm of the level of human neutrophil elastase (HNE) activity measured in sputum • Change in concentration of IL-8 measured in sputum and plasma; concentration of GSH measured in whole blood; the neutrophil count measured in sputum	Nov-08 Feb-11	00809094
11	Glutamine supplementation in CF	n.a.	United States	Not yet recruiting	Dietary supplement: • Glutamine • L-alanine	A|S	II 40	1. R 3. PA 4. DB (Sub, Care, Inv, OA) 5. Prev	• Percent increase in plasma glutamine and GSH redox levels measured at weeks 0, 4, 8, and 12.	Feb-10 Feb-11	01051999
12	Effect of sulforaphane in broccoli sprouts on Nrf2 activation	Chmiel J.F.	United States	Active, not recruiting	Dietary supplement: • Broccoli sprouts	A	n.a. 15	3. SGA 4. OL 5. BS	• Nrf2 activation in NEC • Measures of lipid peroxidation in NEC; GSH from blood lymphocytes; oxidative stress in urine; neutrophil migration into the gingival crevices	Apr-11 Sep-11	01315665

Abbreviations:
A = adult; BC = breath condensate; BNEC = brushed nasal epithelial cells; BS = basic science; C = child; CA = crossover assignment ; Care = caregiver; CF = cystic fibrosis; DB = double blind; EBC = exhaled breath condensate; Eff = efficacy study; FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity; GSH = glutathione; Inv = investigator; MEF25–75 = maximal expiratory flow 25–75; n.a. = not available; NAC = N-acetylcysteine; NEC = nasal epithelial cells; NLF = nasal lavage fluid; NPD = nasal potential difference; NR|E = non-randomized|endpoint; OA = outcomes assessor; OL = open label; PA = parallel assignment; PI = principal investigator; Prev = prevention; R|E = randomized|endpoint; S = senior; Saf = safety study; SB = single blind; SGA = single group assignment; Sub = subject.

[a] Search was done matching the terms "antioxidant therapy" or "Glutathione" with "Cystic Fibrosis".

[b] 1. Allocation; 2. Classification; 3. Intervention model; 4. Masking; 5. Primary purpose.

corrected *Cftr* KO-transgenic mice were given an oral bolus dose of GSH (300 mg/kg) and compared with WT mice for changes in GSH levels in plasma, lung, ELF, and BAL cells. There was a twofold increase in plasma, a twofold increase in lung, a fivefold increase in ELF, and a threefold increase in BAL cell GSH levels at 60 min in WT mice; however, GSH levels only increased by 40% in the plasma, 60% in the lung, 50% in the ELF, and twofold in the BAL cells within the gut-corrected *Cftr* KO-Tg mice. No change in GSH levels was observed in the uncorrected *Cftr* KO mice. These studies suggest that oral GSH administration can increase plasma and lung compartment GSH levels in WT mice and to a lesser extent in gut-corrected *Cftr* KO-Tg animals. It also suggests that oral GSH treatment can boost BAL cell GSH levels. However, since this study failed to show significant increases in serum and lung compartment GSH levels in uncorrected *Cftr* KO mice, it is questionable whether oral GSH administration to CF patients with intestinal malabsorption would benefit from this therapy. It was also shown that GSH is rapidly distributed to the serum and lung compartments. Kariya et al. [118] speculate that other transporter(s) besides CFTR are responsible of the transport of GSH and probably of other dietary molecules to the lung, which may be responsible for dietary deficiencies observed in various lung diseases.

Another strategy is represented by the oral supplementation of high doses of NAC, a well known cysteine donor for the synthesis of glutathione. NAC is considered a safe molecule, which has been used successfully to treat GSH deficiency in a wide range of diseases [119]. As CF mice display defects in GSH export in the ELF comparable to those of patients, they could provide a useful tool to assess the effects of NAC administration on the GSH status. However, only limited studies exploring the effects of NAC on CF animal models have been so far carried out. The effects of NAC have been tested on mucus accumulation, bacterial load, transit, and inflammation in the CF mouse small intestine showing that NAC may reduce intestinal mucus accumulation bacterial overgrowth in the gut [120].

Moreover, NAC has been reported to restore the accumulation of unwanted/misfolded proteins in aggregates that are associated with the CF airway phenotype as a cause of lung inflammation [121]. The mechanism of this NAC-derived effect seems to involve the restoration of beclin 1 expression and activity in the autophagy pathway of the endoplasmic reticulum that was investigated in vivo using Scnn1b-transgenic and Cftr(F508del) homozygous mice. The restoration of this pathway also produced a rescued trafficking of CFTR (F508del) to the cell surface of CF cells obtained from human CF nasal biopsies.

Given the defective GSH metabolism of CF reviewed in the previous sections and in [122], and the reduced response to GSH therapy in CF mice [93], some Authors have investigated the influence of bacterial infections on lung oxidative stress. The effects of *P. aeruginosa* infection on ELF and lung tissue antioxidants and the oxidation of DNA and lipids were investigated in mice challenged with bacterial cells [90]. CFTR-KO (B6.129P2-Cftr^{tm1Unc}) and WT mice were challenged intratracheally with a clinical isolate of mucoid *P. aeruginosa* embedded in agar beads and on the third day of infection BALF and lung tissue were obtained and analyzed for cytokines, antioxidants and enzyme activities [90]. *P. aeruginosa* lung infection increased levels of inflammatory cytokines and neutrophils in the ELF. This corresponded with a marked increase in GSH and in urate levels in the ELF of *P. aeruginosa*-infected WT mice. A twofold increase in urate levels was also observed among lung tissue antioxidants of *P. aeruginosa*-infected WT mice. There were no changes in markers of lung oxidative stress associated with the *P. aeruginosa* lung infection. In contrast to WT mice, the CFTR-KO mice lacked a significant increase in ELF GSH when challenged with *P. aeruginosa*, and this correlated with a decrease in the ratio of reduced to oxidized GSH in the ELF, a marker of oxidative stress. These data would suggest that the lung adapts to infectious agents with elevated ELF GSH and urate. Therefore, individuals with lung diseases associated with altered antioxidant transport, such as CF, might lack the ability to adapt to the infection which may lead to a more severe inflammatory response.

3.2.2. Clinical trials on GSH

The discovery of the defect in GSH export has suggested that therapies able to restore or increase GSH levels in the ASL could counteract the inflammation and oxidative stress conditions typical of CF patients. In an attempt to strengthen extracellular defenses against ROS, some pilot studies have analyzed the effect of GSH inhalation or that of oral GSH prodrug N-acetylcysteine (NAC). All these treatments were well tolerated by the CF patients and most authors were able to measure increased ELF concentrations of GSH in association with some positive clinical outcomes [40,41,73,78,96,122–128]. Although potentially promising, these findings need stronger clinical evidence in that the majority of these were obtained on very limited number of patients investigated in non-randomized controlled trials. This is highlighted in a recently published meta-analysis [129] that provides also a thoroughly analysis of the literature on this aspect of the antioxidant and anti-inflammatory therapy of CF. Among the American CF foundation sponsored trials two phase II trials on inhaled GSH and oral NAC are in progress in US and Germany (ClinicalTrials.gov, Identifier: NCT00506688 and NCT00809094, respectively; Table 1), and safety and tolerability of aerosolized glutathione is also matter of investigation by another (not registered) US trial (more information on this can be found in [130]). Nevertheless, the diffusion among CF patients of NAC preparations for inhalation (Mucomyst®) has increased in recent years.

γ-Glutamylcysteine ethyl ester (GCEE) is another potentially interesting GSH pro-drug, which has proved some efficacy in the amelioration of oxidative stress e.g. in experimental myocardial infarction [131] and central nervous system conditions (see e.g. [132]). However, GCEE has not been investigated in CF yet.

3.3. Limits and potential problems associated to thiol-based therapies

Although the above cited clinical trials may be considered promising attempts to improve the antioxidant levels in the ELF, the actual capacity of these treatments to produce positive clinical effects must be considered with caution. For example, indices of oxidative damage were found to be unaffected by aerosolized GSH treatment [122]. Some in vitro studies have suggested that the reaction of GSH with PCN could produce hydrogen peroxide, with potential exacerbation of oxidative damage [101]. The exact mechanisms of PCN toxicity and the reaction of this toxin with GSH must be better understood in order to evaluate the safety of GSH administration to patients colonized by *P. aeruginosa*.

Inhalation of GSH ensures its direct delivery in the airways, but since GSH can rapidly convert to its oxidized form GSSG, frequent GSH inhalations are required to maintain a high GSH/GSSG. As a consequence of this limit, four separate inhalations have been used in the study carried out by Bishop et al. [123]. Unless justified by clear clinical improvements, these repeated treatments may represent a burden for patients already undergoing complex therapies. Moreover, repeated inhalations of GSH increase the levels of GSSG in ELF [133,134]. In the absence of effective homeostatic mechanisms ensuring the fast recycling of GSSG to GSH, this might produce unwanted effects and even the exacerbation of CF symptoms being GSSG responsible of the S-glutathiolation and functional inhibition of CFTR [124].

In principle, the oral administration of GSH could be considered a safe strategy to prevent GSSG accumulation, but this strategy is likely not feasible due to the substantial inability of this water-soluble molecule to cross biological membranes. Moreover, a study carried out in mice has established that GSH absorption in the gastrointestinal tract, if any, is mediated by CFTR itself, thus excluding the possibility to

improve circulating levels of GSH in CF patients through the dietary supplementation of the antioxidant [127]. The limited absorption of GSH could be overcome by GSH-esters, as in vitro studies have shown that this form of GSH may be specifically useful to rescue mitochondrial defects in cystic fibrosis models [80]. Safety of these GSH derivatives in humans has been poorly investigated.

Two independent studies have shown that the treatment of CF patients with high doses of NAC increases extracellular GSH in sputum [78,125], but contrasting results have been reported concerning the effects of NAC on the concentrations of blood GSH and on the levels of IL-8 and other markers of inflammation. NAC treatments may be useful to modulate the GSH content in cells but it should be reminded that an enhanced cysteine supply cannot lead to an increase of GSH above physiological levels due to the feedback inhibition mechanism of γ-glutamylcysteine synthetase, described in the Section 3.1.1.

3.3.1. Gamma-glutamyltransferase and GSH therapy

So far none of the studies mentioned above has taken into account the fact that GSH is degraded by GGT enzyme activity. GGT concentrations are known to increase several fold in ELF of CF patients, even if the mechanisms for this effect were not determined [135], and this phenomenon parallels the above described decrease of GSH levels in ELF. Preliminary data suggest that a major source of increased ELF GGT is represented by activated neutrophils accumulating in diseased airways (Corti and Pompella, 2011, unpublished observation). Regardless of its origin, it is likely that increased GGT in ELF would degrade locally administered GSH to variable extents, which could contribute to the so far inconclusive results of therapies based on aerosolized GSH. Besides its role in GSH catabolism, GGT has been shown to mediate protein S-thiolation [136], suggesting that GSH administration in the presence of active GGT enzyme might alter CFTR glutathiolation status and function in a potentially unfavorable way. On the other hand, a potential role of GGT in favoring bronchial uptake of antioxidant vitamin C has also been suggested [137].

These controversial findings may add further issues to the question of whether the therapy of lung oxidative stress by aerosolized GSH could be safe in all the CF patients regardless of specific strategies that would ascertain the extent of lung inflammation. These strategies should include the assay of GGT levels in ELF. In principle, once an adverse role of ELF GGT in GSH therapies will be confirmed, the association of GGT inhibitors in the GSH formulations for inhalation could represent a promising pharmacological strategy.

3.4. Malnutrition as a possible cause of defective thiol-dependent antioxidant protection

Successful nutritional interventions strongly impact on the clinical outcome of CF patients [138–140]. Malnutrition by pancreatic insufficiency and other CF-related factors influence the susceptibility to develop recurrent infections and severe inflammatory lesions of the lung tissue. If the onset of a defective (sub-optimal) antioxidant status may represent an underlying component in the clinical effects of malnutrition remains a matter of investigation. Clinicians have to pay particular care to avoid these disturbances and successful protocols of nutritional intervention in CF have been developed, which are essentially aimed to avoid the onset of protein-energy malnutrition (PEM) [141]. Despite this, the risk of developing such an untoward complication in CF infants and children remains high as suggested by the prevalence data registered in some areas. Actually, a prevalence of PEM between 5 and 14% in Moldavian infants was recently reported [142] and PEM is associated with poor outcome and particularly with the risk of developing edema and anemia.

Besides lowered intake and absorption of micronutrient antioxidants discussed in the other sections, malabsorption of dietary protein and excess fecal amino acid losses result in hypoproteinemia/hypoalbuminemia as key biochemical signs of PEM. Hypoalbuminemia is considered to be linked with a cause–effect relationship with oxidative stress and is proposed to influence morbidity and mortality in conditions associated with chronic inflammation and severe oxidative stress [143,144]. The human serum albumin (HAS) molecule contains only a reactive thiol group, e.g. the Cys 34, the importance of which as an antioxidant defense system in blood and for the entire organism is well documented [145]. This is the second main thiol (and the main protein thiol) in the circulation (approx. 2 mmol in the adult organism, assuming 5 l of total blood volume, an Ht of 40% and [HAS] of 45 g/l of plasma), being the RBC GSH the first thiol in blood (near to 5 mmol, assuming the same parameters of above and an average concentration of GSH in packed RBC of 2.5 mM) [146,147]. The antioxidant role of HSA is not only a consequence of the relative abundance of its Cys thiol. A specific capability of acting as a sacrificial target for a series of electrophils and most biologically relevant ROS, i.e. hydrogen peroxide and peroxynitrite has been demonstrated in a series of studies [145,148,149]. Ligand binding activity can contribute to promote antioxidant effects by the HSA molecule [145]. Transitions metals, particularly copper and also iron in the case of iron-overload diseases, bind to HSA. In this way, these are less available to promote the Fenton chemistry and hydroxyl radicals eventually released from this oxidative reaction are mostly directed to the HAS protein sparing more important targets. A free radical-trapping activity of HSA has been also demonstrated and this was proposed to be directed toward both hydrophilic and fat-soluble species. This activity may result from the interaction with other antioxidants such as α-tocopherol [150] and may influence the antioxidant activity of food-derived phenolic antioxidants [151].

However, Cys 34 is considered the main contributor to the antioxidant function of HAS, which plays its role in the antioxidant homeostasis of blood thanks to a complex series of interactions with the metabolism and antioxidant function of the pool of free thiols in plasma and in the circulating RBC, with the latter playing a significant contribution to the extracellular pool of GSH [146] and to the dynamics of inter-organ GSH metabolism in cooperation with liver and other tissues [147]. Immuno-inflammatory cells and the lung tissue are among the main terminals of this metabolism. The RBC contain the entire machinery to synthesize GSH, to restore its redox (by enzymatic reduction of the oxidized form), and to use this as cofactor of Se-GPx and GSH-S-transferase enzymatic activities that are responsible of the detoxification of hydroperoxides and alkylating agents that may form in or enter the RBC cytosol [147]. In this sense, the RBC represents a circulating reservoir of GSH that, in addition to maintaining the redox and respiratory function of Hb, participates to systemic protection of xenobiotics of endogenous and exogenous origin. In consideration of these aspects, the combination of hypoalbuminemia and anemia may exponentially increase the risk of developing oxidative stress in CF patients as it is supposed to occur in other oxidative stress conditions such as chronic kidney disease [55,143,152]. Additional clinical investigation should verify the hypothesis that hypoalbuminemia together with a defective uptake and metabolism of sulfur-containing amino acids and CF-specific defects of the GSH metabolism, may represent a causal risk factor for an impaired antioxidant defense and systemic oxidative stress in CF patients.

3.5. Fat-soluble antioxidants

Pancreatic insufficiency and a diminished bile acid pool cause malabsorption of important essential nutrients and other dietary components in CF. Of particular significance is the malabsorption of fat-soluble antioxidants such as tocopherols, carotenoids and coenzyme Q-10 (Co-Q10), and that of essential fatty acids (EFA).

3.5.1. Vitamin E

Vitamin E therapy in CF has been proposed in several decades of research as a useful approach to overcome both the lower absorption of this fat-soluble micronutrient and the increased antioxidant

demand by the abnormal generation of ROS in CF tissues (see for instance the recommendations by the Cystic Fibrosis Foundation Consensus Conference on nutrition [153]).

The first report of a vitamin E deficiency in CF appeared in literature in 1951 by Filer et al. [154]. In this study, the absorption of the main form of this vitamin, e.g. α-tocopherol, and its ester derivatives was investigated in several subsets of infants and children. Thanks to a simple bioavailability (or tolerance) test, these authors observed that "infants… diagnosed as fibrocystic disease of the pancreas, diarrhea and cirrhosis were characterized by a poor response to the test, i.e., the tolerance curve was low". However, abnormalities were also observed in other subgroups of infants with a variety of disorders not associated with fat or fat-soluble vitamin intolerance, such as sprue, celiac syndrome and lupus erythematosus, which suggests the general observation that malnutrition and inflammatory and degenerative diseases of the GI tract, may lead to absorb tocopherols poorly. At the same time, these authors reported that "Metabolic disorders with associated hypercholesterolemia were observed to give abnormally high values for the area under the curve" and that patients responding poorly to tocopherol absorption test did also in vitamin A absorption tests.

The finding of lowered (lipid uncorrected) levels of vitamin E was confirmed in other studies in which this relative deficiency was found to occur irrespective of pancreatic comorbidity and in association with lowered levels of other liposoluble vitamins such as vitamin A and D [155] regardless of their different liver metabolism and tissue delivery mechanisms [156,157].

Plasma, buccal mucosal cells (BMCs), and breath condensate α-tocopherol decreased significantly with age in association with a decreased respiratory function [5]. This was accompanied by lowered levels of other antioxidants such as vitamin C, and increased oxidative stress markers of different origin such as protein carbonyls, thiobarbituric acid-reactive substances, and F_2-IsoPs.

Clinical symptoms of vitamin E deficiency in CF have not been conclusively investigated. Dolan et al. [158] described that anemia of CF patients is related to vitamin E deficiency and increased peroxide-induced hemolysis of RBC. Other authors, however, observed an increased susceptibility to peroxide-induced hemolysis also in the presence of normal levels of vitamin E [159]. Peters and Kelly [160] observed that RBC vitamin E concentrations were below the normal range in almost all unsupplemented patients and rose into the normal range with a 1-year supplement of 100 mg per day, but not 15 mg per day. Since RBC vitamin E concentration has been shown to correlate well with tissue concentrations of the vitamin in animals, tissue levels of vitamin E are expected to be lower than normal in CF patients.

Bioavailability of fat-soluble vitamin is limited in CF. Vitamin E deficiency of CF is also associated with hypocholesterolemia [7]. As a consequence, the relative deficiency of this vitamin is compensated when the absolute levels are normalized for cholesterol levels, and this suggests a poor transferring of this vitamin in the circulation by a defective lipid and lipoprotein metabolism. Pancreatic insufficiency and the consequent lipid malnutrition cannot completely explain this defect and other, possibly CF-specific, dysfunctions could play a role. Liver metabolism and specific plasma transport systems of this vitamin need further elucidation in CF.

As a consequence of these aspects, it is not presently clear which form and level of supplementation of this vitamin is most appropriate to treat these patients. Using α-tocopherol as a vitamin E supplement, Peters et al. [160] reported that 100 mg per day are required to normalize RBC concentrations. Other authors described unsuccessful supplementation protocols with higher doses and this has led to develop formulations with higher bioavailability in order to achieve better compliance to oral supplementation (see below and the literature reviewed in [161–163]).

Besides absorption and tissue delivery issues, specificity of action is another critical item of antioxidant therapy with fat-soluble agents in CF. As further addressed below, this aspect could be the main limit to a successful use of natural forms of vitamin E in the clinical management of CF inflammation even if a local lung-targeted therapy would be developed according with so far proposed pre-clinical models of aerosolized vitamin E [164,165].

Current pharmacological research is aimed to develop synthetic forms of this and other fat-soluble antioxidants with better radical scavenging properties at the lipid–water interface. Type of ROS target and the sites of action greatly influence the chances of a fat-soluble antioxidant of alleviating oxidative stress in the airways as well as in other organs such as liver and pancreas. These aspects dealing with specificity of action [163,166,167] have stimulated the search of novel vitamin E-derived antioxidants that may help to scavenge radicals at the lipid–water interface of the epithelial cell membrane and surfactant. Amine derivatives of tocopherols and tocotrienols have been recently demonstrated to show higher antioxidant and free radical scavenging activity than α-tocopherol [168]. Further in vitro pharmacological analysis has included toxicity evaluations and the detailed investigation of scavenging of azo- and phenolic radicals with different degrees of hydrophobicity, and the inhibitory activity on IL-8 gene expression and phospholipase activity in CF cells. Comparative evaluation with other synthetic derivatives, such as α-tocopheryl succinate and natural forms of vitamin E, suggested that these amine derivatives are promising antioxidant and anti-inflammatory agents [Galli F. and Pilolli F., unpublished observation] deserving further pre-clinical investigation in CF model systems.

Anti-inflammatory effects of natural and synthetic analogues of vitamin E are also an intriguing pharmacological opportunity currently under investigation by several laboratories [169,170].

Recently, vitamin E supplementation has been at the center of a dispute regarding its safety when used at high dosages in certain populations of patients. An extensive and speculative debate originated on this subject after a meta-analysis study by Miller et al. [171] that examined the largest secondary prevention trials on vitamin E trials in cardiovascular patients finding a significantly increased mortality risk for all the causes (about 4%, 1–8% in the 95% interval of confidence) when the patients were treated with doses >400 IU/die (that are equivalent to 400 mg/die of the synthetic form *allrac*-α-tocopherol and to 185 mg/die of the natural form *RRR*-α-tocopherol). This debate resulted in a careful examination of this meta-analysis study by several other authors and in further revisions of the literature on vitamin E toxicity in humans [see the literature recently reviewed in [163,172–174]] that clearly demonstrated the poor consistency of the conclusions raised with Miller's meta-analysis study and the paucity of the concerns that derived from that. These conclusions have been verified in the recent international symposium on vitamin E of the Society for Free Radical Research, Europe branch of Rome 2009 [173,175]. Vitamin E used as supplement for humans in all its forms (e.g. α-tocopherol and other tocopherols and tocotrienols) is safe in abroad range of intakes [172,176]. The tolerable upper intake level (UL) and the Acceptable Daily Intake (ADI), established by the Joint FAO/WHO Expert Committee on Food Additives for the natural form of vitamin E as α-tocopherol equivalents are of 300 mg/die and 0.15–2.0 mg/kg body weight/die, respectively [172,177].

3.5.2. Carotenoids

Levels of plasma carotenoids such as β-carotene, β-cryptoxanthin, and total lycopene are significantly lowered in CF patients and this was associated with higher susceptibility to lipid peroxidation [5,178–180]. Rust et al. [178] demonstrated that the long-term oral supplementation with 50 mg β-carotene/day (i.e. 1 mg β-carotene/kg BW/day) restored the levels of this carotenoid, while sub-optimal supplementation was observed at doses of 10 mg β-carotene/day or lower, thus confirming the need of high doses of this fat-soluble factor to overcome the limited absorption and thus to achieve plasma concentrations of healthy control subjects. Successful high-dose treatments appear to lower oxidative stress markers such as TBA-MDA

complexes and to correct total antioxidant capacity of plasma. In another study, β-carotene supplementation was observed to decreased lipid peroxide formation as quantitated by malondialdehyde concentrations in plasma (TBA/HPLC method), and to enhance the resistance to copper(II) ion-induced oxidation of low density lipoproteins [180].

At the same time, toxicity issues have been raised for human supplementation with carotenoid formulations and particularly of water-miscible formulations of preformed vitamin A that is regularly supplemented to CF patients, which may increase serum retinol and possible risk of CF-associated liver and bone complications (reviewed in [161,181]). However, β-carotene supplementation seems to be safe since this does not affect plasma concentrations of other carotenoids and retinol, as well as of other fat-soluble vitamins as α- and γ-tocopherol [178]. Recent studies designed to test the clinical efficacy of a CF tailored multivitamin formulation (commercial name AquADEKs®), also tested the safety of this type of formulation and demonstrated that this does not increase vitamin A above the normal levels observed in healthy controls [162,182,183]. The normalization of β-carotene levels obtained in these studies was associated only with minor improvements on respiratory and growth parameters, while the levels of urinary F2-IsoPs used as index of lipid peroxidation were not affected [162]. The surrogate marker of lipid peroxidation MDA was affected together with some selected antioxidant parameters (RBC thiols and superoxide dismutase) in another study in which this multivitamin formulation was preliminarily evaluated in comparison with standard formulations of vitamin E and A [182].

3.5.3. Coenzyme Q-10

Coenzyme Q-10 (Q10) is a well-known electron transporter in the mitochondrial respiratory chain with fundamental role in cellular bioenergetics and scavenging of radical species [184]. This lipophilic substance is present in the circulation at low levels (serum concentrations ≤ 2 μM) mainly as ubiquinol-10, e.g. the reduced form, with an approximate ratio of 95:5 with the oxidized form ubiquinone-10 [185,186]. A mechanism for a preferential distribution and accumulation in mitochondria has been suggested for both the reduced and oxidized forms of CoQ10 that are taken up by the cells in a time- and concentration-dependent. Subcellular localization and trafficking of exogenous Q10 are similar to those of the endogenous form, but were different from that of α-tocopherol that is related with lipid composition, particularly in the mitochondrial and microsomal fractions [184]. Ubiquinol-10 readily oxidizes ex vivo by the reaction with other lipophilic antioxidants such as α-tocopherol and butylated hydroxytoluene [185]. Therefore a higher reduction potential than other physiological fat-soluble antioxidants such as vitamin E and a selective metabolism and cellular trafficking, show peculiar role for the cell CoQ which may also represent an important lipophilic antioxidant in cells and body fluids.

Human cells synthesize this coenzyme through the cholesterol biosynthesis pathway so that more than two thirds of the tissue levels appear to have an endogenous origin; dietary sources provide a contribution to Co-Q10 levels of blood and all solid tissues that varies depending on the dose applied and type of dietary source [187]. However, in the case of oral supplements, it has to be considered that hydrophobicity and large molecular weight of this coenzyme influence its absorption that ultimately is slow and limited [186,187]. Likewise to vitamin E pharmacokinetics [188,189], Co-Q10 showed T(max) of around 6 h that coincides with that of dietary lipids. Elimination is close to that of the more retained form of vitamin E in human body, e.g. α-tocopherol, with a half-life of about 33 h, which suggests poor hepatic metabolism. However, commercially available formulations are reported to be safe even at high doses and solubilized formulations show enhanced bioavailability. In healthy subjects plasma Q10 response to oral ingestion show saturation profiles with a plateau at a dose of 2400 mg and the higher plasma concentrations were found to facilitate uptake by peripheral tissues and also the brain [186,187].

Laguna et al. [190] recently investigated total serum levels of coenzyme Q-10 in a wide population of CF children (n = 381) and estimated their association with clinical outcome. Near to 50% of these CF patients were deficient of Co-Q10 and this defect was significantly more prevalent in patients with pancreatic insufficiency and significantly associated with *P. aeruginosa* colonization in infants (under 24 months of age). Importantly, low Co-Q10 levels correlated to other lipid markers of a poor nutritional status, such as total lipids and also the other fat-soluble antioxidants β-carotene and α-tocopherol, which confirms the presence of a common defect in the absorption and metabolic pathways of this coenzyme with dietary lipids.

The deficit of Co-Q10 may contribute to the impaired energy function of mitochondria of CF tissues and this may exacerbate CF-linked inflammation, infection and cellular stress response of the lung. A systematic analysis of molecular lesions in CF bronchial tissue has been recently carried out by proteomic approach [191]. Comparative evaluation of protein expressional pattern in CF and healthy control tissues has revealed aberrant levels of some mitochondrial and energy-related proteins in CF specimens that included the ubiquinol–cytochrome c reductase complex core protein I and one form of nidogen, a pseudogene of aconitase 2. These changes in CF may reflect molecular changes which could be associated with an altered mitochondrial homeostasis and Co-Q10 redox.

Multivitamin supplements with high bioavailability containing Co-Q10 have demonstrated to correct the deficit of this antioxidant and were preliminarily observed to improve airway inflammation markers in CF patients [183]. However, further clinical investigation failed to demonstrate that such an improved biochemical profile is associated with significant improvements in weight percentile and pulmonary function [162].

3.5.4. Fatty acids

A key pathophysiological role in sustaining inflammation in CF has been attributed to the abnormal polyunsaturated fatty acid (FA) pattern. Abnormalities in FA profiling are potentially linked to CFTR mutation-driven alterations in the absorption and/or metabolism of dietary lipids [192,193], and to the consumption of high oxidizable FA involved in the free radical-mediated lipid peroxidation [7]. Among those alterations, dysregulation of the docosahexaenoic acid and arachidonic acid balance has been extensively studied with reportedly significant reduction of DHA in CF and a parallel increase in the levels of AA and inflammatory indices [193–195]. Actually, AA is the progenitor of both enzymatic- and free radical-derived inflammatory mediators, including leukotrienes, prostaglandins and isoprostanes. On the other hand, n-3 PUFA (eicosapentaenoic acid and docosahexaenoic acid) are involved in the generation of potent mediators, namely resolvins and protectins, which are able to resolve exudates and to act as organ protective and antifibrotic. Secondarily to their anti-inflammatory action, n-3 FA may also produce an antioxidant-like response (e.g. a reduced demand of antioxidants to achieve an optimal control of oxidative pathways). As a consequence, n-3 PUFA have been suggested and widely used as supplements in CF patients usually under the form of fish oil [196–198]. It is worth of note, however, that defective levels of DHA in CF patients was not confirmed in recent studies [7]. It was also shown that patients on DHA supplements did not have increased plasma n-3 FA concentrations, but showed more severe oxidative stress compared to the unsupplemented patients [7]. This observation of an increased risk of oxidative stress in CF subjects receiving n-3 fatty acids supplements has also been described by other authors [199].

Studies reporting increased AA levels in CF patients have been contradicted by others [7,200,201] thus contributing to weakening the pathophysiological role of the altered DHA–AA balance as turn point of an upregulated inflammatory status in CF. In this context, a

recent Cochrane meta-analysis on n-3 supplementation in CF patients [202] highlighted the lack of evidence for a significant correction of the assessed clinical end points (mainly respiratory symptoms) even when inflammatory indices and other laboratory end points were met. Taken together, these data suggest that there is insufficient evidence to draw firm conclusions or recommend routine use of n-3 supplements in CF. Notwithstanding, it is common belief that n-3 supplements provide some benefits for people with CF with relatively few adverse effects and thus their use is not discouraged.

Further alterations in fatty acid metabolism have been highlighted, including the consistent findings of an increase in circulating levels of saturated and monounsaturated fatty acids [7,203,204]. Decreased levels of essential FA (EFA), i.e. the FA that have to be introduced with the diet, correlates with the severity of respiratory insufficiency and the same clinical correlation was observed with altered proportions of FA species converted by the activity of desaturase enzymes (reviewed in [204]). The close relationship between certain fatty acids and oxidative stress, including the negative correlation of C24:0 and linoleic acid with oxysterol levels point to the need of intensive investigation in CF patients of previously neglected lipid species that are emerging candidates in the control of metabolism. Quantitative lipidomic analyses have lead to identify C16:1n7 palmitoleate as a "protective" adipose-derived lipid hormone that strongly stimulates insulin activity in muscle and liver, also suppressing inflammatory cytokine output from mice fat cells [205]. Specific metabolic activities have been also demonstrated by medium chain saturated fatty acids, caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C12:0). Capric acid acts as a direct ligand of PPARγ, using a binding pocket different from the binding pocket of thiazolidinedione or long chain fatty acids [206]. Additional activities of medium chain fatty acids, which are ligands of free fatty acids receptors detected in the immune cells, the gastrointestinal tract, and adipocytes, may contribute to metabolic homeostasis and inflammatory responses [207]. These data underscore the importance of a lipid-mediated "endocrine network", demonstrating how specific alteration of one or few serum lipids would be per se sufficient to influence metabolic homeostasis. Given the relevance of this emerging information and the alteration of lipid metabolism and inflammatory status in CF, fatty acid lipidomics need to be deeply investigated in CF.

Again, these studies have obvious nutritional implications. Saturated fatty acids with chain lengths higher than C18 are poorly absorbed partly because they form insoluble calcium salts [208]. Medium chain saturated fatty acids are well known for being highly absorbed through the intestine, providing rapid delivery of energy via oxidation of the more hydrophilic short chains, and have been suggested to provide proper nourishment in patients with CF [209]. Recent studies have shown that consumption of a high-fat diet rich in medium chain fatty acids, as opposed to long chain fatty acids, does not lead to ectopic fat accumulation in skeletal muscle and liver of both rats and mice [210]. In light of the close association between nutritional status, inflammation and life expectancy in CF patients, the manipulation of dietary lipids in these patients must be further explored as a possible strategy to provide adequate nutrition and better management of oxidative stress.

3.6. Hydrosoluble antioxidants, oligoelements and enzymatic antioxidants

3.6.1. Vitamin C

Vitamin C status in CF patients has been poorly investigated. Early studies suggested a defective vitamin C status that was refractory to oral supplementation [211]. Other and more recent studies showed normal or slightly decreased levels of vitamin C in CF patients as compared to healthy controls, but age- and disease-related decline of this water-soluble antioxidant was reported in these patients [5,212]. In the study of Winklhofer-Roob et al. [212] on mid-European CF patients vitamin C concentrations decreased with age with an estimated rate of 5 μmol/l/yr and vitamin C concentrations < 40 μmol/l were associated with highest indexes of inflammation which is consistent with the hypothesis that optimal levels of vitamin C may influence immuno-inflammatory activity of alveolar macrophages and neutrophils. Other few studies have examined the effect of supplements containing vitamin C on CF inflammation and oxidative stress since the levels of this vitamin do not significantly improve with supplementation (reviewed in [213]). This highlights the common fate that this hydrosoluble vitamin shares with several fat-soluble counterparts in the "micronutrient paradox" of CF patients in which the need for a correction of their status along the progression of the disease is frustrated by the poor efficacy of oral supplementation protocols. Formulations and appropriate supplementation protocols that may produce a better correction of vitamin C status of CF patients are awaited for further clinical evaluation.

3.6.2. Selenium and selenium-dependent peroxidases

Selenium is a trace element with marked electrophilicity [214] that once converted to the organic form of Se-Cys can be introduced in protein structures to play its important role in H_2O_2 metabolism and signaling [215]. As catalytic center of the enzyme GSH-peroxidase (SeGSH-Px), Se plays a crucial role to protect polyunsaturated lipids of plasma membrane and circulating lipoproteins form peroxidative insults. With other Se-proteins such as the high molecular weight thioredoxin reductases, this oligoelement participates to the control of protein thiol-disulfide oxidoreduction and glutathionylation, which regulate signaling pathways of crucial importance in the regulation of immunity and inflammation [16] but also the functioning of other redox-sensitive proteins such as the same CFTR [124].

The activity of SeGSH-Px in blood is considered a functional assessment of selenium status, even if this assumption has some limitations related with the saturation profile that the Se stores show at increasing doses of Se administration. Investigations of the selenium status in CF patients have produced conflicting findings, which may depend on differences in dietary intake, ethnicity and environmental factors in the diverse patient populations (reviewed in [216,217]). CF children have been reported to have lowered blood selenium and RBC SeGSH-Px activity [218,219], normal plasma selenium and lowered RBC SeGSH-Px [220] and even normal levels of both these two parameters [221]. The supplementation with selenium in combination with other antioxidants was observed to increase the concentrations of blood selenium that, likewise β-carotene and fatty acid, were positively correlated with improved lung function [6].

Foucaud et al. [218], observed that a defective selenium status was associated with lowered levels of other antioxidants that contribute to the anti-peroxidative activity of this microelement, such as vitamin E (reviewed in [222]), and the severity of this deficiency was lower in children with pancreatic enzyme replacement and vitamin E supplementation.

Treatments to substitute for exocrine pancreatic insufficiency by pancreatic enzymes from animal sources, such as porcine pancreas, have been confirmed to be a source of Se [220]. These affects RBC SeGSH-Px activity and plasma selenium concentrations, which has to be taken into account when selenium supplements are given to CF patients.

Selenium has been used to develop a series of organoselenium compounds that may open the way to new therapeutic opportunities in CF (see Section 3.9.2). These include GPX mimetic drugs and phase II enzyme inducers, which may provide higher antioxidant activity of ASL and cellular protection effects in the airways.

3.6.3. Zinc and copper

Zinc and copper (Zn and Cu, respectively) are present in many proteins so that a deficiency of these trace metals could have pleiotropic effects in humans. As regards antioxidant systems, these two oligoelements contribute an important role being cofactors of two

isoforms of the superoxide dismutase enzymes, e.g. the extracellular form, e.g. the EC-SOD or Sod 3 and the Cu–Zn-SOD or Sod 1 that is found in several tissues and cells [223]. The EC-SOD exerts its antioxidant role also in the lining fluids of the airways [224].

The notion that CF patients have defective concentrations of blood Zn and Cu is controversial. As far as Cu status is concerned, few data have been produced and are available in the literature suggesting the notion that CF patients develop a moderate copper deficiency [225,226]. That was essentially demonstrated on the bases of a defective activity of Cu-related proteins such as plasma ceruloplasmin, diamine oxidase, and RBC SOD. This defect seems to be refractory to Cu and Zn supplementation (see below).

More advanced studies have been carried out in the case of Zn status in CF. Low plasma zinc concentrations were reported in approximately 30% of young infants with CF identified by newborn screening [227], and an impaired zinc homeostasis in CF patients was described by Easley et al. [228]. This is characterized by poor conservation of the endogenous pool with fecal loss and impaired fractional absorption of zinc, which are the consequences of pancreatic insufficiency and persisting steatorrhea. These studies suggested that in the clinical management of CF patients these defects can be at least in part corrected by exocrine pancreatic enzyme replacement.

A series of studies by Van Biervliet et al. [229,230] demonstrated in a Dutch population of CF infants and children that serum Zn varies in an age-dependent manner, but remained unchanged with respect to healthy control levels. In CF patients no difference in serum Zn concentration between pancreatic-sufficient or pancreatic-insufficient patients was observed and no correlation was found with the nutritional status or height z-score. Importantly, in these studies a significant correlation of serum Zn was observed with the fat soluble vitamins A and E, thus confirming the relationship between the abnormalities of lipid and Zn metabolism in CF. This suggests that co-supplementation of Zn and fat-soluble vitamins should be advised in the presence of pancreatic insufficiency and persisting steatorrhea.

Neve et al. [219] in a study aimed to assess plasma and erythrocyte zinc, copper and selenium in CF children, showed that mean plasma zinc and copper levels were not different from those in age-matched controls which confirms the observations by Van Biervliet et al. described above. However, plasma zinc concentrations decreased in patients with moderate-to-severe growth retardation and with severe pulmonary disease, but very low zinc levels occur sporadically. Erythrocyte zinc and copper levels were significantly higher than normal, while RBC selenium was lower than in age-matched controls. These trace element concentrations in erythrocytes were discussed in relation to the activities of the Cu/Zn-SOD and the Se-enzyme GPx. This observation suggests a compensatory upregulation of the erythrocyte Cu/Zn-SOD by the exposure of erythroid precursors to ROS and/or other CF-related stressors.

Best et al. [225] also studied RBC SOD activity using this as a biological sensor of Cu status in CF patients. A lowered activity of this enzyme was reported in CF together with that of the other Cu-dependent enzyme plasma diamine oxidase, while plasma ceruplasmin showed normal activity. Degradation rates of copper proteins are known to be accelerated in conditions of copper deficiency, which could explain the finding at least in part. Anyway, when Cu and Zn were supplemented to CF patients either separate or in combination (6 weeks of 3 mg copper/d as copper-glycinate and 30 mg zinc/d as zinc-glycinate), any of the copper enzyme activities was affected. Therefore the moderate copper deficiency of CF patients appears to be refractory to the intervention by increased copper and/or zinc intake.

Erythrocyte Cu/Zn-SOD and the plasma levels of Cu and Zn were also measured in the study of Wood et al. [6] in which Australian CF patients (age > 5 years) were treated with a high-dose antioxidant multivitamin formulation containing 200 mg vitamin E (as RRR-α-tocopherol), 300 mg vitamin C (as sodium ascorbate), 25 mg β-carotene (all-trans isomer), 90 μg Se (as selenomethionine), and 500 μg vitamin A (as retinyl palmitate) in oil. Plasma oligoelements, and particularly Zn, were in the normal range at baseline (as compared with the data reported in [229,230]) and were not affected by this multivitamin supplement. The same was found for the activity of RBC SOD and for plasma 8-iso-PGF2α as surrogate biomarker of lipid peroxidation.

In a recent non-randomized small population (n = 21) case–control study in CF children, Zn supplementation was proven to produce positive clinical effects in Zn-deficient patients [196]. The supplementation with 5 mg/kg Zn sulfate/day (maximum 150 mg), significantly decreased the number of infections and increased the forced expiratory volume in 1 s; energy intake and growth parameters also improved. These parameters were unaffected in untreated patients except that in the case of the pulmonary function that decreased significantly. These clinical observations on Zn supplementation need to be confirmed in prospective double-blind randomized control trial.

3.7. Appropriateness and targeting of antioxidant therapies in CF

The choice of the appropriate antioxidant and dose to correct a certain biomarker and its associated biochemical lesion is another important point that appears to have disregarded in many clinical studies. For instance, unlike vitamin E, vitamin C supplementation does not alter F_2-IsoPs levels in humans (reviewed in [74]). This appears to be true also in the antioxidant therapy of CF in which vitamin C (300 mg/day) was administered together with other antioxidants that included a dose of vitamin E of 200 mg/day, without any significant effect on the surrogate biomarker of lipid peroxidation F_2-IsoPs [6]. When carotenoids are used as supplements to prevent the damaging action of ROS in the CF airways, it is noteworthy that these are not particularly good quenchers of peroxyl radicals relative to phenolics and other antioxidants, but are exceptional in quenching singlet oxygen, at which most other phenolics and antioxidants are relatively ineffective. Singlet oxygen is not a radical and does not react via radical mechanisms, but reacts mostly by the addition to double bonds, forming endoperoxides that can be reduced to alkoxyl radicals that initiate radical chain reactions typical of the peroxidative damage of PUFA [38]. In this case the analysis of F_2-IsoPs, lipid peroxide formation and transition metal-induced oxidizability of lipoproteins [162,180] are all appropriate to evaluate the effect of carotenoids in preventing lipid peroxidation.

As far as the dose is concerned, clinical pharmacology of vitamin E as an antioxidant was recently investigated by means of the effect on F_2-IsoPs production, and doses of α-tocopherol of 1600 IU/day or greater were found to be required to statistically affect plasma F_2-IsoPs levels in hypercholeterolemic subjects [176]. Several vitamin E supplementation studies in CF patients have been performed with doses of lower than 300 IU/day regardless of the lowered absorption by pancreatic and liver defects, and the regular supplementation with these doses does not appear to correct lipid oxidation markers in CF [7].

In the clinical practice and planning trials it has to be taken into consideration that, in spite of substantial evidence supporting a higher antioxidant demand in CF, interventions with several antioxidant formulations produce poor responsiveness particularly in the case of fat-soluble antioxidants, which are poorly absorbed [6,162].

Since many oxidants and antioxidants are present in tissues and biological fluids and these have different chemical and physical characteristics, the possibility to produce a successful therapy with a single antioxidant molecule is too far to be realistic. Moreover, antioxidants act by multiple mechanisms in a single system or by a different single mechanism depending on the reaction system responding in a different manner to different radical or oxidant sources. Because multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved, no single

therapeutic approach will effectively prevent damage by multiple ROS sources. The defect of physiological antioxidants may also interfere with antioxidant therapies that are based on one or few exogenous antioxidants.

Moreover, several antioxidant vitamins such as vitamin E and carotenoids have multiple natural forms with different bioavailability, metabolism and bioactivity [157,167,231]. As far as the case of vitamin E supplementation concerns, α-tocopherol was used as unique vitamer in the large majority of supplementation trials so far performed and is the form used in the nutritional management of CF patients. Moreover, several supplements contain the synthetic (or racemic) form of this vitamin that has lower bioavailability than the natural form. Other forms present in nature and abundantly contained in vegetables such as tocotrienols and the less methylated forms of tocopherol are not represented in many of the supplement formulations so far available in clinical centers, and the supplementation with α-tocopherol also limits their bioavailability. These forms show markedly higher susceptibility to hepatic metabolism and biliary excretion with respect to α-tocopherol, e.g. the most represented form in blood and solid tissues, and if co-supplemented, these are easily displaced during liver uptake and excreted with bile by means of competition with the same α-tocopherol. Notwithstanding, these minor forms have been proposed to play important physiological roles showing molecular characteristics, transcriptional effects and antioxidant activities that clearly differentiate them in subfamilies with distinct biological functions. Some of these, such as α-tocopherol, e.g. the second vitamin E form as relative abundance in blood, and its carboxyethyl-hydroxychroman metabolite [189], appear to have health-related anti-inflammatory effects which are particularly relevant in lung protection [232,233]. This has suggested that such a group of "non-α-tocopherol" forms may represent another family of vitamins within the family of vitamin E with an important, but often missed, contribution to the proposed health effects of this vitamin [163].

These aspects may thus limit the possibility to provide CF patients of optimal levels of this vitamin in all its components, even if they are regularly treated with a vitamin E supplement, e.g. with α-tocopherol.

Targeted antioxidant therapy with formulations with higher bioavailability and bioactivity has been anticipated in CF. New antioxidant formulations have been proposed to overcome main limits of antioxidant therapies so far proposed for these patients. Water-miscible α-tocopheryl acetate containing polysorbate, propylene glycol or polyethylene glycol as emulsifiers form micellar structures were suggested to providing greater bioavailability than the fat-soluble counterparts. These were commercialized with the brand names of E-vimin®, Cremophor® EL and Aquasol® E, but despite the original positive expectation [234,235], some studies failed to observe a higher response in vitamin E levels when these were compared with fat-soluble formulations [236,237].

In a pilot study by Papas et al. [183] a micellar formulation of fat-soluble nutrients and antioxidants was found to improve plasma levels of β-carotene, γ-tocopherol and CoQ(10), reducing at the same time some inflammatory markers in induced sputum, e.g. myeloperoxidase and to a lower extent PMN elastase and total cell counts, while lung function and sputum bacterial counts were unaffected. The same group recently confirmed the possibility to increase the absorption of fat-soluble micronutrients using formulations with higher bioavailability specifically designed for malabsorbing patients such as CF patients that include also vitamin K and commercialized with the brand name of AquADEKs® [162]. Despite improved vitamin and micronutrient levels, in this non-randomized, open-label study, AquADEKs® produced only modest improvements in weight percentile and pulmonary function. Another recent pilot observation [182] has suggested the beneficial effect of this formulation on antioxidant and oxidative stress parameters of this oral supplement that surely deserves more clinical investigation by larger randomized controlled trials.

Recent multivitaminic formulations have been designed to contain also the minor forms of vitamin E and specifically γ-tocopherol [238], the expected superiority of which with respect to formulations containing only α-tocopherol needs to be verified. As discussed above in the section dedicated to vitamin E, further advancements in the therapy of CF inflammation and oxidative stress could be based on synthetic forms and natural metabolites of this vitamin that have been recently identified to possess higher antioxidant and also anti-inflammatory activity than α-tocopherol (reviewed in [163,168]).

Pre-clinical investigation of these and other fat-soluble agents is currently addressed to develop formulations and administration protocols that may increase the therapeutic efficacy in the airways. In the antioxidant therapy of lung dysfunction in CF, local administration protocols may overcome the limits of oral and systemic administration protocols, increasing bioavailability and providing targeted approach to inflammation and oxidative stress. Aerosolization of vitamin E and other fat-soluble micronutrients is possible and may allow direct administration in the airways to prevent toxicity of smoke which is closely associated with inflammation and oxidative stress [164,165]. To our knowledge, this as well as other strategies of local administration such as instillation of solutions such as surfactant-like solutions enriched of vitamin E, have not been previously investigated in CF patients and other CF model systems.

On the contrary, inhalation is an administration route widely adopted in the case of GSH and NAC therapy described above. In this context, Cys formulations alternative to NAC have been proposed for use as antioxidant and anti-inflammatory agent for inhalation. Nacystelyn is a lysine adduct of NAC that thanks to a higher protonation equilibrium shows increased water solubility and thus better bioavailability. Nacystelyn has been described to influence IL-8 generation and the inflammatory signaling of bronchial epithelial cells [239] and preliminary clinical evaluation has demonstrated the safety of this drug [240]. Further clinical trials aimed to assess the effect on lung symptomatology of CF patients are needed. Direct administration in the airways could also be adopted for micronutrient vitamins with poor absorption and bioavailability due to GI defects.

3.8. Clinical impact of antioxidant therapy in CF

Huge in vitro and pre-clinical evidence has provided the rationale to support clinical investigation of antioxidant strategies in CF. These should aim to restore the oxidant–antioxidant balance of CF airway challenged by chronic infection and inflammatory cell activation.

Some observational trials have confirmed that antioxidants used as either supplements to the diet or drugs for lung administration by aerosolized formulations, may help in relieving progressive lung damage and other adverse clinical events of CF such as poor growth. So far, few studies have examined with sufficient methodological rigor the clinical efficacy of antioxidant therapy in CF. This was also concluded in a previous review of the literature by Cantin et al. [12] that was published in the beginning of 2006 and is confirmed also in a recent analysis of clinical trials on antioxidant therapy in CF patients [213] that examined the literature until September 2010 using as sources the databases of the Cochrane CF and Genetic Disorders Group CF Trials Register, PubMed, CINAHL and AMED. Useful information was retrieved from just four randomized controlled trials and one quasi-randomized controlled trial on vitamin C, vitamin E, β-carotene and selenium used as supplements administered alone or in combination. Post-hoc data analysis that was possible only in three studies on a total of 87 CF patients, showed the absence of any significant improvement in lung function that was selected as primary outcome together with quality of life that improved in one trial. Secondary outcomes concerning laboratory indices of oxidative stress and antioxidant status showed several improvements. These included an increase of RBC SeGPx by selenium supplementation done as

individual or combined supplementation, and increased levels of all plasma antioxidants, except vitamin C, that were observed in all the trials.

The message from these studies to pediatricians and nutritionists within the clinical staff of CF centers is that in the absence of solid evidence regarding the clinical effectiveness of oral formulations of antioxidant supplements in CF (including PUFA), their use as a routine clinical practice should be considered with caution keeping clear in mind that this should represent just a complement of one of the most critical interventions in the life-long clinical management program of CF patients, e.g. providing an adequate nutriture [140]. In this context, compensating energy and protein malnutrition has to remain the first goal of nutritional management programs. Providing higher intakes of antioxidant micronutrients with food naturally-enriched (or functionalized) of micronutrient antioxidants should be preferred in order to have the higher combination of positive nutritional factors that are lost during refinement procedures and overall industrial processing of dietary supplements.

Measures of good practice in clinical management of micromalnutrition in CF patients would include the support of specialized personnel and laboratories. The centers should have the possibility to ascertain with appropriate nutritional and laboratory tests what the level of deficiency is for the different micronutrients that each patient may suffer from during a dietary program. Monitoring dietary intakes and blood levels of micronutrients is not sufficient to set a personalized nutritional intervention with supplements or functional foods. Sub-optimal intakes and the compliance to nutritional interventions should be verified by means of selected biochemical and clinical criteria. Unluckily, the latter are still not well established for some micronutrients such as the fat-soluble vitamins E (reviewed in [163,173,241]) and K [242,243], and also for trace elements such as Se [244]. For many of the antioxidants used as supplements in humans we still do not know exactly several of the biological effects that these may provide when introduced in a healthy body and we know less what these can do in disease. These aspects hardly impact on the concept of optimal levels of intake that should be identified for each micronutrient on an individual basis. According with the aspects discussed in the previous sections, optimal levels of intake for antioxidant micronutrients are different in CF patients and healthy subjects and vary with age and several individual factors.

Lung administration of antioxidants by means of aerosolized formulations, so far used for GSH therapy (see above), is a promising field of clinical investigation in CF. Toxicity issues are currently the main concern and the subject of clinical evaluation by several groups (described above).

Information on future directions of antioxidant therapy in CF can be inferred by the analysis of pending or recently completed clinical trials that have been searched (December 2011) at ClinicalTrials.gov database matching the terms “antioxidant therapy” or “glutathione” with “cystic fibrosis”. Fifteen trials were retrieved and those directly related to antioxidant therapy (n = 12) are presented in Table 1 and include efficacy and safety studies of inhaled drugs as GSH, sodium pyruvate, NAC, and NO, and the dietary supplements: AquADEKs®, an antioxidant-rich multivitamin supplement, epigallocatechin gallate (EGCG)/tocotrienol, curcuminoids, the amino acid glutamine, sulforaphane in broccoli sprouts. One trial is investigating the flavonoid quercetin as CFTR modulator.

3.9. Emerging antioxidant and anti-inflammatory approaches in CF

Mediterranean foods such as olive oil and red wine or several plant extracts such as herbal tea or grape seed extracts contain a series of natural compounds with important antioxidant and NO scavenging activity that have been widely investigated for the prevention of oxidative stress-related diseases [38,245]. These have been long investigated as template molecules to develop antioxidant and anti-inflammatory drugs. Between the multitudes of natural substances, most investigated ones include for instance tyrosol, hydroxytyrosol, gallic, and caffeic acids, belonging to the family of phenols, while resveratrol of the stilbenes family, epicatechin, kaempferol, and cianidin are examples of flavonoids; examples of fat-soluble phenolics are those of the family of tocols (vitamin E) and carotenoids. All these compounds show different metabolisms and signaling effects [246]. Antioxidant and cell protection mechanisms are associated with different and sometimes concurrent processes as H-atom transfer and electron donation, radical trapping and chelation of transition metals that can be easily investigated at the chemical level [247]. Besides these direct antioxidant effects, metabolic responses and signaling effects of natural compounds are also important to produce second-generation responses that modify the level of antioxidant protection and the potential for ROS detoxification in cells and body fluids. These derive from the transcriptional regulation of antioxidant and pro-oxidant genes that coexist with the modulation of cytokine secretion and inflammatory enzyme activities.

Some of these natural plant-derived antioxidants may provide further pharmacological activities of possible relevance in CF. This is the case, for instance, of the plant flavonoids genistein, apigenin, kaempferol, quercetin and hesperidin [248–251] that fall in the pharmacological category of “potentiators” [252] in that these activate at least to a certain extent CTFR-mediated Cl currents in normal and CF airway epithelium investigated either in vitro or in vivo in humans and animal models. Actually, as prototypal flavonoid-derived potentiator, the tyrosine kinase inhibitor genistein potentiates the gating of wild-type as well as ΔF508 CFTR and G551D CFTR [253], but at higher doses it inhibits CFTR gating, which make this an unlikely clinical candidate. Similar potentiation effects have been reported for the antioxidant curcumin [250] that shows an additive effect with respect to genistein-induced potentiation. Besides CFTR potentiation, which does not appear to have a direct influence on GSH and nutrient transport in the lung tissue, all these natural substances have been used alone or in combination with other agents as in vivo enhancers of lung antioxidant protection [246,254,255]. In the case of quercetin, thiol-reactive phenolic [256], bronchodilation and anti-inflammatory effects by a restoration of the Th1/Th2 balance have been reported in inflammatory syndromes of the lung [255,257–259], but these effects have not been explored in CF. Pyocyanin toxicity (see above in the Section 2) could represent an issue for the use of quercetin and other catechols in infectious diseases of the lung [260].

Other natural antioxidants of potential interest in CF therapy include terpenoids [261] such as terpinen-4-ol, a major constituent of tea tree oil that is being investigated in the therapy of antibiotic resistant organisms, specifically against methicillin-resistant *Staphylococcus aureus* (MRSA) [262], a problem in CF [263]. Other than being lipophilic antioxidants, terpenes can prevent oxidative stress of the CF lung secondarily to the antibiotic effect thereby promoting a better control of immuno-inflammatory pathways and lung antioxidants.

3.9.1. Targeting NF-κB with natural compounds: resveratrol and plant extracts

Natural compounds were long known to be useful in designing anti-inflammatory drugs and some of these could find an application in the control of the transcription factor kappa-B (NF-κB) that may lead to develop advanced pharmacological tools to control inflammation and to produce higher antioxidant protection to CF airways. Inhibitors targeting NF-κB, including transcription factors decoy molecules (TFD), are potent down-regulators of gene expression and release of IL-8 in CF cells infected with *P. aeruginosa* [264,265].

Resveratrol (3,5,4′-trihydroxystilbene, “E” form) is one of the most investigated natural antioxidant with proposed activity as NF-κB inhibitor. This is a phytoalexin present in large quantity in red wine, preferentially in the skin of grapes, being its concentration

50–100 μg/mg of fresh skin and 1.3–3 mg/l of red wine [266]. Furthermore, resveratrol is a constituent of 'Darakchasava' (1.3–6 mg/l), an ayurvedic medicine from India [267]. The antioxidant activity of resveratrol has been reported in several papers and occurs also in lung tissues, suggesting that resveratrol has potential as a therapeutic agent in respiratory disease [268–270]. Resveratrol can be considered one of the most interesting molecules from the natural world exhibiting strong antioxidant activity that at the same time is able to exert anti inflammatory activity through alteration of NF-κB functions and efficient inhibition of pro-inflammatory genes [271,272]. As far as the effects on CF model systems are concerned, Cabrini et al. [26] reported the effects of resveratrol on *P. aeruginosa* infected CF IB3-1 cells. The results obtained demonstrate that resveratrol exhibits minor effects of IB3-1 cell growth and important inhibitory effects on accumulation of IL-8 and GRO-α mRNA, while minor effects on IL-6 gene expression were observed. These data support recently published observations suggesting inhibitory effects of resveratrol in infection-mediated inflammatory processes [273]. Interestingly, resveratrol has been reported as a potent inhibitor of NF-κB activity and a promising anti-inflammatory agent [272,274] also in combination with other molecules [274,275]. However, in vivo translation of this pre-clinical on resveratrol is hard to be foreseen. This natural compound displays poor bioavailability [276] and further characterization of resveratrol pharmacokinetics is troublesome since it is hardly measurable in vivo [277]. Furthermore, the regulation of inflammatory responses by resveratrol has been suggested to be far more complex than simple direct suppression of NF-κB activity. Actually some aspects of the immune response could be enhanced by this natural substance, which may lead to hypothesize broad negative feedback events on immuno-inflammatory pathways [278]. In any case, and with these cautions in mind, the published observations on cellular model systems support the concept that resveratrol and resveratrol-containing formulations deserve further consideration to determine their possible therapeutic values in CF.

Other inhibitors can be identified from systematic studies of natural products and their ability to interfere with NF-κB activity [279–281]. Recent studies have demonstrated that the whole extract of *Aegle marmelos* (Rutaceae) has strong inhibitory effect on the *P. aeruginosa*-dependent IL-8 induction in human CF-derived bronchial IB3-1 cells without affecting cell proliferation [280]. Molecular analysis of components contained in *A. marmelos* extracts revealed that three major compounds, namely 5,6-dimethoxy-1-indanone, 2-hydroxy-cinnamic acid and 5-methoxy psoralen (5-MOP), reproduce the inhibitory effect observed with the whole extract [280]. A further example has been recently published using extracts from bergamot (*Citrus bergamia* Risso). In this study the extracts were characterized and the main detected constituents were assayed for their biological activity [281]. The results obtained demonstrated that the extracts from bergamot epicarps contain components displaying an inhibitory activity on IL-8. Particularly, the most active molecules were bergapten and citropten. These effects have been confirmed by analyzing mRNA levels and protein release in the CF cellular models IB3-1 and CuFi-1 induced with TNF-α or exposed to *P. aeruginosa*.

A parallel strategy linking NF-κB inhibition and down-regulation of NF-κB-dependent functions is described in recently published studies based on a structured-based virtual screening (VS) of differently substituted furocoumarins and analogues against NF-κB, with the objective of selecting molecules able to inhibit the binding of this transcription factor to DNA [282,283]. Novel compounds were identified with this strategy as potent inhibitors of NF-κB dependent biological functions, to be proposed for the control lung inflammation occurring in patients affected by CF [282–284].

3.9.2. Scavengers and regulators of H_2O_2 and NO

3.9.2.1. Lactoferrin and $OSCN^-$. The impaired DUOX/LPO system of CF patients could be the target of therapeutic strategies that may produce concomitant antimicrobial and antioxidant effects. This system described above in Section 2.2 represents an important component of innate immunity with a role in bactericidal defense and regulation of H_2O_2 metabolism of airways [32]. CF patients have also reported to show lowered LF levels in the ASL [37] that together with a CFTR-dependent defective efflux of SCN^- and LPO [36] may create the environment for a sustained flux of H_2O_2 and for the generation of highly reactive Fenton species [11,38]. An ideal therapeutic intervention should restore the LPO-dependent generation of bactericidal $OSCN^-$ from DUOX-derived H_2O_2 and SCN^- in the ASL to control at the same time the microbial biofilm and the flux of H_2O_2 into the ASL. An approach close to this therapeutic mechanism was recently proposed for the drug Meveol®. This is a complex of LF and $OSCN^-$, with proposed in vivo antimicrobial activity [285] to be evaluated in clinical trials for the aerosol treatment of lung infections in CF patients. The role for this drug in the control of H_2O_2 levels of ASL remains to be investigated.

3.9.2.2. Organoselenium compounds. Organoselenium compounds have been proposed as therapeutic agents useful in the antioxidant therapy of inflammatory conditions. These include GPX mimetic molecules such as Ebselen® (2-phenyl-1,2-benzisoselenazol-3(2H)-one) or other and more recently developed molecules that are still in pre-clinical steps of evaluation [286]. Selectively and specificity of action are main issues in the therapeutic mechanism of this candidate antioxidant drug that thanks to the electrophilic properties of Se [214] reacts with thiols to reduce H_2O_2 and other species. Moreover, Ebselen® has been described to act as potent non-competitive inhibitor of extracellular nucleoside diphosphokinase (NDPK) having negligible effect on ecto-ATPase and adenyl kinase activities, which are other players of the metabolism of extracellular nucleotides [287]. This enzyme together with other nucleoside di- and triphosphates contributes to regulate several components of the mucociliary clearance process (MCC) that protects the lung against infections via activation of epithelial purinergic receptors. The inhibition of NDPK by this GPX-mimetic drug may also impact on the energy status of endothelial cells [287]. This aspect could be further investigated as a cellular protection mechanism in the pulmonary epithelium of CF patients, being the control of cell energy of fundamental importance to prevent the activation of death pathways [288].

Further therapeutic mechanisms for these Se-derived drugs may derive from the marked activity as inducers of phase II enzymes such as quinone reductase (QR) and glutathione-S-transferase (GST) [289]. This induction response also observed with other electrophils and redox-active molecules, such as natural phenolic antioxidants, could produce higher cellular protection by the acquisition of an increased capability to detoxify endobiotics generated during oxidative stress and inflammation [246].

3.9.2.3. Melatonin and GSNO as NOx regulators. Increased markers of NO-derived biological damage are present in the CF lung suggesting the abnormal reactivity of NOx in the inflamed lung of CF patients [52]. However, an impaired metabolism and biological function of NO in CF airways (see Section 2.3) suggests a cautious approach to the use of agents that may influence the levels and chemical behavior of NO-derived species (NOx) with possible impact on the physiological roles of NO in vasodilatation, innate immunity and H_2O_2 metabolism of the respiratory tract [290,291]. NO donors also appear to influence CFTR function of alveolar epithelium and gland serous cells [292,293].

Main physiological role of the pineal gland hormone melatonin is the synchronization of circadian rhythms, including the sleep–wake cycle, but several other functions dealing with immunomodulatory, antioxidant and cellular protection effects have been identified [294]. Melatonin receptor stimulation may also influence cyclic AMP signaling and the regulation of CFTR ion channel [295,296].

A recent randomized double-blind placebo-controlled study has examined the effects of short term melatonin administration (3 mg for 3 weeks) on sleep and oxidative stress markers in CF [297]. According with the expected pharmacological effect of this hormonal substance, the treatment was successful in improving sleep indices. At the same time, nitrite levels determined in exhaled breath condensate (EBC) were reduced to suggest a better control of NO metabolism of CF airways (discussed above in Section 2.3), but these effects, however, were not associated with a significant correction of isoprostane as lipid peroxidation marker measured in EBC. Increasing dosages of melatonin could be investigated for the pharmacological treatment of oxidative stress and immune dysfunction of CF patients due to the absence of adverse effects of this hormonal substance in humans.

The S-nitrosation reaction of thiols is another interesting aspect of NO pharmacology [53] that may find an application in the therapy of lung complications of CF. This has been the case, for instance, of the S-nitrosothiol GSNO that has been used safely in human trials [298,299]. This endogenous nitrosothiol with proposed regulatory effect on the CFTR of lung epithelia [292,293], is well tolerated in patients with CF when administered by aerosolization and may produce better oxygenation [299] thereby suggesting that therapy aimed at restoring endogenous GSNO levels in the CF airway may merit further clinical evaluation. Since the administered GSNO decomposes as shown by the increased levels of expired NO in CF patients under treatment with this nitrosothiol [299], further investigation should be addressed to ascertain whether GSNO therapy may merely correspond to a GSH therapy.

NO dependent effects on CFTR function should be considered to evaluate mechanism of action and specificity of antioxidant therapies that target NO metabolism and function in the CF airways.

4. Conclusions and future perspectives

The presence of clinical and biochemical symptoms of inflammation and oxidative stress implies that CF patients have a higher demand of antioxidant protection. The combination of the dysfunctional CFTR with a lowered intake and absorption of dietary antioxidants produce sub-optimal levels of protection from both enzymatic and non-enzymatic defense systems. The former could be associated with a defective glutathione metabolism and lowered intake of sulfur-containing amino acids and oligoelements such as zinc and possibly selenium, while fat-soluble antioxidant vitamins, such as vitamin E and carotenoids, and the water-soluble vitamin C, are lowered than in healthy subjects due to malabsorption and possibly higher consumption.

The increased antioxidant demand in CF patients should be even higher in the presence of complications of the lung, pancreas and liver. Lung comorbidity is particularly important in this context being associated with recurrent infections, which results in the alternation of inflammatory exacerbates and chronic inflammation. These sustain the flux of ROS in the airways and promote the formation of second-generation byproducts by the damage of biomolecules such as PUFA and proteins, which may diffuse also at the systemic level to further promote adverse biological responses and toxic reactions. Pancreatic insufficiency causes malnutrition and may lead to develop endocrine and metabolic defects associated with diabetes of CF. These events can be further aggravated by the concomitance of liver dysfunction and a poor nutritional status. Malnutrition is strongly associated with poor prognosis as assessed by pulmonary function and survival data [138–140]. The more aggressive nutritional interventions seem to produce better clinical outcomes in these patients and the possibility of achieving higher antioxidant protection in well-nourished patients by dietary factors could be a key aspect to explain such a clinical advantage. The assumption that food-derived components may help to prevent the damaging effects of oxidative stress improving the antioxidant defenses, deserves further investigation at the clinical level. This together with the investigation of appropriate laboratory biomarkers could provide critical information to establish intervention criteria for an early nutritional management of newly diagnosed patients [5]. It is expected that a timely secondary prevention strategy could influence the progression of CF symptoms thus affecting with a self-feeding mechanism the same extent of malnutrition and oxidative stress along the life of CF patients. Since several factors contribute to impair the nutritional status of CF patients (such as pancreatic insufficiency, chronic malabsorption, recurrent sinopulmonary infections and progressive lung disease, chronic inflammation, increased energy expenditure, suboptimal intake, multitherapy) careful monitoring of the antioxidant status should be recommended. According with international guidelines, fat-soluble vitamin supplementation is of utmost importance in daily practice together with energy intake requirements and pancreatic enzyme-replacement therapy. Among these, vitamin E, β-carotene and ω-3 FA have observed to alleviate selected biochemical signs of oxidative stress as measured for instance with well established laboratory indices of lipid peroxidation, and in some studies these effects were preliminarily associated with positive clinical outcomes. Notwithstanding, randomized–controlled clinical trials on antioxidant supplements (including ω-3 FA) so far carried out in CF have failed to conclusively demonstrate significant beneficial effects on respiratory symptoms and on the consequent impact that these have on the quality of life of these patients (reviewed in [202,213]), which may provide no support for the use of these supplements in CF. An absence of efficacy in prevention studies on antioxidant supplements has also been observed in other, and possibly less severe, oxidative stress-related conditions, particularly in the secondary prevention of cardiovascular disease and some forms of cancer by vitamin E and other antioxidants such as vitamin C, selenium and β-carotene (reviewed in [163]). Different biases, however, have been identified in randomize large clinical trials that may account for these disappointing findings. These include patient selection criteria (early interventions and primary prevention could have more chances of success than secondary prevention), duration and doses of the treatment, use of wrong antioxidant formulations and administration protocols, absence of verifications of the biological compliance to the treatment, etc. The same biases apply for the few trials carried out in CF patients. Thus, the appropriateness and efficacy of nutritional interventions with natural food-derived or synthetic antioxidants should be verified with respect to the biological pathways of oxidative stress and clinical variables that are identified as end-points. Profiles of blood and tissue antioxidants, as well as of reliable surrogate markers of oxidative stress, have to be selected and determined in highly specialized laboratories with a well-established experience on these analyses.

Well-timed (early) interventions with appropriate antioxidant formulations/protocols need to be proposed for the next generation of trials, and the development of novel CF-tailored antioxidant and anti-inflammatory agents should be promoted.

As a consequence of these considerations, more clinical investigation is awaited to identify future successful approaches to the antioxidant therapy as a measure to further enhance quality of life and the overall clinical outcome of CF patients.

Acknowledgments

We are indebted with the Italian Cystic Fibrosis Research Foundation for the support that it provided with its grant program to the research activity of the authors of this paper. This is a scientific initiative of the “Working Group on Inflammation in Cystic Fibrosis” (Bragonzi A, Bravo E, Cabrini G, Conese M, Cuzzocrea S, Dechecchi MC, De Alessandri A, Evangelista V, Galli F, Gambari R, Lambiase A, Lucidi V, Manca A, Melotti P, Minicucci L, Motta A, Pietraforte D, Pompella A, Quattrucci S, Rizzo R, Romano M, Sorio C, Tesse R) of the Italian Society for Cystic Fibrosis. A particular thank goes to the Prof. Gianni Mastella for the critical revision of the manuscript and for the

stimulating research environment that he has contributed to create within the Italian Cystic Fibrosis Research Foundation.

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