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Docosahexaenoic Acid-Derived
Anti-Inflammatory Mediators
in Sputum of Adults with Cystic Fibrosis:
Clinical and Therapeutic Implications

*Mediatori Anti-Infiammatori
Derivati dall'Acido Docoesaenoico
nell'Escreato di Adulti Affetti da Fibrosi
Cistica:
Implicazioni Cliniche e Terapeutiche*

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1. Abstract

Background. Several reports have shown in patients with cystic fibrosis (CF) a significantly reduction of the levels of docosahexaenoic acid (DHA), which is involved in antioxidant response and in production of mediators identified as important factors during the resolution phase of inflammatory reaction. The deficit of DHA may play a role in the inflammatory cascade of pulmonary disease in CF patients.

The aim of the study was: to determine the levels of the arachidonic acid (AA) metabolites and DHA in sputum of adults with CF subjects, as compared to patients with COPD; to ascertain whether or not DHA supplementation may affect the fatty acid pattern in CF subjects.

Subjects & Methods. We studied 15 CF subjects and 10 COPD patients. CF patients (age range 20 to 40) were recruited at the Cystic Fibrosis Unit of Parma Hospital and the control group of COPD patients were recruited at the University Hospitals of Modena-Reggio Emilia and Parma. At baseline all subjects performed: nutritional status evaluation, severity score evaluation (Shwachman-Kulczycki score), spirometry, exhaled NO measurement, exhaled breath condensate (EBC), sputum induction (SI) to evaluate leukotriene B4 (LTB₄), prostaglandin E2 (PGE₂), 15-hydroxyeicosatetraenoic acid (15-HETE), 17-hydroxydocosahexaenoic acid (17OH-DHA), 15-HETE/17OH-DHA ratio, and blood sample to evaluate DHA/AA ratio and HUFA index in the red cells (*first phase*). During the *second phase*, CF patients performed all evaluations after two weeks of systemic antibiotic therapy or/and after ten weeks with DHA-supplementation and after ten weeks without DHA-supplementation.

Results. As compared to COPD patients, CF subjects showed increased concentrations of LTB₄, PGE₂, 15-HETE. The concentrations of the DHA derived were not different in the two groups. 9 out of 15 CF patients (5 female) were clinically stable and completed *second phase* of the study. After ten weeks of DHA supplementation, CF subjects showed a tendency to decrease in LTB₄ and PGE₂ and to increase in 17OH-DHA, and a significantly reduction in levels. At the end of the washout period, LTB₄, PGE₂, 15-HETE, and 17OH-DHA tended to recover baseline values. After supplementation DHA/AA ratio and

HUFA index were significantly increased. As compared to baseline, 15-HETE/17OH-DHA ratio significantly changed after supplementation.

Conclusion. Our preliminary results showed that in CF patients an impairment in fatty acid metabolism, characterized by increase in AA metabolites and decrease in DHA, was partially corrected by DHA supplementation. A better understanding of these metabolic changes could provide new insights into disease pathophysiology and potentially could identify new biomarkers of disease severity.

2. Riassunto

Razionale. Numerosi studi hanno documentato nella Fibrosi Cistica (FC) una significativa riduzione dei livelli di acido docosaesaenoico (DHA), coinvolto nella risposta antiossidante e nella generazione di metaboliti, quali resolvine e protectine, identificati come importanti fattori nella fase di risoluzione del processo infiammatorio. Tale deficit potrebbe pertanto svolgere un ruolo fondamentale nella progressione della cascata infiammatoria e della malattia polmonare.

Scopo dello studio. In questa fase preliminare ci siamo proposti di: determinare il contenuto dei derivati dell'acido arachidonico (AA) e del DHA nell'escreato di soggetti adulti affetti da FC e verificare se la supplementazione con DHA sia in grado di migliorare il profilo degli acidi grassi. I risultati nei pazienti FC sono stati confrontati con quelli ottenuti in pazienti con bronco-pneumopatia cronica ostruttiva (BPCO).

Materiali e Metodi. Lo studio ha coinvolto 15 soggetti affetti da FC reclutati presso la Struttura Semplice di Fibrosi Cistica dell'Azienda Ospedaliera Universitaria di Parma e 10 soggetti affetti da BPCO reclutati presso la Clinica Pneumologica dell'Università di Parma e di Modena-Reggio Emilia. Il protocollo dello studio è articolato in due fasi. Nella prima fase sono stati valutati nell'escreato i mediatori leucotriene B4 (LTB4), prostaglandina E2 (PGE2), acido 15-idrossieicosatetraenoico (15-HETE), acido 17-idrossidocoexainoico (17OH-DHA), il rapporto 15-HETE/17OH-DHA e negli eritrociti il rapporto DHA/AA e l'indice degli acidi grassi polinsaturi n-3 (HUFA index). Nella seconda fase sono state valutate le variazioni dei medesimi mediatori dopo due settimane di trattamento con antibioticoteraapia ev e/o dopo dieci settimane di supplementazione di DHA nella dieta alla dose di 3 g/die e dopo dieci settimane senza supplementazione.

Risultati. L'esame citologico dell'escreato in condizioni basali ha dimostrato la presenza di una infiammazione di tipo neutrofilico in tutti i pazienti FC. Rispetto ai soggetti BPCO, i pazienti affetti da FC presentano una aumentata concentrazione di LTB4, PGE2 e 15-HETE. Non sono emerse differenze tra i due gruppi per la concentrazione del 17OH-DHA. 9/15 pazienti FC (5 donne) erano in fase di stabilità clinica e hanno ripetuto la valutazione nutrizionale e funzionale e dei mediatori lipidici dopo dieci

settimane di supplementazione di DHA nella dieta e dopo dieci settimane senza supplementazione. Dopo dieci settimane di supplementazione con DHA nei soggetti FC abbiamo osservato nello sputo una riduzione ($X \pm DS$) di LTB₄, PGE₂ e 15-HETE, che per quest'ultimo raggiunge la significatività statistica. Al contrario, il 17OH-DHA aumenta alla fine della supplementazione. Al termine del periodo di wash-out, LTB₄, PGE₂ e 15-HETE aumentano senza raggiungere i valori basali; mentre il 17OH-DHA è sostanzialmente invariato. Inoltre, dopo la supplementazione, l'analisi di composizione degli acidi grassi negli eritrociti ha evidenziato un aumento statisticamente significativo del rapporto DHA/AA e dell'HUFA index. Inoltre, dopo la supplementazione con DHA, il rapporto 15-HETE/17OH-DHA si riduce in modo statisticamente significativo rispetto al basale.

Conclusioni. Questi risultati preliminari dimostrano che nei soggetti FC esiste uno squilibrio del metabolismo degli acidi grassi con aumento dei mediatori derivati dall'AA e riduzione dei derivati del DHA, parzialmente corretto dalla supplementazione dietetica con DHA. La conoscenza dettagliata di queste alterazioni metaboliche potrebbe contribuire a sviluppare specifiche terapie personalizzate sul profilo infiammatorio di ogni paziente.

3. Introduction

More than eight decades have passed since the first description of Cystic Fibrosis (CF) as a deadly childhood disease of the pancreas. Since then, it has been recognized as one of the most common fatal recessive diseases among white persons, usually diagnosed during the first years of life and characterized primarily by pulmonary and gastrointestinal symptoms.

CF in the history

A documented history of CF did not exist until well into the 1930s. Many cases back then of what could have been CF were misdiagnosed as whooping cough, chronic bronchitis or pneumonia. However, there has been a feeling of consciousness of what CF is since the 1700s, as popularized by the German saying, “A child whose forehead tastes like salt when kissed will soon die.”

The 1930s can be said to be the period of discovery for CF. What is considered to be the earliest paper written on the disease was made by Swiss pediatrician Dr. Fanconi. The doctor called the illness “celiac syndrome,” which he defined as changes in the pancreas as observed in children [1]. The term Cystic Fibrosis was coined by Dr. Dorothy Andersen of Babies’ Hospital New York. She also theorized that the condition is caused by deficiency in Vitamin A [2]. Changing theories on the nature of cystic fibrosis marked this decade’s history. Drs. Sidney Farber and Harry Shwachman connected the abnormal secretion of mucus to the disease [3]. The idea that Vitamin A is the underlying cause of CF was challenged by a number of researchers, including Dr. Anderson, the person who proposed it in the first place. In 1949, Lowe et al. postulated that CF must be caused by a defect in a single gene (and therefore a single protein) on the basis of the autosomal recessive pattern of inheritance of the disease [4].

The 1950s saw the beginnings of the sweat test, the standard test now used for diagnosing CF. The test was developed as a result of discoveries made by Dr. Paul di Sant’Agnese during the heat wave in New York in 1950 [5]. In 1955, Dr. Shwachman laid the foundation for the modern way of treating CF, which was early diagnosis, active early treatment and proper nutrition. Moreover, Dr. Archie Norman began studies on high fat diets for treating the disease in London that year [6].

The 1960s is the period when organizations specializing in CF research were formed. These organizations were initiated by parents of children afflicted with the disease, as well as rare patients who lived long enough to see adulthood.

Preliminary work on proper neonatal screenings for diagnosis was the hallmark of the 1970s. This was also the decade that saw the developing of many more specialized clinics for CF, as well as the advocacy

for high-fat diets to treat the condition [7]. The Cystic Fibrosis Foundation in the United States also pioneered the use of the patient registry during this decade.

Further advancements in treating the disease occurred during the 1980s. It was during this decade that the greatest finding on the disease, none other than the discovery of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, was made (this was in 1989) [8]. Use of gene replacement therapies began as part of the treatment for cystic fibrosis. Peter Durie and others in the 1980's demonstrated that the CF pancreas has both chloride and bicarbonate secretion defects [9]. Also, the Food and Drug Administration approved the use of the mucolytic pulmozyne, the first drug designed to target CF.

The total number of patients with CF worldwide is not known, but approximately 30,000 patients are listed in the cystic fibrosis registry in the United States [10]. The median life expectancy of patients with cystic fibrosis is on the rise and has changed dramatically, within a period of three decades, from approximately 10 years to approximately 38 years. This favorable result has been achieved through research; through the development of adequate medications, especially enteric-coated pancreatic enzymes; through the use of prophylactic medications; through treatment by multidisciplinary CF teams; and through early intervention for patients who have pulmonary exacerbations.

CF Epidemiology

CF is a chronic, progressive, and frequently fatal autosomal recessive disorder caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. Heterozygous carriers (those who have inherited only one copy of the altered gene) are asymptomatic. Its estimated heterozygote frequency in white people is up to 1 in 20. Each offspring of 2 heterozygote parents has a 25% chance of developing cystic fibrosis. The worldwide incidence varies from 1 case per 377 in England to 1 case per 90,000 in Hawaii. In the United States, the prevalence is as follows:

- Whites of northern European origin - 1 case per 3,200-3,500 population;
- Hispanics - 1 case per 9,200-9,500 population;
- African Americans - 1 case per 15,000-17,000 population;
- Asian Americans - 1 case per 31,000 population [10].

The disease occurs mostly in Caucasian population, although it affects all races and ethnic groups.

CF Pathogenesis

CF is caused by mutations in CFTR, an apical membrane anion channel involved in epithelial fluid secretion and salt absorption [11]. The most important aspects of CF's pathophysiology arise from alterations in extracellular fluids, such as, premature activation of pancreatic enzymes, increased viscosity of mucus and consequent ductal blockage and irritation.

CFTR gene

CFTR gene, which is responsible for this disorder, encompasses approximately 180,000 base pairs, it contains 27 exons spreading over 250 kb of chromosome 7 (7q31) and it encodes an mRNA of 6.5 kb.

The most common mutation is termed $\Delta F508$ and it's present in approximately 70% of defective CFTR alleles and in 90% of patients with CF in the United States [12]. In Europe, there exists a great heterogeneity in the spread of CF, there is a north-west/south-east gradient (i.e. 88% of $\Delta F508$ cases found in Denmark and 50% in Italy). To explain the spread of this mutation in European population, the hypothesis of a selective advantage of heterozygotes was proposed [12]. Since the discovery of CFTR gene, more than 1200 mutations are described since the duplicating of this gene, out of which 4 (excluding $\Delta F508$) represent more than 2%. The frequency of certain mutations can vary among different geographic groups: as result of a genetic founder effect, W1282X mutation is particularly prevalent among persons of Ashkenazi Jewish descent (48% of CF alleles among the Ashkenazi Jews and only 2% of the total CF alleles) [12].

All types of mutations are represented (missense, frameshift, nonsense, splice, small and large in-frame deletions or insertions), and are distributed throughout the entire gene. The potential of a mutation to contribute to the severity of a CF phenotype depends on multiple factors. The majority of molecular defects of CFTR gene are the point mutations out of which 42% are missense mutations, 24% small insertions/deletions with a frame shift, 16% nonsense mutations, 16% mutations of splicing and 2% deletion of an amino acid. One of the particularities of CFTR gene is the existence of deleted transcripts of one or more exons among normal individuals. These transcripts are due to anomalies leading to

alternative splicing, out of which the most frequent and well-studied is the deleted transcript of exon 9 (9-). The presence or absence of this exon is correlated with a "polymorphism" of sequence of the intron 8 situated near the acceptor site of splicing [13].

Various mutations can be grouped into different classes based on their known or predicted molecular mechanisms of dysfunction and functional consequences for the CFTR protein. The classification, which was first proposed by Tsui [14], has subsequently been expanded and refined to accommodate more data [15]. Although schematic, such classification can often be a good indication of a mutation's severity and provides a rationale for their phenotypic consequences (Tab. 1). In fact, individuals with class I, II and III mutations, on average, have shortened survival compared with those who have "mild" genotypes (class IV, V and VI). The clinical importance of these functional categories is limited because they do not uniformly correlate with specific clinical features or their severity [15].

$\Delta F508$ is a 3-bp deletion in exon 10 causing a loss of phenylalanine at the amino acid position 508 of the protein product and it is categorized as a class II defect. The defective protein retains substantial chloride-channel function in cell-free lipid membranes. When synthesized by the normal cellular machinery, however, the protein is rapidly recognized as misfolded and is degraded shortly after synthesis, before it can reach its crucial site of action at the cell surface. Like $\Delta F508$, several other clinically important mutations — such as N1303K, G85E, and G91R — lead to misfolded CFTR protein that is prematurely degraded [13].

About 5% to 10% of CFTR mutations are due to premature truncation or nonsense alleles (designated by "X," such as G542X, a class I mutation). Other CFTR mutations encode properly processed, full-length CFTR protein that lacks normal ion-channel activity. For example, the G551D mutation (class III) is believed to possess little or no chloride-channel function in vivo because of abnormal function of a nucleotide-binding domain, resulting in disordered regulation. The A455E mutation (class IV) exhibits only partial CFTR ion-channel activity, a feature that probably explains a less severe pulmonary phenotype. Other mutation classes include reduced numbers of CFTR transcripts (class V) and defective CFTR stability at the cell surface (class VI) [13].

Table 1: classification of CFTR molecular anomalies and its effects on the CFTR protein morphology and functions [15]

Mutation Class	Type of Mutation	Effects on Protein	Example
Class I	This class includes the nonsense mutations and those that produce a premature stop codon (anomalies of splicing and frameshift mutations). In certain cases the mutated mRNA is unstable and doesn't produce the protein. In other cases, the abnormal protein produced will probably be unstable and degrade rapidly.	Defective Protein Synthesis (Net Effect: No CFTR Protein at the Apical Membrane)	G542X
Class II	A number of mutations alter the maturation of the protein and thus the transport of these proteins to the plasma membrane.	Abnormal Processing and Trafficking (Net Effect: No CFTR Protein at the Apical Membrane)	ΔF508
Class III	These mutations are frequently situated in the ATP binding domain (NBF1 and 2) and disturbing the regulation of Cl ⁻ channel.	Defective Regulation (Net Effect: Normal Amount of Nonfunctional CFTR at the Apical Membrane).	G551D
Class IV	Certain segments of membrane spanning domains participate in the formation of an ionic pore. The missense mutations situated in these regions produce a correctly positioned protein that has a cAMP dependant Cl ⁻ channel activity.	Decreased Conductance (Net Effect: Normal Amount of CFTR with Some Residual Function at the Apical Membrane)	R117H, R334W, R347P
Class V	Various mutations may be associated with reduced biosynthesis of fully active CFTR due to partially aberrant splicing (3849+10kbC→T), promoter mutations or inefficient trafficking.	Reduced Synthesis/Trafficking (Net Effect: Reduced Amount of Functional CFTR at the Apical Membrane)	A455E
Class VI	Truncation of the C-terminus of CFTR leads to the marked instability of an otherwise fully processed and functional variant. These are usually nonsense or frameshift mutations causing a 70- to 100-bp truncation of the C-terminus of the CFTR.	Decreased Stability (Net Effect: Functional but Unstable CFTR Present at the Apical Membrane).	Q1412X, 4326delTC, 4279insA

CFTR protein

The CFTR gene encodes a transmembrane protein with a symmetrical, multi-domain structure, consisting of two membrane-spanning domains, two nucleotide-binding domains and a central, highly charged regulatory domain (R) with multiple phosphorylation consensus sites. The principal function of CFTR is that of chloride transport at the apical membranes of epithelial cells but it has also been implicated in many other processes such as regulation of other ion channels, membrane trafficking, pH regulation and apoptosis.

CFTR is synthesized and assembled in the endoplasmic reticulum (ER). During the earliest steps of this process, nascent chain-ribosome complexes are targeted to the ER membrane, and transmembrane segments are precisely oriented and integrated into the lipid bilayer [16]. Additional biogenesis events involve the packing of transmembrane helices, folding of cytosolic domains, and finally, assembly of these domains into a mature tertiary structure [17]. This process is mediated by specialized cellular machinery that includes the Sec61 translocation complex and cytosolic (hsp70, hsp40) as well as ER (calnexin) chaperones that assist folding and prevent aggregation of folding intermediates [18]. CFTR maturation is thus a stepwise and compartmentalized process that coordinates folding of different protein domains in the lipid environment of the ER membrane, the oxidizing environment of the ER lumen and the reducing environment of the cytosol.

CFTR is the only known anion channel that links an enzymatic activity (adenosine triphosphate (ATP) hydrolysis) to opening and closing of the pore (channel gating). CFTR owes this property to its origin as an ATP-binding cassette transporters (ABC transporter), many others of which are active transport ATPases or pumps [19].

ABC-transporter are members of a protein superfamily that is one of the largest and most ancient families with representatives in all existent phyla from prokaryotes to humans. ABC proteins were known to function as mediators of organic solute transport and included, for example, the genes that encode multidrug resistance (i.e., MDRs, or P-glycoprotein genes), a gene that encodes chloroquine resistance in

Plasmodium falciparum, and a number of prokaryotic and eukaryotic small nutrient and molecular transporters [20]. ABC transporters are transmembrane proteins that utilize the energy of ATP hydrolysis to carry out certain biological processes including translocation of various substrates across membranes and non-transport-related processes such as translation of RNA and DNA repair. They transport a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids and sterols, and drugs [19]. Proteins are classified as ABC transporters based on the sequence and organization of their ABC domain(s). The common feature of all ABC transporters is that they consist of two distinct domains, the transmembrane domain (TMD) and the nucleotide-binding domain (NBD). The TMD, also known as membrane-spanning domain (MSD) or integral membrane (IM) domain, consists of alpha helices, inserted in the membrane bilayer. It recognizes a variety of substrates and undergoes conformational changes to transport the substrate across the membrane [19]. The NBD or ATP-binding cassette (ABC) domain, on the other hand, is located in the cytoplasm and it has a highly conserved sequence. The NBD is the site for ATP binding. The structural architecture of ABC transporters consists minimally of two TMDs and two ABCs. Two ATP molecules bind in pockets at the interface of an NBD dimer; each binding pocket is lined with residues from both NBDs. ATP binding to both sites creates or stabilizes the NBD dimer with an associated rearrangement of the translocation pathway by a proposed tweezer-like mechanism. For an exporter, this involves a shift from an inward-facing conformation with high substrate affinity to an outward conformation with low substrate affinity. Long cytosolic loops connect the NBDs to the TMDs and mediate the coupling between ATP binding and structural rearrangements of the TMDs. The transporter is reset to the inward-facing conformation following ATP hydrolysis at one or both sites [21].

Like this generic ABC transporter, CFTR also possesses two TMDs and two NBDs, binds two ATP molecules at the interface of an apparent NBD dimer, and exhibits ATPase activity, even if predominately at one site [21]. Channel opening is normally associated with ATP binding at both composite sites, which promotes or stabilizes an NBD1-NBD2 dimer that can be detected functionally or biochemically by cysteine cross-linking. What makes CFTR different from typical ligand-gated channels is the exceptionally slow rate of ATP unbinding ($k_{off} < 0.2 \text{ s}^{-1}$), which is due presumably to the tightness of the NBD1-

NBD2 dimer. This is where the enzymatic activity of CFTR comes in; ATP hydrolysis and subsequent product release destabilize the dimer and increase the probability of channel closure [22].

Numerous laboratories have now established that CFTR conducts chloride across the cell membrane. It down-regulates transepithelial sodium transport, in particular the epithelial sodium channel; it also regulates calcium-activated chloride channels and potassium channels and may serve important functions in exocytosis and the formation of molecular complexes in the plasma membrane [23].

Clinical manifestations

Mutational heterogeneity and environmental factors appear responsible for highly variable involvement of the lungs, pancreas, and other organs. A list of presenting manifestations is overlong, although pulmonary and gastrointestinal presentation predominant (Table 2).

Respiratory tract

Chronic infection in CF is limited to the airways. In human lung, dense, tenacious secretions obstruct the distal airways and submucosal glands, which express CFTR [24]. Ductular dilatation of these glands (associated with obstruction by mucus) and the plastering of airway surfaces by viscous, neutrophil-dominated mucopurulent debris are among the pathological hallmarks of the disease. Glandular hyperplasia in submucosal regions is prominent and enclosed by peribronchiolar inflammation with scar tissue. Submucosal gland ducts are inconspicuous in lungs of patients without CF, whereas luminal dilatation by mucus is one of the earliest visible changes in lungs of CF children. Pathogens such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Staphylococcus aureus*, and *Haemophilus influenza* become well established within firmly fixed airway secretions in patients affected and are not effectively eradicated. *P. aeruginosa*, specifically adapts to the pulmonary microenvironment in CF patients through the formation of biofilms and the production of a capsular polysaccharide (an alginate product) that inhibits penetration by antipathobiologic microbial agents and confers the mucoid phenotype [25].

Pulmonary inflammation is another important cause of the decline in respiratory function in CF patients and may precede the onset of chronic infection. Elevated levels of interleukin-8 (IL-8), interleukin-6 (IL-6), tumor necrosis factor α (TNF- α), and leukotriene B4 (LTB-4), along with reduced levels of anti-inflammatory cytokines and proteases, have been found in the airways of patients affected. Toll-like receptors (TLR), which recognize a variety of inflammatory mediators (i.e. neutrophil elastase, bacterial lipopolysaccharide, and other microbial products), partially mediate inflammatory effects by activating the transcription factor nuclear factor- κ B (NF- κ B), which governs a molecular pathway that induces the

production of inflammatory mediators and cytokines [26]. These and other inflammatory molecules (such as mannose-binding protein and α 1-antitrypsin) influence the progression of respiratory disease [27].

Recently, an elevated ratio of arachidonic acid (AA) to docosahexaenoic acid (DHA) was found in mucosal scrapings from CF patients, as compared to scrapings from normal people and from patients with inflammatory bowel disease; thus, the altered ratio cannot be explained by systemic inflammation alone [28].

Cough is the most important symptom of pulmonary involvement. At first, the cough may be dry and hacking, but eventually it becomes movable and productive. In adults, the cough is most prominent on arising in the morning or after sporting activity. Expectored mucus is often purulent. Some patients remain asymptomatic for long time or seem to have prolonged but intermittent acute lung infections. Other acquire a chronic cough in the first weeks of life, or they have pneumonias repetitively. Extensive bronchiolitis is attended by wheezing, which is a frequent symptom during the first years of life. Exacerbations of lung symptoms, presumably owing to more active airways infection and bronchiectasis, eventually require repeated hospitalizations for intravenous antibiotic treatment. Cor pulmonale, acute respiratory failure and death eventually occur unless lung transplantation is accomplished [29].

Spirometry has long been the accepted standard in disease follow up. Forced expiratory maneuvers such as forced expiratory volume in the first second (FEV₁) and forced vital capacity (FVC) are well understood, and almost universally FEV₁ is used to define mild (60% - 70% of predicted), moderate, and severe (40% - 30%) disease. Although the coefficient of variability for FEV₁ in normal people is reported to be around 2–3% [30], it is much higher in CF patients [31]. Flows at lower lung volumes (i.e., the forced expiratory flow during the middle half of the forced expiratory maneuver [FEF_{25%-75%}]), which may reflect obstruction of small airways, is the site of interest. The measurements are extremely technique and effort dependent. Some patients find such maneuvers difficult, and they are not routinely performed in 5 years old young children. The measurements also lack sensitivity, particularly in mild, early stages of disease or when looking for small changes in response to an intervention, and there is currently a very slow rate of decline (1–2% per year) in the CF population [32]. This means that, though patients who are

deteriorating rapidly or over a short time period can be easily identified, observing any improvement on this rate of decline in an individual patient will be difficult. Finally, although FEV₁ has historically been used in defining severity, there is some evidence to suggest it is not a very useful tool on which to base prognosis. In fact, FEV₁ is no longer included in the lung allocation score as part of transplant-waiting-list assessment in the United States [33].

Lung volumes (measured with pletismography) and diffusion capacity are listed in some of the current consensus documents for annual assessment, and are performed in the majority of modern centers, at least in Europe. Similarly to spirometry, they require cooperation and recent data suggest that lung-volume values add very little to spirometry for the majority of CF patients [34].

The Lung Clearance Index (LCI) uses multiple-breath wash-out of a non-absorbable gas (nitrogen and, more commonly now, sulfur hexafluoride) to measure ventilation inhomogeneity owing to airway narrowing from inflammation or mucus obstruction. The patient inhales a low concentration of sulfur hexafluoride, via either mask, until the concentration in the airways is in equilibrium with the concentration administered (*wash-in phase*). The supply is then switched off and, during continued tidal breathing, wash-out is monitored. Gas analyzers include conventional mass spectrometer or photoacoustic and ultrasonic-based technologies. Wash-out is defined as the point when the sulfur hexafluoride reaches 1/40th of its original concentration. Patients with more severe disease take longer to wash out phase, because gas is trapped in airways; therefore, they have a higher LCI. One advantage of the LCI is that it has a quite narrow range of normal values, which obviates the requirement for age/size-adjusted normal values [35]. The technique is easy to perform (requires only tidal breathing, and no additional coordination, cooperation, or forced maneuvers) and it can be performed at all ages (including infancy and pre-school ages); it is repeatable, reproducible, and more sensitive at the early stages of disease than spirometry [36-38]. Finally, it is as sensitive as forced expiratory maneuvers in infants and correlates better with structural changes on high-resolution computed tomography (HRCT) than does FEV₁ [39]. An important disadvantage of the LCI is that completely obstructed lung regions do not contribute to the

overall measurement because the inhaled gas does not spread those regions. In CF patients who have totally obstructed lung regions, the LCI could underestimate respiratory disease severity [40].

CF sputum contains high levels of inflammatory cells, pro-inflammatory cytokines, and proteolytic enzymes; some of these appear to correlate with other measurements of pulmonary involvement, such as spirometry, although whether they cause the pulmonary damage is less clear. Cytokines can be measured reproducibly, even in sputum from young children, and numerous of these are reduced by treatment with conventional intravenous antibiotics and other treatments [41-42]. A study that found changes in sputum cytokines after nebulized heparin found no corresponding changes in spirometry, which may suggest either that this technique is more sensitive to detect change than conventional lung function, or may simply reflect the fact that changes in lung function may occur in advanced disease [43]. Promisingly, results suggest that spontaneous sputum and samples obtained via induction methods based on nebulization of hypertonic saline are not significantly different with respect to inflammatory markers [44]. Interpreting these data is rather problematic, due to the varied and often unvalidated methods used in the processing stage. For example, mucolytics such as dithiothreitol are often used. Dithiothreitol, by cleaving disulphide bonds, affects the levels of proteins and may adversely affects reagents in the assay system [45]. A second concern relates to the lack of standardization of nature and concentration of protease inhibitors used. Both of these issues may be specific to the type of assay used and the substance measured. There is an urgent need to address these methodological issues, specifically for CF sputum, before this test can be routinely used within the clinical context.

Much interest has focused on the observation that the level of exhaled NO (FeNO) is reduced in CF. Given the anti-inflammatory and anti-infective properties of NO, some think it may play an important primary role in CF pathophysiology—a hypothesis supported by the low level of NO synthase messenger ribonucleic acid in relatively undamaged airways [46]. An alternative view is that NO production is itself adversely affected by inflammation and that low NO level is secondary to CF lung disease. NO level is extremely low in primary ciliary dyskinesia, a disease with a generally much better outlook than CF. A recent clinical trial of orally administered L-arginine (an NO donor) with CF subjects used exhaled NO as

an outcome and reported a sustained increase in NO production, although this was not mirrored by any significant effect on lung function [47]. However, no studies have addressed longitudinal change or correlations with other clinical variables, which would support this measure as a useful monitoring tool in the clinic.

Exhaled Condensate can be collected simply by asking a patient, even a quite young child, to exhale into a cold tube during tidal breathing. The condensate contains a small (but variable and undetermined) volume of airway-lining fluid, the pH of which is peculiarly low in CF [48]. However, this technique appears to lack sufficient sensitivity for use with an individual subject [49, 50].

Intestinal and biliary tract

Pancreatic insufficiency is the most frequent gastrointestinal manifestation in CF. More than 85% of affected patients show evidence of maldigestion from exocrine pancreatic insufficiency. Symptoms include recurrent, bulky, greasy stools and failure to gain weight even when food intake appears to be large. Nearly 40% of patients display nutritional failure by the criterion of weigh/height less than the 10th percentile [51]. Characteristically, stools contain readily visible droplets of fat. A protuberant abdomen, decreased muscle mass, poor growth, and delayed maturation are typical physical signs. The diagnosis of pancreatic insufficiency is a clinical one. The most effective test to confirm the diagnosis is to measure faecal elastase, which is low in people with pancreatic insufficiency [53]:

- Normal >200 mcg/g stool;
- Mild/moderate pancreatic insufficiency 100-200 mcg/g stool;
- Severe pancreatic insufficiency <100 mcg/g stool;
- CF pancreatic insufficiency <15 mcg/g stool.

This is not affected if children are already taking pancreatic enzymes. In patients who have residual exocrine pancreatic function, recurrent acute pancreatitis occurs occasionally. In addition to exocrine pancreatic insufficiency, evidence for hyperglycemia, polyuria, glycosuria and weight loss may appear.

Moreover, 8% of 11-17 years old patients and 18% of 18-24 years old patients have insulin-dependent diabetes [54].

Meconium ileus (MI) at birth, distal intestinal obstruction syndrome (DIOS, formerly designated “meconium ileus equivalent”), and constipation are all consequences of the increased viscosity of intestinal mucus and the prolonged intestinal transit time in CF [55].

In 13-17% of newborn infants with CF, the ileum is completely obstructed by meconium. MI is unique to CF and is clearly influenced by genetic factors, because a large twin study reports that homozygous twins show a greater concordance for MI than heterozygous twins [56]. Abdominal distension, emesis, and failure to pass meconium appear in the first 24-48 hours of life. Abdominal radiographs show dilated loops of bowel with air-fluid levels and, frequently, a collection of “ground glass” material in the lower central abdomen. Rarely, meconium peritonitis results from intrauterine rupture of the bowel wall and can be detected radiographically by the presence of peritoneal or scrotal calcifications. Meconium plug syndrome occurs with increased frequency in infants with CF but is less specific than MI [55].

Survival in patients with MI has improved dramatically; some authors report that survival through the first year of life increased from 55% in CF patients born between 1958 and 1972 to 96% in those born between 1973 and 1987 [57]. More recent articles even report no differences in survival between MI and non-MI patients [58, 59] In contrast, the long-term outcome of pulmonary function in MI patients is less uniform between the MI and non-MI groups. Some studies suggest that MI patients have a worse pulmonary phenotype, when corrections for the advantage of early detection and treatment were made [60, 61].

After the neonatal period, DIOS emerges, characterized by complete or incomplete intestinal obstruction of viscid fecal accumulation in the terminal ileum and proximal colon [55]. Characteristically, DIOS patients have abdominal pain, distension, and vomiting in combination with a right lower quadrant mass, which is palpable and usually seen on plain abdominal radiography. An important differential diagnosis of DIOS is constipation. However, in contrast to DIOS, symptoms are usually milder and have longer standing. Each condition is frequently seen in CF patients, with constipation in particular being greatly

underdiagnosed in CF [62]. The incidence (2.2–6.2 episodes per 1000 patient-years) and lifetime prevalence (7–8%) of DIOS in childhood is low [63]. Two studies report that DIOS incidence and lifetime prevalence increase as patients become older [64, 65]; indeed, incidence (23.3 episodes per 1000 patient-years) and lifetime prevalence (14–16%) are higher in adult patients. Additionally, DIOS is recurrent medical issue, because 20% of all pediatric patients experienced more than one episode during a 5-year observation period [64, 65]. Some studies report an increased frequency of severe CFTR genotypes in DIOS [63], whereas others report no differences [64], and a large twin study shows equally low concordance rates for DIOS in heterozygous and homozygous twins [66]. After lung transplantation, DIOS is frequently seen; about 10% to 20% of CF lung transplant patients develop at least one DIOS episode early in the post-transplant period [67]. The transplantation period is characterized by dehydration, immobility, and opiate use; all of these could promote fecal impaction in the ileo-cecum, eventually leading to complete intestinal obstruction in selected patients. Therefore, starting preventive laxative treatment in these high-risk patients after lung transplantation could be considered. Most DIOS episodes can be treated conservatively with intensive laxative treatment (oral laxatives and/or enema or polyethylene glycol lavage), and most large studies report low numbers of surgical interventions [62].

Less frequent gastrointestinal manifestation include intussusception, fecal impaction of the cecum with an asymptomatic abdominal mass, and duodenal inflammation with epigastric pain. In adults, acid or bile reflux with esophagitis symptoms is common. Rectal prolapse occurs much less frequently as the result of earlier diagnosis of pancreatic insufficiency and initiation of pancreatic enzyme replacement treatment.

Clinical manifestations of fat-soluble vitamin deficiencies have been noted:

- neurologic dysfunction (dementia, peripheral neuropathy) and hemolytic anemia owing to **vitamin E deficiency**;
- bleeding diathesis and hypoprothrombinemia owing to **vitamin K deficiency**;
- decreased bone density and rickets (rarely) owing to **vitamin D deficiency**;
- night blindness owing to **vitamin A deficiency**.

Manifestation of biliary tract involvement can include icterus, ascites, hematemesis from esophageal varices, and evidence of hypersplenism. Liver disease occurs independent of genotype but is associated with MI and pancreatic insufficiency. Evidence for liver dysfunction is most often detected during the first 15 years of life (> 30% of CF patients). Biliary colic secondary to cholelithiasis may occur in the second decade or later. A neonatal hepatitis-like picture and massive hepatomegaly owing to steatosis have been documented [68].

Genitourinary tract

Sexual development is often delayed and adolescent females may experience secondary amenorrhea, especially during respiratory exacerbations. The female fertility rate is diminished, owing to frequent cervicitis and accumulation of tenacious mucus in the cervical canal. Pregnancy is generally well tolerated in patients with good pulmonary function; on the contrary, pregnancy may accelerate pulmonary progression in those with advanced lung involvement [69].

More than 95% of males suffer from bilateral absence of the vas deferens and other forms of obstructive azoospermia, because of failure of development of Wolffian duct structures. The incidence of inguinal hernia, hydrocele, and undescended testis is higher than general population [70].

Genotype and Phenotype in CF

Studies of clinical phenotype in CF in correlation with CFTR genotype have discovered a very complex relationship. A CFTR genotype is only a predisposition for CF disease, which will be expressed to various penetrance and translated into CF pathophysiology. The content of the 'black box' linking genotype and clinical outcome is slowly being unraveled by a growing number of studies examining CFTR defects, by evaluating their molecular and cellular effects and ultimately relating them to a clinical phenotype [13]. In fact, of more than 850 mutations reported to the CF Consortium, a small part have been evaluated at the transcript or protein level and some of them have also been tested for their functional effects [12].

The extent by which CF phenotype is determined by specific CFTR mutations varies significantly from organ to organ.

Pulmonary involvement

There was considerable variation in severity of pulmonary disease in CF, even within patients belonging to the same genotype group. Among studies using more sophisticated assessment of CFTR mutations, such as those classifying their molecular mechanisms, or extent of the CFTR chloride channel function, some have shown statistically significant correlations between CFTR genotypes and pulmonary status [71, 72] while others have failed to detect a significant association [73, 74].

However, an important longitudinal study demonstrated a significant difference in the rate of decline of pulmonary function between patients with pancreatic sufficiency and pancreatic insufficiency mutations [75]. Pancreatic sufficiency phenotype is predominantly associated with mild CFTR mutations, which retain some residual chloride channel activity. The strongest association between mild pulmonary disease and a specific CFTR mutation was found for the missense allele A455E [76]. When this mutant variant was expressed in a heterologous cell system, slightly reduced Cl currents were identified with normal conductance and gating properties. However, its presence in the apical membrane was significantly reduced, with incomplete processing and degradation [77]. Of interest, in vivo studies showed no correction of the bioelectric properties in nasal epithelial cells of patients with this mutation [78]. If the

same is valid for lower airways, other mechanisms may exist for lightening the pulmonary involvement in patients with this genotype. There may be other specific mutations consistently associated with a mild lung presentation, like the G551S allele, but the minor number of patients analyzed do not consent for a definite conclusion [79, 80].

Other studies suggest that early respiratory outcome in CF children may depend on specific CFTR mutations progression of pulmonary disease. Poor or no genotype correlations in more advanced stages of CF lung involvement may be due to an influence of modifying factors in the later phase of the disease (i.e. environmental factors, *P. aeruginosa*-induced chronic inflammation), setting conditions for a slower or faster evolution [81-82].

Gastrointestinal involvement

Of the several gastrointestinal manifestations in CF, the exocrine pancreas is most profoundly and commonly affected (pancreatic insufficient is in 85% of CF patients) [12]. There is a strong association between specific CFTR alleles and exocrine pancreatic function. A small proportion (2–3%) of patients experience gradual transition from pancreatic sufficient to pancreatic insufficient. Preclinical knowledge of CFTR genotype (newborn screening) consents an early treatment intervention in patients with two severe alleles predictive of pancreatic insufficient. There is emergent evidence that identification of patients through newborn screening programs and providing early nutritional support as well as pancreatic enzyme supplements in CF children with pancreatic insufficient is important for evolution [13].

Expression of other less common gastrointestinal complications (MI, liver disease, diabetes) is not genotype dependent. However, manifestation of these symptoms is almost exclusively observed in patients with pancreatic insufficient and severe CFTR genotypes [83].

Other organs

The sweat glands were examined by measurements of sweat chloride concentration (standard sweat testing) in CF patients with different CFTR mutations. The patients were evaluated either according to classes of mutations or specific genotypes. Some studies [73] showed that definite mild alleles (R117H;

A455E; 3849+10kbC→T) from class IV or V tend to be associated with significantly lower Cl sweat levels than those for severe alleles ($\Delta F508$; 621+1G→T; G542X; R553X, etc.). Interestingly, although most severe genotypes produce high but similar sweat Cl concentrations, some are significantly associated with much higher chloride secretion (i.e., 621+1G→T)[79].

Gender-specific expression of the CF phenotype which involves the reproductive tract in men with CF is manifested by bilateral absence of the vas deferens or other forms of obstructive azoospermia. The majority of males with CF (95%) suffer from this form of infertility [84]. Therefore it appears that the male reproductive tract is extremely sensitive to CFTR mutations. Indeed, the clinical spectrum affecting the male reproductive tract and associated with defects in the CFTR gene is extending beyond CF in the form of isolated congenital bilateral absence of vas deferens (CBAVD). Typically, the genotype in those patients consists of at least one very mild mutation uncharacteristic for CF patients. The most interesting genotypic feature for these patients is a high prevalence of the RNA splice variant: IVS8-5'T (5'T) [85-87]. The frequency of 5'T allele in CBAVD was found to be up to 6 times higher than in general population and is present in about 40–50% of patients [88-90]. Additional insights into potential mechanisms underlying the differential phenotypic expression in CF and CBAVD patients were gained by analyzing CFTR transcripts in nasal epithelium and vas deferens in normal individuals with different T-tract genotypes [91].

Besides CBAVD, a higher than expected frequency of CF mutations/variants has been observed in other more common forms of infertility, including obstructive azoospermia and oligospermia [92]. Unlike observations in patients with CBAVD, men with obstructive azoospermia rarely harbored two distinct CFTR mutations. It has recently been proposed that in absence of a well-defined CFTR mutation, frequent polymorphic CFTR variants may predispose to obstructive azoospermia and perhaps other CFTR-associated diseases [93-94]. Although the CFTR defect has not been generally implicated in spermatogenesis, a recent report [95-97] suggested that the sperm maturation process can be delayed in CBAVD patients. Overall, it seems that male reproductive tract is the most sensitive system of all CFTR-affected tissues to even very minor defects in CFTR function.

Diagnosis

The diagnosis of CF has been based on a positive quantitative sweat test ($\text{Cl} \geq 60 \text{ mEq/L}$) in association with 1 or more of the following: typical chronic obstructive lung disease, documented exocrine pancreatic insufficiency, or a family history of CF [98].

The sweat test, using pilocarpine iontophoresis to collect sweat and chemical analysis of its chloride content, is the gold standard for the confirmation of CF diagnosis. The procedure requires care and accuracy. The collection methods are the Gibson-Cooke procedure and the Macroduct sweat collection system. In both, the sweat is stimulated by iontophoresis (3mA of electric current) with pilocarpine, after which it is collected with paper filter or gauze (Gibson-Cooke) or in a microbore tube (Wescor). Then, the sample is analyzed to determine the concentration of sodium chloride. For reliable results, the minimal acceptable sweat volume is 75 mg in the Gibson-Cooke procedure and 15 μL for the Macroduct system [98]. Testing may be difficult in first two weeks of life because of low sweat rates, but is recommended any time after the first 48 hours of life. Positive results should be confirmed; a negative result should be repeated if suspicion of CF remains. The sweat test should always be interpreted in the clinical context. The measurement of sodium is useful as a quality control. Highly discordant values indicate errors in the collection or analysis. A chloride concentration $> 60 \text{ mmol/L}$ is consistent with a diagnosis of CF, when 1 or more other criteria are present. Chloride values between 40 and 60 mmol/L have been reported at all ages in cases of typical involvement. False negative test results may occur in young patients with hypoproteinemic edema. False positive test may occur when sweat test is performed on pathologic or contaminated skin and in case of conditions associated with elevated concentrations of sweat electrolytes (untreated adrenal insufficiency, hereditary nephrogenic diabetes insipidus, hypotirodism, hypoparathyroidism, fucosidosis, malnutrition) [98].

The identification of mutations known as the cause of CF in each of the CFTR genes, confirms the diagnosis. However, the finding of only one mutation or no mutations in the CFTR gene does not rule out a diagnosis of CF [98]. Cases of non-classic CF in which there is no evidence of mutation in the CFTR genes have been reported [99].

Several commercial laboratories test identifies $\geq 90\%$ individuals who carry two CF mutations. The analysis of mutations presents high specificity and low sensitivity for confirming a diagnosis of CF. In fact, there are a great number of mutations (over 1000) that are known to cause CF, and the commercial panels available for this analysis only study a minority of those mutations [98].

Most affected newborns can be identified by determination of immunoreactive trypsinogen and restricted DNA testing on blood spots, associated with confirmatory sweat analysis (95% of sensitivity). There not convincing evidence that early diagnosis and prenatal screening improve pulmonary outcome or survival [100].

CF score of severity

Monitoring of disease progression is evaluated through spirometry and others test of pulmonary function, chest radiography and tomography, and clinical evaluations. However, there is some scores to assess disease severity in CF, such as Shwachman-Kulczycki (SK) score.

The SK score, published in 1958, was the first most important score to assess the severity of CF. It was developed based on a study which observed 105 patients for 5 years, exposing the need to measure CF severity, and to provide a perception of the overall clinical status of the patient [101]. This score is based on clinical and radiological evaluation and, despite its subjectivity, it is still widely used. The SK score is divided into four classes: general activity; physical examination; state of nutrition; and radiological findings. Each class has five possible subscores, according to the degree of severity. The scores of the four domains are summed to obtain the final score, from which the impairment of the patient is categorized as excellent (86-100), good (71-85), average (56- 70), poor (41-55) or severe (<40) (Table 3).

Since its publication, many other scoring systems for different aspects of CF have been developed, mainly radiological, functional and clinical scores [102]. However, the SK score is still the most used score, despite its lack of systematic application and its failure to consider the lung function test. In recent study, Stollar demonstrated that the SK score correlated strongly and significantly with FEV₁, chest radiography and tomography and with the 6-MWT [103].

Table 2: Shwachman-Kulczyki score

Score	Points	General activity	Physical examination	Nutrition	Radiological findings
Excellent (86-100)	25	Full normal activity. Plays, plays ball. Goes to school regularly.	Normal. No cough. Normal HR and RR. Clear lungs. Good posture.	Weight and height above the 25th percentile. Normal stool. Good muscle mass and tone.	Clear lung fields.
Good (71-85)	20	Irritability. Tires at end of day. Good school attendance.	Normal HR and RR at rest. Rare cough. Clear lungs. Minimal emphysema.	Weight and height 5-20th percentile. Stool slightly abnormal.	Minimal accentuation of bronchovascular markings. Mild emphysema
Average (56-70)	15	Needs to rest during the day. Tires easily after exertion. Fair school attendance.	Occasional cough, sometimes in the morning. Slightly increased RR. Medium emphysema. Mild clubbing.	Weight and height above 3rd percentile. Abnormal stool, poorly formed. Abdominal distension. Muscle hypotrophy.	Moderate emphysema. Increased bronchovascular markings.
Poor (41-55)	10	Dyspnea after short walks. Rests frequently.	Frequent, productive cough, chest retraction. Moderate emphysema. May have chest deformities. Clubbing 2 to 3+.	Weight and height below 3rd percentile. Abnormal stool. Marked reduction of muscle mass.	Moderate emphysema. Areas of atelectasis. Areas of mild infection. Bronchiectasis.
Severe (≤40)	5	Orthopnea. Bedridden.	Severe coughing spells. Periods of tachypnea and tachycardia and extensive pulmonary alterations. Can show signs of right heart failure. Clubbing 3 to 4+.	Marked malnutrition Abdominal distension. Rectal prolapse.	Extensive alterations. Obstructive phenomena. Infection, atelectasis, bronchiectasis.

Treatment of CF

CF is a systemic disease, so its treatment plan should be comprehensive and linked to close monitoring and early intervention. The use of the multidisciplinary approach model to treat CF patients is based on the observation that the creation of comprehensive care centers for CF is related to the progressively better prognosis of the patients. Despite the great advances in the knowledge regarding CF, the treatment of the disease continues to focus on resolving symptoms and correcting organ impairment [104].

Initial intervention after diagnosis should be intensive and include baseline assessment, initiation of treatment, clearing of lung involvement, and education of patients and parents. The goal of therapy is to maintain a stable condition for prolonged periods [98].

Pulmonary impairment is the principal cause of morbidity and mortality and an appropriate therapeutic approach can slow its progression. The standard therapeutic regimen for the pulmonary manifestations includes antibiotic therapy, that is the mainstay of therapy designed to control progression of lung infection. Indications to an oral antibiotic therapy include the presence of respiratory tract symptoms and identification of pathogenic organism in sputum cultures. The usual course of therapy is greater than two weeks, and the dosages for some antibiotics are often 2 to 3 times amount recommended for minor infectious. In fact, it is difficult to achieve effective drug levels of many antibiotics in lung secretions and CF patients have higher clearance rates [98]. Wherever possible, the choice of antimicrobials drug should be guided by in vitro sensitivity testing. For the patients who has progressive or acute respiratory infections, intravenous antibiotic therapy is indicated. This treatment is usually initiated in the hospital but may be completed at home or within ambulatory bases, with permanent intravenous access. The usual course of systemic antibiotic treatment is at least 14 days, and requires two drug therapy, especially in case of *Pseudomonas* infection. The positive culture always must guide the choice of antibiotic treatment [105].

Some studies have reported that, in addition to their antimicrobial activity, macrolides have immunomodulatory properties in several respiratory diseases. The anti-inflammatory action of macrolides was firstly discovered in Japan in the 1980s, when treatment with macrolides significantly increased

survival in patients with diffuse panbronchiolitis, a severe chronic lung disease with intense neutrophilic inflammation [106]. The most important effects of macrolides are the following: inhibition of pro-inflammatory cytokine synthesis, inhibition of neutrophil migration to sites of inflammation, inhibition of leukocyte degranulation, reduction of eosinophilic inflammation, activation of macrophage phagocytic activity and increasing mucociliary transport. Other described actions include reduced goblet cell secretion, reduced bronchoconstriction induced by a decreased release of endothelin-1, and inhibition of the cholinergic response in the airway smooth muscle [107]. An important study evaluate the effect of azithromycin (250 mg/day) vs placebo for 3 months in 60 clinically stable CF patients. Pulmonary function levels remained constant in patients with azithromycin, whereas, in patients receiving placebo, there was a significant decline in lung function. In addition, patients receiving azithromycin had significantly fewer total days of i.v. antibiotic treatment [108]. On the contrary, a chronic use of macrolides in CF is significantly related to the emergence of bacterial resistance to this class of antibiotics [109].

Aerosol treatment is important to deliver medication and hydrate the lower respiratory tract. Often, anti-inflammatory drugs (such as corticosteroids or glutathione or N-acetyl-cysteine) and bronchodilators (such as albuterol or other β agonists) are employed, especially after chest physical therapy [110]. Human recombinant DNase, given as a single daily aerosol dose, improves pulmonary function and decrease rate of pulmonary exacerbations, in particular in advanced lung disease after 12 months on continuous therapy [111]. Hypertonic saline aerosol is reported to improve mucus clearance and increase FEV₁. Instead, its benefits are quite variable and inferior to that documented with recombinant DNase [111]. Finally, aerosol delivery of antibiotics offers an option for continuous and home delivery of additional agents. In particular, inhaled tobramycin has been studied widely: given twice daily on alternative months, *Pseudomonas* density in sputum decrease after 6 months [112]. It is documented that fewer hospitalization are required and pulmonary function can improve by $\geq 10\%$. Other used inhaled antibiotics such as colistin must be used twice a day, but bronchospasm may complicated this aerosolized therapy [113].

Airway clearance therapy is a fundamental mainstays of CF management. Chest physical therapy (PT) can be particularly useful in this patients because they accumulate secretion in small airways, even before the

onset of symptoms. Chest PT include: autogenic drainage; modified autogenic drainage, active cycle of breathing, forced expiration, use of oral oscillatory devices, positive expiratory pressure using a mask, high frequency thoracic compressions, vest-type mechanical percussors and intrapulmonary percussive ventilation [114]. CF patient should be guided in the choice of techniques combinations and should be instructed in order to perform correctly these techniques, especially within children. Chest PT is recommended from 1 to 4 times a day, guided by severity of lung dysfunction and by patient's necessity. Although immediate improvement of lung function generally cannot be demonstrated after PT, cessation of this physical therapy in CF patients results in deterioration of pulmonary function within three weeks [115].

Routine physical activity increases airway clearance and slow the rate of decline in lung function, improves cardiovascular performance, increases functional capacity, and improves quality of life [116]. Pulmonary rehabilitation programs provide benefits in CF patients, especially in case of low free fat mass [117].

Acute and chronic respiratory failure occurs in CF patients after prolonged deterioration of lung function, usually as result of a severe viral or other infectious disease. Since a long-standing $\text{PaO}_2 < 50$ mmHg promotes the development of right side hearth failure, it is recommended low-flow oxygen therapy [98]. Some CF patients also present hypoxemia only during exercise or sleeping. Oxygen therapy during exercise is indicated if oxygen saturation drops below 90%. Nocturnal oxygen therapy is indicated if the oxygen saturation is lower than 90% for 10% or more of total sleep time. Noninvasive mechanical ventilation can improve gas exchange in patients with hypercapnia, awaiting a lung transplant [118].

Lung transplantation is the first option for end-stage lung involvement. Due to the supplicative complications of CF, most widely used technique is bilateral lung transplant through a sequential bilateral surgical procedure using a cadaver donor. The criteria for patient candidacy are: $\text{FEV}_1 < 30\%$ than the predicted; severe hypoxemia and/or hypercapnia; progressive functional impairment or increase in the duration and frequency of hospital treatment for exacerbations; life-threatening pulmonary complications such as hemoptysis. An increased antibiotic resistance of bacterial pathogens and chest infection with B. cepacia or non-tuberculous mycobacteria are negative predictors of outcome. Lung transplantation has

demonstrated one-year survival of about 90%, but the five-year survival is only 50%, most probably because of the development of chronic rejection as bronchiolitis obliterans [119].

There is particular interest in gene therapy, that consists in restoring CFTR function in CF patients through insertion of a normal copy of the gene. However, much has been documented from gene therapy trials and the airways present many problems to successful gene therapy, more so than many other organs [120]. Although we cannot currently supplement mutant CFTR alleles with a normal copy by gene therapy, small molecules have been identified that can modulate mutant CFTR channel such that its function may be closer to normality. The first approved CFTR modulator, ivacaftor, is indicated for CF patients with a specific mutation. Ivacaftor potentiates the CFTR channel by increasing the probability of channel opening, it is associated with improvements in lung function at 2 weeks that were maintained through 48 weeks. Substantial improvements can be also observed in the risk of pulmonary exacerbations, in patient-reported respiratory symptoms, weight, and in concentration of sweat chloride [121].

One prospective treatment is stem-cell therapy. However, knowledge of pulmonary stem cells is quite limited, and such researches are still preliminary [122].

Pancreatic enzyme therapy in patients with pancreatic insufficiency is associated with increase in the rate of fat absorption, reduction in stool frequency and improvement in stool consistency, with weight increase. The initial dose is determined by the weight of the patient and level of dietary fat intake. The recommended dose is 500 to 1,000 lipase units/kg/meal, which may be increased if clinical signs of pancreatic insufficiency (steatorrhea) persist. The maximum daily dose is 2,500 lipase units/kg/meal or 10,000 lipase units/kg/day, based on reports of the occurrence of DIOS in patients receiving high-dose pancreatic enzymes [123]. If steatorrhea persists, the concomitant use of proton pump inhibitors or histamine H₂-receptor inhibitors may be considered to reduce pancreatic enzyme inactivation. Treatment aims at a normal intake of fat and other dietary nutrients, and proper development and growth (height and weight improvement). Cases in which steatorrhea persists even after administration of high-dose pancreatic enzymes should be investigated for other diseases, such as celiac disease, parasitosis, food allergy, among others [123].

Because pancreatic insufficiency results in malabsorption of fat-soluble vitamins, it is recommended daily vitamins supplementation (A, D, E, K). Additional doses may be required when low serum levels are documented or when they became symptomatic [124-126].

Many children at diagnosis time have nutritional deficit and an adequate diet plays an important role in the clinical course of CF. Malnutrition has many adverse effects on pulmonary functions including decreased ventilatory drive, impaired pulmonary muscle function, decreased exercise tolerance, and altered pulmonary immune response. Nutritional intervention should begin early, and can decrease deterioration of the lung function and have a positive effect on survival. Every CF patient should be regularly evaluated in order to monitor the nutritional state and ensure an adequate caloric intake. The recommendation includes a high-fat diet, with 35% to 40% of the calories coming from fat (120% to 150% of the estimated minimum daily requirement) [127].

An estimated approximation of the energy needs can be made using the following equation:

$$\begin{aligned} \text{total energy spending} = & \\ & \text{basal metabolic rate} \\ & \times 1.1 \text{ (poor absorption factor)} \\ & \times 1.5-1.7 \text{ (activity factor)} \\ & + 200 \text{ to } 400 \text{ kcal/day} \end{aligned}$$

Most CF patients have an increased caloric need when infective respiratory exacerbations occur and in case of advanced lung disease. These patients can be monitored by determining the 3-day intake and by anthropometric evaluation (body mass index, arm circumference, mid-arm muscle circumference, triceps skinfold thickness, and weight loss percentage), analysis of body composition (electrical bioimpedance), and peripheral muscle strength determination.

The goal is to maintain a body mass index of 20-25 kg/m², while a body mass index lower than 19 kg/m² indicates significant malnutrition and a more aggressive nutritional intervention is needed [127]. For all age categories, a special attention must be given if stunting is evident, defined as ht/age of <90% or height centile <0.4th (Table 3).

In end-stage phase, weight stabilization requires nocturnal feeding via nasogastric tube or percutaneous enterostomy or short-term intravenous supplementation [128].

Table 3: three class of severity in nutritional status.

Nutritional Status	<5 years	5-18 years	> 18 years	Intervention
Normal nutritional state	Wt/ht 90-110%	Wt/ht 90-110%	BMI 19-25 kg/m ² and/or no recent weight loss	<i>Preventative counselling</i>
Light malnutrition	Wt/ht 85-89% or weight loss over 4 months or plateau in weight over 6 months	Wt/ht 85-89% or weight loss over 6 months or plateau in weight over 6 months	BMI <19 kg/m ² or 5% weight loss over more than 2 months	<i>Consider supplements</i>
Severe malnutrition	Supplements tried and either wt/ht <85% or weight falling 2 centile positions	Supplements tried and either- wt/ht <85% or weight falling 2 centile positions	Supplements tried and either- BMI <19 kg/m ² or >5% weight loss over more than 2 months	<i>Aggressive nutritional support</i>

Docosahexaenoic Acid (DHA)

Fat is an essential component of the diet [129]. On a fat-free diet, rats do not grow or reproduce. Essential fatty acids include linoleic acid and its omega-6 derivative, arachidonic acid (AA). More recently, the vital role of alpha-linolenic acid and its omega-3 derivative, docosahexaenoic acid, has been recognized [129].

Omega-3 (ω -3) fatty acids are polyunsaturated fatty acids (PUFAs) and essential components for the growth of higher eukaryotes [130]. Nutritionally, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are the most important fatty acids belonging to this group of bioactive compounds.

DHA can be synthesized from alpha-linolenic acid or obtained directly from maternal milk or fish oil. Its structure is a carboxylic acid with a 22-carbon chain and six cis double bonds; the first double bond is located at the third carbon from the omega end (Fig. 1). Its trivial name is cervonic acid, its systematic name is all-cis-docosa-4,7,10,13,16,19-hexa-enoic acid, and its shorthand name is 22:6(n-3) in the nomenclature of fatty acids. DHA is a primary structural component of the human brain, cerebral cortex, skin, sperm, testicles and retina. DHA is the most abundant omega-3 fatty acid in the brain, in sperm and retina. DHA comprises 40% of the polyunsaturated fatty acids (PUFAs) in the brain and 60% of the PUFAs in the retina [131]. Fifty percent of the weight of a neuron's plasma membrane is composed of DHA. In the brain, DHA modulates the carrier-mediated transport of choline, glycine, and taurine, the function of delayed rectifier potassium channels, and the response of rhodopsin contained in the synaptic vesicles, among many other functions.

Cold-water oceanic fish oils are rich in DHA. Most of the DHA in fish and multi-cellular organisms with access to cold-water oceanic foods originates from photosynthetic and heterotrophic microalgae, and becomes increasingly concentrated in organisms the further they are up the food chain [131]. Many marine microalgal strains have oil contents of between 10–50% and produce a high percentage of total lipids (up to 30–70% of dry weight) [130]. The accumulation of fatty acids is closely linked to microalgal growth stages, functioning as an energy stockpile during unfavorable conditions or cell division. Omega-3 is accumulated due to its high energy content, as well as the good flow properties crucial for cellular

functions. DHA is also commercially manufactured from microalgae; *Cryptocodinium cohnii* and another of the genus *Schizochytrium* [132-133].

DHA is essential for the growth and functional development of the brain in infants. DHA is also required for maintenance of normal brain function in adults. DHA deficiencies are associated with fetal alcohol syndrome, attention deficit hyperactivity disorder, CF, phenylketonuria, unipolar depression, aggressive hostility, and adrenoleukodystrophy. Epidemiological studies have shown that omega-3 fatty acid consumption reduces Alzheimer disease risk and DHA modifies the expression of Alzheimer-like brain pathology in mouse models [134-135]. Several studies have also shown a strong correlation between fish consumption and reduction in sudden death from myocardial infarction. The reduction is approximately 50% with 200 mg day⁻¹ of DHA from fish [137].

Moreover, children who ate fresh, oily fish more than once per week had a significantly reduced risk of present asthma. No other food groups were associated with any risk difference for asthma [140]. In another study, 39 asthmatic children were supplemented with omega-3 or omega-6 fatty acids. Significant differences were seen in plasma phospholipid fatty acids and TNF- α production was lower than baseline in the omega-3 group. No significant differences were found in clinical outcome measures in this study [138]. Broughton studied asthmatic patients that sequentially given a diet with a 10:1 or 2:1 ratio of omega-6 to omega-3 PUFA for 1 month each. The patients were then stressed with methacholine inhalation. Methacholine-induced respiratory distress increased with low omega-3 PUFA ingestion [140]. In fact, leukotriene synthesis from AA is involved in the asthma process through vasoconstriction and mucus secretion and it is inhibited by long-chain omega-3 PUFA.

Humans originally consumed a diet rich in the omega-3 fatty acids and poor in saturated fatty acids because wild and free-range food animals have much higher contents of omega-3 fatty acids than the present-day commercial livestock. The dietary supply of fatty acids previously contained a 1:1 ratio of omega-6 to omega-3 polyunsaturated fatty acids. The present ratio in the US is greater than 10:1, causing a deficiency of the omega-3 fatty acids. The excess of omega-6 fatty acids stimulates the formation of AA, the fatty acid precursor of prostaglandins and other eicosanoids that are involved in inflammation [140].

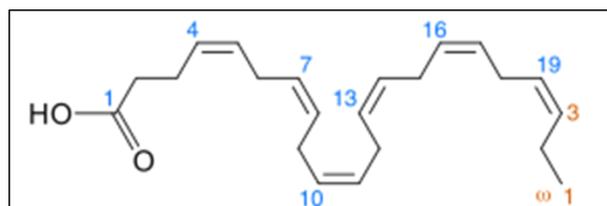


Figure 1: DHA's structure

DHA in CF

Several reports have indicated the presence of specific fatty acid alterations in CF patients. When this phenomenon was first noted more than 40 years ago, the primary abnormalities identified were decreased linoleic acid (LA) levels in different organs and tissues. Since pancreatic insufficiency is present in more than 80 percent of CF patients, these abnormalities have been initially presumed to be secondary to fat malabsorption and therefore not a primary abnormality due to CFTR mutations [141].

In later 30 years, some studies have given support to the theory that the essential fatty acid abnormalities in patients with CF may have a fundamental role in the symptoms and progression of the disease [142]. It is well documented that in newborn with CF, identified by neonatal screening or by MI manifestations, low LA concentrations are present at birth and are more pronounced in infants who present with MI [143]. Abnormal metabolism of fatty acids has been described by several groups, and increased release of AA, the most important metabolic product of LA, has been documented in vitro systems [144]. Later studies demonstrated additional significant alterations in the levels of fatty acid in the blood and tissues of CF patients, most notably decreased levels of DHA [145].

AA is the substrate for inflammatory metabolites, such as prostaglandin E2 (PGE2), thromboxane A2 (TXA2), leukotriene B4 (LTB4), and the cysteinyl leukotrienes. In the process of the transformation of different groups of longchain fatty acids, an increase of one group of fatty acids, those of the omega-3 series, has been shown to slow the transformation of omega-6 fatty acids. There is both an increase in AA and a marked increase of the prostanoids, which might contribute to the inflammation that characterizes CF [147]. Since DHA, in an in vitro system, has been shown to inhibit the synthesis of prostanoids but not of leukotrienes, a decrease in DHA might contribute to the increased prostanoid synthesis in CF [148].

Over the last 10 years, several lipid mediators DHA-derived, such as resolvins and protectins, arising from the coordinated activity of 5-, 15-, and other lipoxygenases, have been identified as important factors in the resolution phase of the inflammatory reaction, raising considerable interest on the potential deficit of these lipid mediators in chronic inflammatory conditions. These new compounds possess potent actions in controlling the resolution of inflammatory exudates: the term resolvins, resolution phase interaction products, was introduced to signify that the new structures are endogenous, local-acting mediators possessing potent anti-inflammatory and immunoregulatory properties. At the cellular level, these include reducing neutrophil infiltration and regulating the cytokine-chemokine axis and reactive oxygen species, as well as lowering the magnitude of the inflammatory response [148]. The protectin family, neuro-protectin when generated in the neural tissue [148], was named after the potent anti-inflammatory as well as protective actions demonstrated in animal models of stroke and Alzheimer's disease [149]. Both families of lipid mediators are potent local-acting agonists of endogenous anti-inflammation that promote resolution. Protectin D1 and its immediate precursor 17S-hydroxy-docosahexaenoic acid were identified in exhaled breath condensates from healthy subjects, and significantly lower concentrations were detected in exhaled breath condensates from subjects with asthma exacerbations [150], providing evidence for endogenous PD1 as a pivotal counter-regulatory signal in airway inflammation and pointing to new therapeutic strategies for modulating inflammation in the lung.

These data, along with the observation that mice with a targeted deletion of the CFTR gene have an abnormal ratio AA/DHA in pancreatic tissue, have led to the speculation that abnormalities in the metabolism of AA and DHA may be important in evolution of CF. Freedman et al. have shown that diet with high doses of DHA in CF-knockout mice corrects the fatty acid abnormality, reverses the histologic changes in the pancreas and ileum, and decreases neutrophil and the eicosanoids levels in mice with *Pseudomonas* lipopolysaccharide-induced pneumonia [151]. These authors also demonstrated an increased ratio AA/DHA in nasal and rectal epithelium, tissues that express CFTR, in patients with CF and pancreatic sufficiency, who have normal assimilation of micronutrients and macronutrients. It is important to note that the ratio in nasal mucosal scrapings from obligate heterozygotes was intermediate between values in subjects with CF and healthy control subjects [152].

Moreover, recently Tetaert et al demonstrated that lung infection by *P. aeruginosa* induced a dysregulation of the large mucins (such as Muc5b and Muc4). These modifications of mucin expression are modified by dietary long-chain fatty acids with a beneficial suppressive effect of PUFAs confirming [153]. This strongly supports that malnutrition can compromise lung defenses against *P. aeruginosa* colonization. Therefore, it seems rational to try to normalize plasma abnormal levels of essential fatty in CF patients.

Aims of the study

DHA and his mediators are important factors in the resolution phase of the inflammatory reaction, raising considerable interest on the deficit of these lipid metabolites in FC and its systemic chronic inflammatory condition. We hypothesized that mediators derived-DHA could be significantly lower in airway of CF patients, as compared to another neutrophilic inflammatory disease, such as COPD, and that this condition could be reversible by DHA oral supplementation.

The aim of the study was, therefore, to determine the levels of the AA and DHA-metabolites in sputum of adults with CF subjects, as compared to patients with COPD; to ascertain whether or not DHA supplementation may affect the fatty acid pattern in CF subjects.

4. Materials and Methods

We studied 15 CF subjects and 10 COPD Gold stage 2-3 patients (not age-matched). CF patients (age range 20 to 40 years) were recruited at the Cystic Fibrosis Unit of Parma Hospital and the control group of COPD patients were recruited at the University Hospitals of Modena-Reggio Emilia and Parma. All patients with CF were diagnosed by evidence of CFTR dysfunction (elevated sweat test) and/or identification of two pathological CFTR mutations (INNO- LiPA CFTR19®). The inclusion criteria were as following: genotype $\Delta F508$ homozygous, mild/moderate pulmonary disease ($FEV_1 \geq 40\%$ predicted value), and pancreatic insufficiency. All patients was clinically stable and following standard CF therapy.

All subjects recruited performed at baseline: nutritional status evaluation, severity score evaluation (SK score), spirometry, exhaled NO measurement, SI, EBC, and their blood sample were drawn to evaluate their fatty acid profile (*first phase*). In *second phase*, CF patients performed all evaluations after ten weeks with DHA-supplementation and after ten weeks without DHA-supplementation.

Nutritional status was assessed and expressed as BMI (kg/m^2).

The pulmonary function was measured by body plethysmograph (B3Box Biomedin, Padua, Italy) and oxygen saturation (SatO_2) was measured by pulse oxymetry (Nellcor N-395).

FeNO was measured according to the ATS/ERS guidelines [154] using a stationary chemiluminescence analyzer (NIOX, Aerocrine AB, Solna, Sweden), which has been approved by the U.S.A. Food and Drug Administration for use in asthma management [155]. FeNO measurement was performed at the same time of day to allow a possible circadian rhythm effect. In detail, FeNO measurement was performed asking the subjects, who was seated in the upright position without a nose clip, to inhale nitric oxide-free air through a filter connected to the device deeply to total lung capacity and then to exhale for 10s at a constant pressure guided by a visual cue to stabilize flow rate. All tests were performed at an exhalation pressure of 10-20 cm H₂O, to maintain a fixed flow-rate of 50 ml/s. Measurements were repeated after a brief rest period until two acceptable values (± 2.5 p.p.b. for measurements < 50 p.p.b. and $\pm 5\%$ for

measurements ≥ 50 p.p.b.) were performed (maximum six attempts). The mean of two adequate values for each subject was recorded for analysis.

SI was performed in accordance with the European Respiratory Society task force [156, 157]. FEV₁ and FVC were measured at baseline and after inhalation of salbutamol (200 µg by metered dose inhalers). After that, subjects were asked to rinse their mouth. Subjects inhaled sterile hypertonic saline (NaCl, 3%, prepared by the hospital chemist) nebulized with an ultrasonic device (Heyer Orion 1, BAD EMS; mean volume output: 2.40 ml/min) for four cycles of 5 minutes each. After each cycle and when needed they were asked to rinse their mouth and cough in to a plastic container. Three flow volume curves were performed before and after each inhalation and the best FEV₁ was recorded. Induction of sputum was stopped if FEV₁ value fell by at least 15% from baseline or if troublesome symptoms occurred. The collected sputum samples were processed [158]. The volume of the sputum sample was measured and an equal volume of dithiotreitol 0.1% was added and incubated at 37°C for 30 min. Ten microlitres of the homogenised sample was used to determine the total cell count and results were expressed as number of cells/ml. The remaining sputum was centrifuged at 2000 rpm for a 5 min period. The supernatant was aspirated, and cell pellets will be re-suspended in PBS (2000 rpm for 10 min), cytocentrifuged at 600 rpm for 10 min and stained with May-Grunwald-Giemsa. Only samples with less than 20% squamous cell contamination, to exclude salivary contamination, and more than 50% viable cells was considered suitable. Cell count was performed on at least two slides for an overall differential count of 800 nucleated nonsquamous cells and was reported as the relative numbers of eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells, expressed as a percentage. The percentage of squamous cells was reported separately.

EBC is a non-invasive method [159] for studying the composition of airway lining fluid and provides biological insight into the pathogenesis of respiratory disorders. Based on the currently available evidence and the consensus of the expert panel for EBC collection, EBC was collected by using Cosmed Rtube (Cosmed, Milan, Italy). The subject breathed through a mouthpiece and two-way non rebreathing valve.

They were asked to breathe at a normal frequency and tidal volume, for a period of 10 min. Condensate, at least 1 ml, was collected, transferred to Eppendorf tubes and immediately stored at -80°C .

Samples were kept at -80°C until transferred to the Milano unit for analysis. After thawing, EBC and sputum supernatant samples (1 ml) were added with stable isotope labeled internal standards ([d4]LTB₄, [d4]PGE₂, [d8]5-HETE, and [d5]LXA₄ 2.5 ng each), centrifuged to remove particulate, acidified with acetic acid (final concentration 0.01%) and extracted using preconditioned polymeric solid phase extraction cartridges (Strata-X, 33 μm Polymeric Reversed Phase, Phenomenex, Torrance, CA). After washing with ultrapure water, DHA- and arachidonic acid-derived metabolites were eluted using methanol/water, 90/10, v/v (0.5 mL), and the eluate taken to dryness using a rotary vacuum evaporator (SpeedVac, Thermo Scientific, Waltham, MA). Upon reconstitution in 40 μL HPLC solvent A (8.3 mM acetic acid buffer to pH 5.7 with ammonium hydroxide) plus 20 μL of HPLC solvent B (acetonitrile/methanol, 65:35, v/v), an aliquot of each sample (20 μL) was injected onto a C18 HPLC column (Ascentis 150 x 2 mm, 3 μm , Supelco, Bellefonte, PA) and eluted at the rate of 400 $\mu\text{L}/\text{min}$ with a linear gradient from 45% solvent B, which was increased to 75% in 12 min, to 98% in 2 min, then held for 11 min before re-equilibration at 45% B for 10 min. The HPLC effluent was directly infused into a triple quadrupole mass spectrometer (6460, Agilent) equipped with electrospray ion source for mass spectrometric analysis in the negative ion mode using multiple reaction monitoring (MRM) for the specific m/z transitions: 343-281 for 17(S)OH-DHA (the precursor of both resolvins and protectins), 359-206 for protectin D1 and for its isomer I 10(S),17(S)-DiHydroxyDocosaHexaenoic acid, 375-141 for resolvin D1, 335-195 for LTB₄, 319-219 for 15-HETE (a marker of 15-lipoxygenase activity), 351-271 for PGE₂, 327-116 for [d8]5-HETE, 339-197 for [d4]LTB₄, 359-275 for [d4]PGE₂ and 356-222 for [d5]LXA₄. (Pioselli et al, 2010). Quantitation was performed using isotope dilution of the internal standards and data analyzed using MassHunter software. Standard curves were obtained using synthetic LTB₄, PGE₂, resolvin D1 and 10(S),17(S)-DiHydroxyDocosaHexaenoic acid (isomer I of protectin D1), 15-HETE and 17OH-DHA (Cayman Chem, Ann Arbor, MI). The peak-area ratios of every compound to the relevant deuterated internal standard was calculated and plotted against the amount of the synthetic standards. Calibration lines were calculated by the least squares linear regression method and the correlation coefficient r^2 was

always better than 0.99. To calculate the concentration of any given analyte the peak-area ratio to the relevant internal standard was calculated and read off the corresponding calibration line. Detection limit varied between 1 pg to 25 pg depending on the analyte. Optimization of declustering potential, collision energy and CXP, was carried out for each metabolite directly injecting 1-5 ng of synthetic standard using the same eluent used for the analysis.

For the determination of Fatty Acid composition of erythrocytes membrane phospholipids, blood samples were collected in 10% sodium heparin and centrifuged at 200 g for 18 min. The lower fraction was additionally centrifuged at 800g for 18 min and the pellet washed twice with phosphate buffer containing 0.1M NaH₂PO₄ (5:1 v/v). Cells were lysed with water, followed by spotting on preconditioned absorbing strips (Rise et al, 2010). Fatty acid methyl esters were prepared by transesterification with 3N methanolic HCl (Supelco). Methyl esters were analyzed on a GC 1000 (Dani, Monza, Italy) gas chromatograph, equipped with a flame ionization detector, using an Omegawax 320 column (Supelco). Peaks were identified by using pure reference compounds. The fatty acids will be quantified by referring to an internal standard (19:0) [160].

The results of exhaled NO concentrations, total and differential cell counts in sputum samples, respiratory function parameters, fatty acid profile and pro- and anti-inflammatory mediators concentrations in EBC and sputum were analyzed for potential correlations within each group of subjects.

Statistical analysis

Data are reported as mean \pm standard deviation (SD), unless otherwise specified. The distribution of variables was assessed by means of Kolmogorov-Smirnov Goodness-of-Fit test. Comparisons for each parameter among the different groups were carried out, and the statistically significant difference was set at $p < 0.05$.

5. Results

We studied fifteen patients (9 female) affected by FC, aged between 15 and 40. Table 4 shows the demographic data, SK score, pulmonary function and nutritional status for patients in this study.

Table 4: demographic, anthropometric and functional characteristics of CF patients at baseline

No	Patients	BMI (kg/m ²)	Age (years)	Gender (F/M)	FEV ₁ (%)	FEV ₁ /FVC (%)	SK SCORE
1	B.A	20	32	F	46	62	80
2	C.D.	22.3	36	F	46	67	50
3	C.E.	20.4	27	F	81	87	75
4	F.G.	21.7	37	M	85	73	75
5	I.M.	24	40	M	71	78	85
6	L.L.	20	26	M	65	87	65
7	M.L.	20.4	23	F	53	58	80
8	M.E.	22.2	31	M	99	93	75
9	P.S.	19.2	29	F	28	52	70
10	P.A.	19	30	F	68	83	60
11	R.S.	19.9	32	F	74	67	75
12	R.A.	22.5	23	M	72	89	75
13	V.M.	19.8	15	F	93	86	55
14	V.L.	20	29	F	72	80	70
15	Z.C.	21.4	29	M	94	98	60
Mean		20.9	29.3		69.8	77.3	70
SD		± 1.4	± 6.2		± 19.9	± 13.6	± 10
Ratio (F/M)		9/6					

All 15 CF patients completed first phase. 4 out of 15 subjects (3 female) had recruitment of chronic inflammatory condition and performed complete evaluations of nutritional status, functional status and EBC/SI analysis after two weeks of systemic antibiotic therapy. 9 out of 15 patients (5 female) were stable and completed second phase after ten weeks with DHA-supplementation and after ten weeks without DHA-supplementation.

Characteristic inflammatory status of CF patients were neutrophilic and table 5 shows cytological analysis on SI at baseline.

Table 5: cytological analysis on SI of CF patients at baseline

No	Patients	Epithelial cells (%)	Macrophages (%)	Neutrophils (%)	Lymphocyte (%)	Eosinophil (%)
1	B.A	0	13	84	1.5	1.5
2	C.D.	0	16	79	1	2
3	C.E.	1	14	83	2	1
4	F.G.	2	31	67	0	0
5	I.M.	1.5	11	85	2.5	0
6	L.L.	0	16	83	0.5	0.7
7	M.L.	0.4	15	83	0.4	2
8	M.E.	0.3	11.3	86.6	0.2	1.5
9	P.S.	0	11	84	2	3
10	P.A.	1	35	60	0.5	3.5
11	R.S.	1	17	79	3	0
12	R.A.	0	19	77	3	1
13	V.M.	2	30	63	4	1
14	V.L.	3	13	81	0	3
15	Z.C.	1	19	77	2	1
	Mean	0.7	16.2	80.2	1.6	1.3
	SD	± 0.9	± 7.3	± 8.1	± 1.2	± 1.61

Direct comparison of CF subjects with COPD subjects clearly showed markedly increased concentrations of LTB₄, PGE₂ and 15-HETE in CF subjects. Strikingly, the concentrations of the DHA derived 17OH-DHA were very similar in the two groups, showing that a rather large unbalance of AA-derived inflammatory mediators over the DHA-derived metabolites was occurring in CF patients (Fig. 2).

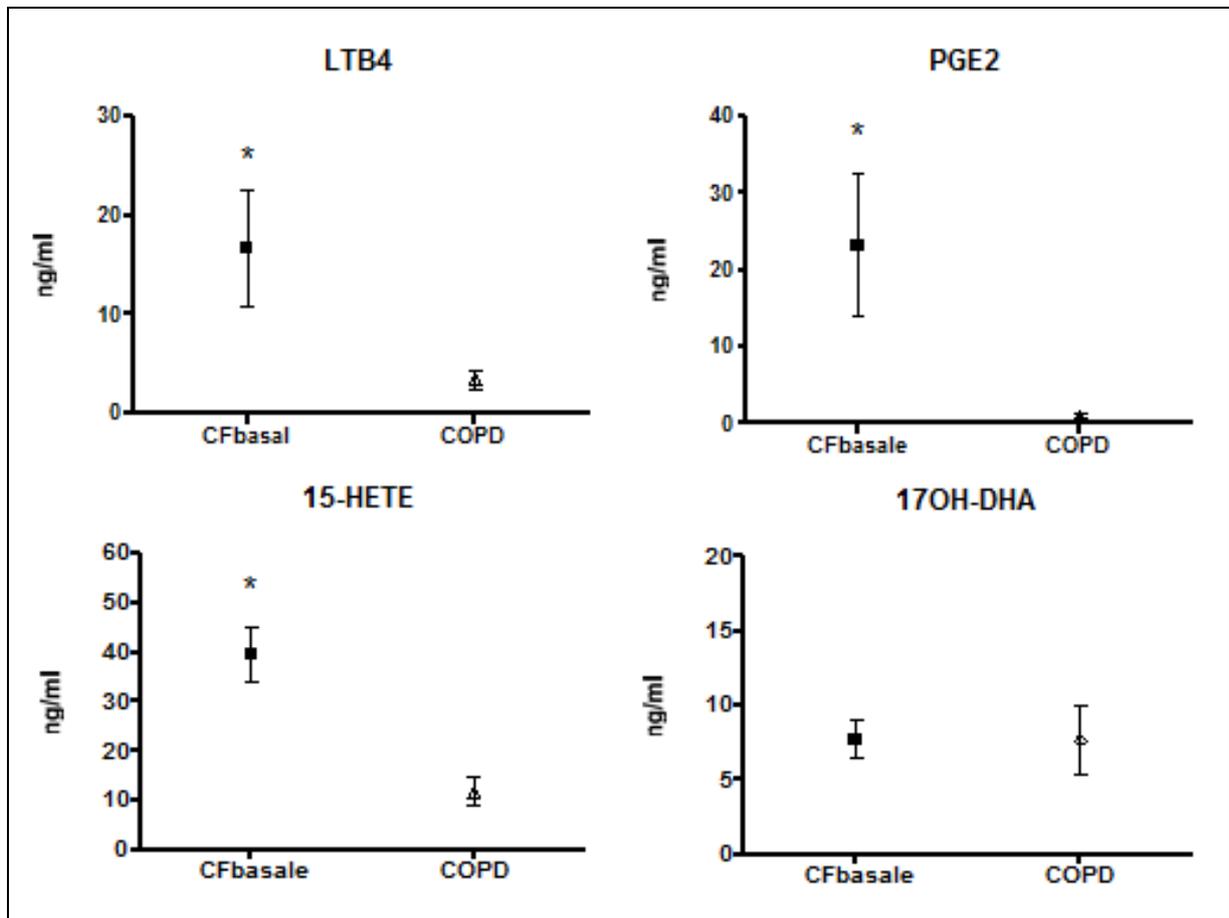


Figure 2: concentrations of LTB4, PGE2, 15-HETE and 17OH-DHA in CF subjects and in COPD subjects

The sputum concentrations of 5- and 15-lipoxygenase metabolites LTB₄ and 15-HETE showed a decreasing trend at the end of the DHA supplementation, a decrease that was statistically significant for 15-HETE (Fig. 3A and 3C). Decreased concentrations, when compared to basal values, were also observed at the end of the washout period, although the difference did not reach statistical significance. A similar trend (although less pronounced) was observed for the cyclooxygenase product PGE₂ (Fig. 3B). On the contrary, and in agreement with the observed enrichment of membrane phospholipids in DHA, 17OH-DHA showed a trend toward an increase in concentrations at the end of the DHA supplementation (Fig. 3D), resulting in a significant correction of the unbalance between AA and DHA-derived lipid mediators observed under basal conditions.

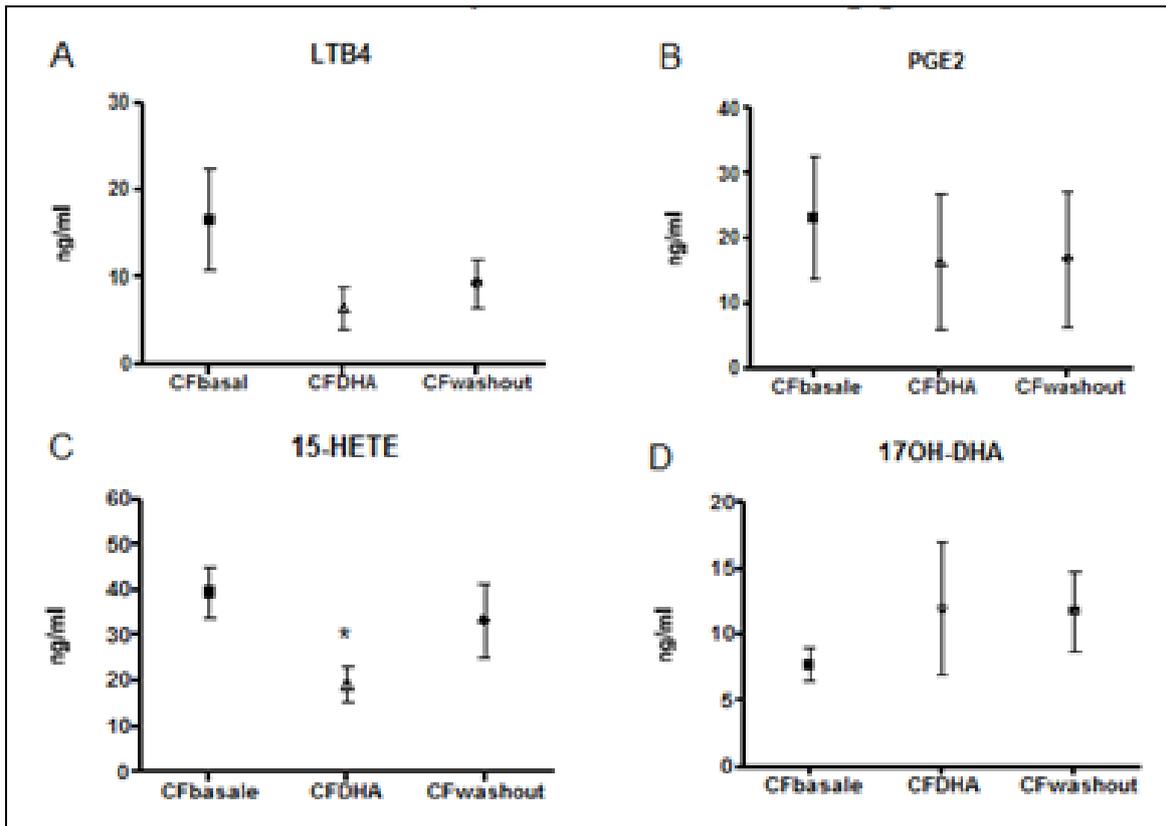


Figure 3: concentrations of LTB4, PGE2, 15-HETE and 17OH-DHA in CF subjects after ten weeks of DHA supplementation

Table 6 shows the mean concentrations (\pm SD) of LTB4, PGE2, 15-HETE, 17OH-DHA and ratio 15-HETE/17OH-DHA in CF subjects after 10 weeks with DHA supplementation and after 10 weeks without DHA supplementation.

Table 6: mean concentrations (\pm SD) of LTB4, PGE2, 15-HETE, 17OH-DHA and ratio 15-HETE/17OH-DHA in CF patients at visit 1, at visit 2 and at visit 3.

	LTB4	PGE2	15-HETE	17-OH-DHA	RATIO 15-HETE/ 17-OH-DHA
V1 (baseline)	16,18 \pm 22,57	19,74 \pm 31,05	35,63 \pm 16,54	7,18 \pm 4,68	6,70 \pm 5,99
V2 (after diet with DHA)	6,58 \pm 7,55	16,31 \pm 32,61	19,03 \pm 12,95	11,98 \pm 15,94	2,70 \pm 1,65
V3 (after diet without DHA)	8,80 \pm 8,91	12,23 \pm 18,06	30,08 \pm 21,03	11,52 \pm 9,76	11,52 \pm 9,76

Analysis of fatty acid composition in the subgroup (8 subjects) that received 10 weeks of DHA supplementation showed a significant increase in the DHA/AA ratio, as well as in the n-3 highly unsaturated fatty acid (HUFA) index, representing the percentage of n-3 fatty acids over the total amount of HUFA present in red blood cells phospholipids (Fig 4).

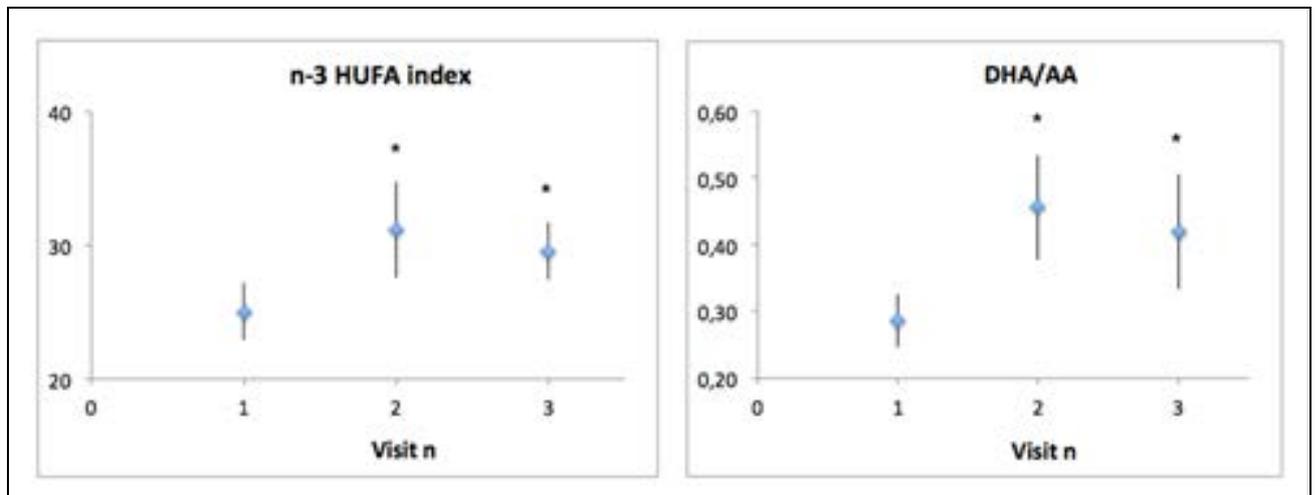


Figure 4: DHA/AA ratio and HUFA index after supplementation

Indeed, DHA supplementation in CF subjects significantly decreased the ratio between the concentrations of 15-HETE and 17OH-DHA to values that were not different from that observed in COPD subjects (Fig. 5).

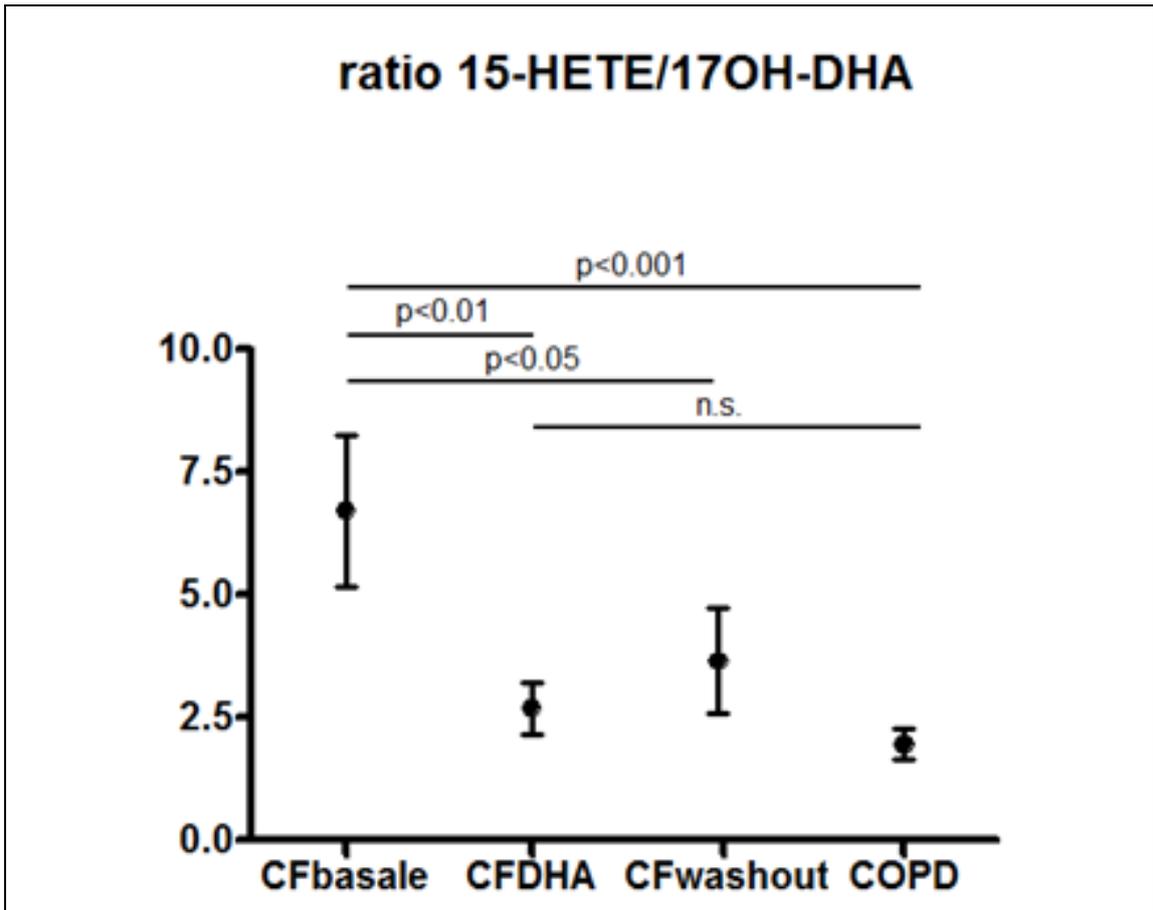


Figure 5: ratio between the concentrations of 15-HETE and 17OH-DHA in CF subjects, before and after DHA supplementation, and COPD subjects

6. Discussion

The essential fatty acids, in particular omega-3, are precursors to important lipid mediators, that are pro-resolving and anti-inflammatory. These pro-resolving lipid mediators (including resolvins and protectins) appear to play a physiological role in ending inflammation [161]. They stimulate the uptake of apoptotic PMNs [162] and activate endogenous antimicrobial defense mechanisms [163] as well as clearance on mucosal surfaces [164].

In fact, the acute inflammatory response is generally a self-limited mechanism, initiated by PNM in response to damage or infection. Excessive inflammatory responses can lead to chronic disorders, owing to PMN-derived pro-inflammatory mediators, including LTB₄ and PGE₂. Within inflammatory exudates, neutrophils can change phenotypes to generate anti-inflammatory mediators, derived from essential fatty acids, to promote a resolution of the inflammatory battle and to return homeostatic health state [165]. These protective mediators include the EPA-derived resolvin E-series, and DHA-derived resolvin-D series and protectins. Resolvins are generated in human whole blood with enzymatic conversion of DHA to 17-hydroxydocosahexaenoic acid (17OH-DHA). Generation of these compounds is significantly increased in the presence of aspirin [166,167]. These discoveries reinforce the evidence that the resolution of acute inflammation is not passive, but an dynamically regulated response. Of interest, the production of protectins and resolvins, when administered in vivo can accelerate this process and the return to tissue homeostasis [168]. At the cellular level, these include reducing neutrophil infiltration and regulating the cytokine-chemokine axis and reactive oxygen species, as well as lowering the magnitude of the inflammatory response [148].

Some authors studied an oxygen-induced retinopathy murine model and they compared the effects of omega-3 vs omega-6 PUFAs, on retinal angiogenesis in wild-type mice and in mice that overexpressing the fat-1 gene, to determine whether EPA, DHA, and AA regulate retinal vaso-obliteration and neovascularization in vivo [169]. Fat-1 gene converts omega-6 PUFA into omega-3, resulting in elevated tissue levels of omega-3 PUFA within the fat-1 overexpressing mice. A protective outcome against uncontrolled angiogenesis, it was found in the retina when there was a inferior ratio of omega-6/omega-3

PUFA. Wild-type mice lacking the fat-1 transgene had more diffuse vaso-obliteration and more severe retinal neovascularization, if compared with fat-1 mice [170]. Of interest, in mice fed omega-3 PUFA, there were markers of biosynthesis of neuroprotectin D1 and resolvina E1 in the retinal tissues. In wild-type mice, a supplementation of resolvina D1, resolvina E1 or neuroprotectine D1 gave protection from vaso-obliteration and neo-angiogenesis. Resolvina E1 is also protective in periodontal disease in rabbits, where it appears to stimulate regeneration [171,172]. A recent study showed that increased intake of omega-3 PUFAs alleviates obesity-induced insulin resistance and advanced hepatic steatosis in obese mice [173]. These beneficial effects were associated with up-regulation of genes involved in insulin sensitivity, glucose transport, and insulin receptor signaling in adipose tissue and liver.

Little is known about the role of resolvins and protectines in lung disease, but as resolvins have been shown to exercise anti-inflammatory effects in models of peritonitis and renal ischaemia–perfusion injury [174,175], it is likely that resolvins would also be lung protective and promote the resolution of airway damage and inflammation. Grau-Carmona et al demonstrated that, in septic patients with acute lung injury or ARDS, an enteric feeding of supplements enriched with omega-3 PUFA improves clinical outcomes, including time to liberation from mechanical ventilation and discharge from the intensive care unit [176]. Moreover, protectin-D1 and its immediate precursor 17OH-DHA, have been identified in EBC from healthy subjects, and significantly lower concentrations were detected in EBC from subjects with asthma exacerbations [177].

The essential fatty acids and their proportion have been documented to have also a remarkable effect on membrane receptor function, transmembrane signaling mechanisms, phospholipase activation, calcium release, ion channels, and gene expression. Disturbances of this complex interactive system can have central importance for cell function. Actually, the molecular mechanism by which CFTR regulates fatty acid metabolism is unknown, but several studies show that the decreased DHA levels may be important in the excessive chronic inflammatory response in CF [145]. Freedman et al. have previously shown that oral supplementation of high doses of DHA to CF–mice corrects the fatty acid abnormality and decreases neutrophil levels in mice with pseudomonas lipopolysaccharide–induced pneumonia [151].

It is important to studying essential fatty acids and determining whether correction of DHA defect represents a treatment for CF. Cutting-edge analytical methods in mass spectrometry were applied to the analysis of AA and DHA-derived lipid mediators in EBC and sputum samples, under the hypothesis that the known deficit in DHA correlate with a decreased concentrations of DHA-derived anti-inflammatory and pro-resolution mediators within the airways of CF subjects. The analysis EBC yielded a very limited number of samples in which all the metabolites of interest could be detected. On the contrary almost all the sputum samples resulted in significant amounts of several lipid mediators, such as LTB₄, PGE₂ and 15-HETE as well as the precursor of protectins and resolvins 17OH-DHA.

In agreement with previous studies [141, 167], we observed a quite significant unbalance between AA-derived pro-inflammatory mediators and the precursor of protectins and resolvins 17OH-DHA in the airways of CF subjects. DHA supplementation caused an overall decrease in the concentrations of pro-inflammatory mediators such as 15-HETE and LTB₄, suggesting that the decreased concentrations of DHA observed in CF subjects could indeed cause a lack of proresolving lipid mediators. Moreover, DHA supplementation strikingly corrected the observed unbalance between AA- and DHA-derived compounds, providing evidence supporting a potential anti-inflammatory activity of n-3 PUFA supplementation in CF subjects.

For the first time, this study had compared these lipid mediators in FC patients to COPD patients, another prevalently neutrophilic chronic inflammatory condition, and had demonstrated that a DHA diet was able to correct fatty acid impaired pattern. We acknowledge that the small sample size of a pilot study can represent an limitation. Moreover, it is possible that a longer DHA supplementation can reveal some important clinical and functional correlation with these mediators.

The results of this pilot study open up a novel therapeutic approach based on the oral treatment with these endogenous anti-inflammatory molecules. In future, it is important to study long-term DHA-supplementation in more numerous CF population, as compared to COPD patients, healthy smokers and patients with asthma. In more wide CF population, it will be possible to correlate molecular data to clinical and functional data.

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