Contribution to the Study of the Role of Arachidonic Acid Metabolism in Airway Inflammatory Diseases

(Nasal polyps, Asthma and Cystic Fibrosis)

Suha Said Mohammad Jabr

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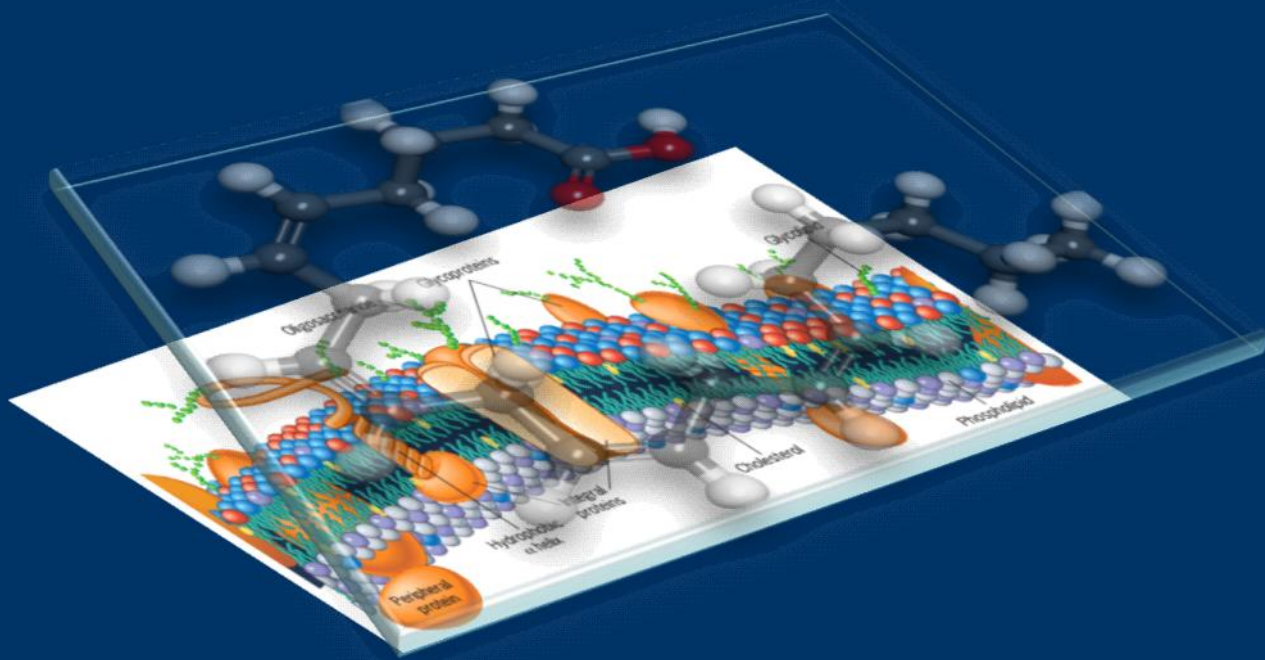

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CONTRIBUTION TO THE STUDY OF THE ROLE OF ARACHIDONIC ACID METABOLISM IN AIRWAY INFLAMMATORY DISEASES (Nasal polyps, Asthma and Cystic Fibrosis)

A thesis presented by

SUHA SAID MOHAMMAD JABR

In partial fulfillment of the requirements for the degree of

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SUPERVISOR

Prof. Dr. CÉSAR PICADO

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Barcelona

ID BAPS

Universitat de Barcelona
Facultat de Medicina
Departament de Medicina
Casanova 143, 08036 Barcelona

Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)
Rosselló 149-153 ·
08036 Barcelona

The undersigning, certify that the present work has been carried out by SUHA SAID JABR under their supervision as part of the requirements for the award of his PhD degree at the Department of Medicine, Faculty of Medicine at the University of Barcelona (UB). That work has been carried out at the Clinical and Experimental Respiratory Immunoallergy Institute (IDIBAPS), Hospital Clinic.

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SUPERVISOR

Prof. Dr. CÉSAR PICADO

Abstract

CONTRIBUTION TO THE STUDY OF THE ROLE OF ARACHIDONIC ACID
METADOLISM IN AIRWAY INFLAMMATORY DISEASES
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Suha Jabr

Eicosanoids are derived from the fatty acids that make up the cell membrane and nuclear membrane. They begin as a single poly unsaturated fatty acid, the arachidonic acid. AA is produced from membrane phospholipids and then it can be enzymatically metabolized through the cycloooxygenase pathway into different eicosanoids including prostaglandins. They have various roles in inflammation, and many diseases including asthma, and cystic fibrosis.

Fibroblasts from nasal polyps of asthma patients have reduced expression of cyclooxygenase-2 and production of prostaglandin (PG) E_2. It is hypothesized that the reported alterations are due to alterations in the availability of AA. So we aimed to determine the fatty acid composition of airway fibroblasts from healthy subjects and from asthma patients with and without aspirin intolerance.

In patients with cystic fibrosis there is a relationship between prostanoid production and cystic fibrosis (CF) genotype severity, and also with the severity of the phenotype expression determined by the presence or absence of pancreatic insufficiency. We aimed to assess the relationship in patients with cystic fibrosis between prostanoid production and lung function values, pancreatic function as a measure of CF severity, and genotype severity. And to assess the relationship between PGE-M and PGD-M urinary metabolites of PGE_2 and PGD_2 and CF severity.

Since eicosanoids and their precursor AA have a crucial role in physiology and pathology, it is very important to identify and quantify the amount that is produced by the cells and tissues in order to identify better the targets for pharmaceutical intervention. They need a special method for isolating them and a specific and sensitive instrument for identifying, and quantifying them. Gas chromatography was used for the analysis of fatty acids in human nasal fibroblasts culture, and the high performance liquid chromatography tandem mass spectrometry was used for the identification and quantification of prostaglandins metabolites (Tetranor-PGEM and Tetranor-PGDM) in human urine.

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List of abbreviations

AA	Arachidonic acid
AERD	Aspirin exacerbated respiratory disease
AIA	Aspirin induced asthma
ANOVA	Analysis of variance
ATA	Aspirin tolerant asthma
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
cAMP	Cyclic adenosine monophosphate
CBAVD	Congenital bilateral absence of the vas deferens
CF	Cystic fibrosis
CFRDM	Cystic fibrosis-related diabetes mellitus
CFTR	Cystic fibrosis transmembrane conductance regulator
CID	Collision-induced dissociation
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
Cr	Creatinine
CRS	Chronic rhinosinusitis
CRSwNP	Chronic rhinosinusitis with nasal polyp
$CRTH_2$	Chemoattractant Receptor expressed on Th_2 cells
CV	Coefficient of variation
CYP	Cytochrome
CysLT	Cysteinyl leukotriene
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's modified eagle medium
DP	Prostaglandin D2 receptor
DPA	Docosapentaenoic acid
ECD	Electron capture detector
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienic acid
EFA	Essential fatty acid
ELISA	Enzyme-linked immunosorbent assay
EP	Prostaglandin E2 receptor
EPA	Eicosapentanoic acid
ESI	Electrospray ionisation
FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
FLAP	5-lipoxygenase activating protein
FEV1	Forced expiratory volume in one second
FID	Flame ionization detector
FP	Prostaglandin F receptor

FTICR	Fourier transform ion cyclotron resonance
FVC	Forced vital capacity
GC	Gas chromatography
GLA	Gamma-linolenic acid
GLC	Gas-liquid chromatography
GPCR	G protein-coupled receptor
GSH	Reduced Gluthatione
HETE	Hydroeicosapentanoic acid
HPETE	Hydroxyeicosapentanoic acid
HIV	Human immune deficiency virus
Ig	Immunoglobulin
IL	Interleukin
IP	Prostacyclin receptor
LA	Linoleic acid
L-ASA	Lysine acetylsalicylate
LC	Liquid chromatography
LOD	Limit of detection
LOX	Lipoxygenase
$LPLA_2$	Lysosomal Phospholipase A_2
LT	Leukotriene
LX	Lipoxin
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUFA	Monounsaturated fatty acid
m/z	Mass to charge ratio
NBF	Nucleotide binding fold
NICI	Negative-ion chemical ionization
NM	Nasal mucosa
nm	Nanometres
NP	Nasal polyp
NSAIDs	Nonsteroidal anti-inflammatory drugs
PA	Pseudomona aeruginosa
PAF	Platelet activating factor
PAF-AH	Platelet-activating factor acetylhydrolase
PBS	Phosphate buffered saline
PG	Prostaglandin
PGD2	ProstaglandinD2
PGE2	ProstaglandinE2
PGEM	Tetranor-prostaglandin E metabolite
PGDM	Tetranor-prostaglandin D metabolite
PLA_2	Phospholipase A_2
PC	Phosphatidylcholine
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
QoL	Quality of life
RBC	Red blood cells

RT-PCR	Reverse transcription polymerase chain reaction
SCOT	Support-coated open tubular
SD	Standard deviation
SEM	Standard error of the mean
SFA	Saturated fatty acid
SRM	Selected reaction monitoring
TGF-	Transforming growth factor beta
TNF	Tumor necrosis factor
TOF	Time-of-flight
TP	Thromboxane receptor
TX	Thromboxane
UFA	Unsaturated fatty acid
WCOT	Wall-coated open tubular

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I. INTRODUCTION

CHAPTER 1

Respiratory System

1.1 The Airways

The main purpose of the respiratory system is to supply the body with oxygen and remove carbon dioxide to promote gas exchange between our body and the external environment. The airways are a continuous and large structure that extends from nasal vestibule to alveoli. There is an imaginary line that divides airways into two subdivisions: upper and lower airways.

The upper airways, also known as the upper respiratory system, include the nose, the nasal cavity, the paranasal sinuses, the nasopharynx and the oropharynx. On the other hand, the lower respiratory system (lower airways) consists in the larynx, trachea, bronchi, and lung.

1.2 The Nose

The nose, included in the upper respiratory tract, participates in several functions related to respiration, providing the necessary air resistance for the proper function of the lung and preparing inhaled air by filtering, warming, and moistening it before reaching the lungs.

The nose is a double organ composed of two nasal cavities divided by a septum. In these cavities there are three prominent structures called upper, middle, and lower turbinates. Both nasal septum and turbinates are covered by a respiratory mucosa. This organization is essential for the functions in which the nose is involved: respiration, inhaled air humidification, inhaled air clearance by mucociliary transport, immunological response, and voice resonance and modulation.

The vascularisation of the nose comes from the external and internal carotid arteries while innervations can be sensitive, parasympathetic, sympathetic, and non-adrenergic non-cholinergic M (Mygind N an Bisgaard, 1990).

1.2.1 Nasal Mucosa

Histology. Nasal mucosa is composed of a respiratory epithelium, a basement membrane, and a submucosa (**Figure 1.1**).

- ***Epithelium****.* This is a pseudostratified columnar ciliated epithelium containing goblet, basal, ciliated, and non-ciliated cells. In addition, immune cells, inflammatory cells, and phagocytic cells migrate to, remain within, or transit through it to the lumen.

- ***Basement membrane****.* The basement membrane consists predominantly of types III, IV, and V collagen, type V laminin, and fibronectin, produced by epithelial cells and subepithelial fibroblasts.

- ***Submucosa****.* Under the epithelium and separated by the basement membrane, there is the submucosa containing: a) an external area rich in blood fenestrated capillaries, b) submucosal glands, composed of both serous and mucous cells and glandular ducts which drain secretions to the nasal lumen, and c) venous sinusoids that form the erectile tissue. In the connective tissue around submucosal glands, there is a blood vessels net responsible for the nasal congestion and decongestion. Finally, there exists a bone structure in which nasal mucosa is attached (Mygind N and Bisgaard, 1990).

Physiology. In physiological situations, the respiratory epithelium is covered by a mucus layer, containing an upper *gel* layer that traps inhaled particles, and a lower *sol* layer in which epithelial cell cilia are embedded. The main role of this mucus is to cover and protect the respiratory tract by trapping pathogens and irritant substances and to facilitate their removal by mucociliary clearance. This function is carried out by the action of epithelial-cell cilia that are embedded in the mucus gel phase and sweep it along with a coordinated "beat". Chronic increases in volume and viscosity of the mucus layer impair clearance and contribute to the pathophysiology of hypersecretory conditions of the airways, for
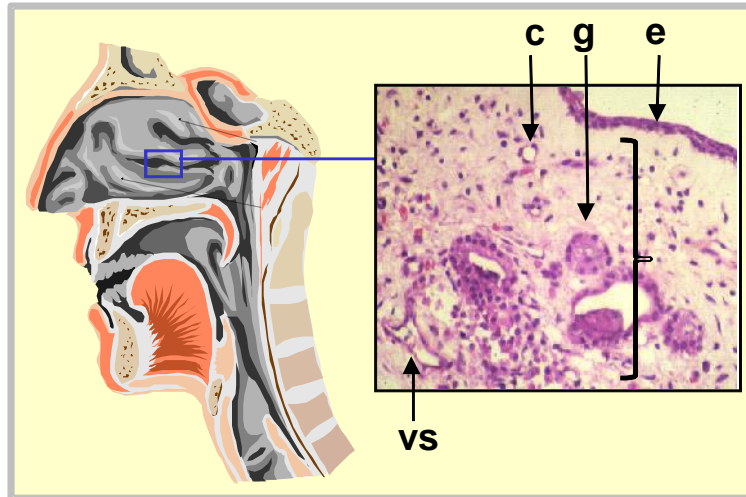
Figure 1.1. Nasal Mucosa Histology. The epithelium (e), the submucosa (sm), submucosal glands (g), venous sinusoids (vs), and capillars (c), are shown in this tissue section. Hematoxilin-eosine staining. Magnification 100X.

instance asthma and chronic rhinosinusitis (Mygind N and Bisgaard, 1990).

In addition, the nasal mucosa through its epithelium plays other functions such as physical barrier, transport, secretion, and inflammatory modulation (Mygind N and Bisgaard, 1990).

Table 1.1. Functions of sinonasal epithelium.

Functions	Mechanisms	Final result
1. Physical protection	- Via intercellular adhesion complexes (tight junctions, desmosomes) · Via mucin secretion	- Selective absorption - Humidification and warming of inhaled air - Entrapping of noxious agents
2. Transport	- Via cilia beats	- Transport of mucus from the lung to the throat
3. Secretion	- Via its cellular types	- Mucins, cytokines, adhesion molecules, growth factors
4. Target of pro-inflammatory and anti-inflammatory agents	- Via specific receptors	- Response to cytokines, glucocorticoids, chromones, antihistamines

CHAPTER 2

Airway Inflammatory Diseases

2.1 Chronic Rhinosinusitis with Nasal Polyposis

2.1.1 Definition and Classification

Chronic rhinosinusitis (CRS), including nasal polyps, is defined as an inflammation of the nose and paranasal sinuses characterized by two or more symptoms, one of which should be either nasal blockage/obstruction/ congestion or nasal discharge, and with/out facial pain/pressure, with/out reduction or loss of smell (Fokkens, 2012).

Chronic rhinosinusitis with or without nasal polyps is often considered one disease entity, because it seems impossible to clearly differentiate both entities. Chronic rhinosinusitis with nasal polyps is considered a subgroup of chronic rhinosinusitis (Fokkens, 2012).

Figure 2.1. endoscopic image of a nasal polyp.

Nasal polyps are edematous masses originated from the middle meatus that cause long-term symptoms, in particular nasal obstruction, sense of smell reduction (hyposmia) or even anosmia, rhinorrhea, and facial pain. The typical history of patients suffering from nasal polyposis is similar to perennial rhinitis (Fokkens, 2012) (**Figure 2.1**).

Nasal polyposis. May be divided into different subgroups based on clinical aspects, etiology, histopathology and mediators' content. The more general classification is the following :

1. Unilateral nasal polyps:

     a.   Antrochoanal polyps, a commonly large isolated unilateral cyst-like non-eosinophilic formation.

   2.   Bilateral nasal polyps:

     a.   Idiopathic unilateral or bilateral, mostly eosinophilic polyps without involvement of the lower airways.

     b.   Bilateral eosinophilic polyps with concomitant asthma and/ or aspirin sensitivity.

     c.   Polyposis associated to other diseases such as cystic fibrosis, Churg-Strauss syndrome, Kartagener syndrome, etc.

2.1.2 Histopathology of Nasal Polyps

Histomorphologically, polyp tissue reveals frequent epithelial damage, a thickened basement membrane, edematous stromal tissue, a reduced number of glands and vessels, and no visible neural structures. It is therefore assumed that denervation of nasal polyps causes a decrease in secretory activity of the glands and induces an abnormal vascular permeability, leading to an irreversible tissue oedema (Fokkens, 2012).

Nasal polyps contain a great amount of inflammatory cells, specially eosinophils, lymphocytes, and mast cells. These cells, together with structural cells (fibroblasts and epithelial cells), release molecules such as histamine, cytokines, chemokines, transcription factors, and eicosanoids that act as inflammatory mediators playing a crucial role in the persistent eosinophilic inflammation observed in nasal polyps. Additionally, these inflammatory mediators are involved in the stimulation of mucus hypersecretion, specifically increasing mucin expression and secretion. The final presentation of this mucus hypersecretion is rhinorrhea, a common symptom in patients suffering from nasal polyposis (Fokkens, 2012).

Nasal polyps have been divided in 4 subclasses attending to their different histology, and the wrong-called "allergic" polyp is the one that characterize the nasosinusal polyposis. This is oedematous, eosinophillic, and the most common type. The presence of edema in the stroma, goblet cell hyperplasia, increased eosinophil and plasma cell content in the stroma, and a thick basement membrane are common features of this polyp subtype. In addition, they are bilateral and represent around 80% of the nasosinusal polyps (Fokkens, 2012).

2.1.3 Inflammatory Cells in Nasal Polyps

Nasal polyp contain a number of inflammatory cells include mast cells, eosinophils, T cells Lymphocytes and B cells Lymphocytes **Figure 2.2**.

Mast cells. Nasal polyps contain a great number of mast cells. After mast cell activation, degranulation occurs in the form of histamine, serotonin, platelet activating factor (PAF), leukotrienes, and prostaglandins. Mast cells also produce cytokines (IL-4, IL-5, and IL-6) that activate adhesion molecules, induce eosinophilic infiltration, and perpetuate inflammation (Fokkens WJ, 2012).

Eosinophils. The infiltration of the nasal polyp mucosa by eosinophils is a hallmark of nasal polyposis . The eosinophillic infiltrate is due to several causes: a) increased production of eosinophils in the bone marrow induced by growth factors; b) eosinophil chemotaxis induced by cytokines, adhesion molecules, and chemoattractants; c) *in situ* eosinophil activation; and d) increased eosinophil survival. Eosinophil migration occurs from the submucosa to the basement membrane and to the epithelium. Once in the tissue, the activation and survival of eosinophils will be increased by cytokines and other mediators.

T cells Lymphocytes. These cells, found activated in nasal polyps, promote eosinophilic inflammatio together with other inflammatory cells. They represent a mixed population, consisting in CD4+ and CD8+ cells, and show a mixed

Th1/Th2 profile. In nasal polyps, T lymphocytes prevail over B lymphocytes, while T suppressors (CD8+) prevail over the T helper cells (CD4+).

B cells Lymphocytes. These cells are responsible for the production of the immunoglobulin E (IgE) involved in the early allergic reaction through activation and degranulation of mast cells.
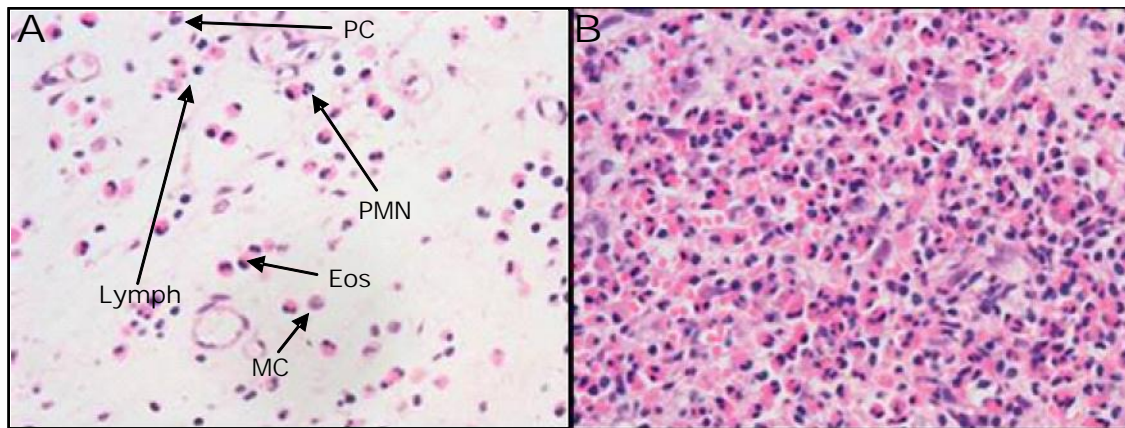
Figure 2.2. Inflammatory cell infiltrate in nasal polyps. A) Poorly infiltrated nasal polyp. Eos: Eosinophils; Lymph: Lymphocytes; PMN: Polymorphonudears; MC: Mast cells; PC: Plasma cells. B) Massive infiltration mainly by eosinophils in nasal polyp tissue. Hematoxiline-eosine staining. 400X magnification.

2.1.4 Inflammatory Mediators in Nasal Polyps

Histamine. This inflammatory mediator, released after mast cell activation, and degranulation, has strong effects on smooth muscle constriction, and increases vascular permeability and edema (Mullol J, Picado C, 2013).

Cytokines. They are responsible for the induction of intercellular signaling, by activation of membrane specific receptors that leads to cellular proliferation, cellular differentiation, cellular chemotaxis, growing, and Ig secretion modulation (Mullol J, Picado C, 2013).

Chemokines. They promote the chemotaxis of inflammatory cells such as lymphocytes, monocytes, and eosinophils . RANTES, and eotaxin-1, -2, -3, and -4 are the main chemokines found in nasal polyps (Mullol J, Picado C, 2013).

Eicosanoids. They are products of arachidonic acid metabolism, which include 2 large mediator families, leukotrienes and prostanoids. Due to hyperproduction or failure in the degradation processes, eicosanoids can accumulate and become involved in the pathogenesis of nasal polyposis. The enzyme cyclooxigenase (COX) metabolizes arachidonic acid into prostaglandin H_2, source of other prostaglandins, prostacyclins, and tromboxans while lipoxigenases metabolize AA into leukotrienes, lipoxins, and hydroxyeicosatetraeinoic acids (HETEs) (Mullol J, Picado C, 2013).

2.1.5 Clinical Aspects and Diagnosis

Symptoms in acute and chronic rhinosinusitis as well as in CRS with nasal polyps are similar, although the symptom pattern and their intensity may vary. The general symptoms are: nasal blockage, congestion or stuffiness, nasal discharge or postnasal drip, facial pain or pressure, and reduction/loss of sense of smell. Nasal polyps may cause nasal congestion, which can be a feeling of pressure and fullness in the nose and paranasal cavities. This is typical for ethmoidal polyposis, which in severe cases can cause widening of the nasal and paranasal cavities demonstrated radiologically. Disorders of smell are more prevalent in patients with nasal polyps than in other chronic rhinosinusitis patients (Fokkens, 2012).

Clinically, nasal polyp diagnosis is based on clinical symptoms and on endoscopy and CT-scan of the paranasal sinuses showing the presence of endoscopically visible bilateral polyps growing from the middle meatus into nasal cavities, and affecting etmoidal and maxillary sinuses. During the last decade more attention has been paid not only to symptoms but also to their effect on patient's quality of life (QoL) (Alobid I, 2005).

2.1.6 Epidemiology and Co-morbidities

The exact prevalence on nasal polyposis in the general population is not known, because there are few epidemiological studies and their results depend on the study population selected and the diagnostic methods used. Data published by the American General Health Survey show that patients seeking for medical advice owing to chronic rhinosinusitis-related symptoms represent the 14.7% of the American population, although nasal polyposis affect 2 to 5% of the general population. Other relevant publications mention nasal polyposis prevalence of 4.3% in the general population. The incidence is higher in men than in women and significantly increases after the age of 40 years. Nasal polyps occur more frequently in subgroups of patients such as asthmatics, aspirin sensitive and cystic fibrosis patients (Fokkens, 2012).

2.2 Asthma

2.2.1 Definition and Epidemiology

Asthma is an inflammatory disorder of the conducting airways which undergo distinct structural and functional changes, leading to non-specific bronchial hyperresponsiveness (BHR) and airflow obstruction that fluctuates over time. Asthma is one of the most common chronic conditions in Western countries, affecting 1 in 7 children and 1 in 12 adults, and is responsible each year for 1500 avoidable deaths, as well as 20 million lost working days. A recent assessment of asthma across Europe (Brussels declaration) has identified substantial unmet clinical needs which, in the 10% of patients with severe disease, account for approximately 50% of health costs. Thus, in spite of recent advances in the detection and treatment of the condition, asthma remains the cause of significant morbidity and economic burden (Eder, 2007; Fanta, 2009).

2.2.2 Etiopathogenesis

The key feature of bronchial asthma is the development of airway inflammation and BHR in the form of a heightened bronchoconstrictor response, not only to the allergen to which an individual is sensitized, but also to a range of non-specific stimuli, such as environmental pollution and cold air (Fanta, 2009).

Asthma behaves as a spectrum of disorders initiated at different stages throughout life by a range of environmental factors interacting with a susceptible genetic background. At its simplest, asthma is divided into allergic (extrinsic) and nonallergic (intrinsic) subtypes, but even within each of these two broad categories, there exists considerable heterogeneity with respect to underlying mechanisms, clinical and physiological manifestations, response to treatment, and natural history. The majority of asthma cases are associated with T_H2-type T-lymphocyte-driven cell recruitment and mediator release involving mast cells, eosinophils, basophils, and macrophages that contribute to the chronic, subacute, and acute inflammatory responses (Fanta, 2009).

Most, but not all, asthma is associated with atopy. However, asthma prevention has not been achieved with allergen-reduction strategies; once established there is no cure and there are currently no medications that can alter the natural history of the disease. Emerging evidence highlights the complexity of asthma, in particular the iterative nature of the underlying interactions between innate and adaptive immune mechanisms in which virtually every signal emanating from one cellular compartment provokes an answering response from the other. To further complicate this picture, the local mesenchyme can also interpose signals to fine-tune immune responses to optimally meet local microenvironment needs. Perturbation of the balance between these interlinked innate and adaptive immune pathways is increasingly believed to be the basis for disease expression and in the specific case of atopic asthma, the prototypic example of this being acute exacerbations triggered by viral infections (Fanta, 2009).

2.2.3 Treatment

Management is primarily directed towards suppressing airway inflammation with inhaled corticosteroids and relieving bronchoconstriction with bronchodilators. Apart from corticosteroids, the only oral medications in widespread use are cysteinyl leukotriene (LT) receptor 1 antagonists that inhibit the bronchoconstrictor and inflammatory actions of LTC_4, LTD_4 and LTE_4. All of these therapies exert their effect downstream of the origin of asthma. So, there is an urgent need to identify the underlying basis of asthma, understand the complex genetic and environmental influences, and develop appropriate treatment strategies (Picado, 2008; Fanta, 2009).

Asthma may start at any time in life; however the majority of cases begin in early childhood (Eder, 2007), and, although it may spontaneously remit, longitudinal studies reveal that later relapses frequently occur.

Severe irreversible airflow obstruction may develop despite apparently appropriate use of controller therapy, as advocated by international and national management guidelines (Picado 2008; Fanta, 2009). That is not to say that widespread adherence to anti-inflammatory controller therapy does not influence long-term outcomes of asthma; indeed, when treatment adherence is high, asthma mortality and morbidity can be dramatically reduced.

The problem is that, in most countries of the world, treatment adherence is low. Many patients only take their anti-inflammatory treatment when they are symptomatic and stop when their symptoms subside. This approach works well in mild asthma, but not in those with more severe disease where regular use of inhaled corticosteroids, often in large doses and in combination with long-acting $_2$-bronchodilators, is required for disease control (Picado, 2008; Fanta, 2009).

2.3 Aspirin and other Non-steroidal Antiinflamatory Drugs Hypersensitivity

Aspirin-induced asthma is a distinct clinical syndrome characterized by the triad apirin sensitivity, asthma and nasal polyposis (Picado, 2006), and starts with a prolonged episode of nasal congestion, rhinorrea, and hyposmia with persistent mucosal inflammation. Physical examination often reveals nasal polyps. Bronchial asthma and aspirin intolerance develop subsequently. The intolerance appears after ingestion of aspirin when an acute asthma attack occurs, often accompanied by rhinorrhea and conjunctival irritation (Picado, 2006).

Aspirin induced-asthma affects about 10% of asthmatic patients, this percentage increasing to 20% in severe asthmatic patients (Bochenek, 2013). In addition, in patients with chronic rhinosinusitis undergoing endoscopy surgery reveals that 11-20% of them have aspirin sensitivity, being this fact an indirect marker of the severity of polyposis in this group of patients (Picado 2006; Bochenek, 2013). The prevalence of nasal polyps in aspirin sensitive asthmatics may be over 60-70%, as compared to less than 10% in the population of aspirin-tolerant asthmatics (Mullol, 2013).

Diagnosis of NSAIDs hypersensitiviy is usually stablished taking into account the clinical history of two or more asthma attacks precipiated by different NSAIDs (Bochenek, 2013). When the diagnosis is unclear confirmatory chelenge tests with Lysine-aspirin are usually performed. Various challenges tests can be used such as nasal, brochial or oral tests. The nasal test is very safe and it is the usually recommended test to confirm or exclude NSAID intolerance (Casadevall, 2000).

2.4 Cystic Fibrosis

2.4.1 Definition and Epidemiology

Cystic fibrosis (CF) is the most common severe genetic disease, with an incidence rate varying from 1 per 2000 to 1 per 6500 living newborn babies. Defective expression of the CF transmembrane conductance regulator (CFTR) in CF epithelial cells is associated with mucus hypersecretion, inflammation, and infection that begin in early life and lead to a marked cyclical airway obstruction and infection responsible for the morbidity and mortality in patients with CF (O'Sullivan, 2009).

In patients suffering from CF, nasal polyposis is associated with a higher prevalence than in the general population, this prevalence ranging from 6 to 48%. In addition, 92 to 100% of CF patients present radiologic signs of sinonasal disease (Feuillet-Fleux, 2011). Fifty percent of the children between 4 and 16 years of age suffering nasal polyposis present CF (Feuillet-Fleux, 2011).

Most people with cystic fibrosis also have digestive problems. Some affected babies have meconium ileus, a blockage of the intestine that occurs shortly after birth. Other digestive problems result from a build-up of thick, sticky mucus in the pancreas. The pancreas is an organ that produces insulin (a hormone that helps control blood sugar levels). It also makes enzymes that help digest food. In people with cystic fibrosis, mucus blocks the ducts of the pancreas, reducing the production of insulin and preventing digestive enzymes from reaching the intestines to aid digestion. Problems with digestion can lead to diarrhoea, malnutrition, poor growth, and weight loss. In adolescence or adulthood, a shortage of insulin can cause a form of diabetes known as cystic fibrosis-related diabetes mellitus (CFRDM) (O'Sullivan, 2009).

Cystic fibrosis used to be considered a fatal disease of childhood. With improved treatments and better ways to manage the disease, many people with cystic

fibrosis now live well into adulthood. Adults with cystic fibrosis experience health problems affecting the respiratory, digestive, and reproductive systems. Most men with cystic fibrosis have congenital bilateral absence of the vas deferens (CBAVD), a condition in which the tubes that carry sperm (the vas deferens) are blocked by mucus and do not develop properly. Men with CBAVD are unable to father children (infertile) unless they undergo fertility treatment. Women with cystic fibrosis may experience complications in pregnancy (O'Sullivan, 2009; Zielenski, 2000).

2.4.2 Etiopathogenesis

Mutations in the CFTR gene disrupt the function of the chloride channels, preventing them from regulating the flow of chloride ions and water across cell membranes. The CFTR gene provides instructions for making the CFTR regulator protein. This protein functions as a channel across the membrane of cells that produce mucus, sweat, saliva, tears, and digestive enzymes. The channel transports negatively charged particles called chloride ions into and out of cells. The transport of chloride ions helps control the movement of water in tissues, which is necessary for the production of thin, freely flowing mucus. The CFTR protein also regulates the function of other channels, such as those that transport positively charged particles called sodium ions across cell membranes. These channels are necessary for the normal function of organs such as the lungs and pancreas (Zielenski, 2000).

Cystic fibrosis is inherited in an autosomal recessive pattern, which means both copies of the gene in each cell have mutations. The parents of an individual with an autosomal recessive condition each carry one copy of the mutated gene, but they typically do not show signs and symptoms of the condition (Zielenski, 2000).

More than 1,000 mutations in the CFTR gene have been identified in people with cystic fibrosis. Most of these mutations change single protein building blocks (amino acids) in the CFTR protein or delete a small amount of DNA from the

CFTR gene. The most common mutation, called delta F508, is a deletion of one amino acid at position 508 in the CFTR protein. The resulting abnormal channel breaks down shortly after it is made, so it never reaches the cell membrane to transport chloride ions. The occurrence of delta F508 mutations varies considerably between different populations and geographical locations with lowest reported incidence reported in Tunisia (17.9%) and the highest in Denmark (90%). In Spain is found in around 50% of diagnosed patients Zielenski, 2000; Alonso, 2006). 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 (**Figure 2.3**).

Class I: Defective protein synthesis (Net effect: no CFTR protein at the apical membrane). Mutations that belong to this category are associated with lack of biosynthesis or defective biosynthesis producing abnormal protein variants (truncation, deletion, etc). No functional CFTR is present at the apical membrane of the epithelial cells and as a consequence phenotypic effects (clinical expression) of class I mutations tend to be severe. Examples include nonsense (G542X), frameshift (3659delC) or severe splicing (1717–1G   A) mutations.

Class II: Abnormal processing and trafficking (Net effect: No CFTR protein at the apical membrane). These gene variants fail to properly process the protein to a mature form and also fail to transport the protein to the apical membrane. Because of their absence in the membrane, these mutant CFTR variants are associated with severe CF phenotypes. The most common mutation responsible for CF is a deletion of a single amino acid, F508del. Another one is the missense mutation N1303K, which substitutes an asparagine residue for a lysine at position 1303.

Class III: Defective regulation (Net effect: Normal amount of non-functional CFTR at the apical membrane). Mutations of this class affect the regulation of CFTR function by preventing ATP binding at the nucleotide binding domains
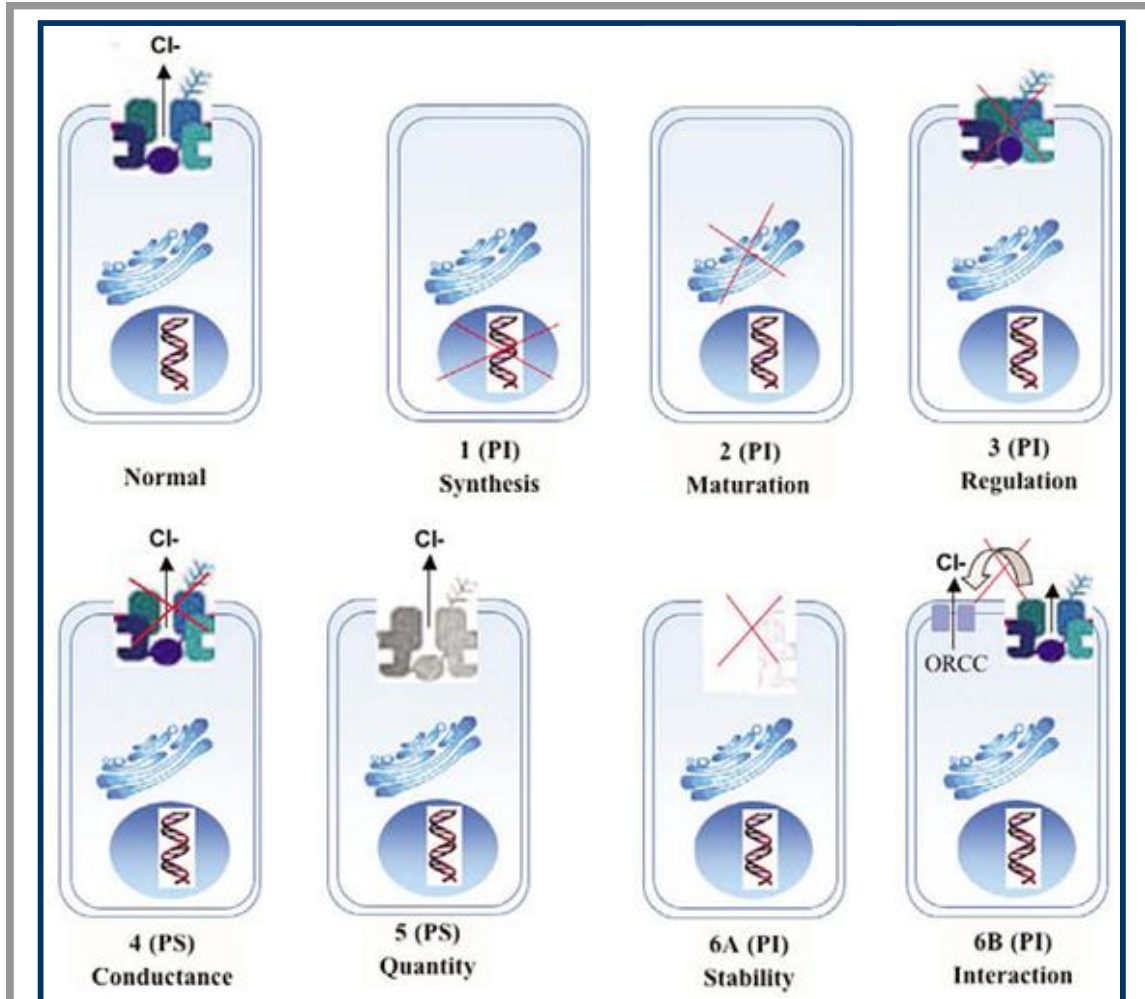
Figure 2.3. Molecular consequences of CFTR mutations (Adapted from Welsh and Smith, 1993 and modified by Claustres, 2005). **Class1**: mutations interfere with CFTR production. **Class 2**: mutations affect protein maturation. **Class 3**: mutations affect channel regulation. **Class 4**: mutations affect chloride conductance. **Class 5**: mutations reduce the levels of a normally functioning CFTR. **Class 6**: mutations decrease stability of CFTR present or affect the regulation of other channels.

required for channel activation. These mutant CFTR variants are usually associated to severe/moderate CF phenotypes, such as missense mutations G551D.

Class IV: Decreased conductance (Net effect: Normal amount of CFTR with some residual function at the apical membrane). These mutations are implicated in forming the pore of the channel. The CFRT variants of these mutations retain a

residual function and are usually associated to mild CF phenotypes, such as missense mutations R117H or R347P.

Class V: Reduced synthesis/trafficking (Net effect: Reduced amount of functional CFTR at the apical membrane). Various mutations may be associated with reduced biosynthesis of fully active CFTR such as (3849+10kbC T, 2789+5G A or inefficient trafficking (A455E). These mutations result in reduced expression of functional CFTR channels in the apical membrane and are usually associated to mild CF phenotypes.

Class VI: Mutations decrease stability of CFTR present or affect the regulation of other channels. Recently, two new classes of mutants have been added. In the class that mutations decrease stability of CFTR; mutants lack the last residues of CFTR (Q1412X, S1455X, 4279insA, 4326delTC), which dramatically reduces the apical stability of the protein (Haardt, 1999). In the class that affect the regulation of other channels; mutants are unable to interact properly with other ion channels, such as missense mutation G551D and the ORCC (outwardly rectifying chloride channel) (Fulmer, 1995). Although they have been investigated in a few patients, most class 6 mutants should be considered as severe (Zielenski, 2000; Claustres, 2005).

The availability of the CFTR gene sequence and the development of increasingly efficient mutation detection methods allow for detailed genotype characterization among various CF patient cohorts grouped according to selected genetic or clinical features. These studies have demonstrated that the degree of correlation between CFTR genotype severity and CF phenotype varies between its clinical components and is highest for the pancreatic status and lowest for pulmonary disease.

There appears to be a graduated risk of developing pancreatic insufficiency and pancreatitis, according to genotype severity, in patients with CF who carry mild/mild, mild/moderate-severe or moderate–severe/moderate-severe alleles

(Zielenski, 2000;). An association between the severity of CFRT mutation and lung disease has been reported in some studies, resulting in a lower baseline forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) and a faster decline in lung function (Zielenski, 2000). In contrast, other surveys failed to find any relationship between the risk of poor lung function and CFTR genotype severity (Zielenski, 2000;). **Table 2.1** shows the main CFTR mutations as related to the risk of developing pancreatic insufficiency, which is considered the clinical parameter (phenotype) that best correlates with genotype severity.

Table 2.1. Main CFTR mutation class and severity as related to pancreatic status.

Severe Mutations		Mild Mutations	
W128X	5T-12TG	L206W	3272-26A<G
I507del	N1303K	3272-26A>G	D110H
G542X	124del23kbp	F587I	D565G
F508del	M1137V	R117H	G576A
N1303K	W1282X	3449+10kbc>T	D1152H
1677delTA	R553X	A455E	V232D
G551D	711+1G>L206W	G178R	D1270N
2188AA>G	394delTT	R352Q	711+3A>G
Q890X	R560T	R117C	
E1308X			

CHAPTER 3

Fatty Acids and Arachidonic Acid

3.1 Fatty Acids and Arachidonic Acid Metabolism

3.1.1 Cell Membrane Fatty Acids

Cell Membrane. Cell membrane is a membrane that surrounds the cell, defines its boundaries and protects the integrity of the interior of the cell maintaining certain substances inside the cell while let others outside the cell (Alberts, 2008; Berg, 2012). Cell membranes have additional functions such as providing a selectively permeable barrier, concentrate membrane-associated molecules, transporting solutes, enabling ion and creation of concentration gradients across membranes, responding to external signals, and energy transduction. (Gilbert, 2000; Karp, 2010).

Cell Membrane Composition. The cell membrane structure consists of a very thin film of lipid and protein molecules held together mainly by noncovalent interactions (**Figure 3.1**), (Alberts, 2008).
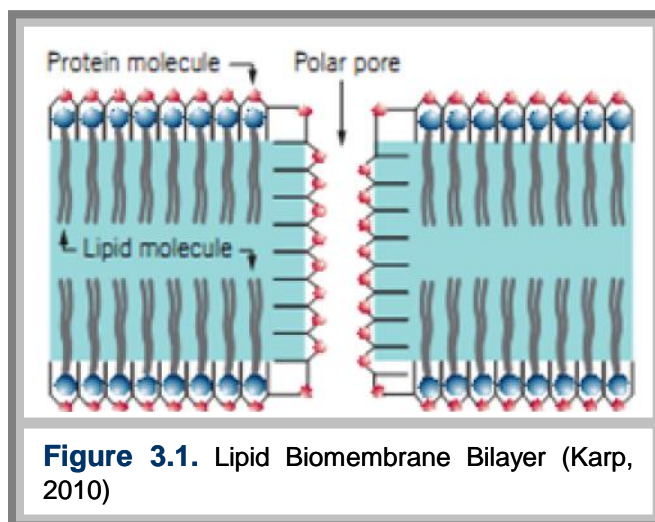
Figure 3.1. Lipid Biomembrane Bilayer (Karp, 2010)

Cell membrane consists largely of lipids together with proteins embedded in the lipid bilayer which provides the basic structure of almost all biological membranes (**Figure 3.2**).

Figure 3.2. A representation of the Biomembrane. The two leaflets of the bilayer contain different types of lipids as indicated by the differently colored head groups. (Adapted from Karp, 2010).
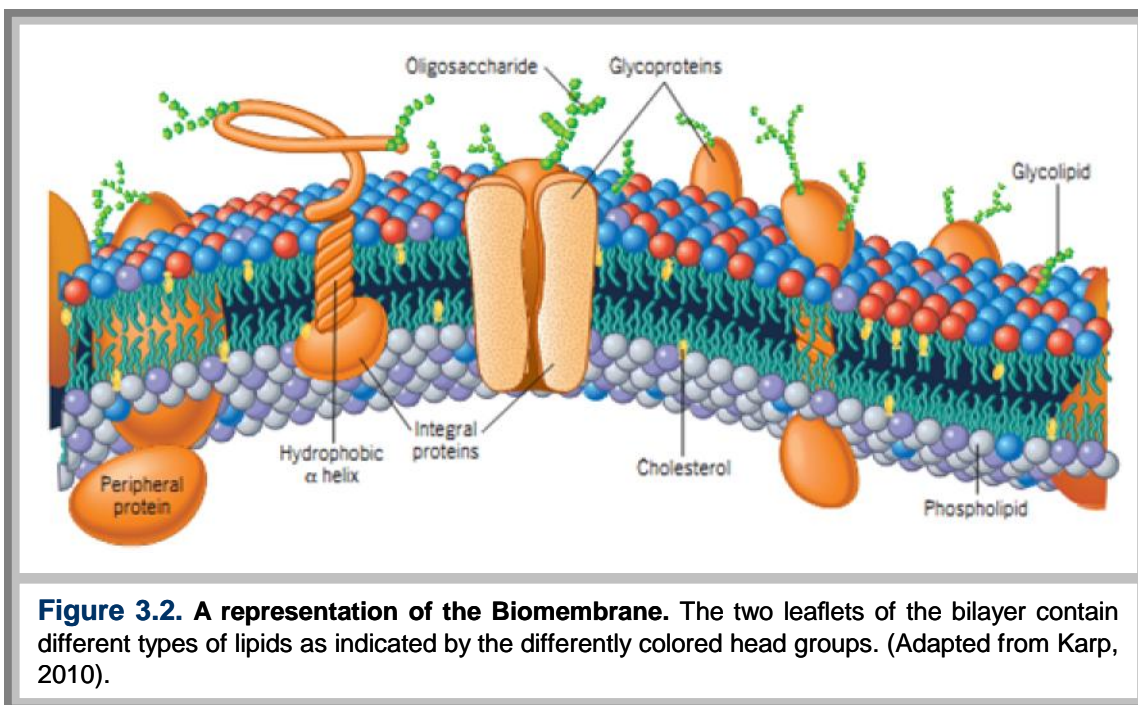
Membrane Lipids. Membranes contain a wide diversity of lipids, all of which are amphipathic containing both hydrophilic and hydrophobic regions (Karp, 2010, Berg, 2012). Lipids are water-insoluble biomolecules that are generally hydrophobic in nature and highly soluble in organic solvents such as chloroform (Berg, 2012).

In 2005, the International Lipid Classification and Nomenclature Committee under the sponsorship of the LIPID Metabolites And Pathways Strategy (LIPID MAPS, www.lipidmaps.org) Consortium developed and established a "Comprehensive Classification System for Lipids" based on well-defined chemical and biochemical principles. This classification system, that have defined eight categories of lipids and numerous classes and subclasses to allow the description of lipid molecular species, its details are reviewed and updated to its suggested nomenclature and structure-drawing recommendations for lipids. Lipids are classified into major six categories in mammalian sources: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, and prenol lipids (Fahy et al., 2005; Fahy et al., 2009).

Phospholipids are the major class of membranes lipids. Phospholipids are abundant in all biological membranes. A phospholipid molecule is constructed from four components: one or more fatty acids, a platform to which the fatty acids are attached, a phosphate, and an alcohol attached to the phosphate (**Figure 3.3**). The fatty acid components provide a hydrophobic barrier, whereas the remainder of the molecule has hydrophilic properties that enable interaction with the aqueous environment (Berg, 2012).

Figure 3.3. Schematic structure of a phospholipid and the orientation of phospholipids in membrane (Adapted from Alberts, 2008).
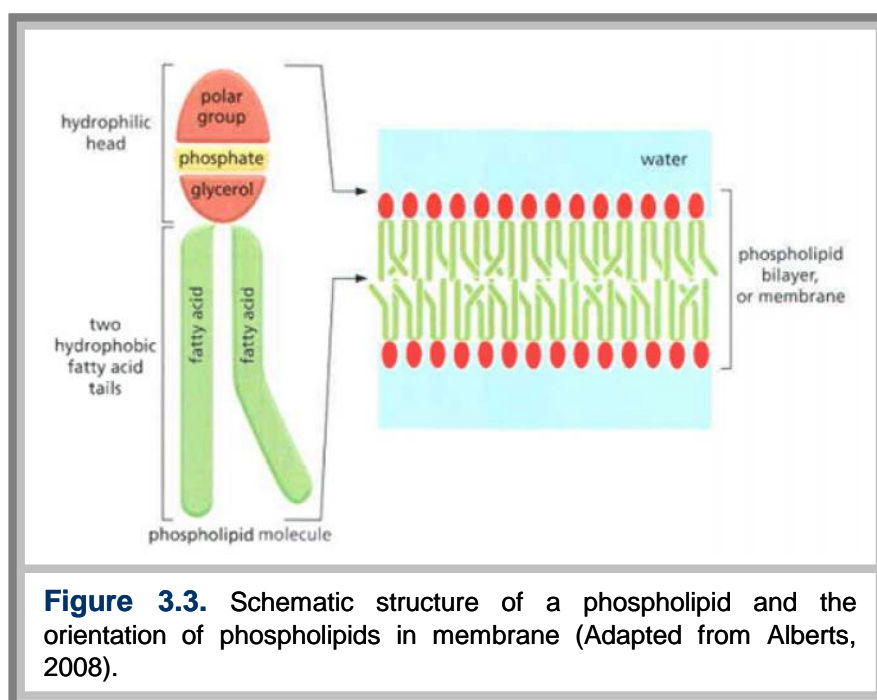
Cell Membrane Fatty Acid Composition. The fatty acyl structure represents the major lipid building block of complex lipids and therefore is one of the most fundamental categories of biological lipids. However, only traces of free fatty acids are present in free form in tissues and cells (Fahy et al., 2005). As the simplest unit, fatty acids can be transferred between or removed from other lipids, a process that is fundamental to a wide range of metabolic actions and physiological functions (Lawrence, 2010). The fatty acyl group in the fatty acids and conjugates class is characterized by a repeating series of methylene groups that impart hydrophobic character to this category of lipids (Fahy et al., 2005).

Fatty acids, such as Myristic acid, (**Figure 3.4**) are long hydrocarbon chains of various lengths ranging from 4 to 36 carbon long (C_4 to C_{36}) and degrees of unsaturation (number of double bonds) terminated with carboxylic acid groups (Berg, 2012). A number of fatty acids are listed in **Table 3.1**.

Figure 3.4. (Myristic acid, a saturated fatty acid with 14 carbon atoms).
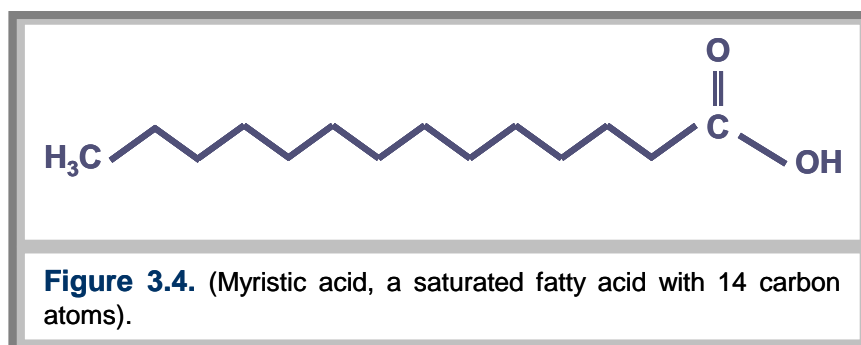
Fatty acids present in cells typically vary in length from 14 to 20 carbons. Fatty acids can be sub classified into straight chain saturated fatty acids (those lack the double bonds), such as Myristic acid (**Figure 3.4**), those possessing double bonds are unsaturated. In the fully saturated compounds, free rotation around each carbon–carbon bond gives the hydrocarbon chain great flexibility; the most stable conformation is the fully extended form, in which the steric hindrance of neighbouring atoms is minimized. These molecules can pack together tightly in nearly crystalline arrays, with atoms all along their lengths in van der Waals contact with the atoms of neighbouring molecules (Lehinger, 2004). Naturally occurring saturated fatty acids are mainly of even chain length between C4 to C24 (Gunstone, 2007).

In unsaturated fatty acids, a cis double bond forces a kink in the hydrocarbon chain. Fatty acids with one or several such kinks cannot pack together as tightly as fully saturated fatty acids, and their interactions with each other are there-fore weaker. Because it takes less thermal energy to disorder these poorly ordered arrays of unsaturated fatty acids. Naturally occurring fatty acids have double bonds in the cis configuration (Lehinger, 2004). **Figure 3.5** shows animations of the structures of some typical saturated and unsaturated fatty acids.

Unsaturated fatty acids may contain one or more double bonds. Fatty acid molecule that contains only one double bond along the carbon chain is called monounsaturated fatty acids (MUFAs), and those that contain two or more double bonds are called polyunsaturated fatty acids (PUFAs). Two classes of PUFAs exist: omega-6 (-6) and omega-3 (-3). humans, like all mammals, are unable to totally synthesize -3 or -6 PUFAs and must obtain them from dietary sources (Mostofsky, 2001).

Figure 3.5. The packing of fatty acids into stable aggregates. The extent of packing depends on the degree of saturation. Palmitic and stearic acids are two representations of the fully saturated acid in its usual extended conformation. Each line segment of the zigzag represents a single bond between adjacent carbons. (b) The cis double bond in oleic, linoleic, -linolenic, and arachidonic acids does not permit rotation and introduces a rigid bend in the hydrocarbon tail. All other single bonds in the chain are free to rotate.(Adapted from Campbell, 2009).

3.1.2 Functions of Fatty Acids

The most important function of fatty acids in cells is in the construction of cell membranes. These thin sheets enclose all cells and surround their internal

organelles. They act also as targeting molecules as they are attached to many proteins so that they are directed to their appropriate place in membranes. Fatty acids are stored as triacyglycerols (esters of glycerol and fatty acids), they act as energy-storage molecules (Alberts, 2008, Yaqoob, 2003). The fatty acids are precursors for signaling molecules such as prostaglandins. These essential fatty acids in human diet like linoleic and linolenic acids are also necessary for the health (Neitzel, 2010, Yaqoob, 2003).

3.1.3 Omega 3 and Omega 6 Fatty Acids

Polyunsaturated fatty acids (PUFAs) contain more than one double bond between pairs of adjacent carbon atoms.

There are two classes of PUFAs, the first is omega 3 fatty acids (also called –3 fatty acids or n–3 fatty acids) where the term omega or n signifies the location of the first double bond in relation to the number of carbon atoms beginning from the methyl end. The second class of PUFA is omega 6 (–6 or n-6) where the first double bond located between the sixth and seventh carbon atoms from the methyl end. Both classes are considered as essential fatty acids (EFAs), because they cannot be synthesized de novo in animal cells and must therefore be obtained from the diet (Yaqoob, 2003). -3 and –6 fatty acids are not interconvertible in the human body and are important components of practically all cell membranes.

These two classes of PUFAs should be distinguished because they are metabolically and functionally distinct and have opposing physiological functions (Simpoulos, 1991, Mostofsky, 2001).

There are two essential fatty acids, linoleic and -linolenic acid. Linoleic acid is an n-6 PUFA, described by its shorthand notation of 18:2n-6, which refers to an 18-carbon fatty acid with two double bonds. -Linolenic acid is an n-3 PUFA with

Table 3.1. Some naturally occurring fatty acids in animals.

Saturated Fatty Acids			
Common Name of the Acid	**Number of C- atoms**	**Degree of Unsaturation***	**Formula**
Lauric	12	0	$CH_3(CH_2)_{10}CO_2H$
Myristic	14	0	$CH_3(CH_2)_{12}CO_2H$
Palmitic	16	0	$CH_3(CH_2)_{14}CO_2H$
Stearic	18	0	$CH_3(CH_2)_{16}CO_2H$
Arachidic	20	0	$CH_3(CH_2)_{18}CO_2H$
Behenic	22	0	$CH_3(CH_2)_{20}CO_2H$
Lignoceric	24	0	$CH_3(CH_2)_{22}CO_2H$
Unsaturated Fatty Acids			
Palmitoleic	16	$16:1^{9}$	$CH_3(CH_2)_5CH=CH(CH_2)_7CO_2H$
Oleic	18	$18:1n9^{9}$	$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$
Linoleic	18	$18:2n6^{9,12}$	$CH_3(CH_2)_4CH=CH(CH_2)CH=CH(CH_2)_7CO_2H$
-Linolenic	18	$18:3n3^{9,12,15}$	$CH_3(CH_2CH=CH)_3(CH_2)_7CO_2H$
Arachidonic	20	$20.4n6^{3,8,11,14}$	$CH_3(CH_2)_4(CH=CH\ CH_2)_4(CH_2)_2CO_2H$
Eicosapentaneoic	20	$20:5n3^{5,8,11,14,17}$	$CH_3CH_2(CH=CH\ CH_2)_5(CH_2)_2CO_2H$
Docosahexanoic	22	$22:6n3^{4,7,10,13,16,19}$	$CH_3CH_2(CH=CH\ CH_2)_6CH_2CO_2H$

*Degree of Unsaturation refers to the number of double bonds. The superscript indicates the position of double bonds. For example, [9] refers to a double bond at the ninth carbon atom from the carboxyl end of the molecule. (Adapted from Campbell, 2009).

Role of Arachidonic Acid in Nasal polyps, Asthma and Cystic Fibrosis

a shorthand notation of 18:3n-3, describing an 18-carbon fatty acid with three double bonds.

Both essential fatty acids can be further elongated and desaturated in animal cells forming the n-6 and n-3 families of PUFAs. The metabolism of the -6 and -3 fatty acids is competitive because both pathways use the same set of enzymes (**Figure 3.6 and 3.7**).

The major end-product of the -6 pathway is arachidonic acid (AA). While the major end-products of the -3 pathway are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Yaqoob, 2003, Gunstone, 2007, Lawrence, 2010).

Fatty acids are initially synthesized as saturated fatty acids, and then specific enzymes are responsible for desaturating them. These enzymes known as desaturases that put double bonds at specific positions (Lawrence, 2010). Humans and other mammals have genes containing the information for making desaturases enzymes that place double bonds at positions 5, 6, and 9 in the fatty acid chain (between C5 and C6, between C6 and C7, or between C9 and C10) (McGarry, 2002). These are named delta-5-, delta-6-, and delta-9-desaturase, respectively.

Desaturation is not random, because desaturase enzymes have a preference for certain fatty acids; they select a fatty acid with a certain number of carbon atoms in the chain and a certain number of double bonds already present. The delta-6-desaturase has a preference for fatty acids with eighteen carbons and more than one existing double bond. Linoleic acid and linolenic acid are the most common polyunsaturated fatty acids in the diet that are desaturated by the delta-6-desaturase. The metabolism of these polyunsaturated fatty acids usually does not stop there. In a separate synthetic step, the chain can be further elongated by addition of two carbon atoms at a time by a process known as elongation. If the starting EFA is linoleic (18:2n-6), the final product of this reaction sequence has
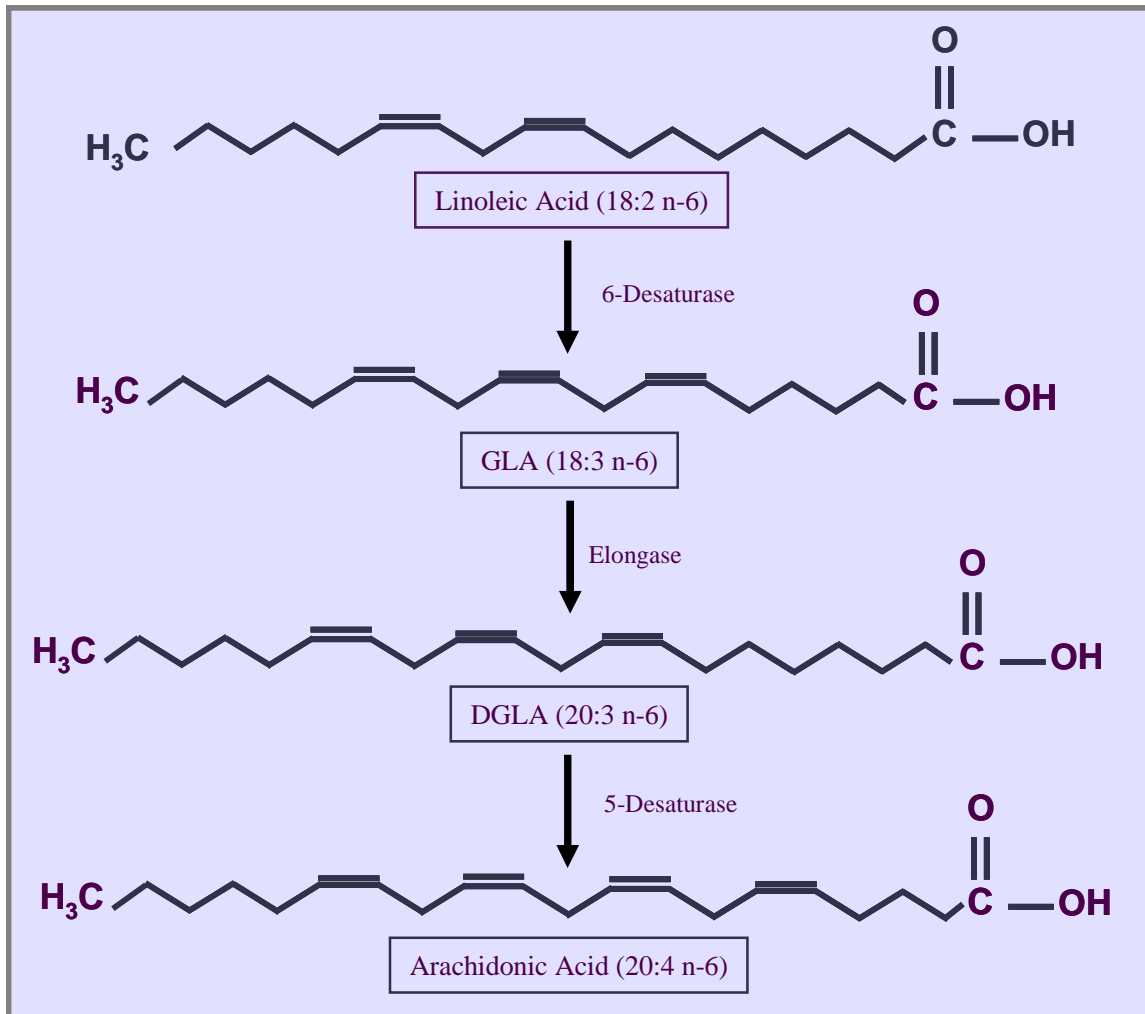
Figure 3.6. Metabolism of linoleic acid to arachidonic acid. This involves initial desaturation at carbon 6, followed by elongation to increase the chain length by two carbons (through a series of enzyme-catalyzed reactions), and final desaturation at carbon 5 forming the n-6 family of PUFAs. GLA, -linolenic acid; DGLA, dihomo- -linolinic acid.

twenty carbon atoms now and four double bonds at positions 5, 8, 11, and 14 relative to the acid group, this is arachidonic acid. On the other hand, Linolenic acid (18:3n-3) is metabolized to its corresponding twenty-carbon fatty acid, which has five double bonds at positions 5, 8, 11, 14, and 17. This is known as eicosapentaenoic acid or EPA. The conversion of EPA to DHA occurs by a metabolic pathway involving an extra elongation step and then removal of the two carbons that were just added (Lawrence, 2010).

Role of Arachidonic Acid in Nasal polyps, Asthma and Cystic Fibrosis
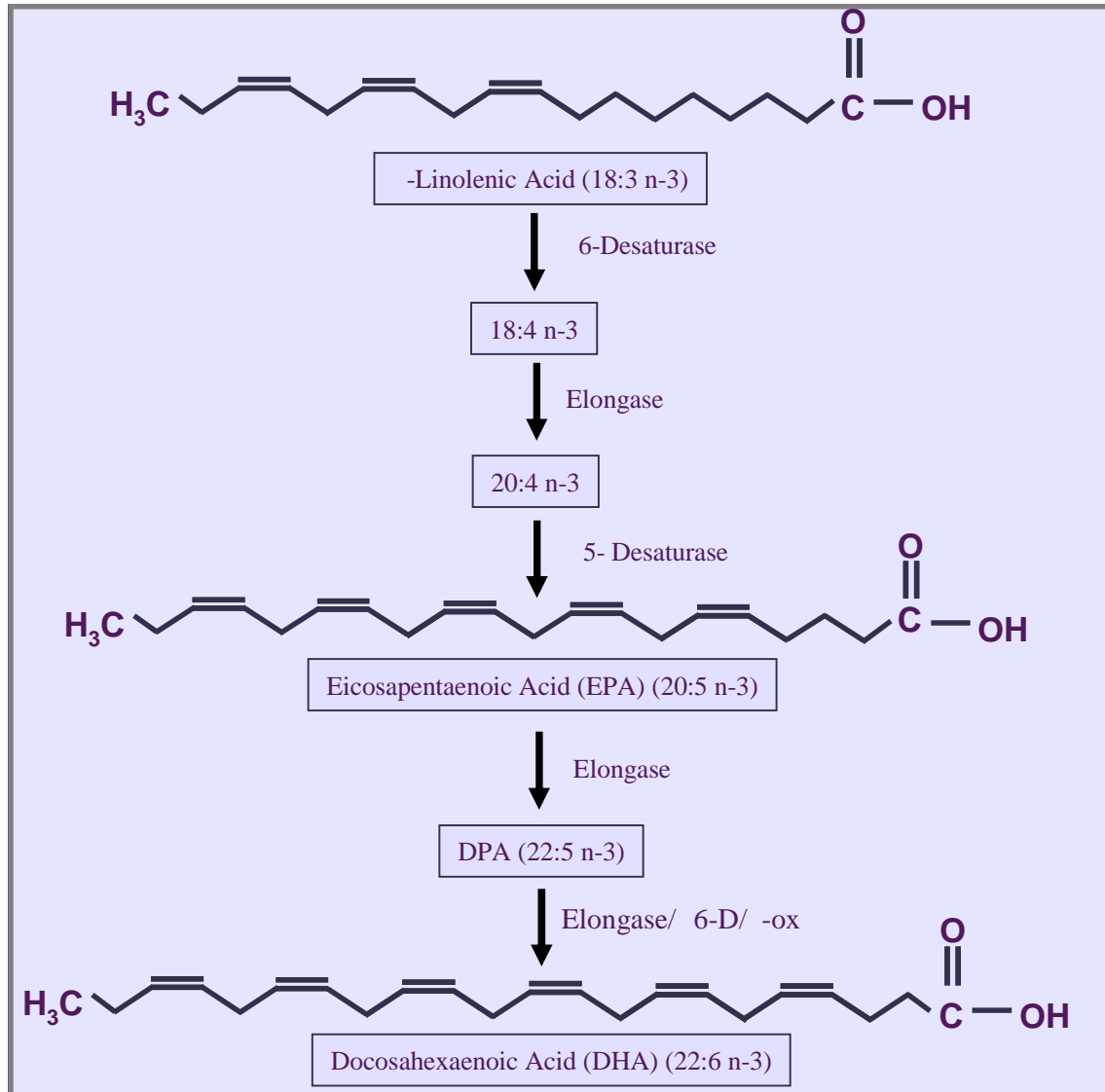
Figure 3.7. Metabolism of -linolenic acid to docosahexaenoic acid (DHA). Polyunsaturated fatty acids can be elongated and desaturated by several sequential steps in animal cells forming the n-3 family of PUFAs. DPA, docosapentaenoic acid; D, Desaturase; -ox, fatty acid -oxidation to remove 2 caron atoms from the chain.

The -6: -3 Ratio and its Biological Significance. Because PUFAs are essential in growth and development, they should be included in the diets of all human. They are important components of all cell membranes and their composition to a great extent dependent on the dietary intake (Simopoulos, 1991). The -3 fatty acids are abundant in fish oils but are also found in varying proportions in leafy green vegetables and in some vegetable oils, such as flaxseed, walnut, canola, and soy oils, as well as in wheat germ. Many vegetable

oils have an abundance of the ⍵-6 polyunsaturated fatty acid known as linoleic acid. It is converted in the body to arachidonic acid, which is involved in a multitude of physiological effects (James, 2000, Lawrence, 2010).

Another important consideration when it comes to dietary sources of poly-unsaturated fatty acids is to get a balance of ⍵-3 and ⍵-6 varieties. Several sources of information suggest that humans evolved on a diet with a ratio of ⍵-6 to ⍵-3 fatty acids of approx 1, whereas today this ratio is approx 10–20 : 1 indicating that the diets are deficient in ⍵-3 fatty acids like the western diets. Therefore, appropriate amounts of dietary ⍵-6 and ⍵-3 fatty acids need to be considered in making dietary recommendations. Their balance is imporatnt for normal development. ⍵-3 fatty acids (EPA and DHA) in the body can be quite different from those of ⍵-6 (AA) in metabolic and physiological functions, but in some cases EPA competes with arachidonic acid as the raw material for metabolism to biologically active eicosanoids (Mostofsky, 2001).

DHA is the major structural lipid constituent in all cell membranes possessing marked functions in retinal and neuronal tissues. It is essential for the central nervous system during development and plays a role in visual system structure and function (Stubbs 1992, Bazan, 1992). As an ⍵-3 long-chain PUFA, DHA functions to modulate production, activation, and potency of bioactive molecules. Similarly, the ⍵-6 long chain PUFA, AA also functions to modulate production, activation, and potency of bioactive molecules including eicosanoids. Whereas, DHA exhibits anti inflammatory properties, AA serves as a precursor for inflammatory mediators and is considered pro inflammatory (Gil, 2002).

The polyunsaturated fatty acids found in most vegetable oils are a double-edged sword. In addition to lowering serum cholesterol, they are metabolized to a wide range of highly potent bioactive substances, such as prostaglandins, thromboxanes, and leukotrienes, that evoke beneficial physiological actions throughout the body. The high levels of ⍵-6 vegetable oils consumed in industrialized nations are being blamed for a wide range of diseases, including

cardiovascular and coronary heart diseases (Harris, 2006, Simopoulos, 2009), asthma (Black and Sharpe 1997), diabetes (Berry 1997), and inflammatory diseases such as rheumatoid arthritis, ulcerative colitis, and psoriasis (Heller et al. 1998, Gil, 2002).

3.2 Eicosanoids

Eicosanoids is a large family of metabolites. They represent a class of molecules that are chemical messengers that carry information of cell activation from one cell to another and thereby they have been termed as lipid mediators. These cellular messenger molecules have a diverse set of important physiological and pathophysiological roles and coordinate events between cells so that proper tissue function can result (Curtis-Prior, 2004).

Eicosanoids are signaling molecules made by oxidation of twenty-carbon essential fatty acids. The first step in the synthesis of eicosanoids is the release of free arachidonic acid from its storage site in membrane phospholipids (Curtis-Prior, 2004). Although eicosanoids are not only generated from the arachidonic acid pathway, but also from pathways originating from eicosapentaenoic and dihomo- -linolenic acids (Levin, 2002, Wada, 2007). Once released from membranes free arachidonic acid is both metabolized to eicosanoids and reincorporated into phospholipids. Release of arachidonic acid from phospholipids can occur through a number of phospholipase enzymes (Marks, 1999). These enzymes can be considered as five types: cytosolic PLA_2 ($cPLA_2$), secreted PLA_2, calcium-independent PLA_2 ($iPLA_2$), platelet-activating factor acetylhydrolase (PAF-AH), and lysosomal PLA_2 ($LPLA_2$) (Schaloske, 2006).

Cytosolic phospholipase A_2, a major enzyme of eicosanoid biosynthesis, is activated by MAPkinase through phosphorylation to release arachidonic acid from glycerophospholipids (Marks, 1999). However, there is typically little substrate available for the initial enzyme that oxygenates arachidonic acid, since

arachidonic acid is conserved in the cell sequestered in glycerophospholipids and triglycerides and the cell regulates the quantity of arachidonic acid very tightly through activation of CoA synthase and CoA transeferase (Curtis-Prior, 2004). Eicosanoids are short-lived; they are not stored in cells but rapidly metabolized, i.e. they either hydrolyze spontaneously (such as thromboxane A_2 and prostacyclin) or undergo rapid metabolic inactivation by enzymatic dehydrogenation, -hydroxylation and fatty acid -oxidation. Thus their effects are locally expressed (Marks, 1999).

3.2.1 Eicosanoid biosynthesis from arachidonic acid

AA can be metabolised via three principal pathways to form an important class of bioactive lipid mediators, collectively termed eicosanoids that are released from the source cell and act at nanomolar concentrations in an autocrine/ paracrine manner on target cells. These family products are prostaglandins (PGs) and thromboxane (collectively termed prostanoids), formed by cyclooxygenase (COX); leukotrienes (LTs) and lipoxins (LXs) by lypooxygenase (LOX); and epoxyeicosatrienic acids (EETs) by cytochrome P450 enzymes (Stables, 2011).

Figure 3.8 indicates the three pathways of AA metabolism. This class of bioactive lipid mediators has been intensely studied because of their contribution to the inflammatory response in diseases such as arthritis and asthma.

AA-derived eicosanoids exert complex control over a wide range of physiological processes that have effects in health and disease. **Table 3.2** shows eicosanoids and their effects in different organs or cells of the human body.

To facilitate a systems biology approach to eicosanoid analysis, the biosynthetic enzymes and metabolic products identified in humans to date have been described here by focussing on their general signaling mechanisms.

Figure 3.8. Biosynthesis of eicosanoids from AA pathway. AA is released from membrane phopholipids by phospholipases, especially cytosolic phospholipase A2 (cPLA2). Free AA can be converted to bioactive eicosanoids through the cyclooxygenase (COX), lipoxygenase (LOX) or P-450 epoxygenase pathways. LOX enzymes (5-LO, 12-LO, 15-LO) catalyse the formation of LTs, 12(S)hydroperoxyeicosatetraenoic acids and lipoxins (LXs), respectively. COX isozymes (constitutive COX-1 and inducible COX-2) catalyse the formation of PGH2, which is converted by cell-specific PG synthases to biologically active products, including PGE2, PGF2a, PGI2 and TXA2, known collectively as prostanoids. The P-450 epoxygenase pathway catalyses the formation of hydroxyeicosatetraenoic acids (HETEs) and epoxides.

3.2.2 Eicosanoids Receptors

Eicosanoids exert their biological effects by binding to membrane receptors. They activate them at, or close to, the site of their formation. Because of their importance in the pathogenesis of several immunological and inflammatory diseases, the focus will be on the prostanoids receptors. Endogenously produced PGs can undergo facilitated transport from the cell through known prostanoid

Table 3.2. Physiological effects of major eicosanoids

Eicosanoids	Effect	Organs or Cells
PGF2, TXA2, LTC4, LTD4	Vasoconstriction	Vessels
PGI2, PGE2, PGD2	Vasodilation	
PGE1, PGI2	Anti-aggregation	Platelets
TXA2	Pro-aggregation	
PGF2 , TXA2, LTC4, LTD4	Bronchconstriction	Bronchi
PGI2, PGE2	Bronchodilation	
PGF2 , PGE1	Nausea, diarrhoea	Intestines
PGF2 , PGE1	Motility	
PGE2, PGI2	Inhibition og gastric acid secretion	Stomach
PGE2, PGF2	Motility	
PGE2, PGF2, TXA2	Contraction, parturition	Uterus
PGH2, PGE1, PGI2	Filtration and renal blood flow	Kidney
PGE1, PGE2	Increase in hypothalamic and pituitary hormone secretion	Hypothalamic and pituitary axis

(Reproduced from Harizi, 2008)

transporters or other carriers to exert autocrine or paracrine actions on a family of prostanoid membrane receptors. There are at least nine known prostanoid receptor forms in mouse and man (**Figure 3.9**). Four of the receptor subtypes bind PGE_2 (EP1, EP2, EP3, and EP4), two bind PGD_2 (DP_1 and DP_2), the PGF receptor, FP; the PGI receptor, IP; and the TXA_2, TP (Harizi, 2008, Stables, 2011). The prostanoid receptors have been recently cloned and are members of the G-protein coupled receptor seven transmembrane domain receptor family (GCPR) (Griswold, 1996), with the exception of DP2 (also known as CRTH2) which is a member of the chemoattractant receptor family (Xue, 2005).

DP receptors are not exclusively expressed on any cell or tissue but are

Figure 3.9. The prostanoid biosynthesis response pathway indicating prostanoids receptors. (Adapted from Smyth, 2009).
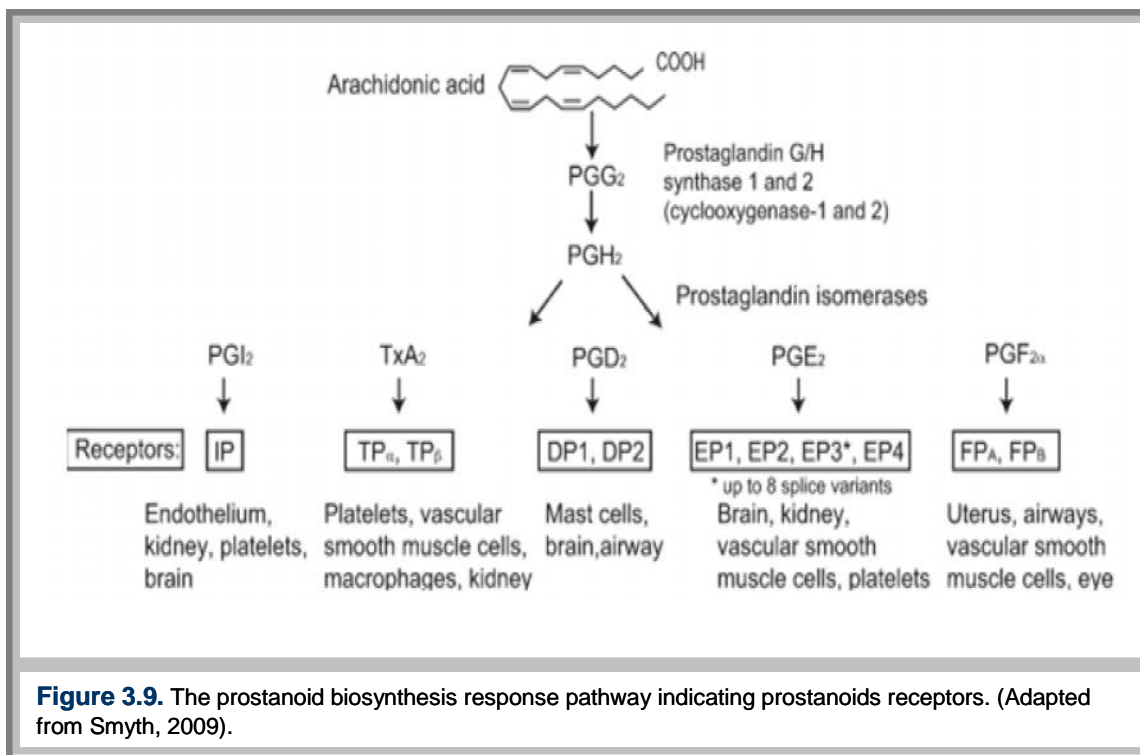
coexpressed along with other prostanoid receptors on platelets, vascular smooth muscle, and nervous tissue. They are largely inhibitory in nature (e.g., inhibition of platelet aggregation) (Griswold, 1996).

EP receptors mediate an impressive list of both inhibitory and stimulatory responses. EP1 receptor expression is generally low compared to the other EP receptors; however significant expression occurs on smooth muscle from various sources where they mediate contraction (Coleman, 1987). EP2 receptors on smooth muscle appear to mediate relaxation as well as inhibition of secretion from mast cells and basophils (Coleman, 1990). It has been described that EP2 playing a role in mediating the biological functions of COX-2/PGE2, particularly cell proliferation, angiogenesis, and apoptosis of several tissues (Regan, 2003, Sonoshita, 2001, Kamiyama, 2006). It mediates the antagonistic effect of COX-2 on TGF- signaling during mammary tumorigenesis (Tian, 2010). Different studies indicate that EP_3 is involved in smooth muscle contraction, the constriction of vessels, venules and airway, and the sensation of pain (Griswold, 1996). Relative activation of this receptor regulates the intracellular level of cAMP

and in this way conditions the response of the platelet to aggregating agents (Iyu´, 2010, 2011). The EP4 receptor plays a direct role in mediating endothelial cell functions in vitro and, most importantly, promotes angiogenesis in vivo (Rao, 2007). Also, EP4 plays a role in altering colonic epithelial barrier integrity (Lejeune, 2010). It also contributes to inflammatory pain hypersensitivity (Lin, 2007).

Recent study suggested that EP4 together with EP3 signaling contributes to lymphangiogenesis in proliferative inflammation, possibly via induction of VEGF-C and VEGF-D, and may become a therapeutic target for controlling lymphangiogenesis (Hosono, 2011).

FP receptors are highly expressed in the kidney (Abramovitz, 1995). They are also particularly highly expressed in the corpus luteum and interaction at this receptor mediates leuteolysis. Where they may mediate contraction and in the lung there, they may mediate bronchoconstriction (Griswold, 1996).

IP receptors are localized on platelets and vascular smooth muscle and provide a homeostatic inhibition of vascular tone and platelet aggregation. In addition, the vascular IP receptors are confined to the arterial circulation (Griswold, 1996). IP receptors also mediate hyperalgesia through their presence on sensory afferent nerves (Birrell, 1993). It is of interest that mRNA encoding IP receptors is rich in the thymus suggesting a possible immunoregulatory role (Namba, 1994).

TP receptors have an "opposing" role to IP receptors and they are distributed in vascular smooth muscle and platelets where they mediate excitatory activity. TP receptors also occur in airway smooth muscle and mediate contraction. The presence of TP receptors on immature thymocytes, where an interaction at the TP receptor on T cells may mediate apoptosis (Griswold, 1996).

3.3 Eicosanoids in Health and Disease

The diverse biological actions of eicosanoids on almost every cell reflect the central role of these mediators in maintenance of physiological homeostasis, of cell adhesion, vasomotion and organ functions. Eicosanoids were historically considered as terminal mediators, causing symptoms such as fever, pain, edema, smooth muscle contraction and inflammation. They also play a fundamental role in pathological processes and disease. Imbalance of the major lipid signalling pathways contribute to disease progression and chronic inflammation, autoimmunity, allergy, cancer, atherosclerosis, metabolic and degenerative diseases (Bruegel, 2009).

3.4 Cyclooxygenase Pathway

The hydrolysis of esterified arachidonic acid by phospholipase A_2, to yield free arachidonate is the initial rate-limiting step in the generation of eicosanoids including prostanoids (PGs of the E_2, D_2, F_2, I_2, and thromboxane A_2) (Griswold, 1996).

Cyclooxygenases (also termed prostaglandin H synthases) are enzymes which catalyze the initial two steps in the biosynthesis of prostanoids, preferentially using arachidonic acid as a substrate. The first step consists of a bisoxygenation of arachidonic acid yielding the hydroperoxy endoperoxide prostanoids G_2 (PGG_2) by the cyclooxygenase (COX) activity proper. In the second step PGG_2 is reduced to the hydroxy endoperoxide PGH_2 by the peroxidase activity of the enzyme. PGH_2 is a highly unstable endoperoxide, which functions as an intermediate substrate for the biosynthesis of biologically active prostanoids PGE_2, PGF_2, PGD_2, PGI_2, and thromboxane A_2 (TXA_2) that are generated by reduction and isomerization catalyzed by various synthases and isomerases (Marks, 1999).

Interestingly, the coupling of PGH_2 synthesis to its transformation to PGs and TX by downstream enzymes is intricately orchestrated in a cell-specific fashion. That is, any given prostanoid-forming cell tends to form only one of these compounds as its major product. Thus, for example, in brain and mast cells, PGH_2 is converted to PGD_2 by cytosolic enzyme PGD synthase. PGH_2 can alternatively be converted to PGF_2 by PG synthase, which is mainly expressed in the uterus. Vascular endothelial cells produce PGI_2 or prostacyclin from PGH_2 by means of the PGI or prostacyclin synthase, and platelets release TXA_2 from the same precursor (PGH_2) as the PGs through the action of the enzyme TX synthase. Both PGI_2 and TXA_2, have a very short half-life (30 seconds and 3 minutes, respectively) and are rapidly hydrolyzed to the inactive compounds TXB_2 and 6-keto-PGF_1 , respectively. Finally, PGE_2 is formed in many cell types by the enzyme PGE synthase. (Claria, 2003).

3.4.1 Comparison of COX-1 and COX-2

There are two main isoforms involved in the conversion of AA, COX-1 and COX-2. COX-1 is constitutively expressed in most cells and tissues. In particular, it is implicated in maintaining the protective lining of the stomach mucosa, regulating the renal blood flow and mediating platelet aggregation at sites of vascular injury (Griswold, 1996, Monnier, 2005). COX-2 is usually undetectable, it is absent from most normal tissues, except for some specific regions in the brain, in the kidney and in the uterus. It is rapidly induced when cells are challenged by a variety of stimuli including inflammatory cytokines (Ristimaki, 1994, Herschman, 1994, Monnier, 2005), such as Tumor Necrosis Factor (TNF), and Interleukin (IL)-1 or -6, in leukocytes, in particular monocytes/macrophages, as well as in stromal, epithelial and endothelial cells (Monnier, 2005). Although not exclusive, it is generally accepted that COX-1 is involved in cellular housekeeping functions necessary for normal physiological activity whereas COX-2 acts primarily at sites of inflammation (Stables, 2010). So, the distinction is not entirely accurate, since COX-1 can be induced or upregulated under certain conditions and COX-2 has

been consistently shown to be constitutively expressed in organs, such as the brain and the kidneys (Claria, 2003).

COX-1 and COX-2 are isoenzymes that differ also in gene expression. Since isoenzymes are genetically independent proteins, the genes in humans for the two enzymes are located on different chromosomes and show different properties. The COX-1 gene is located on chromosome 9, COX-2 is encoded by a gene on chromosome 1 (Dannhardt, 2001). Both genes also differ in size; the gene size for COX-1 is 22 kb contains 11 exons as opposed to COX-2 that has a relatively small size of 8.3 kb containing only 10 exons (Dannhardt, 2001, Herschman, 1994). However, mRNA size favors COX-2 (2.8 vs 4.1 kb). Perhaps one of the most interesting differences is the presence of AUUUA motifs in the 3' UTR as there are in COX-1 message and 22 repeats in the COX-2 message. In addition, multiple 5' transcriptional elements including NF-kB, AP-2, and NF-IL-6 exist in COX-2 but are not present in the COX-1 gene (Herschman, 1994).

3.5 Cyclooxygenase Metabolites

3.5.1 Prostaglandins

Prostaglandin E2 (PGE2). Prostaglandin E2 (PGE$_2$), the main inflammatory PG, may be the best characterized signaling molecule within the eicosanoid class. As PGE2 can act through its four different receptors (EP1-4) and the regulation of expression of the various subtypes of EP receptors on cells by inflammatory agents or even PGE$_2$ it self enables PGE$_2$ to affect tissues in a very specific manner (Narumiya, 2001). PGE$_2$ plays crucial roles in various biological events such as neuronal function, female reproduction, vascular hypertension, tumorigenesis, gastrointestinal, and kidney function (Kobayashi, 2002, Serhan, 2003). Moreover, of the cyclooxygenase metabolites, PGs and mostly PGE2 are

endogenous mediators with potent biological activities in the pathogenesis of many inflammatory, autoimmunity, allergic diseases and cancer (Harizi, 2008).

During inflammation, the levels of PGs are increased. PGE_2 is synthesized in substantial amounts at sites of inflammation where it acts as a potent vasodilator and sinergistically with other mediators such as histamine and bradykinin causes an increase in vascular permeability and edema (Hata, 2004). Moreover PGE_2 is a central mediator of febrile response triggered by the inflammatory process and intradermal PGE_2 is hyperalgesic in the peripheral nervous system (Dinarello, 1999). PGE_2 is thought to be a major PG species working in autoimmune diseases such as rheumatoid arthritis pathogenesis that is produced during inflammatory responses (Park, 2006, Sano, 2011), and also mediates some inflammatory responses and bone resorption as well as activation of osteoclasts (Sano, 2011) and osteoarthritis (Park, 2006).

The synthesis of PGE_2, takes place in several different cellular types within the airways, including epithelial and smooth muscles cells, follicular dendritic cells, fibroblasts, monocytes alveolar macrophages, and pulmonary endothelial cells also produce it (Taha, 2000, Ozaki, 1987, Widdicombe, 1989, Sheller, 2000).

PGs might act as both proinflammatory and anti-inflammatory mediators depending on the context, which is due in part to the array of EP receptors with different signal transduction pathways (Harizi, 2008). Both proinflammatory and antiinflammatory activities of PGE_2 have been reported in patients with chronic obstructive pulmonary disease (COPD) (Profita, 2010). In allergic reactions that result in an inflammatory response, mast cells are among the cells that involved during these reactions. They release chemical mediators majorly histamine which dilate vessels and triggers the inflammatory response (Ninnemann, 1988). PGE_2 protects against histamine as well as methacholine that are bronchoconstrictor agents (Manning, 1989). PGE_2 also protects against exercise-induced, allergen-induced, and aspirin-induced bronchoconstriction (Park, 2006). Within the immune system, PGE_2 modulates the function of T cells by suppressing their

proliferation (Minakuchi, 1990) and inhibits cytokine production of macrophages (Scales, 1989).

PGE_2 has anti-asthmatic and anti-inflammatory effects. It is concluded that one major effect of PGE_2 on the airways includes inhibition of stimulation-evoked responses such as mediator secretion from epithelium and inflammatory cells. In addition to its effects on inflammatory cells and its ability to cause relaxation of airway smooth muscle, PGE_2 may also inhibit the release of acetylcholine from airway cholinergic nerves. The normal response to PGE_2 appears to be bronchodilation. However, Inhalation of PGI_2 and PGE_2 may sometimes also cause bronchoconstriction and can trigger transient cough, but the mechanisms have not been established (Mark, 1999). Elevated sputum PGE_2 concentrations have been found in patients with chronic cough and eosinophilic bronchitis patients, with differences found in concentrations of PGE_2 in eosinophilic bronchitis patients that were more than those in asthmatics (Sastre, 2008).

Prostaglandin D2 (PGD2). Prostaglandin D2 is a structural isomer of PGE_2, and early studies of D-series prostaglandins regarded them as side-products of E-series biosynthesis. Whereas the prostane ring on PGE_2 has a 9-keto and 11-hydroxy moiety, the positions of these substituents are reversed on PGD_2 (Ito, 1989). PGD_2 is a proinflammatory mediator in human airways released by different types of cells including Th2 lymphocytes, macrophages, dendritic cells and mainly mast cells during asthmatic attacks that could contribute to an enhanced allergic response. PGD_2 has been shown to be increased in bronchoalveolar lavage fluid from asthmatic patients (Park, 2006). It is known to cause bronchoconstriction in both humans and animals (Hardy, 1984).

Two types of PGD_2 receptors have been identified to behave differently in the allergic reaction; DP1 and CRTH2/DP2. The DP1 receptor belongs to the prostanoid receptor family that consists of eight types and subtypes of receptor, each specific to an individual prostanoid. PGD_2 is likely involved in multiple

aspects of allergic inflammation through these dual receptor systems, DP1 and CRTH2/DP2. DP1 receptor is present on mast cells and eosinophils that may mediate production of effecter molecules that contribute to the asthmatic phenotype or predisposition (Park, 2006). CRTH2/DP2 is preferentially expressed in Th2 cells, eosinophils, and basophils in humans; it mediates effects such as eosinophil chemotaxis, actin polymerization, CD11b expression, and L-selectin shedding (Spik, 2005). It is also known that PGD_2 can cause airway smooth muscle contraction via the TP receptor (Coleman, 1989).

Prostaglandins have been shown to be produced in physiologically relevant levels in both the periphery and the central nervous system (CNS), with PGD_2 being the most abundant prostaglandin in the brain (Hiroshima, 1986).

Prostaglandin F2 (PGF₂). There are three Prostaglandin F_2 (PGF_2) biosynthetic enzymes that have been cloned and characterized in humans: Prostaglandin PGE2 9-ketoreductase, which catalyzes the conversion of PGE_2 to PGF_2 (Hayashi, 1990), Prostaglandin F (PGF) ethanolamide (prostamide F) synthase catalyzes the reduction of PGH2 to PGF_2 (Moriuchi, 2008), provided that human PGFS plays an important role in the pathogenesis of allergic diseases such as asthma (Suzuki-Yamamoto, 1999) and the conversion of PGD_2 11-ketoreductase to PGF_2 (Watanabe, 2002).

One PGF2 specific receptor has been cloned, a GPCR termed FP, which upon binding ligand results in an elevation of intracellular calcium (Abramovitz, 1994). Airflow obstruction and airway plasma exudation induced by instilled PGF_2 may be mediated via activation of TP receptors (Arakawa, 1993). PGF_2 inhalation resulted in bronchoconstriction in healthy and asthmatic subjects (Smith, 1975). Patients with asthma developed a marked and prolonged bronchoconstriction effect after PGF_2 inhalation suggesting that after inhalation a constriction and closure of the central and peripheral airways did occur in these patients leading to air trapping (Patel, 1976). In addition to its bronchoconstrictor effect; PGF_2

directly or indirectly causes airway relaxation, predominantly in large airways (Fish, 1983).

3.5.2 Prostacyclin (PGI2)

Prostaglandin I2 (PGI2) is formed by the prostacyclin synthase. The endoperoxide, PGH2 is the most proximal biosynthetic substrate known for PGI2 (Pace-Asciak, 1983). PGI2 is a prostanoid that has predominant pro-inflammatory effects which are important for the generation of oedema and pain accompanying inflammation (Park, 2006). It is produced during the allergic reaction in human lung (Dahlen, 1983), it enhances vasodilation (Kaley, 1985), it involves in smooth muscle relaxation in pulmonary vessels (Szczeklik, 1980), inhibits of increased vascular permeability (Schutte, 2001), it inhibits platelet aggregation (Pace-Asciak, 1983). In experimental models, it inhibits allergic mediator release and eosinophil recruitment (Gorgoni, 2001).

PGI2 binds the G-coupled protein receptor IP (Boie, 1994). PGI2 causes peripheral and central hyperalgesia when bound to IP receptors by reducing the threshold of nociceptor sensory neurons to stimulation (Stables, 2011). In addition to IP receptor, PGI2 activates also the EP2 receptor (Wheeldon, 1993). In addition to have proinflammatory effect, PGI2 also exerts immunosuppressive effects through upregulation of intracellular Camp (Aronoff, 2006). For example; it reduces the ability of inflammatory leukocytes to phagocytose and kill microorganisms. Also inhibits the production of downstream proinflammatory mediators (Xu, 2008).

3.5.3 Thromboxanes

There are two major thromboxanes: thromboxane A2 (TXA_2) and thromboxane B2 (TXB_2). TXA_2 is rapidly metabolized to the stable inactive metabolite TXB_2 (Funk, 2001). Thromboxane synthase; a cytochrome P-450 protein enzyme family member, catalyzes the rearrangement of PGH2 to TXA2 (Hecker, 1989).

TXA_2 has opposite biologic properties to those of PGI_2. TXA_2 is a powerful casconstrictor and aggregator of platelets. TXA_2 amplifies the effects of other, more potent, platelet agonists such as thrombin. The TP-$G_q$signaling pathway elevates intracellular Ca^{2+} and activates protein kinase C, facilitating platelet aggregation and TXA_2 biosynthesis. Activation of G_{12}/G_{13} induces Rho/Rho-kinase-dependentregulation of myosin light chain phosphorylation leading to platelet shape change. A single point mutation in the human TP results in a mild bleeding disorder (Katzung, 2012). TXA_2 also causes vascular smooth muscle contraction (Salzman, 1989).

Thromboxane A2 receptors (TPs) are widely distributed among different organ systems and have been localized on both cell membranes and intracellular structures. There are two separate TP isoforms: TP which is broadly expressed in numerous tissues, and TP which may have a more limited tissue distribution (Huang, 2004).

3.6 Lipoxygenase Metabolites

AA can also be metabolized through the lipoxygenase (LOX) pathway. There are three enzymes that are involved in this pathway: 5-, 12-, and 15-LOX (Picado, 2006).

3.6.1 The 5-Lipoxygenase (5-LOX) Pathway

Metabolites from the 5-lipoxygenase (5-LOX) pathway (LTC4, LTD4, and LTE4) are called slow-reacting substances of anaphylaxis (Lewis, 1980). 5-LO is mostly found in cells that are involved in inflammatory response such as eosinophils, basophils, neutrophils, leukocytes, platelets, mast cells, and macrophages (Stables, 2011, Picado, 2006, Gohil, 2010). Thus the 5-LO has a major role in inflammation due to its involvement in leukotriene (LT) synthesis.

Oxygenation of arachidonic acid (AA) at position C-5 of the molecule is performed by two proteins; 5-LO activating protein (FLAP) and 5-LO. The FLAP transports arachidonic acid into the cytosol to be acted on by the enzyme 5-LO. The product of 5-lipoxygenation of AA, leukotriene A4 (LTA4), is released by granulocytes. LTA4 further hydroxylated to LTB4. LTB4 is a potent chemotactic agent for leucocytes and may be important in mediating the inflammatory process in asthmatic airways. The enzyme leukotriene-C4 synthase (LTC4S), alternatively converts LTA4 to Cysteinyl-LTs (Cys-LTs). LTC4 is exported to the extracellular space where it forms LTD4, which in turn cleaved to form LTE4, the 6-cysteinyl analog of LTC4 (Gohil, 2010). Furthermore, LTC4, LTD4 and LTE4 are together form the Cys-LTs. LTC4, LTD4 and LTE4 are potent constrictors of the smooth muscle of the airways and may also contribute to the bronchial hyperresponsiveness characteristic of asthma (Arm, 1988). They are capable to enhance responsiveness to histamine in asthmatic airways (Hickey, 1991).

Two GPCR receptors have been implicated in cysteinyl leukotriene signaling, cysLT1 and cysLT2. The expression of cysLT1 appears to be highest in inflammatory cells, it mediates mucus secretion, and oedema accumulation in airways (Lynch, 1999), it also has a powerful bronchoconstricting effects and a role in asthma exacerbations (Kanaoka, 2004), indeed, an overexpression of $cysLT_1$ receptor was indicated in aspirin-sensitive patients with chronic rhinosinusitis (Sousa, 2002). CysLT2 contributes to inflammation, vascular permeability, and tissue fibrosis in lung (Stables).

3.6.2 The 12/15-Lipoxygenase (12/15-LOX) Pathways

The 12/15-Lipoxygenase (12/15-LO) enzyme catalyzes the transformation of free arachidonic acid to 12-hydroperoxy-eicosatetraenoic acid (12-HPETE) and 15-HPETE. These products are reduced to the corresponding hydroxy derivatives 12-HETE and 15-HETE by cellular peroxidases (Funk, 2006). 12/15-LO has been shown to oxidatively modify the key lipid components of LDL (Zhao, 2004). While

humans produce mainly 15-HETE, Mice make predominantly 12-HETE (Funk, 2006).

A classification of 12/15- LOX types has been proposed. These types are named according to their levels in the specific cells, platelet-type 12-LOX (12-LOX-p), epidermis-type 12-LOXs (12-LOX-e and 12R-LOX), leukocyte-type 12/15-LOX (12/15-LOX-l) and 8/15-lipoxygenase (8/15-LOX-2) (Kuhn, 2006).

Although less well characterized, the 12-lipoxygenase as well as the 15-lipoxygenase pathway also play an important role in the progression of human inflammatory diseases such as psoriasis, atherosclerosis, osteoporosis, diabetes and cancer (Schneider, 2005, Kuhn, 2006). It was suggested that LOX metabolites such as 12-HETE are critical in prostate cancer (Yang, 2012), and is implicated in colorectal and thyroid cancer (Prasad,2012). It is not known whether 12-HETE plays any role in lung diseases (Picado, 2006). The 15-HETE contributes to pulmonary vascular remodeling and pulmonary vascular resistance by regulating not only proliferation but also inhibiting apoptosis (Li, 2009, Wang, 2010).

Mammalian 15-LOX may be subclassified into reticulocyte-type and epidermis-type enzymes. The reticulocyte-type 15-LOX is expressed in a variety of mammalian cells and tissues such as human reticulocytes, human eosinophils, human airway epithelial cells, polymorphonuclear leukocytes, alveolar macrophages-inflamed tissue, vascular cells, uterus, placenta and various cells of the male reproductive system. The epidermis-type 15-LOX is expressed in the prostate, the lung and in cornea (Marks, 1999).

3.6.3 Lipoxins (LXs)

The interaction between 5-lipoxygenase and 15-lipoxygenase on arachidonic acid metabolism generates a new series of biologically active metabolites described as lipoxins (Serhan, 1984). There are two major isomers of LXs,

lipoxin A4 (LXA4) and lipoxin B4 (LXB4), and two more epimers, 15-epi-LXA4 and 15-epi-LXB4. Lipoxins can be generated by human neutrophils, eosinophils, or platelets during inflammatory responses. During the biosynthesis of lipoxins from arachidonic acid, leukotriene biosynthesis is blocked. Thus, there exists inverse relationship between leukotriene and lipoxin biosynthesis. The 15-lipoxygenase is abundant in lung tissue and that LXA4 has been detected in the bronchoalveolar lavage fluid of patients with asthma and other lung diseases suggests that LXA4 may be a potential mediator or modulator of inflammation in the lung. Moreover, inhalation of LXA4 in asthmatic subjects inhibited the bronchoconstrictor response to LTC4 (Gohil, 2010). Aspirin intolerant asthmatics seem to exhibit decreased capacity for generation of lipoxins (Sanak, 2000).

Lipoxins have diverse effects on leukocytes, including activation of monocytes and macrophages and inhibition of neutrophil, eosinophil, and lymphocyte activation. Both lipoxin A and lipoxin B inhibit natural killer cell cytotoxicity. They also exert coronary vasoconstrictor effects in vitro (Katzung, 2012 CH 18).

Both LXs and 15-epi-LXs have been identified and proven to exert beneficial effects in various experimental models of inflammation and human diseases, such as glomerulonephritis, ischemia/reperfusion injury, cystic fibrosis, periodontitis, acute pleuritis, asthma, wound healing processes in the eye, colitis, inflammation-induced hyperalgesia in rats, various cutaneous inflammation models and microbial infection in mice (Stables, 2011).

3.7 The Cytochrome P450 (CYP)

Cytochrome P-450 (CYP) designates a group of heme-containing enzymes, which absorb light at 450nm after reduction and treatment with carbon monoxide. This characteristic property is due to the heme group with its iron in ferric state bound to a cysteine residue. Therefore, they are called heme-thiolate proteins.

They can catalyze oxygenation of both foreign and endogenous compounds, such as fatty acids, steroids and bile acids, retinoids, prostaglandins and leukotrienes (Marks, 1999). They are found in the liver, brain, kidneys, lung, and heart (Stables, 2011). It has been indicated tat AA is metabolized via CYP P450 enzymes in the liver and kidney to epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatetraenoic acids (DiHETEs). It was revealed that CYP enzymes also catalyze in other tissues the formation of 19- and 20-hydroxyeicosatetraenoic acids (19- and 20-HETE) and 7-, 10-, 12-, 13-, 15-, 16-, 17-, and 18-HETEs from AA. These compounds play critical roles in the regulation of renal, pulmonary, and cardiac function and vascular tone (Roman, 2002).

Besides the EETs role in regulating vascular tone, they have antiinflammatory actions and prevent adhesion of activated leukocytes to the vascular wall (Node, 1999). EETs inhibit platelet aggregation induced by AA and vascular injury by inhibiting the formation of thromboxane (Fitzpatrick, 1986). It has become known that CYP metabolites of AA also play an important role in the regulation of airway resistance and pulmonary vascular tone (Zhu, 2000). The concentrations of EETs and DiHETEs in homogenates of human lung and in bronchoalveolar lavage fluid are relatively high. EETs are capable to relax bronchial rings, and similarly, 20-HETE relaxes human bronchi preconstricted by histamine (Zeldin, 1996). Recently, it was reported that EETs display hyperalgesic bioactions during experimental inflammatory pain (Inceoglu, 2008). EETs/DHETs may function as novel endogenous GPCR competitive antagonists, inducing vaso/broncho dilation, and, potentially, additional anti-inflammatory actions via direct TP inhibition (Behm, 2009).

3.8 Leukotrienes in Health and Disease

Leukotrienes owe their names to the fact that they are predominantly formed by leukocytes and that all of these molecules contain three

conjugated double bonds (Samuelsson, 1979). Pathologically, LTs contribute to a variety of inflammatory and allergic diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, allergic rhinitis, bronchial asthma, cancer, atherosclerosis and osteoarthritis (Werz, 2005). There are some medications for the treatment of asthma on the market that interfere with the synthesis or the action of leukotrienes. Zileuton, is an inhibitor of 5-LO and interferes directly with the biosynthesis of LTA4. It is used for the maintenance treatment of asthma. Montelukast and Zafirlukast are selective antagonists of the cysteinyl leukotriene receptor CysLT1 in the lungs and the bronchial tubes. They act by reducing the bronchoconstriction, resulting in less inflammation (Drazen, 1999, Haeggström, 2010).

Despite their pathophysiologic role, LTs are now recognized to be important participants in the host response against infection. LT deficiency is also observed in a number of clinical conditions that are associated with impaired microbial clearance (Human immune deficiency virus [HIV] infection, mal nutrition, cigarette smoking, vitamin D deficiency and post-bone marrow transplantation). LTB4 may also possess anti-inflammatory properties (Stables, 2011).

There are several observations that indicate the presence of a complex change in the AA metabolism of NSAID-intolerant asthmatic patients. Additionally, these alterations affect practically COX and LOX pathways (Picado, 2006). In aspirin-intolerant subjects, COX inhibition triggers the synthesis of bronchoconstrictor and vasoactive cys-LTs, LTC4, LTD4, and LTE4, by the 5-lipoxygenase (5-LO)/LTC4 synthase pathway (Adamjee, 2006). Aspirin hypersensitivity is likely to be mediated by a deviation of the arachidonic acid metabolic pathway toward excessive leukotriene production. The basal production Cys-LTs is 2 to 10-fold higher in AIA. It was found that people with aspirin-induced asthma are not sensitive only to aspirin, but also showed cross sensitivity to all NSAIDs that inhibit cyclooxygenase (COX) enzymes including ibuprofen, diclofenac, and naproxen (Gohil, 2010).

3.9 Fatty Acids and Arachidonic Acid Metabolism in Chronic Rhinosinusitis, Nasal Polyposis and Asthma

3.9.1 Fatty Acids

The concentration of free AA in resting cells is commonly described as "low". In some inflamed tissues, such as the skin of patients with psoriasis, free AA is abundant, but in healthy skin it is scarcer (Ikai, 1999). Similar results have been reported in fibroblasts from hypertrophic scars as compared to normal dermis (Nomura, 2007). These observations suggest that the level of AA increases under conditions of inflammation and remodelling, thereby facilitating the subsequent synthesis of PGs, leukotrienes and lipoxins.

To our knowledge, only one *ex vivo* study has assessed the fatty acid composition in the cells of asthma patients (De Castro, 2007). The authors reported significantly lower levels of AA in platelets isolated from asthma patients, compared with those obtained from health subjects. Platelets are not the ideal cells to explore fatty acids and arachidonic acid metabolism because they have very litte if any COX-2, a finding that may significantly limit the ability of platelets to transform AA into prostanoids under conditions of inflammation (Riondino, 2008).

3.9.2 Arachidonic Acid Metabolism

Various studies have assessed the regulation of arachidonic acid metabolism in asthma and in asthma associated to NSAIDs-hypersenstivity. Several anomalies in the regulation of the cyclooxygenenase, 5.lipoxygenase and trans-cellular metabolic routes have been reported.

3.9.3 Cyclooxygenase pathway

Various studies have reported low PGE_2 production in CRSwNP of patients with and without aspirin intolerance compared to control nasal mucosa (Perez Novo, 2005, Roca-Ferrer, 2011). The deficient production of PGE_2 was greater in aspirin-intolerant than in aspirin-tolerant patients (Perez-Novo, 2005, Roca-Ferrer, 2011).

Given the dependence of PGE_2 production on COX-1 and COX-2 activity, the expression of both enzymes has been assessed in nasal mucosa and in nasal polyps. COX-1 is usually considered an enzyme that responds to the physiological needs of cells but that is not involved in inflammatory responses (Simmons, 2004). Some studies, however, have shown mild increases in its expression in inflamed tissues (Simmons, 2004). A substantial up-regulation of COX-1 has been reported in CRSwNP associated to cystic fibrosis – a finding which suggests that airway COX-1 is sensitive to inflammatory stimuli (Roca-Ferrer, 2006). However, other studies on the regulation of COX-1 in CRSwNP of patients with and without aspirin intolerance have yielded contradictory results. There are studies that reported no differences at baseline in COX-1 expression between nasal mucosa and NP from either ATA or AIA (Picado, 1999, Adamusiak, 2010), however when the studies are performed using proinflammatory stimulation a lower COX-1 expression have been reported in fibroblasts of nasal polyps derived from AIA patients compared with healthy nasal mucosa (Roca-Ferrer, 2011) .

Since COX-2 expression increases in inflammatory processes, it would be expected that changes in PGE_2 levels detected in nasal polyps would be accompanied by a concomitant alteration in the expression of COX-2. In line with the results reported with PGE_2, COX-2 expression has been shown to be down-regulated in nasal polyps of patients with and without aspirin hypersensitivity (Perez-Novo, 2006, Roca-Ferrer, 2011, Mullol, 2002, Adamusiak, 2010). However, other studies could not find any difference in the expression of COX-2

in the nasal polyps of aspirin-intolerant, aspirin-tolerant and control subjects (Adamjee, 2006).

There are no clear explanations to account for the reported discrepancies in the regulation of the two COX enzymes in asthma and nasal polyps. Differences in the methods used in the identification and quantification of enzymes probably contribute to explain the reported discrepancies. In general, the studies that used RT-PCR and western blot techniques found low expression of the enzymes, while those using immunohistochemistry show contradictory results, with low or similar expression of COX-2 in nasal polyps compared with nasal mucosa. It has been suggested that discrepancies may result from the use of polyclonal or monoclonal antibodies (Sampson, 1998). The accuracy of this methodology can also be limited in studies of tissues samples with very different cells composition, such as healthy nasal mucosa and inflamed nasal polyps infiltrated by numerous inflammatory cells. To overcome these technical limitations a recent study assessed COX-2 expression, with ELISA, Western blot, and immunostaining in isolated and stimulated fibroblast derived from nasal mucosa and nasal polyps from both aspirin-tolerant and aspirin-intolerant patients. The three methods confirm the low expression of COX-2 expression in nasal polyps from aspirin-tolerant and aspirin-intolerant patients (Roca-Ferrer, 2011).

The expression of the four PGE2 G-protein-coupled receptor designated EP1-PE4 has been assessed in CRSwNP. A reduced expression of EP2 receptor has been reported in nasal inflammatory cells and in fibroblast of nasal polyps of aspirin-intolerant patients (Adamusiak, 2010, Ying, 2006, Roca-Ferrer, 2011). Since most of the anti-inflammatory effects of PGE_2 appear to be induced through the stimulation of the EP2 receptor (Sugimoto, 2006, Vancheri 2004), it may well be that the association of the reduced release of PGE_2 – a substance with anti-inflammatory properties in the airways – with the low expression of the PGE_2 receptor involved in the transmission of anti-inflammatory signals might contribute to the intensification of the inflammatory process in the upper airways

of patients with nasal polyps, especially in those with associated aspirin-intolerance (Roca-Ferrer, 2011).

3.9.4 Lipoxygenase Pathway

There are studies reporting increased production of cysteinyl leukotriene (CysLT) in CRSwNP of asthma patients with and without aspirin hypersensitivity (Higashi, 2012). When the production of CysLT is assessed by measuring the urinary levels of leukotriene E4, various studies have demonstrated a significant higher urinary excretion of LTE4 at baseline in patients with CRSwNP and aspirin intolerance compared with CRSwNP from aspirin-tolerant patients (Higashi, 2012). The baseline levels increase even further when aspirin-intolerant patients are challenged with oral, intravenous and intranasal aspirin (Higashi, 2012, Micheletto, 2006, Picado, 1992).

Interestingly, a recent study has demonstrated that severe aspirin-tolerant asthmatics in comparison with mild/moderate aspirin-tolerant asthma patients, also exhibited a significantly higher urinary LTE4 concentration at baseline (Higashi, 2004). This observation suggests that overproduction of CysLT is closely associated with asthma severity and the presence of CRSwNP. Therefore, an increased production of CysLTs should not be considered as a condition exclusively associated to aspirin-hypersensitivity. Since most patients with aspirin-intolerant asthma suffer from a severe form of the disease (Szczeklik, 2000), it is possible that the increased production of CysLT production detected in these patients is related, at least in part, to the severity of the associated asthma.

A recent study found a significant decrease in the urinary LTE4 concentrations after endoscopic sinus surgery in both CRSwNP aspirin-tolerant and CRSwNP aspirin-intolerant asthma patients, probably as a consequence of the elimination of a great amount of CysLT producing cells in the sinus (Higashi, 2004). This

observation suggests that CRSwNP is one of the most important factors involved in CysLT overproduction in asthma (Higashi, 2012).

The increased production of CysLT in CRSwNP is associated with the increased expression of the LTC4 synthase enzyme, and both CysLT production and LTC4 synthase expression are significantly correlated with the intensity of the eosinophilic infiltration present in nasal polyp tissue (Perez-Novo C, 2006, Adamusiak, 2012). CysLT levels in patients with CRSwNP positively correlate with IL-5 concentrations, a cytokine that primes eosinophils and thereby increases the biosynthesis capacity of these cells to produce CysLT (Perez-Novo, 2006). All in all, these observations suggest that there is a close link between CysLT overproduction and accumulation of activated eosinophil in CRSwNP (Higashi, 2012).

Pharmacological studies in human subjects provide evidence for the existence of at least three functional receptors for CysLT, however only two have been identified the so called CysLT1 and CysLT2 receptors (Laidlaw, 2012). CysLT1 has been detected in eosinophils, mast cells, macrophages, neutrophils and vascular cell in human nasal mucosa. CysLT2 receptors are broadly distributed in leukocytes, heart tissue, brain, adrenal and vasculature (Laidlaw, 2012).

Elevated numbers of nasal inflammatory cells expressing the CysLT1 has been reported in CRSwNP of patients with aspirin intolerance (Ying 2006, Adamusiak, 2010) . Desensitization with lysine-aspirin selectively reduced the number of leukocytes expressing the CysLT1, suggesting that downregulation of CysLT1could represent a mechanisms producing therapeutic benefit (Sousa, 1997).

A recent study has also reported an increased expression of CysLT2 receptor in nasal polyps from aspirin-intolerant asthma patients compared with aspirin-tolerant asthmatics (Adamusiak, 2010). Because data on the role of CysLT2

receptor in asthma and rhinitis have not been reported, the biological significance of this observation is not clear.

3.9.5 Transcelular Metabolism (Lipoxins)

The levels of LXA4 has been reported higher in patients with CRSsNP compared with control nasal mucosa but decreased in CRSwNP of aspirin-intolerant patients compared with the levels found in CRSwNP aspirin-tolerant asthmatics (Perez-Novo, 2006). A recent study reported that the urinary levels of 15-epi-LXA4 were significantly lower in aspirin-intolerant asthmatics compared with aspirin-tolerant patients and control nasal mucosa (Yamaguchi, 2011). These findings appear to indicate a deficient regulation of anti-inflammatory arachidonic acid metabolites generated through the trancelular pathway in patients with CRSwNP and aspirin-intolerance.

In summary, several observations support the notion that changes in the regulation of arachidonic acid metabolism are involved in the pathophysiology of CRSwNP especially when CRSwNP is associated with aspirin-intolerance. There are studies reporting changes at different levels of the Cox pathway including: very low production of PGE_2, alterations in the regulation of both Cox-1 and Cox-2 under inflammatory conditions, and low expression of the EP2 receptor. Moreover, there are studies that have shown that the 5-LO pathway is very active in CRSwNP, especially when CRSwNP is associated with aspirin-intolerance, resulting in an increased production of CysLTs. In contrast with EP2 receptor, the CysLTs receptor CysLT1 is upregulated in CRSwNP, a finding that probably contributes to maximize the proinflammatory effects of CysLT (Picado, 2006).

All in all, the impaired regulation of substances (PGE_2) and receptors (EP2) with anti-inflammatory effects, associated to the increased production of proinflammatory products (CysLT) and upregulation of their receptors (CysLT1) probably contributes to enhance and perpetuate the inflammatory process present in CRSwNP. Alterations in the arachidonic acid metabolism might also

contribute to the development of aspirin exacerbated respiratory disease (AERD). The mechanisms involved in the reported altered regulation of arachidonic acid metabolism remain to be clarified.

3.10 Fatty Acid and Arachidonic Acid Metabolism in Cystic Fibrosis

3.10.1 Fatty acid metabolism

Kuo et al. (Huo, 1962) described that CF was characterized by low linoleic acid (LA) concentrations in plasma and tissues and Underwood et al. (Underwood, 1972) in addition found low docosahexaenoic acid (DHA) concentrations in different tissues at autopsy in patients with CF. For many years the lipid abnormality was mainly referred to as a consequence of the fat malabsorption caused by the pancreatic insufficiency present in 85% of the patients. That causality is still presented in many reviews and textbooks where the fatty acid abnormalities are mentioned. Some interest in the abnormality was raised in 1975, when Elliot and Robinson (Elliot, 1975) described a reversal of the pancreatic dysfunction in a newborn with CF by administration of Intralipid, a finding which could not be confirmed in a larger study by the same author (Elliot,1976). In the following years many studies with supplementation of fatty acids were performed, usually with little influence on the clinical course (Rusoffsky, 1983), but with some physiological improvements, like reduction of steatosis and normalization of renal functions (Strandvik,1996). Studies in animals confirmed that many symptoms in CF could be due to essential fatty acid deficiency (Strandvik, 1988, Strandvik 1996), but with the identification of the gene and discovery of the cystic fibrosis transmembrane conductance regulator (CFTR) in 1989, much more recent research has concentrated on the chloride channel and the search for a successful gene therapy (Amstrong, ,2005, Strandvik, 1996).

However new interest in the area of fatty acids in CF was raised when Freedman et al. (Freedman, 1999) showed that the morphological abnormalities in pancreas and ileum of genetically modified mice displaying the symptoms of CF could be normalized by high doses of DHA. In a series of studies Freedman's group confirmed the abnormality of low DHA and a number of studies were performed with supplementation of DHA to patients with CF without any clinical improvement (Strandvik, 1996). In one study an improvement was seen in lung function by 8 months of supplementation with both eicosapentaenoic acid (EPA) and DHA, but in another study negative effects were found in adults with CF after 3 months of regular intravenous administration of n-3 fatty acids (Strandvik, 1996). Thus the fatty acid abnormality has turned out to be more complicated than predicted.

Hypothesis about increased AA release. Before the discovery of the CFTR gene defect in CF, it was found that prostaglandin synthesis was not decreased in patients with CF, as expected in patients with real or potential essential fatty acid deficiency, this led to investigate if the low LA concentration seen in CF might be an effect of increased turn-over. It was therefore investigated the release of arachidonic acid (AA) from blood mononuclear cells in patients with CF and controls and was found that the AA release could be inhibited in healthy controls by dexamethasone but not in the patients (Freedman, 1999). Interestingly some pilot studies with NSAIDs could not inhibit urinary prostaglandin excretion in patients with CF (Strandvik, 1996). CF is characterized by an increased inflammatory status and, although chronic bacterial colonization with repeated infections and chronic inflammation contributes, increasing evidence has been presented that prior to bacterial colonization there is inflammation of the bronchial mucosa, characterized by a neutrophil-dominated state (Casrlstedt-Duke, 1986). .Some studies also report increased inflammation of the intestinal mucosa (Norkina, 2004). CFTR dysfunction has been related to increased expression of phospholipase A_2 (PLA_2) and increased expression and activity of cyclooxygenase-2, resulting in increased production of PGE_2 (Medjane, 2005). Since the release of AA by phospholipases is rate-limiting for the eicosanoid

synthesis, an abnormal activity of PLA_2 liberating AA from the phospholipids in the membranes would be a possible mechanism to explain the inflammatory state of patients with CF (Strandvik, 1996).

Increased AA concentration was found in bronchial secretions from patients with CF in comparison with non-CF patients infected with pseudomonas (Strandvik, 1996).. Increased activity of PLA_2 has been reported in plasma from patients with CF and increased production and excretion of prostanoids have been reported in the patients in the bronchial secretions (Strandvik, 1996). In that compartment, however, it is difficult to know what is secondary to the bacterial colonization and the confirmation of increased prostanoid excretion in other compartments was therefore a support for a general increase in the AA cascade (Strandvik, 1996).. An abnormally high AA release in CF has been confirmed in different systems explaining the high turn-over of n-6 fatty acids which results in the observed low concentrations of LA (Strandvik, 1996). On the other hand, blocking of the Cl-conductance reduced the incorporation of labelled LA in phospholipids in cultured human airway epithelial cells and a low uptake was also seen in red blood cells (RBC) from patients with CF and in cell lines with the F508delta mutation (Strandvik, 1996). It is not quite clear if this reduced incorporation was real or a result of increased turn-over rate. It has also been suggested that AA can inhibit Cl-channels, including the CFTR, probably by electrostatic interaction. Depending on whether the CFTR mutations are affecting or not positively charged amino acids in the side chain, this inhibitory effect can be modified, which might have an influence on the phenotype (Strandvik, 1996).

The CFTR protein forms an integral membrane complex, where the first nucleotide binding fold domain (NBF-1) has been shown to predominantly bind to phosphatidylserine (PS), but with the F508delta mutation the binding affinity to phosphatidylcholine (PC) is increased (Strandvik, 1996). It is interesting that choline uptake has been shown to be increased in CF cells more than twofold both in the basic state and by different stimuli, indicating a higher turn-over of PC. The molecular species of PC are also different in peripheral blood

mononuclear cells from CF patients and in tissue samples from CF mice compared to controls (Strandvik, 1996). PC is synthesized from phosphatidylethanolamine (PE) or de novo by the cytidine diphosphocholine pathway, which requires choline from the diet. The synthesis from PE is dependent on the methionine–homocysteine cycle and thereby via betain and 5-methyltetrahydrofolate related to reduced gluthatione (GSH), an important antioxidant, which is decreased in CF which has been referred to a defective transport by the mutated CFTR (Strandvik, 1996). Innis and Davidson (Innis, 2008) have suggested that an impaired methyl metabolism might be a link between the pathological regulation of GSH and the impaired PC metabolism, especially since PC derived from PE has higher DHA content than PC derived from the cytidine diphosphocholine pathway. Support for this hypothesis was found in a child with CF, who was supplied with 5-methyltetrahydrofolate from 2 months to 6 years of age, and thereby increased her plasma DHA markedly above levels in an age-matched control (Strandvik, 1996). Although this result was not confirmed in a study where 31 children with CF were supplied with 5-methylhydrofolate and vitamin B_{12} for 6 months supplementing choline for 14 days to patients with CF improved the PC/PE ratio in RBC phospholipids (Strandvik, 1996). An overview of the relation between folate and the PC synthesis is given in (**Figure 3.10**).

Although there is strong evidence for increased AA release with a concomitant increase of its metabolic products in CF, explaining many symptoms and at least part of the inflammation, the intriguing question remains, what is the hen and what is the egg in the association between CFTR and the increased AA release?.

3.10.2 Relationship between CFTR and Arachidonic Acid Metabolism

Several studies have demonstrated overproduction of PGE_2 in CF patients. Very high levels of PGE_2 have been reported in the bronchoalveolar lavage, saliva,

Fig. 3.10. Scheme showing the influence of defective cystic fibrosis transmembrane conductive regulator (CFTR) on phospholipase A2 (PLA2) in the membranes and the resulting increased arachidonic acid (AA) release, increasing the demand for linoleic acid (LA). The increased release of AA will have many effects. This also results in increased turn-over of phosphatidylcholine (PC), increasing de novo synthesis from choline. If this is insufficient the synthesis from phosphatidylethanolamine (PE) will increase with increased demand for docosahexaenoic acid (DHA). (Adapted from Strandvik, 2010).

urine, sputum, and exhaled air of patients with CF (Lucidi, 2008, Zakrzewski, 1987, Yang, 2012, Strandvik, 1996). A recent study has also found the presence of PGD_2 in the sputum of CF patients (Yang, 2012). As discussed before various mechanisms may be involved in the increased prostanoid release in CF, including an increased activation of the enzyme phopholipase A2 (Medjane, 2005). Whether a defective CFTR can also be directly indirectly involved on the altered AA metabolism was a matter of speculation (Carlstedt-Duke, 1986).

Interestingly, recent studies for the first time provide evidence that alterations in CFTR can be directly involved in the regulation of the COX pathway (Chen, 2012). Moreover, *in vitro* and experimental animal studies have demonstrated that CFTR dysfunction induces and enhances AA metabolism, leading to an

increased expression of COX-2, which results in PGE_2 overproduction (Medjane, 2005, Chen, 2012). Chen J et al. (Chen, 2012) demonstrated that a CFRT defect leads to COX-2 induction in a CF line and CFTR-/- mice, resulting in increased production of PGE_2.

In keeping with these experimental observations, a previous study reported a marked up-regulation of COX-2 in the nasal polyps obtained from CF patients (Roca-Ferrer, 2006).

Moreover, various studies have identified a positive feedback loop from PGE_2 to COX-2 that is present in various cells types (Hinz, 2000, Faour, 2008). This helps maintain the continuous production of PGE_2 in normal tissues.

Chen J et al. (Chen, 2012) have proposed that CFTR acts as a negative regulator of PGE_2-mediated inflammatory responses and serves as a protective mechanism that prevents excessive tissue damage derived from enhanced production of PGE_2 during inflammatory responses.

The altered regulation of CFTR in CF would result in a deficient dampened/offset regulatory mechanism of the autocrine loop, involving PGE_2-mediated COX-2 induction; this would lead to an overproduction of PGE_2, which contributes to the perpetuation of the noxious inflammatory cycle in CF tissues (Chen, 2012).

Strandvik et al (Strandvik, 1996) reported, for the first time, an increased urinary excretion of PGE-M -a stable urine metabolite of PGE2- in CF patients, revealing an increased *in vivo* formation of PGE_2. However, they could not find any relationship between the severity of genotype alteration with the amount of PGE_2 produced by patients, probably because they studied a limited number of patients (14 patients) and all but one were patients who suffered the severe form of the disease.

All in all, these observations suggest that by mechanisms still not known, a defective CFRT gene is directly involved in the up-regulation of COX-2 that results in an increased production of PGE_2.

CHAPTER 4

Techniques
And Methodologies

4.1 Quantification of Eicosanoids and PUFAs

Eicosanoids are derived from the fatty acids that make up the cell membrane and nuclear membrane. They begin as a single poly unsaturated fatty acid, the arachidonic acid. From this single molecule, hundreds of biologically active signalling metabolites can be generated in a very short time. They are not stored within cells, but are synthesized as required. They have various roles in inflammation, fever, regulation of blood pressure, blood clotting, immune system modulation, control of reproductive processes, tissue growth, and many diseases including asthma, cystic fibrosis, chronic obstructive inflammatory disease, cardiovascular disease, and cancer. Since they have a crucial role in physiology and pathology, it is very important to identify and quantify the amount that is produced by the cells and tissues in order to identify better the targets for pharmaceutical intervention. Since the eicosanoids are products of a single species, AA, and represent, for the most part, the addition of various oxygen species, the hundreds of eicosanoids have very similar structures, chemistries, and physical properties. Also the endogenous levels of eicosanoids are quite low. Because of their identity and the small amount that is produced, the identification and quantitation of all eicosanoids in a single biological sample are a challenging task, they need a special method for isolating them and a specific and sensitive instrument for identifying, and quantitating them. Several analytical methods for the measurement of different eicosanoids metabolites (prostaglandins, leukotrienes, and thromboxanes) in biological samples were established with advantages and limitations of each method (Kindahl, 1983). Immunoassays such as enzyme immunoassay (ELISA) (Kumlin, 1996), bioassay and radioimmunoassay (Zakrzewski JT, 1987, Peskar, 1988) were the most widely used assays. Quantification with these methods requires specific antibodies for each eicosanoid. Since few eicosanoids have commercially avilable antobodies, this limit the number of eicosanoids that can be quantitated by these methods. And since only one eicosanoid can be determined by one assay this make this technique inefficient. Quantification with chromatographic and ionization

techniques were developed in order to improve the limitations of immunoassays techniques and allow to analyze multiple eicosanoids. In particular the development of tandem mass spectrometry and negative-ion chemical ionization (NICI), in gas chromatography (Schweer, 1994, Tsikas, 1998) and also advances in methodologies for solid-phase extraction and sample purification by thin-layer chromatography and more recently, high-performance liquid chromatography mass spectrometry have been made (Song, 2007, Neale, 2008, Zhang, 2011). In this thesis, studies by analytical methods for eicosanoids based on gas chromatography (GC) for the analysis of fatty acids in human nasal fibroblasts culture, and the high performance liquid chromatography tandem mass spectrometry (HPLC-MS-MS) for identification and quantification of prostaglandins metabolites (Tetranor-PGEM and Tetranor-PGDM) in human urine.

4.2 Gas Chromatography (GC) for the Analysis of Fatty Acid

Gas-liquid chromatography (GLC) is a technique that is used usually for the analysis of fatty acid composition in biological samples.

Preparation a sample for GC analysis. Conventionally, preparation of samples for GC involves two separate procedures: extraction and methylation. In order to extract lipids from tissues, it is necessary to find solvents which will not only dissolve the lipids readily but will overcome the interactions between the lipids and the tissue matrix (Christie, 1989). Lipids are usually extracted from cells or tissue homogenates by using organic solvents such as chloroform/methanol (Folch, 1957). Before the fatty acid components of lipids can be analyzed by GC, it is necessary to convert them to low molecular weight non-polar derivatives, such as methyl esters. Esterification of lipids can be carried out with several reagents based on acid-catalyzed or base-catalyzed reactions. Boron trichloride

(or trifluoride) in methanol can be used to prepare fatty acid methyl esters (FAMEs) with the reaction is slower when trifluoride is used as a catalyst (Brian, 1968). Esterification of the fatty acid prior to analysis avoids the interaction of the long chain fatty acid with the capillary column phase, as the esterified compound gives better peak shape, more reproducible quantitation and improved separation of the large number of structural isomers.

Sample injection system. For optimum column efficiency, the sample should not be too large, typically around 10^{-3} µL in capillary GC. The temperature of the sample port is usually about 50°C higher than the boiling point of the least volatile component of the sample. There are several types of sample introduction systems available for GC analysis. Among these types are the gas sampling valves, split and splitless injectors (Kitson et.al., 1996). For capillary GC, split/splitless injection is used.

The Carrier gas. To transfer the sample from the injector, through the column, and to the detector, a carrier gas (mobile phase) such as hydrogen, helium or nitrogen is used. It must be an inert and cannot be adsorbed by the column stationary phase (Kitson, 1996). The choice of carrier gas is often dependant upon the type of detector which is used.

GC Columns. In a gas chromatograph, separation occurs within a heated hollow tube, the column. The column contains a thin layer of a nonvolatile chemical that is either coated onto the walls of the column (capillary columns) or coated onto an inert solid that is then added to the column (packed columns) (Kitson, 1996). Capillary columns can be one of two types: wall-coated open tubular (WCOT) or support-coated open tubular (SCOT) (Christie, 1989). The use of long capillary columns has been frequently used in human tissue fatty acid analysis (Bohov, 1984). Modern, commercially available fused-silica capillary columns offer excellent separation of FAMEs from biological samples. Very polar stationary phases give excellent separation of all FAMEs but have relatively low thermal stability, resulting in long retention times. Non-polar phases have a much greater

thermal stability but inferior selectivity. For many analyses, phases of intermediate polarity, which combine the advantages of a relatively high resolution capability with relatively high thermal stability, are the most suitable (Eder, 1995).

GC Detectors. A large number of detectors operating on different principles have been developed for use in gas chromatography, but only a few of these continue to be used to a significant extent. Flame ionization detector (FID) and electron capture detector (ECD) the most widely used. The flame ionization detector is now almost universally adopted as it can be used with virtually all organic compounds, and has high sensitivity and stability, a low dead volume, a fast response time and the response is linear over an extremely wide range. This detector is simple to construct and operate, and it is highly reliable in prolonged use (Christie, 1989). With the FID the substances to be detected are burned in a hydrogen flame and are thus partially ionized. Because only hydrogen burns in the flame, only radical reactions occur. No ions are formed. If organic substances with C-H and C-C bonds get into the flame, they are first pyrolysed. The carbon-containing radicals are oxidised by oxygen and the OH radicals formed in the flame. The excitation energy leads to ionisation of the oxidation products. Only substances with at least one C-H or C-C bond are detected (Hübschmann, 2009).

Analysis and Identification. FAMEs can be identified by comparison of their retention times with those of individual purified standards containing accurately known amounts of methyl esters of saturated and unsaturated fatty acids. FAMEs can be quantitated by peak areas via calibration factors, and absolute concentrations can be determined by adding an internal standard (Eder, 1995). The area under of each peak is proportional to the amount of each component in the lipid mixture in the sample expressed as a percentage composition. **Figure 4.1** shows an example of a chromatogram with different peaks of fatty acid methyl esters.
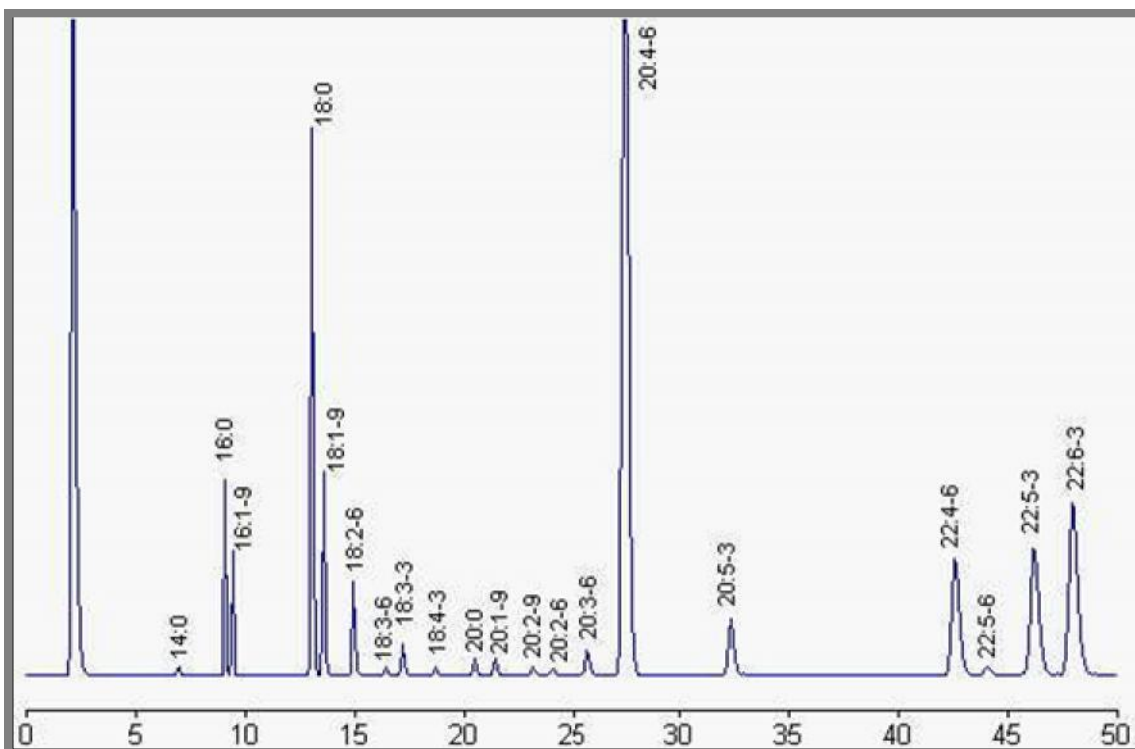
Figure 4.1. A chromatogram corresponding to the fatty acid profile of liver phospholipids. Fatty acids are represented by their number of carbon atoms : number of double bonds - series (n-x). (Adapted from http://www.cyberlipid.org).

4.3 Liquid Chromatography and Mass Spectrometry

4.3.1 Analytical Methods for Eicosanoids Quantitation

Prostaglandins play an important role in the pathogenesis of many diseases due to the production of multiple prostaglandins during disease that have diverse biological functions. Therefore, the investigation of them requires using specific assays for measuring the levels of prostaglandins in different biological fluids that are sensitive due to their low concentrations and selective due to their structural similarity of the different eicosanoids.

Compared with gas chromatography, antibody based assays such as ELISA and RIA have been developed to provide higher throughput measurement of prostaglandins in biological samples (Noguchi, 1995, Proudfoot, 1995). However, antibody based assays suffer from cross-reactivity with related compounds resulting in reduced selectivity leading to ambiguous and possible misleading results (Cao, 2008).

A combination of gas chromatography (GC) or LC with MS provides a quantitative analysis with high sensitivity and selectivity. While gas chromatography-mass spectrometry (GC-MS) provides suitable sensitivity and selectivity for prostaglandin measurement (Baranowski, 2002, Tsikas, 1998) and for simultaneous analysis of multiple eicosanoids, it requires laborious sample preparation including tedious purification and derivatization steps, and consequently is quite time-consuming technique especially in the case of analyzing a large number of samples. Furthermore, the thermal instability of many eicosanoids. Therefore, it is not an ideal analytical technique.

To overcome the limitations of GC-MS and immunoassays for prostaglandin measurements, LC-MS (Koda, 2004, Yue, 2007) and LC-MS/MS (Schmidt, 2005, Zhang, 2011) based methods have evolved as powerful tools for measuring prostaglandins in biological samples because of their high sensitivity, high selectivity and simplicity of sample preparation.

Mass spectrometry (**Figure 4.2**) is the key technique in the quantification of eicosanoids, Because of their sensitivity, mass spectrometers are considered as very powerful detectors. The principle of mass spectrometry is that ions in gas phase are separated according to their mass to charge ratio (m/z) and subsequently detected. First, the compounds must be transformed to yield ions in gas phase before the analysis by mass spectrometry since these samples analytes present in the liquid phase.
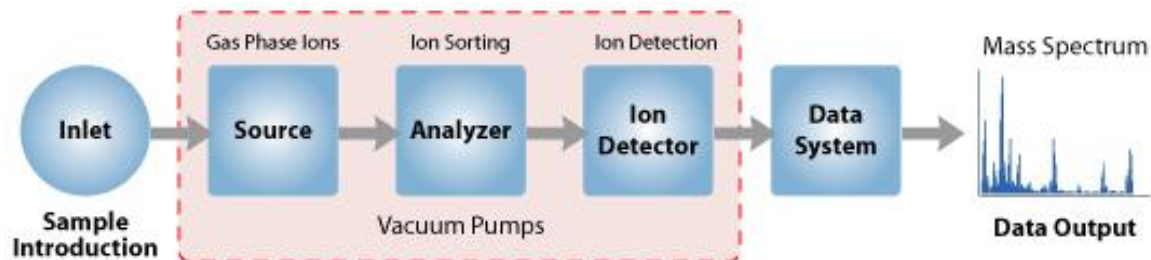
Figure 4.2. Components of a mass spectrometer.

Several ionization techniques are used to produce fragmentation of the analyte ion, among them and the most commonly used nowadays is the electrospray ionization (ESI) (Manisali, 2006) that let the coupling of MS with LC capable of producing gas phase ions from analytes in aqueous solution (Whitehouse, 1985). In ESI the transfer of ionic species from solution into the gas phase involves the dispersal of a fine spray of charge droplets, followed by solvent evaporation then ion ejection from the highly charged droplets. The emitted ions are introduced into a mass analyser, for subsequent analysis of molecular mass and measurement of ion intensity. The precursor ions of interest can be mass selected and further fragmented in a collision cell. The fragment ions can then be mass analysed by a second mass analyser of a tandem mass spectrometer system. Several types of mass analyzers are available including quadrupole and tandem quadrupole, time-of-flight (TOF) analysers, ion trap instruments and fourier transform ion cyclotron resonance (FTICR) mass spectrometers. For the quantitative analysis of eicosanoids quadrupole and triple quadrupole instruments are the most commonly used because they combine a sufficiently high mass accuracy with a high sensitivity (Ho, 2003). After the fragments of the selected ions are analyzed in the second analyzer, the ions are detected from the last analyzer and their abundance are measured with the detector that converts the ions into electrical signals.

In the present study, LC-MS/MS methodology was used for quantitative analysis of PGE_2 and PGD_2 metabolites to quantify the levels of urinary tetranor-PGEM and tetranor-PGDM, by using a preparative method with one simple derivatizing

step and one purification step before the analysis of prostaglandin metabolites by MS.

II. HYPOTHESIS AND OBJECTIVES

II.1 HYPOTHESES

This thesis establishes two hypotheses:

First hypothesis (**Study 1**: Fatty acid composition of cultured fibroblasts derived from healthy nasal mucosa and nasal polyps).

Second hypothesis (**Study 2**: Quantification of major urinary metabolites of PGE_2 and PGD_2 in cystic fibrosis: correlation with disease severity).

Background observations to develop the first hypothesis

1. Arachidonic acid metabolites are involved in inflammatory responses. It is generally accepted that in inflammatory diseases there is an increased activity in the cyclooxygenase and 5-lipoxygenase pathways that results in an increased production of prostanoids such as prostaglandin E2 and leukotrienes.

2. The expected increase in the expression of COX-2 and the subsequent increase in PGE_2 production are not observed in the inflamed tissue of nasal polyps from asthma patients, especially in those with non-steroidal anti-inflammatory drugs hypersensitivity.

3. In contrast with nasal polyposis associated with asthma, and according to the generally accepted dogma, the nasal polyps obtained from in cystic fibrosis patients, also an inflammatory airways disease, have an increased expression of COX-2 that results in am enhanced production of PGE_2.

4. An abnormally high AA release in CF has been confirmed in different systems, explaining the observed high concentrations of PGE_2 in various secretions and fluids (saliva, bronchoalveoalar lavage, blood).

5. Given the association of an abnormal high AA release with an enhanced PGE_2 production in cystic fribrosis, it is reasonable to speculate that the abnormally low release of PGE2 in nasal polyposis associated to asthma could be due a reduced AA availability.

First Hypothesis

The hypothesis establishes that the reported alteration in the production of some prostanoids such as PGE_2 in the nasal polyps of asthma patients, particularly in those with aspirin intolerance is, at least in part, due to alterations in the availability of AA.

Background observations to develop the second hypothesis

1. Cystic fibrosis (CF) is a genetic disease caused by a defective expression of the CF transmembrane conductance regulator (CFTR).

2. Various mutations can be grouped into different severity classes based on their known or predicted molecular mechanisms of dysfunction and functional consequences for the CFTR protein.

3. In vitro and experimental animal studies have demonstrated that CFTR dysfunction induces and enhances AA metabolism, leading to an increased expression of COX-2, which results in PGE_2 overproduction.

4. There are not studies assessing the relationship between severity of CFTR dysfunction and PGE_2 production.

Second Hypothesis

We hypothesize that in patients with cystic fibrosis there is a relationship between prostanoid production and CF genotype severity, and also with the severity of the phenotype expression determined by the presence or absence of pancreatic insufficiency.

II.2 OBJECTIVES

II.2.1 General Objectives

To contribute to elucidate the role of arachidonic acid metabolism in airway inflammatory diseases.

II.2.1 Specific Objectives

- First Study

Primary objective: To determine and compare the fatty acid composition of airway fibroblasts from healthy subjects and those from nasal polyps of asthma patients with and without aspirin intolerance.

Secondary Objective: To compare the fatty acid composition of nasal fibroblasts obtained from healthy subjects, aspirin tolerant and aspirin intolerant patients before and after stimulation with aspirin-D,L-Lysine, and interleukin-1 .

- Second Study

Primary objective: To assess the relationship in patients with cystic fibrosis between prostanoid production and lung function values, pancreatic function as a measure of CF severity, and genotype severity.

Secondary objective: To study the usefulness of the assessment of prostanoid production by measuring the urinary levels of PGE-M and PGD-M, terminal metabolites of PGE_2 and PGD_2.

III. RESEARCH WORK

Study 1.
Fatty Acid Composition of Cultured Fibroblasts Derived From Healthy Nasal Mucosa And Nasal Polyps

1. ABSTRACT

Background. Fibroblasts from nasal polyps (NP) of asthma patients have reduced expression of cyclooxygenase 2 (COX-2) and production of prostaglandin E_2 (PGE$_2$). We hypothesised that the reported alterations are due to alterations in the availability of arachidonic acid (AA).

Objective. The objective was to determine the fatty acid composition of airway fibroblasts from healthy subjects and from asthma patients with and without aspirin intolerance

Methods. We analyzed the fatty acid composition of cultured fibroblasts from non-asthmatics (n=6) and from aspirin-tolerant (n=6) and aspirin-intolerant asthmatics (n=6) by gas chromatography-flame ionization detector. Fibroblasts were stimulated with acetyl salicylic acid (ASA).

Results. The omega-6 fatty acids dihomo-gamma-linolenic acid (C20:3) and AA (C20:4) and omega-3 fatty acids docosapentaenoic acid DPA (C22:5) and docosahexaenoic acid DHA (C22:6) were significantly higher in NP fibroblasts than in fibroblasts derived from nasal mucosa. The percentage composition of the fatty acids palmitic acid (C16:0) and palmitoleic acid (C16:1) was significantly higher in fibroblasts from patients with NP and aspirin intolerance than in fibroblasts derived from the nasal NP of aspirin-tolerant patients. ASA did not cause changes in either omega-3 or omega-6 fatty acids.

Conclusions. Our data do not support the hypothesis that a reduced production of AA in NP fibroblasts can account for the reported low production of PGE$_2$ in nasal polyps. Whether the increased proportion of omega-3 fatty acids can contribute to reduced PGE$_2$ production in nasal polyps by competitively inhibiting COX-2 and reducing the amount of AA available to the COX-2 enzyme remains to be elucidated.

2. INTRODUCTION

Asthma is a syndrome characterized by the presence of chronic inflammation, resulting in airway obstruction and bronchial hyper-responsiveness that causes wheezing, coughing, and dyspnea (Busse, 2001). Nasal polyposis (NP) is a chronic inflammatory disease of the sinus mucosa usually seen in association with chronic rhinosinusitis (CRS) and asthma (Fokkens, 2007). The pathogenesis of CRS with NP is related to an altered inflammatory state that results in a tissue remodelling process (Pawliczak, 2005).

Aspirin-intolerant asthma (AIA) is a distinct syndrome characterized by asthma, CRS, NP, and aspirin sensitivity. Aspirin sensitivity may be present in 5 to 10% of the asthmatic population (Stevenson, 2006, Picado, 2006). The pathophysiological mechanism of AIA is only partially understood and appears to be related to anomalies in the metabolism of AA (Stevenson, 2006, Picado, 2006).

AA is produced from membrane phospholipids by the action of phospholipase A2 enzymes; it can then be converted into different eicosanoids. AA can be enzymatically metabolized by three main pathways: P-450 epoxygenase, cyclooxygenases (COXs) and lipoxygenases (LOXs). The LOXs convert AA into leukotriene (LT) A_4, which is the precursor of LTB_4 and cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) (Harizi, 2008). The COX pathway produces PGG_2 and PGH_2, which are in turn converted into prostaglandins (PGEs) and thromboxanes. There are two isoforms of the COX enzymes: prostaglandin H synthase-1 (PGHS-1), also known as COX-1, is generally constitutively expressed and is typically considered a "house-keeping" gene; prostaglandin H synthase-2 (PGHS-2), also known as COX-2, is usually only expressed under inflammatory conditions (Simmons, 2004).

Eicosanoids are released from cells during hypersensitivity reactions and are involved in the clinical manifestations of rhinitis and asthma. LTs are potent pro-

inflammatory mediators and this can explain why anti-LTs are beneficial in asthma and rhinitis (Harizi, 2008). PGs might act as both pro-inflammatory and anti-inflammatory mediators, depending on the context; this is partly due to the level of expression of the four PG receptors in the cells involved in the response (Simmons, 2004). The COX pathway is the major target for non-steroidal anti-inflammatory drugs (NSAIDs), the most popular medications used to treat fever, pain, and inflammation (Simmons, 2004). However, and in contrast with other inflammatory diseases such as arthritis (Simmons, 2004) and cystic fibrosis (Konstan, 2008), inhibition of COX with aspirin or NSAIDs does not provide any salutary effect to asthma patients. In fact, for a subset of patients with asthma, ingestion of NSAIDs induces bronchoconstriction and nasal obstruction (Stevenson, 2006, Picado, 2006). Interestingly, selective COX-2 inhibitors are usually well tolerated by AIA (Stevenson, 2006, Picado, 2006).

These observations suggest that eicosanoids can be differentially regulated in asthma, unlike other inflammatory airway diseases (Picado, 2006). In patients with asthma, and especially in AIA, various data support the existence of an altered regulation of the COX pathway (Kowalski, 2000, Pujols, 2004, Perez-Novo, 2005, Roca-Ferrer, 2006). PGE_2 levels have been reported to be low in the nasal polyps of asthma patients, as well as in nasal-polyp and bronchial fibroblasts from asthmatic patients, particularly those with aspirin sensitivity (Perez-Novo, 2005, Pierzchalska, 2003, Kowalski, 2000, Roca-Ferrer, 2011). As PGE_2 production mostly depends on the level of COX-2 induction under conditions of inflammation, it should be expected that the low production of PGE_2 detected in asthma and nasal polyps would be accompanied by a similar, concomitant alteration in the expression of COX-2. Accordingly, lack of up-regulation of COX-2 in the nasal polyps of asthma patients, both with and without aspirin sensitivity, has been reported in various studies (Perez-Novo, 2005, Roca-Ferrer, 2011, Mullol, 2002).

The mechanisms involved in the abnormal production of prostaglandin E_2 in nasal polyps, and in particular in those associated with AIA, are still unclear (Picado, 2006).

Various studies have shown that the activity of COX-1 and COX-2 enzymes is controlled differentially by regulating the amount of AA available to the enzymes (Harizi, 2008). As PGE_2 production by COX-1 and COX-2 is dependent, at least in part, on the availability of AA, it could be possible that alterations in the AA supply may account for the anomalies in PGE_2 production reported in nasal polyps and AIA. A number of observations support the notion that fibroblasts are more than just structural cells with no other physiological or modulator functions (Smith, 1997). Fibroblasts can contribute to the regulation of inflammatory and immunological responses by producing various growth factors, cytokines and eicosanoids (Olsson, 2003).

The hypothesis of the present studies establishes that the reported alteration in the production of some prostanoids such as PGE_2 in the nasal polyps of asthma patients, particularly in those with aspirin intolerance, is at least partly due to alterations in the availability of AA.

3. MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), and phosphate buffered saline (PBS), were purchased from (Lonza, Spain), 0.05%Trypsin–0.02% Ethylenediaminetetraacetic acid from (Invitrogen, United Kingdom), 6-well culture plates, 96-well culture plates, 75-cm^2 culture flasks and 150-cm^2 culture flasks were obtained from (NUNC, Wiesbaden, Germany), Cryoprotective media from (Lonza, Walkersville, USA), Aspirin-D,L-Lysine from (Bayer, Germany), interleukin-1 from (R&D systems, USA). Penicillin-streptomycin and fetal bovine serum (FBS) from Invitrogen Corporation (Paisley, Scotland, UK), Amphotericin B from Squibb (Esplugues de Llobregat, Catalonia, Spain). Chloroform, Methanol, n-Hexane, boron-triflouride methanol (BF_3-MeOH) were obtained from (Merck, Barcelona, Spain). For the determination of total protein; Protein Assay Kit: Protein Standard Solution (Lowry Reagent, Folin & Ciocalteau's Phenol

Reagent) from (Sigma, St. Louis, USA). Triton X-100 from (Sigma Chemical Co., St. Louis, USA), Pefabloc SC from (Roche Diagnostics, Mannheim, Germany), Hepes Buffer Solution at 0.05M from (Gibco-Invitrogen, Stockholm, Sweden), and bovine serum albumin (BSA) from (Sigma Chemical Co., St. Louis, USA).

Study Population and Tissue Handling

Nasal polyp tissue was obtained from 12 asthmatic subjects (6 aspirin-tolerant, and 6 aspirin-intolerant). The main demographic data and clinical characteristics of the study population are shown in **Table III.1**. The subjects were referred to the Hospital Clínic of Barcelona for sinus surgery. The study subjects were selected on the basis of a medical history consistent with severe chronic NP, as described in the EP3OS document (Fokkens, 2007), and of documented disease in their sinuses, as shown via CT scan. The diagnosis of asthma was established from the clinical history and the demonstration of a reversible bronchial obstruction. Diagnosis of aspirin intolerance was based on a clear-cut history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs (NSAIDs) and confirmed by aspirin nasal challenge in patients with an isolated episode of NSAID-induced asthma exacerbation, according to a method previously reported (Casadevall, 2000). Nasal mucosa from 6 subjects in each study undergoing nasal corrective surgery was used as control.

Table III.1. Characteristics of study subjects.

Characteristics	Controls	Asthma Aspirin Tolerant	Asthma Aspirin Intolerant
N	6	6	6
Age (years) Mean ± SD	53 ± 19	60 ± 16	68 ± 9
Min.-Max	28-77	44-87	59-82
Gender (M/F)	(4 / 2)	(5 / 1)	(1 / 5)
Atopy (yes/no)	(0 / 6)	(1 / 5)	(1 /5)

N, Number; SD; standard deviation, M; male, F; female.

All patients with NP were on intranasal glucocorticoid therapy that was discontinued at least five days before surgery. None of the patients were on oral glucocorticoid therapy at the time of surgery, nor had they received any systemic glucocorticoids for at least one month prior to surgery. No subjects from the nasal mucosa control group had a history of nasal or sinus disease, nor had they received glucocorticoids for any reason. None of the subjects had suffered from an upper respiratory infection during the two weeks prior to surgery.

The subjects were asked for their permission and informed consent was given to study pathological specimens under a protocol approved by the human investigations committee of our hospital.

Fibroblasts Cell Culture

Specimens obtained during nasal endoscopic surgery were cut into 3 x 3 mm fragments and placed in six-well plates containing DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 µg/ml amphotericin B. After a period of about 3 weeks, when fibroblast growth was established, tissue fragments were removed and the first passage was performed. Cultures were washed three times with PBS and incubated for 5 min with 0.05% trypsin and 0.02% EDTA. The reaction was stopped by the addition of growth medium, cells were collected by centrifugation (1800 rpm, 5 min), seeded in two 75-cm^2 flasks and grown up to 90% confluence (duplicate per sample). Cells from the sixth passage were used in the study of fibroblasts lipid composition. When the confluence reached up to 90%, cells were washed with PBS and incubated with DMEM without FBS for 24 hours, and then the medium was changed for another quantity of DMEM without FBS. For samples stimulated with aspirin; 0.5 mg/dl of aspirin were added, for samples stimulated with interleukin-1 ; 10µg/ml were added, the samples then were left for 24 hours in culture before the lipid extraction.

Lipid Extraction

Total lipids were extracted from fibroblast cells using methanol-chloroform containing 1% BHT according to the method of Bligh and Dyer (Bligh, 1959). The cells were washed three times with ice-cold Ca^{2+}, Mg^{2+}, free PBS; then it was left and the cells were harvested by scraping. The cells were pelleted by centrifugation at 1,200 rpm at 4ºC for 5 min and the PBS was removed. The cells were washed once with 10 ml ice-cold PBS and centrifuged at 1,200 rpm at 4ºC for 5 min, then the PBS was removed. 300 µl of ice-cold PBS was added to the sample, and then an aliquot sample of 50µl was taken to determine the total protein. The rest of the pellets and PBS were transferred to a glass tube and centrifuged at 2,500 rpm at $20^{o}C$ for 5 min and the PBS was removed. Two ml of distilled water were added, then the sample was sonicated to assure further cell lysis, and then centrifuged at 2,500 rpm at 4ºC for 10 min; the supernatant was discarded. The pellets were resuspended by 1 ml of physiological serum then the chloroform-methanol (2:1) were added, then the tube contents were centrifuged and the lower phase was collected and dried under nitrogen. 1ml of 14% BF3/MeOH reagent was added. The mixture was heated at $100^{o}C$ for 1 hour. Then it was cooled to room temperature and methyl esters were extracted twice in the hexane phase, following the addition of 1 ml H_2O. The aliquot was evaporated to dryness under nitrogen and re-diluted with 75 µL of n-hexane.

4. ANALYTICAL METHODS

Gas Chromatography Conditions

Gas chromatography analyses were performed using an Agilent 7890A system (Agilent Technologies, Spain) equipped with a flame ionization and autosampler. Separation of fatty acid methyl esters was carried out on a SupraWAX-280 capillary column (30m × 0.25µm × 0.25mm I.D.) coated with a stationary phase (polyethylene glycol 100%) from Teknokroma (Barcelona, Spain). The operating

conditions were as follows: the split-splitless injector was used in split mode with a split ratio of 1:10. The injection volume of the sample was 1μL. The injector and detector temperatures were kept at 220^OC and 300^OC, respectively. The temperature program was as follows: initial temperature 120^OC for 1 min., increased at 15 ^{O}C/min to 210^OC and held at this temperature for 42 min (total running time: 49 min.). For safety reasons, helium was used as the carrier gas, with a head pressure of 300 kPa that referred to a linear velocity of 27.5 cm/s at 140^OC. Detector gas flows: H_2, 40 ml/min; make-up gas (N_2), 40ml/min; air, 450ml/min. Data acquisition processing was performed with HP-chemstation software.

Analysis of Fatty Acid Methyl Esters (FAMEs)

Samples were determined in duplicate. The identities of sample methyl ester peaks were determined by comparison of their relative retention times with those of well-known standards. The results are expressed in relative amounts (percentage of total fatty acids).

The fatty acid composition of fetal bovine serum was also analyzed. The same batch of a commercial fetal bovine serum (FBS) was used in all the experiments to prevent any interference of culture procedure in the fatty acid composition of fibroblast.

Determination of Total Protein Concentrations

The total protein concentrations were quantified using a microplate spectrophotometer (Thermo Multiskan EX). Sample protein concentrations were compared with those of standard protein concentrations and the absorbance was measured at 630 nm.

Whole proteins were obtained by taking 50 μl of an aliquot sample from the cell suspension that used to determine the composition of fatty acids; the cell

suspensions were then centrifuged at 1,200 rpm for 5 min at 4°C and resuspended in 0.1 ml ice cold lysis buffer (Complete™ protease inhibitor cocktail tablet in 50 ml of 0.05 M Hepes buffer solution, 0.05% v/v Triton X-100, and 625 µM PMSF). The cells were sonicated twice for 15 seconds in a sonifier from Branson (Danbury, CT, USA). The samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatants were removed. The protein concentration of the cell lysates was measured using a modified Lowry method, with bovine serum albumin (BSA) concentrations as the protein standard. Briefly, the standards were prepared by the dilution of *Protein Standard Solution* in deionized water to a final volume of 250µL, as shown in **Table III.2**. The blank was prepared adding 250µL of water. Samples were analyzed at 1:50 dilution in water and the Lowry Reagent was added and mixed in each tube and left for 20 minutes. Then the Folin & Ciocalteau's Phenol Reagent Working Solution was added and mixed in each tube to allow colour to develop during 30 minutes. The content of each tube was transferred to a 96-well plate and all samples were quantified in duplicates and averages were used to perform the quantification. Absorbancies were read at 630 nm in the spectrophotometer.

Table III.2. Standards preparation for calibration curve.

Protein Concentration(µg/mL)	Protein Standard Solution (µL)	Water (µL)
0	0	250
50	31.2	218.7
100	62.5	187.5
200	125	125
300	187.5	62.5
400	250	0

Statistical Analysis

Results were described by means frequencies and percentages for qualitative variables and mean ± Standard Deviation (SD) or Standard Error of mean (SE). Data were analyzed using ANOVA models, adjusting the effect of studied factor by level of protein. For these inferential analyses, a non-parametrical approach by means rank transformation was applied.

Statistical significance was considered at $P<0.05$. Analyses were carried out using the SPSS program for MS Windows (version 15).

5. RESULTS

Fatty acid composition in nasal mucosa and nasal polyps

The fatty acid composition of fibroblasts from control nasal mucosa and nasal polyps are shown in **Table III.3**.

The omega-6 fatty acids, dihomo-gamma-linolenic acid (C20:3) and AA (C20:4), and the omega-3 fatty acids, DPA (C22:5) and DHA (C22:6), were significantly higher in nasal polyp fibroblasts than in fibroblasts derived from nasal mucosa. In contrast, oleic acid (C18:1), gamma-linolenic acid (C18:3), and eicosadienoic acid (C20:2) were significantly lower in nasal polyp fibroblasts than in those cultured from nasal mucosa.

Fatty acid composition was also analyzed and compared according to the biochemical classification into monosaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA). The percentage of MUFA was significantly lower in nasal polyp fibroblasts compared

Table III.3. Changes in the fatty acid composition of total lipids from fibroblasts in different patient groups.

Fatty Acid	NM N = 6	NP N = 12	NP-ATA N = 6	NP-AIA N = 6
14:0	1,38 ± 0,76	1,58 ± 0,93	1,44 ± 1,04	1,72 ± 0,82
14:1	0,15 ± 0,09	0,1 ± 0,03	0,09 ± 0,03	0,10 ± 0,03
16:0	17,34 ± 2,53	17,78 ± 2,96	16,74 ± 3,80	18,83 ± 1,11[f]
16:1	2,87 ± 0,74	3,02 ± 1,06	2,55 ± 1,17	3,50 ± 0,69[d]
18:0	16,72 ± 0,95	16,80 ± 1,67	17,25 ± 1,87	16,35 ± 1,36
18:1	33,38 ± 2,77	31,83 ± 2,78[c]	31,80 ± 3,71	31,86 ± 1,45
18:2n6	2,15 ± 1,14	1,80 ± 0,64	1,60 ± 0,53	2,01 ± 0,70
18:3n6	0,63 ± 0,38	0,40 ± 0,20[c]	0,36 ± 0,21	0,43 ± 0,19
18:3n3	0,24 ± 0,14	0,18 ± 0,10	0,19 ± 0,10	0,16 ± 0,10
20:0	0,34 ± 0,09	0,31 ± 0,09	0,32 ± 0,09	0,30 ± 0,09
20:1n9	1,51 ± 2,16	1,58 ± 3,52	2,40 ± 4,90	0,76 ± 0,35[f]
20:2n6	2,36 ± 0,56	1,61 ± 0,51[a]	1,81 ± 0,45	1,41 ± 0,51[e]
20:3n6	1,50 ± 0,38	1,73 ± 0,35[a]	1,64 ± 0,40	1,81 ± 0,26
20:4n6	5,97 ± 2,12	7,20 ± 1,55[a]	7,39 ± 1,91	7,00 ± 1,10
20:3n3	0,16 ± 0,11	0,18 ± 0,19	0,23 ± 0,26	0,14 ± 0,03
20:5n3	0,66 ± 0,24	0,68 ±0,37	0,76 ±0,40	0,61 ±0,34
22:0	0,77 ± 0,20	0,69 ±0,45[a]	0,79 ± 0,63	0,58 ± 0,05[f]
22:1n9	3,57 ± 1,80	3,03 ± 1,84	2,79 ± 2,47	3,27 ±0,85[f]
22:5n3	1,83 ± 0,40	2,11 ± 0,39[c]	2,11 ± 0,40	2,11 ± 0,39
24:0	1,86 ± 0,49	1,74 ± 0,38	1,77 ± 0,50	1,71 ± 0,20
22:6n3	2,69 ± 0,72	3,41 ± 1,10[c]	3,44 ± 1,14	3,38 ± 1,10
24:1	1,90 ± 0,54	2,24 ± 1,07	2,53 ± 1,37	1,96 ± 0,55[f]
SFA	38,41 ± 2,69	38,90 ± 2,72	38,32 ± 3,53	39,49 ± 1,44
MUFA	43,39 ± 1,75	41,81 ± 2,75[b]	42,16 ± 3,54	41,45 ± 1,65
PUFA	18,20 ± 3,41	19,29 ± 2,62	19,53 ± 3,47	19,06 ± 1,43
TOTAL UFA	61,59 ± 2,69	61,10 ± 2,72	61,68 ± 3,53	60,51 ± 1,44
SFA/UFA	0,63 ±0,07	0,64 ± 0,07	0,63 ± 0,09	0,65 ± 0,04
Omega 3	5,59 ± 1,09	6,56 ± 1,65	6,73 ± 1,78	6,40 ± 1,54
Omega 6	12,61 ± 3,02	12,73 ± 2,14	12,80 ± 2,67	12,66 ± 1,51
6/ 3	2,33 ± 0,68	2,05 ± 0,57	2,00 ± 0,56	2,10 ± 0,60

Values given as mean ± standard deviation (SD). MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UFA, unsaturated fatty acids; SFA, saturated fatty acids. NM, nasal mucosa; NP, nasal polyp; NP-ATA, nasal polyp asthmatic aspirin tolerants; NP-AIA, nasal polyp asthmatic aspirin intolerants. [a] $P< 0.001$, [b] $P< 0.01$, and [c] $P< 0.05$, as compared to mucosa. [d] $P< 0.005$, [e] $P< 0.02$, and [f] $P< 0.05$, as compared to NP-ATA. [d] $P< 0.005$, [e] $P< 0.02$, and [f] $P< 0.05$, as compared to NP-ATA.

with fibroblasts derived from nasal mucosa, whereas the percentage composition of PUFA in nasal polyp was significantly higher than in mucosa. There were no differences in the percentage of SFA (**Table III.3**).

The comparison between the fatty acid composition of fibroblasts from aspirin-tolerant and aspirin-intolerant patients is also shown in **Table III.3**. The percentages of fatty acid composition in palmitic acid (C16:0), and palmitoleic acid (C16:1) were significantly higher in fibroblasts from patients with nasal polyps and aspirin intolerance than in fibroblasts derived from nasal polyps of aspirin-tolerant patients. In contrast, the percentage of eicosadienoic acid (C20:2 n6) was significantly lower in fibroblasts from aspirin-intolerant patients, compared with those cultured from aspirin-tolerant subjects.

Effects of aspirin on fatty acid composition

The effect of aspirin on the fatty acid composition of fibroblasts from nasal mucosa and nasal polyps is shown in **Table III.4**. There was only one statistically significant difference in the percentage composition of 20:3n6, between the mean of an induced aspirin fibroblast and its basal status in aspirin-tolerant patients. The lipid changes in fibroblasts from asthmatic and non-asthmatic patients were accompanied by changes in the amount of total proteins used to normalize the fatty acid composition.

Effects of Interleukin-1ß on Fatty Acid Composition

The effect of Interleukin-1β on the fatty acid composition of fibroblasts from nasal mucosa and nasal polyps is shown in **Table III.5**.

There was a significant difference before and after stimulation with IL-1 in aspirin-tolerant and aspirin-intolerant asthmatics fatty acid composition. In non-asthmatics, the fatty acid contents increased significantly in palmitic acid (C16:0)

Table III.4. Significance of the mean difference of fatty acid composition between control subjects and subjects stimulated with aspirin from fibroblasts of different group of patients.

Fatty Acid	NM N = 6	NP N = 12	NP-ATA N = 6	NP-AIA N = 6
16:0	0,03 ± 1,31	-1,44 ± 1,17	-0, 38 ± 2,30	-0,19 ± 0,43
16:1	-0,16 ± 0,46	-0,14 ± 0,38	-0,04 ± 0,62	-0,08 ± 0,43
18:0	-0,46 ± 0,55	0,47 ± 0,63	0,29 ± 0,91	0,27 ± 0,85
18:1	0,83 ± 1,52	0,96 ± 1,13	2,76± 1,83	-0,23 ± 0,90
18:2n6	-0,34 ± 0,70	-0,12 ± 0,24	0,13 ± 0,25	-0,31 ± 0,44
18:3n6	-0,09 ± 0,24	-0,09 ± 0,08	-0,13 ± 0,12	-0,03 ± 0,12
18:3n3	0,06 ± 0,08	-0,04 ± 0,04	-0,02 ± 0,06	-0,08 ± 0,06
20:2n6	0,26 ± 0,34	0,13 ± 0,22	0,36 ± 0,23	-0,03 ± 0,32
20:3n6	-0,16 ± 0,20	0,24 ± 0,13	0,55 ± 0,20[a]	-0.09 ± 0,14
20:4n6	-0,19 ± 1,07	0,88 ± 0,64	1,66 ± 1,13	-0,16 ± 0,63
20:3n3	-0,11 ± 0,06	-0,09 ± 0,08	-0,18 ± 0,15	0,00 ± 0,02
20:5n3	0,12 ± 0,12	0,07 ± 0,14	0,05 ± 0,23	0,08 ± 0,21
22:5n3	-0,08 ± 0,25	0,12 ± 0,15	0,25 ± 0,20	-0,02 ± 0,24
22:6n3	0,06 ± 0,46	0,67 ± 0,46	0,63 ± 0,68	0,55 ± 0,67
Omega 3	0,05 ± 0,70	0,72 ± 0,67	0,72 ± 1,03	0,53 ± 0,99
Omega 6	-1,24 ± 1,55	1,04 ± 0,87	2,57 ± 1,52	-0,62 ± 0,86
6/ 3	0,05 ± 0,07	0,01 ± 0,07	-0,05 ± 0,09	0,07 ± 0,11

Values given as a mean difference of fatty acid composition percentages before and after aspirin stimulation ± standard error of mean (SEM). NM, nasal mucosa; NP, nasal polyp asthmatics; NP-ATA, nasal polyp asthmatic aspirin tolerant; NP-AIA, nasal polyp asthmatic aspirin intolerant. [a] P< 0.05 as compared to the basal status of the same sample before aspirin stimulation.

($p<0.05$), and alpha-linolenic acid (C18:3n3) ($p<0.02$), while decreased significantly in dihomo-gamma-linolenic acid (C20:3n6) ($p<0.05$), arachidonic acid (C20:4n6) ($p<0.05$) and eicosatrienoic acid (C20:3n3) ($p<0.05$). In aspirin-

Table III.5. Significance of the mean difference of fatty acid composition between control subjects and subjects stimulated with interleukin-1beta from fibroblasts of different group of patients.

Fatty Acid	NM N = 6	NP N = 12	NP-ATA N = 6	NP-AIA N = 6
16:0	3.00 ± 1.37[a]	0.66 ± 1.17[b]	-0.25 ± 2.32	1.72 ± 0.43[c]
16:1	0.39 ± 0.48	0.28 ± 0.38	0.46 ± 0.62	0.23 ± 0.43
18:0	-0.65 ± 0.58	-0.28 ± 0.63	-0.44 ± 0.92	-0.50 ± 0.84
18:1	1.60 ± 1.59	-1.25 ± 1.13	3.56 ± 1.84	-0.34 ± 0.89
18:2n6	-0.54 ± 0.73	-0.04 ± 0.24	0.21 ± 0.25	-0.24 ± 0.43
18:3n6	0.13 ± 0.25	-0.05 ± 0.08	-0.04 ± 0.12	-0.04 ± 0.12
18:3n3	0.15 ± 0.09[b]	-0.02 ± 0.04	-0.02 ± 0.06	-0.02 ± 0.06
20:2n6	0.08 ± 0.36	0.16 ± 0.22	0.43 ± 0.23[a]	0.01 ± 0.32
20:3n6	-0.39 ± 0.21[a]	0.16 ± 0.13	0.36 ± 0.21	-0.07 ± 0.14
20:4n6	-2.00 ± 1.12[a]	0.50 ± 0.64	0.77 ± 1.14	0.01 ± 0.63
20:3n3	-0.14 ± 0.07[a]	-0.12 ± 0.08	-0.27 ± 0.15[a]	0.02 ± 0.02
20:5n3	0.14 ± 0.13	0.02 ± 0.14	-0.05 ± 0.23	0.08 ± 0.20
22:5n3	-0.31 ± 0.26	0.00 ± 0.15	0.01 ± 0.21	-0.03 ± 0.24
22:6n3	-0.29 ± 0.48	0.25 ± 0.46	0.33 ± 0.69	0.01 ± 0.66
Omega 3	-0.45 ± 0.73	0.13 ± 0.67	0.00 ± 1.03	0.05 ± 0.98
Omega 6	-2.72 ± 1.62	0.73 ± 0.87	1.73 ± 1.54	-0.33 ± 0.85
6/ 3	0.07 ± 0.08	-0.02 ± 0.07	-0.08 ± 0.09	0.01 ± 0.11

Values given as a mean difference of fatty acid composition percentages before and after interleukin-1beta stimulation ± standard error of mean (SEM). NM, nasal mucosa; NP, nasal polyp asthmatics; NP-ATA, nasal polyp asthmatic aspirin tolerant; NP-AIA, nasal polyp asthmatic aspirin intolerant. [a] $P < 0.05$, [b] $P < 0.02$, [c] $P < 0.002$ as compared to the basal status of the same sample before aspirin stimulation.

tolerant asthmatics, there was a significant increase in fatty acid contents of eicosadienoic acid (C20:2n6) ($p<0.05$), while there was a decrease in fatty acid contents of eicosatrienoic acid (C20:3n3) ($p<0.05$).

In aspirin-intolerant asthmatics, there was a significant increase in the fatty acid contents of palmitic acid (C16:0) ($p<0.002$). In fibroblasts of patients with nasal polyps, fatty acid contents increased significantly in palmitic acid (C16:0) ($p<0.02$).

Fatty acid composition of foetal bovine serum (FBS)

The fatty acid composition of FBS is shown in **Table III.6**. The fatty acids C16:0, C18:0 and C18:1n9 were found in high percentages, while linoleic acid, arachidonic acid, and docosahexanoic acid were present in lower amount. As mentioned before, the same batch of a commercial FBS was used in all the experiments to prevent any interference of culture procedure in the fatty acid composition of fibroblast.

6. DISCUSSION AND CONCLUSIONS

The concentration of free AA in resting cells is commonly described as "low". In some inflamed tissues, such as the skin of patients with psoriasis, free AA is abundant, but in healthy skin it is scarcer (Ikai, 1999). Similar results have been reported in fibroblasts from hypertrophic scars as compared to normal dermis (Nomura, 2007). These observations suggest that the level of AA increases under conditions of inflammation and remodelling, thereby facilitating the subsequent synthesis of PGs, leukotrienes and lipoxins.

We used fibroblasts from nasal polyps to study the impact of inflammation and aspirin intolerance in fatty acid composition, because previous studies have shown very low production of PGE_2 in nasal polyps from both aspirin-tolerant and aspirin-intolerant patients (Perez-Novo, 2005, Kowalski, 2000, Roca-Ferrer, 2011). Similarly, other studies have also shown a significantly low production of PGE_2 in cultured bronchial fibroblasts from AIA (Pierzchalska, 2003).

Table III.6. Total fatty acid composition of the fetal bovine serum used.

Fatty acid	Mean ± SD	Fatty acid	Mean ± SD
14:0	2.45 ± 0.45	20:2n6	0.24 ± 0.02
14:1	0.39 ± 0.05	20:3n6	1.81 ± 0.07
16:0	24.97 ± 0.14	20:4n6	5.31 ± 0.20
16:1	2.81 ± 0.09	20:3n3	0.57 ± 0.01
18:0	14.04 ± 0.08	20:5n3	0.97 ± 0.04
18:1n9	23.86 ± 1.03	22:0	1.07 ± 0.05
18:1n7	5.44 ± 0.16	22:1n9	0.57 ± 0.02
18:2n6	6.74 ± 0.18	22:5n3	0.39 ± 0.01
18:3n6	0.50 ± 0.04	24:0	1.08 ± 0.12
18:3n3	0.53 ± 0.03	22:6n3	3.48 ± 0.18
20:0	0.41 ± 0.03	24:1	1.54 ± 0.20
20:1n9	0.75 ± 0.04		

The sample was analyzed in triplicate.

Previous studies have shown that PGE_2 production in inflammatory situations is directly related to the level of expression of COX-2 (Harizi, 2008). Other studies have also reported that the induction of COX-2 by inflammatory mediators in human lung fibroblasts does not simply result in an increase in all the prostanoids that a given cell can produce. Instead, there is a shift in the balance of PGs toward the preferential production of prostacyclin and PGE_2 (Brock, 1999). Based on these observations, we reasoned that the altered production of PGE_2 in airway fibroblasts from nasal polyps of asthma patients might be due, at least in part, to an insufficient amount of AA available to the COX-2 enzyme.

Our results do no support this hypothesis. In fact, the percentage of AA present in fibroblasts derived from an inflamed tissue (nasal polyps) was significantly higher that in those cultured from non-inflamed nasal mucosa. These results concur with others that show an increased production of AA in airway inflammatory processes such as cystic fibrosis, a disease characterized by a

chronic inflammatory process affecting both the lower (bronchiectasis) and upper airways (chronic rhinosinusitis and nasal polyps). After performing nasal tissue biopsies, significantly higher levels of AA in cystic fibrosis and asthma patients compared with healthy control subjects were reported (Freedman, 2004). Interestingly, it was also found that COX-2 mRNA and protein were markedly up-regulated in NP from cystic fibrosis patients (Roca-Ferrer, 2006); these findings contrast with the lack of expression of COX-2 mRNA and COX-2 protein in NP from asthma patients. As expected, in cystic fibrosis the increased release of AA, together with the up-regulated COX-2, results in an enhanced production of PGE_2, as has been demonstrated in saliva, exhaled air and urine (Lucidi, 2008, Strandvik, 1996, Rigas, 1989).

To our knowledge, only one *ex vivo* study has assessed the fatty acid composition in the cells of asthma patients (De Castro, 2007). In contrast with our study, the authors reported significantly lower levels of AA in platelets isolated from asthma patients, compared with those obtained from health subjects. The reasons for this discrepancy are unclear, although it is most probably explained by the differences in the cells, fibroblasts and platelets that were used. Significantly, COX-2 platelets make a very limited contribution to prostanoid production (Riondino, 2008).

Our study's second finding was that, in addition to the increased production of omega-6 fatty acid AA, there were also higher levels of omega-3 fatty acids DPA (C22:5) and DHA (C22:6) in the fibroblast derived from NP. The membranes of most cells contain large amounts of AA, compared with other potential prostaglandin precursors, including eicosapentaenoic acid (EPA); so, our finding explains why AA is usually the principal precursor of eicosanoid synthesis, and also why the series-3 prostaglandins (PG_3) that have EPA as their precursor are formed at a slower rate than series-2 prostaglandins (PG_2). These differences usually result in an increased production of omega-6, in contrast with the low formation of omega-3 in inflammatory diseases. When omega-3 exists in high amounts in cells, it can decrease the levels of AA in the membranes of

inflammatory cells, so there will be less substrate available for the synthesis of pro-inflammatory eicosanoids (Harizi, 2008, Calder, 2008). In addition, EPA competitively inhibits the oxygenation of AA by cyclooxygenases (Obata, 1999). Overall, by means of various mechanisms, omega-3 fatty acids can reduce the production of prostanoids such as PGE_2.

Based on these observations, several studies have evaluated the effects of diet manipulation in the treatment of inflammatory diseases, including those, such as asthma, that affect the airways. The metabolism of PUFA is highly dependent on the availability of lipid precursors. The AA pool for eicosanoids can only be slowly influenced by dietary omega-6 PUFA. In contrast, the omega-3 PUFA pool is usually smaller and can be modified more rapidly by dietary omega-3 PUFA supplementation (Galli, 2009).

Various uncontrolled fish oil trials have shown clinical benefits in asthma. However, a more recent report covering a large number of studies concluded that "no definitive conclusion can yet be drawn regarding the efficacy of omega-3 FA supplementation as a treatment for asthma" (Schachter, 2004). Another review and meta-analysis concluded that it is unlikely that supplementation with omega-3 plays an important role in the prevention of asthma and allergic diseases (Anandan, 2009).

Our finding of an increased production of omega-3 DHA fatty acid in fibroblasts coming from an inflamed nasal tissue contrasts with previous studies showing the opposite effect. A very low production of DHA in the nasal tissue of cystic fibrosis patients (Freedman, 2004), and platelets of asthma patients (De Castro, 2007) was reported. The reasons for these differences are unclear.

Recent studies support the notion that PGE_2 exerts anti-inflammatory rather than pro-inflammatory actions in the lung (Vancheri, 2004). We are tempted to speculate that the increased production of omega-3 in nasal polyps competitively inhibits the oxygenation of COX-2 and results in a reduced production of the anti-

inflammatory PGE_2. If this hypothesis is true, any attempt to increase the amount of cell-membrane omega-3 fatty acids by increasing the dietary intake of EPA would result in deleterious rather than salutary effects in asthma patients, particularly in AIA.

Interestingly enough, a diet supplemented with fish oil for 6 weeks was associated with a reduction in prostanoid production, a mild clinical deterioration and increased bronchial obstruction in a group of aspirin-intolerant asthma patients (Picado, 1988). This observation concurs with a recent study reporting that fish oil supplementation in a mouse model of asthma led to a significant suppression of the production of PGE_2, associated with both an enhanced lung inflammatory response and an increased release of pro-inflammatory cytokine IL-5 and IL-13 (Yin, 2009). All in all, these results bring into question the proposed potential protective role of fish oil supplementation in the treatment of airway inflammatory processes such as asthma and chronic rhinosinusitis with nasal polyps.

Aspirin-intolerant asthma is characterized by a persistently elevated production of cysteinyl leukotrienes (Cys-LTs) in a steady state, due to the up-regulation of the 5-lipoxygenase pathway enzymes (Stevenson, 2006). The release of Cys-LTs is further enhanced when these patients are challenged with aspirin and suffer an episode of bronchoconstriction (Stevenson, 2006). The mechanism responsible for the increased release of Cys-LTs is only partially known, although the inhibition of COX-1 appears to be the crucial precipitating event (Stevenson, 2006, Picado, 2006). In contrast, selective COX-2 inhibitors are usually well tolerated, do not increase the release of Cys-LTs and do not cause broncho-constriction (Stevenson, 2006). Diversion of AA from the COX-1 pathway to the 5-LO pathway has been suggested as an explanation for the increased release of Cys-LTs after aspirin exposure (Stevenson, 2006). Whether this AA diversion is also accompanied by an increase in the release of AA from membrane phospholipids is a possibility that has not yet been examined. We assessed the effects of aspirin on fatty acid composition in fibroblasts from aspirin-tolerant and

aspirin intolerant patients and could not find any change in the levels of AA. This finding suggests that aspirin does not enhance the release of 5-lipoxygenase metabolites by increasing the amount of AA available to the lipooxygenase enzymes. We compared the fatty acid composition of fibroblasts derived from NP of aspirin-tolerant and aspirin-intolerant patients and we found only an increase in the percentage composition of palmitic acid (C16:0).

Interleukin 1 beta (IL-1) is a pro-inflammatory cytokine produced by a variety of cells in the body (Dinarello, 1996). It plays an important role in a number of chronic and acute inflammatory diseases including asthma. It was reported that bronchoalveolar lavage fluid from patients with symptomatic asthma contains significantly elevated levels of a number of proinflammatory cytokines including IL-1 (Mattoli, 1991). An increased production of PGE2 is marked upon stimulation of cultured human airway smooth muscle cells by IL-1 . This release of PGE2 after IL-1 treatment was accompanied by a corresponding increase in COX activity (Pang, 1997).

Our results show a decrease production in the fatty acid composition of IL-1 -induced-cells from healthy subjects in the fatty acids: C20:3n6, 20:4n6, and 20:3n3.

When the culture medium contains an adequate supply of lipids, fatty acids are suppressed, and most of the cellular lipids are derived from the material that is taken up from the medium (Spector, 1980). As shown in **Table III.6**, the FBS in the medium that we used in cell culture containing different amounts of various fatty acids with the highest percentage in C16.0 then C18:1n9, then less amount but considerable in C18:0, C20:4n6, C18:1n7, C18:2n6.

Since it was found that the serum in the culture medium contains considerable amount of fatty acids that may affect cultured cells, we supplied the medium with only 4% FBS, the cells grow and proliferate normally but this low percentage may

affect the morphology of the fibroblast cells. Further studies are required to determine the role of all fatty acids in this type of cells.

In summary, the objective of this study was to investigate whether the previously reported low release of PGE_2 in fibroblasts from asthma patients could be caused by a reduced supply of AA. Our data shows an increased production of the omega-6 AA and the omega-3 DPA and DHA fatty acids in nasal polyp fibroblasts compared with nasal mucosa fibroblasts. Whether the increased production of omega-3 fatty acids can contribute to reducing PGE_2 production in nasal polyps by competitively inhibiting COX-2 and reducing the levels of AA available to the COX-2 enzyme in the membranes of the inflammatory and structural cells remains to be elucidated.

Study 2.
Quantification of Major Urinary Metabolites of PGE$_2$ And PGD$_2$ in Cystic Fibrosis: Correlation With Disease Severity

1. ABSTRACT

Background. Prostaglandins (PG)E2 and (PG)D2 are cyclooxygenase (COX) products that are important in human pathophysiology. They play a crucial role in many diseases including cystic fibrosis (CF). Cystic fibrosis transmembrane conductance (CFTR) alterations are involved in the overproduction of prostaglandins (PG) in CF *in vitro*.

Objective. We assessed the relationship between PGE-M and PGD-M urinary metabolites of PGE_2 and PGD_2 and CF severity.

Methods Twenty-four controls and 35 CF patients were recruited. PGE-M and PGD-M levels were measured by liquid chromatography/mass spectrometry and results were expressed as median and 25th-75th interquartile of ng/mg creatinine (Cr).

Results. PGE-M (15.63;9.07-43.35ng/mgCr) and PGD-M (2.16;1.43-3.53ng/mgCr) concentrations were higher in CF than in controls: PGE-M, (6.63;4.35-8.60ng/mgCr); PGD-M (1.23; 0.96-1.54ng/mgCr). There was no correlation between metabolite levels and spirometric values. Patients with pancreatic insufficiency (n=29) had higher PGE-M levels (19.09;9.36-52.69ng/mgCr) than those with conserved function (n=6) (9.61; 5.78-14.34ng/mgCr). PGE-M levels were associated with genotype severity: mild (7.14;5.76-8.76, n=8), moderate (16.67;13.67-28.62 ng/mgCr, n=5) and severe (22.82;10.67-84.13ng/mgCr).

Conclusions. Our study confirms the key role of CFTR in the regulation of the cyclooxygenase pathway of arachidonic acid metabolism found in *in vitro* studies. The assessment of PGE-M and PGD-M by LC-MS/MS assay is precise and accurate. The method can detect 40pg of both metabolites. The measurement of PGE-M and PGD-M may serve as potential biomarkers in

inflammatory conditions and   may be useful to assess CF severity and to evaluate efficacy of therapies aimed to improve a defective CFTR.

2. INTRODUCTION

Cystic fibrosis (CF) is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR). One hallmark of CF is the early development of progressive lung disease, characterized by chronic bacterial infection and inflammation. It was long accepted that bacterial infections give rise to pro-inflammatory substances produced by various cells types, particularly neutrophils, which are responsible for the lung inflammatory process observed in CF (Lyczak, 2002). Various studies have suggested, however, that inflammation may even occur when no infection is present (Verhaeghe, 2007). High levels of various inflammatory cytokines and mediators have been detected in several fluids and secretions from CF patients in stable clinical conditions, without any evidence of active infection (Lucidi, 2008, Noah, 1997, Eickmeier, 2010). It has been suggested that lung inflammation apparently independent of infections may be related to abnormalities in the mechanisms that regulate inflammatory responses, associated with a defective CFTR (Hunter, 2010). These anomalies may contribute to the progression of lung damage.

CFTR mutations have been grouped into different classes, based on their predicted functional consequences for the CFTR protein (Tsui, 1992). Class I mutations affect biosynthesis, resulting in an absence of CFTR protein in the apical membrane. Class II mutations affect protein maturation through abnormal processing and trafficking, resulting in no detectable CFTR in the apical membrane. Class III mutations result in a normal amount of non-functional CFTR protein in the apical membrane. Class IV mutations affect chloride conductance, resulting in a normal amount of CFTR with some residual function in the apical membrane. Class V mutations result in a reduced synthesis of CFTR protein. Class I, II, and III mutations are usually associated with pancreatic insufficiency,

and are thus considered severe mutations, while Class IV and V mutations are usually associated with normal or slightly altered pancreatic function (Ooi, 2011, Kristidis, 1992). There appears to be a graduated risk of developing pancreatic insufficiency and pancreatitis, according to genotype severity, in patients with CF who carry mild/mild, mild/moderate-severe or moderate–severe/moderate-severe alleles (Ooi, 2011, Kristidis, 1992). An association between the severity of CFTR mutation and lung disease has been reported in some studies, resulting in a lower baseline forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) and a faster decline in lung function (Cleveland, 2009, de Gracia, 2005). In contrast, other surveys failed to find any relationship between the risk of poor lung function and CFTR genotype severity (Lai, 2004).

The eicosanoid pathway plays a crucial role in inflammation by producing bioactive products that modulate both the onset and resolution of inflammation. Arachidonic acid (AA) formation from the phophoslipid cell membrane is followed by the metabolism of AA by cyclooxygenases (COX) and lipoxygenases, leading to the production of prostaglandins (PG) and leukotrienes, respectively. These lipid mediators are involved in the regulation of many aspects of inflammation, including vascular permeability, chemotaxis of inflammatory cells, and induction of pro-inflammatory cytokines, growth factors and enzymes; they therefore play a crucial role in several lung diseases, such as chronic obstructive respiratory disease, asthma, and CF. Prostanoids such as PGE_2, and PGD_2 are among the many substances produced by a variety of structural and inflammatory cells (Smith, 2000).

COX is the key enzyme involved in the conversion of AA into PGs. Two COX isoforms have been identified: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and it is involved in the regulation of physiological processes, whereas COX-2 expression is inducible and therefore implicated in inflammatory diseases (Smith, 2000). In a previous study we reported that COX-1 and, more particularly, COX-2 expression are up-regulated in the nasal polyps of CF patients (Roca-Ferrer, 2006).

Several studies have demonstrated overproduction of PGE_2 in the bronchoalveolar lavage, saliva, urine, sputum, and exhaled air of patients with CF (Lucidi, 2008, Zakrzewski, 1987, Yang, 2012, Strandvik, 1996). A recent study has reported the presence of PGD_2 in the sputum of CF patients (Yang, 2012). Various mechanisms may be involved in the increased prostanoid release in CF, including an increased activation of the enzyme phopholipase A2 (Medjane, 2005), associated with the direct or indirect effect of a defective CFTR on AA metabolism (Carlstedt-Duke, 1986).

Interestingly, recent studies provide evidence that alterations in CFTR can be directly involved in the regulation of the COX pathway (Chen, 2012). Chen J et al. (Chen, 2012) demonstrated that a CFTR defect leads to COX-2 induction in a CF line and CFTR-/- mice, resulting in increased production of PGE_2. Moreover, various studies have identified a positive feedback loop from PGE_2 to COX-2 that is present in various cells types (Hinz, 2000, Faour, 2008). This helps maintain the continuous production of PGE_2 in normal tissues.

Chen J et al. (Chen, 2012) have proposed that CFTR acts as a negative regulator of PGE_2-mediated inflammatory responses and serves as a protective mechanism that prevents excessive tissue damage derived from enhanced production of PGE_2 during inflammatory responses. The altered regulation of CFTR in CF would result in a deficient dampened/offset regulatory mechanism of the autocrine loop, involving PGE_2-mediated COX-2 induction; this would lead to an overproduction of PGE_2, which contributes to the perpetuation of the noxious inflammatory cycle in CF tissues (Chen, 2012). All in all, these observations suggest that the measurement of PGE_2 production may help us assess the severity of the altered regulation of CFTR.

Since the direct measurement of PGE_2 and PGD_2 in biological fluids is often difficult, due to the chemical instability of these prostanoids, it is generally accepted that the most accurate index of endogenous prostanoid production in humans is the measurement of excreted urinary metabolites. Tetranor PGEM

(tPGE-M) and tetranor PGDM (tPGD-M) are metabolites of PGE_2 and PGD_2, respectively; they are present in urine and accurately reflect the biosynthesis of the two prostanoids (Duffield-Lillico, 2009, Song, 2008). Strandvik et al (Strandvik, 1996) have reported, for the first time, an increased urinary excretion of PGE-M in CF patients, revealing an increased in vivo formation of PGE_2.

The similarities in eicosanoid structure and chemical characteristics require a technique that is efficient to isolate and unambiguously identify the individual eicosanoid species. The tPGE-M and tPGD-M are two metabolites having extremely similar chemical structure (**Figure III.1**) and identical MRM transitions in tandem mass spectrometry.Their measurements and identification need a developed technique that is selective and sensitive. A developed method that allows sensitive measurement of both metabolites in urine samples was reported (Zhang, 2011). High performance liquid chromatography (LC) in conjunction with electrospray ionization-mass spectrometry (ESI-MS) allow a large number of eicosanoids in an individual sample to be measured in a short time by LC-MS-MS (Deems, 2007) with the presence of ESI, which allows the eicosanoids to be analyzed by MS directly from an aqueous sample. Here in our study we present a protocol for identifying and quantitating the urinary levels of tPGE-M and tPGD-M in cystic fibrosis patients with LC-MS/MS. In the present study we aimed to assess the relationship in patients with cystic fibrosis between prostanoid production - measured by the urinary levels of PGE-M and PGD-M - and lung function values, pancreatic function as a measure of CF severity, and genotype severity.
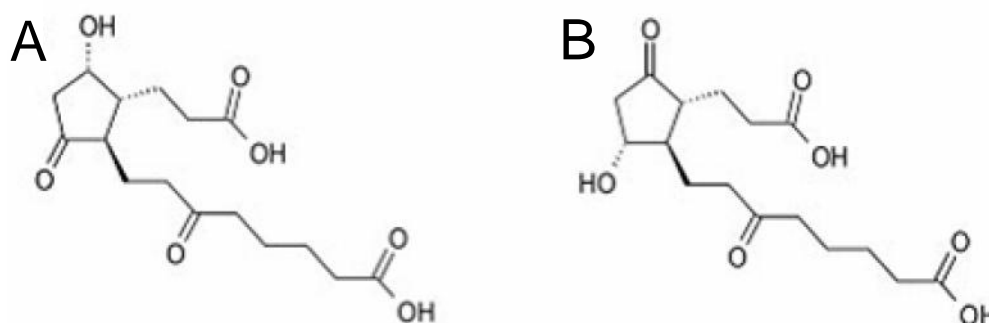
Figure III.1. (A) Structure of Tetranor-PGDM, **(B)** Structure of Tetranor-PGEM.

Role of Arachidonic Acid in Nasal polyps, Asthma and Cystic Fibrosis

3. MATERIALS AND METHOD

Chemicals

PGE-M (9,15-dioxo-11 -hydroxy-13,14-dihydro-2,3,4,5-tetranor-prostan-1,20-dioic acid), tPGE-M-d6 (11 -hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostan-1,20-dioic acid-17,17,18,18,19,19-d6), PGD-M (9 -hydroxy-11,15-dioxo-13,14-dihydro-2,3,4,5-tetranor-prostan-1,20-dioic acid), PGD-M-d6 (9 -hydroxy-11,15-dioxo-2,3,4,5-tetranor-prostan-17,17,18,18,19,19-d6-1,20-dioic acid) were purchased from (Cayman Chemical, USA). C-18 Sep-Pak (Waters, Milford, MA, USA). Methoxyamine HCl was obtained from Sigma–Aldrich (St. Louis, MO, USA). All chemical solvents used in these studies were of HPLC grade and were purchased from EM Science (Gibbstown, NJ, USA).

Subject characteristics

Twenty-four healthy subjects and 35 patients with stable CF were included in the study. The study had a cross-sectional design. Patients who regularly attended the Cystic Fibrosis Unit of Hospital Vall d'Hebron, Barcelona, Spain, were invited to participate in the study.

The diagnosis of CF was based on clinical data, an abnormal sweat test (sweat chloride >60 mmol/L), and genotypic characteristics consistent with CF. CF patients were in a stable clinical condition, defined as the absence of fever, frequent coughing, increased sputum volume, and symptoms of upper respiratory tract infection. Healthy control children were recruited from the families of the hospital workers. BMI was calculated as weight in kg/height in m^2. The demographic characteristics of patients and controls are shown in **Table III.7**.

The study protocol was approved by the Ethics Committee of our Institution (Hospital Vall d'Hebron). The patients gave their informed consent.

Table III.7. Demographic and clinical characteristics of study population.

Characteristics	Cystic fibrosis	Controls	P value
Subjects (n)	35	24	
Age (y), median (25th-75th)	10 (6-14)	9.5 (6-14.5)	NS
Male/Female, (%)	20/15 (58.5)	15/9 (62)	NS
BMI (kg/(height in m)^2)	17.90 (15.40-19.20)	18.40 (16.70-20.50)	NS
FEV$_1$ % predicted	95.7 (86-107)		
Pancreatic sufficiency (n) (%)	6 (16%)		
Patients colonized by PA (n) (%)	11 (31%)		

Values expressed as medians and interquartile range (25th-75th interquartiles) or percentages (%). BMI=body mass index. FEV$_1$= forced expiratory volume, one second. NS=not significant. PA= *Pseudomonas aeruginosa*. Y=year.

Lung and Pancreatic Exocrine Function Tests

All the CF patients had undergone evaluation of exocrine pancreatic function via the 72-hour fecal fat test (detection of steatorrhea) and fecal elastase (values <15 mg/g stool denote pancreatic insufficiency) that are routinely used to monitor the development of pancreatic insufficiency in specialized CF clinics.

Forced expiratory volume in one second (FEV$_1$) and forced vital capacity (FVC) were measured by spirometry in 33 patients, and the best of three maneuvers, expressed as percentage of predicted values, were chosen.

CFTR Mutations and Severity

The patients were genotyped using methods reported elsewhere (Alonso, 2006). They were classified into three groups (mild, moderate and severe) based on the predicted functional consequences of the CFTR protein alteration, and on the accepted premise of the dominant phenotypic effect conferred by the milder of the 2 CFTR mutations. Patients carrying Class I and II mutations in their alleles

were considered severe, those carrying Class I or II mutations in one allele associated with a Class III in the second allele were classified as moderate, and, finally, those patients with Class I or II mutations associated with a Class IV or V were considered mild. The distribution of patients according to severity is shown in **Table III.8**.

All the patients with a severe genotype (n= 22) had pancreatic insufficiency. All but one of the patients classified as moderate (n=5) also had an altered pancreatic exocrine function. In contrast, all but three of the patients carrying a phenotype considered mild (n=8) had the pancreatic function preserved.

Sample Collection and Preparation

Urinary samples were collected from the participants and kept at -80C$^{\circ}$ until analysis. Participants who have used non-steroidal anti-inflammatory drugs 72 hours prior to urinary collection were not eligible for the study. The PGE-M and PGD-M concentrations in urine were measured according to the method of Murphy (Murphy, 2004). Urinary samples were prepared as the following; to 1mL of urine, PGE-M-d6 and PGD-M-d6 internal standards were added then the mixture was acidified to pH 3 with 1 M HCl, the endogenous metabolites PGE-M and PGD-M and their deuterated internal standards were then converted to the O-methyloxime derivatives by treatment with 0.5 mL of 16% (w/v) methyloxime HCl in 1.5 M sodium acetate buffer pH 5. Following an hour incubation, the aqueous sample was diluted with 10 mL water adjusted to pH 3, the mixture was then applied to SPE cartridges of C-18 Sep-Pak (Waters, Milford, MA, USA) that had been preconditioned with 5 mL methanol and 5 mL water (pH 3). The Sep-Pak was washed with 20 mL water (pH 3) and 10 mL heptane. The endogenous PGE-M and tPGD-M and their deuterated standards were then eluted from the Sep-Pak with 5 mL ethyl acetate, and any residual aqueous material was removed from the eluate by aspiration. The eluate was then evaporated under a stream of nitrogen. The dried residue was resuspended in 50 µL of mobile phase A for injection into LC-MS/MS.

Table III.8. Mutation class and severity, and their association with exocrine pancreatic function.

Mutations	Mutation class	Severity	Number	Pancreatic
W128X/W128X	I/I	Severe	1	0
I507/Q890X	I/I	Severe	1	0
F508del/ G542X	II/I	Severe	2	0
F508del/2188AA>G	II/I	Severe	1	0
F508del/N1303K	II/I	Severe	3	0
F508del/1677delTA	II/I	Severe	1	0
F508del/2188AA>G	II/I	Severe	1	0
F508del/F508del	II/II	Severe	10	0
F508del/Q890X	II/II	Severe	1	0
F508del/E1308X	II/II	Severe	1	0
F508del/5T-12TG	II/III	Moderate	2	0
G542X/G85V	I/III	Moderate	1	0
F508del/124del23kbp	II/III	Moderate	1	0
G542X/M1137V	I/III	Moderate	1	1
I507/L206W	I/IV	Mild	1	0
F508del/L206W	I/IV	Mild	4	2
711+1G>L206W	I/IV	Mild	1	1
N1303K/3272-26A>G	I/IV	Mild	1	1
F508del/F587I	II/V	Mild	1	1

Creatinine Analysis

The urinary PGE-M and PGD-M levels in each sample are normalized using the urinary creatinine level of the sample and expressed in ng/mg creatinine. Urinary

creatinine levels are measured using a test kit from Enzo Life Sciences.

Analysis of Sample by LC-MS/MS

LC was performed on a 2.0 x 50 mm, 1.7µm particle Acquity BEH C18 column (Waters Corporation, Milford, MA, USA). Mobile phase A was 95:4.9:0.1 (v/v/v) 5 mM ammonium acetate: acetonitrile: acetic acid, and mobile phase B was 10.0:89.9:0.1 (v/v/v) 5 mM ammonium acetate:acetonitrile:acetic acid. Samples were separated by a gradient of 85–76% of mobile phase A over 6 min at a flow rate of 200µl/min prior to delivery to a ThermoFinnigan TSQ Quantum Vantage triple quadrupole mass spectrometer operating in the selected reaction monitoring (SRM) mode and equipped with an ESI source. The mass spectrometer was operated in the negative ion mode with a capillary temperature of 210 °C, a spray voltage of 3.0 kV, and a tube lens voltage of 118 V. The source collision-induced dissociation (CID) was set at 10 eV.

The mass transitions monitored for endogenous PGDM or PGEM and that of the internal standards were m/z 385 336 and 391 339, respectively were collisionally activated at 22eV under 1.5mT argon gas. Quantification of endogenous PGE-M and PGD-M used the ratio of the mass chromatogram peak areas of the m/z 336 and 339 ions. Results were normalized according to urinary creatinine concentration.

Statistical analysis

The PGE-M and PGD-M concentrations in urine are expressed as medians and interquartile range (25th-75th interquartiles). The Kruskall-Wallis test, followed the by Mann-Whitney U test, was used to compare groups. Correlation was expressed as Spearman coefficient. Data were considered to be significant if p 0.05.

3. RESULTS

Identification of Prostaglandin Metabolites by LC-MS/MS

There are four chromatographic peaks, apparently two major of PGEM isomers and two smaller of PGDM isomers, were observed in the urine samples of normal human and CF patients as shown in the mass chromatogram of the SRM analysis in **Figure III.2**.

In **Figure III.2A & B** (upper panel) the transition m/z 385   336 was monitored, the two major peaks represent the endogenous tPGE-M while the two smaller represent the endogenous PGD-M in a urine sample from normal human and cystic fibrosis patient, respectively. Two peaks were monitored due to the presence of more than one PGE-M and PGD-M methyloxime isomers. In **Figure III.2A** (lower panel), the transition m/z 391   339 for the deuterated internal standard (PGE-M-d6) was monitored, and also two peaks were observed

Assay Validation

The lower limit of detection for both metabolites (PGE-M and PGD-M) is in the range of 40 pg (signal/noise ratio of 4:1), which is about 100-fold lower than the levels in normal human urine. The assay is highly precised and accurate. The accuracy of the assay is 93%.

Urinary PGE-M and PGD-M levels in Normal Human

Normal healthy males excrete 7-40µg of tetranor PGE-M over a 24h period (Hamberg, 1972). Whereas the levels of tetranor PGDM were found to be 1.5 ± 0.3 ng/mg Cr (Song, 2008). The daily excretion of PGE-M has been reported to be higher in men than in women (Seyberth, 1976, Murphy, 2004).
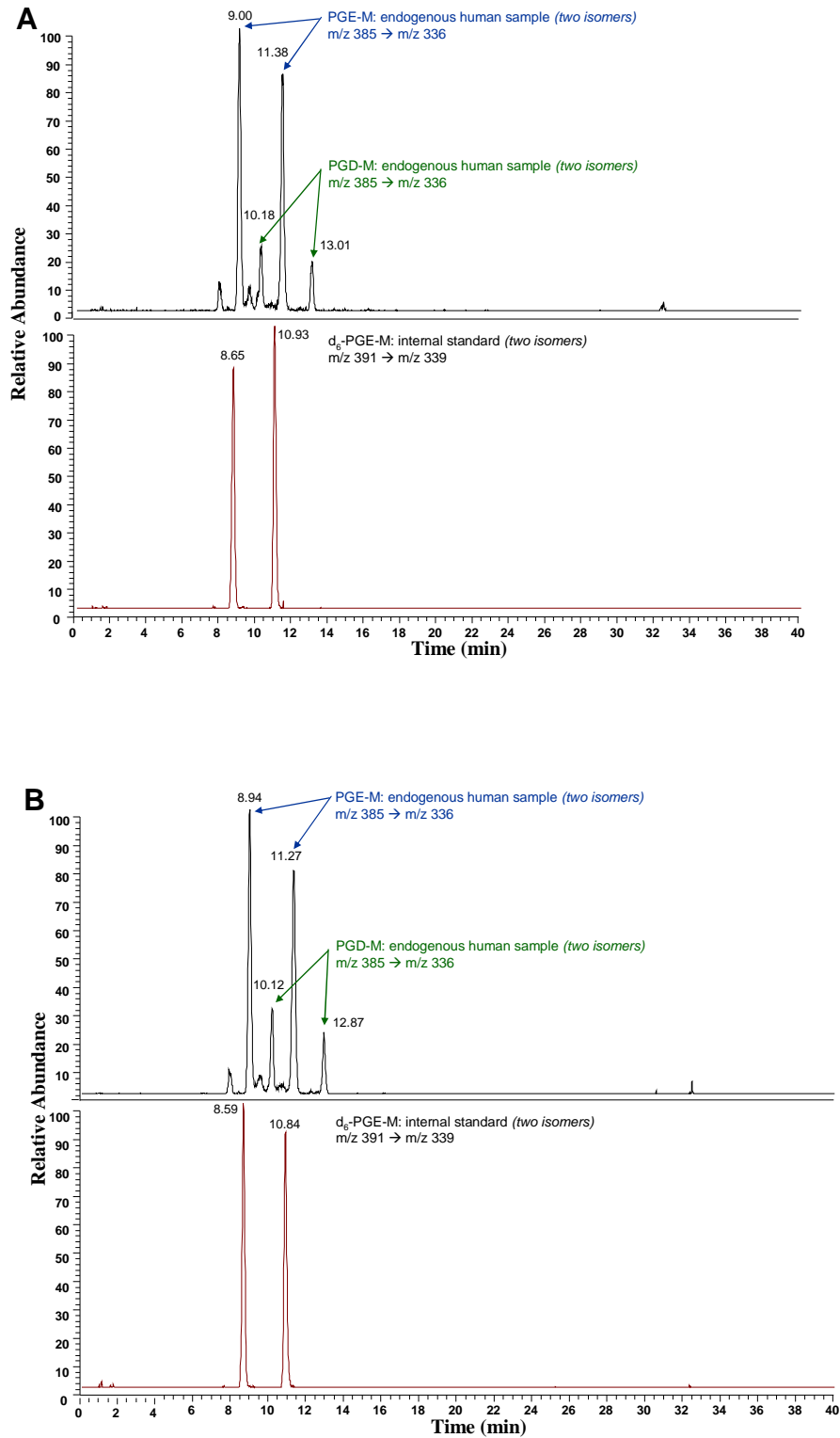
Figure III.2. **HPLC-MS/MS of major urinary PGE2 and PGD2 metabolites**. Selected reaction monitoring chromatogram of endogenous PGE-M and PGD-M (upper panel), in urine from a **(A)** normal human, **(B)** patient with cystic fibrosis. Transition of PGE-M-d6 is shown in both lower panels.

To detect the range of normal values for excretion of PGE-M and PGD-M, 24-h urine collections were obtained from 24 healthy subjects (15 men and 9 women) and were analyzed. We found that the levels of urinary PGE-M in normal, healthy men are 8.4 ± 4.9 ng/mg Cr while levels in normal, healthy women are slightly lower averaging 6.0 ± 2.1 ng/mg Cr. On the other hand, the levels of urinary PGD-M in man and women are approximatly the same, 1.4 ± 0.8 and 1.1 ± 0.3 ng/mg, respectively.

Stability of PGE-M and PGD-M in Human Urine during Storage

The PGE-M is chemically unstable (Seyberth, 1976) and may degrade and undergo dehydration after 24h at room temperature (Neale, 2008), While PGD-M was stable at room temperature for 24h (Zhang, 2011). Recently, it was detected that both were stable at 4°C for 24h, at–80ºC for 37days and after going through three freeze/thaw cycles (Zhang, 2011). However, it was reported that PGE-M in urine is stable when stored for at least 12 months at -70°C and for at least four days at 4°C (Murphy, 2004).

Urine samples that used in the study were stored at -80ºC for about 2 months days before the measurement of prostaglandin metabolites.

Analysis of PGE-M and PGD-M in Samples from Healthy Subjects and CF Patients

The mass spectral analysis of human urine revealed the abundance level of endogenous tetranor PGE-M relative to tetranor PGD-M.

Compared with those in healthy subjects, the PGE-M concentrations were markedly and significantly elevated in the urine of CF patients (**Figure III.3.A**). Similarly, PGD-M urinary levels were also significantly higher in CF patients than in healthy controls (**Figure III.3.B**). The PGE-M and PGD-M urinary concentrations were significantly correlated in both healthy and CF patients

(**Figure III.4**). There were no differences in urinary cr levels between controls (1.10; 0.80-1.45 mg/ml) and patients (0.97; 0.55-1.31 mg/ml).

A

B
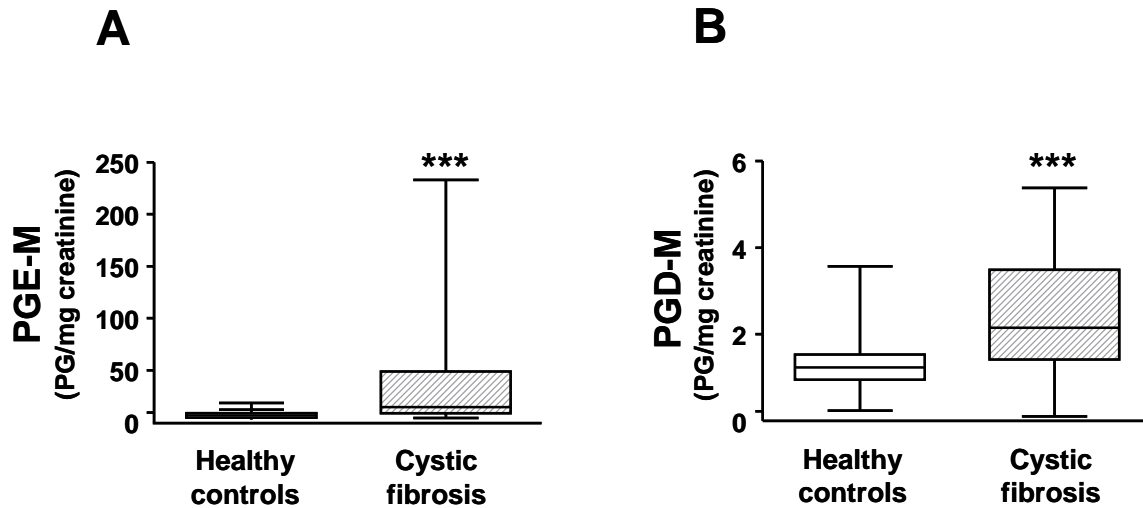
Figure III.3. **(A)** PGE-M and **(B)** PGD-M concentrations in urine of healthy controls and cystic fibrosis patients. ***P<0.001 compared to healthy controls.
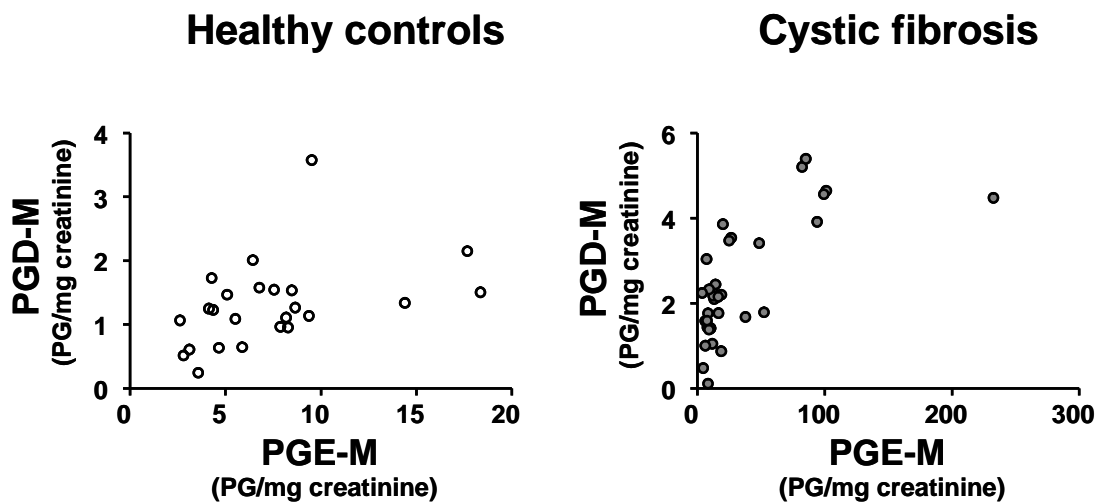
Figure III.4. Correlation between PGE-M and PGD-M urine concentrations in (A) healthy controls and (B) cystic fibrosis patients. In healthy controls P<0.01, in CF P<0.0001.

Correlations with parameters of severity

FEV$_1$ values decreased in parallel with severity. FEV$_1$ % of predicted was 93% (range 44-120) in patient carrying the severe genotype, 98% (range 90-109) in patients with moderate and 102% (range 80-117) in those with the mild genotype, but differences were not statistically different.

There was no correlation between PGE-M and PGD-M urine levels and spirometric values (FEV$_1$, FVC) (**Figure III.5**).

In patients with pancreatic insufficiency (n=29) there were higher PGE-M urinary levels (19.11: 9.95-57.85 ng/mgCr) than in patients with conserved pancreatic function (n=6) (8.64; 5.78-14.34 ng/mgCr) ($p<0.01$). Similarly, the PGD-M levels in patients with pancreatic insufficiency were higher (2.19:1.58-3.88 ng/mgCr) than in those with conserved pancreatic function (1.59:0,73-2.68 ng/mgCr) but the difference was not statistically significant.

All patients colonized by *Pseudomonas aeuriginosa* carried severe phenotypes. There were no differences in either the PGE-M or PGD-M levels between patients colonized by *Pseudomonas aeruginosa* (n=11) (PGE-M, 13.53:8.77-38.11ng/mgCr; PGD-M1.63:1.39-3.65ng/mgCr) and those who were not (n=24) (PGE-M:16.89:9.17-50.80ng/mgCr; PGD-M: 2.21:1.59-3.69ng/mgCr).

Correlations between Prostanoid Production and CFTR Gene Mutations

When the urinary levels of PGE-M levels were compared in healthy controls and patients with mild, moderate and severe genotypes, it was found that there were no differences between healthy controls and patients carrying the mild genotype. In contrast, there were statistically significant differences between moderate ($p<0.01$) and severe genotypes and healthy controls ($p<0.001$). There were also

statistically significant differences between the severe genotype and moderate
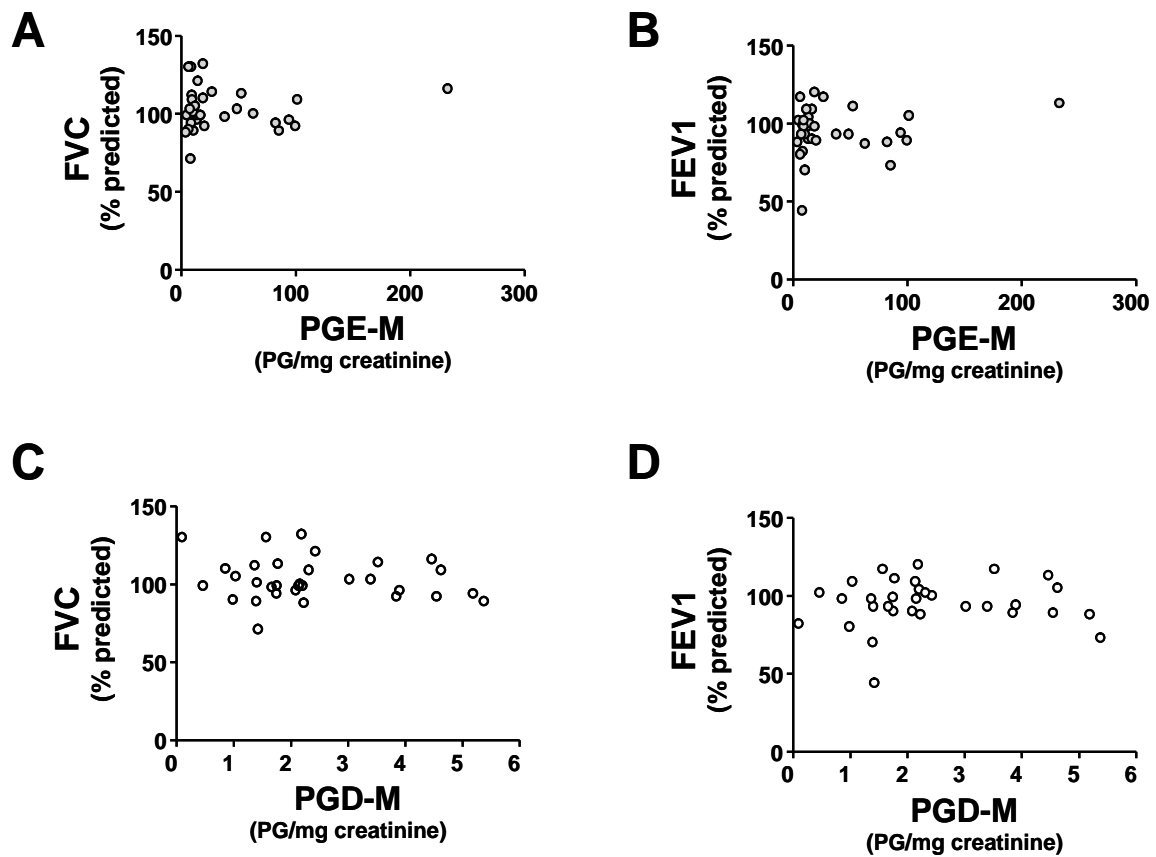
Figure III.5. Correlation between PGE-M and PGD-M urine levels and spirometric values (FEV$_1$, FVC).

(p<0.01) and mild genotypes (p<0.001), and also between moderate and mild genotypes (p<0.01) (**Figure III.6A**). There were also statistically significant differences in PGD-M levels between healthy controls and severe genotypes, but not between healthy controls and patients carrying the mild and moderate genotypes (near significance p<0.065). There were no differences, however, in urinary PGD-M values between the three groups of patients as regards genotype severity (**Figure III.6B**).
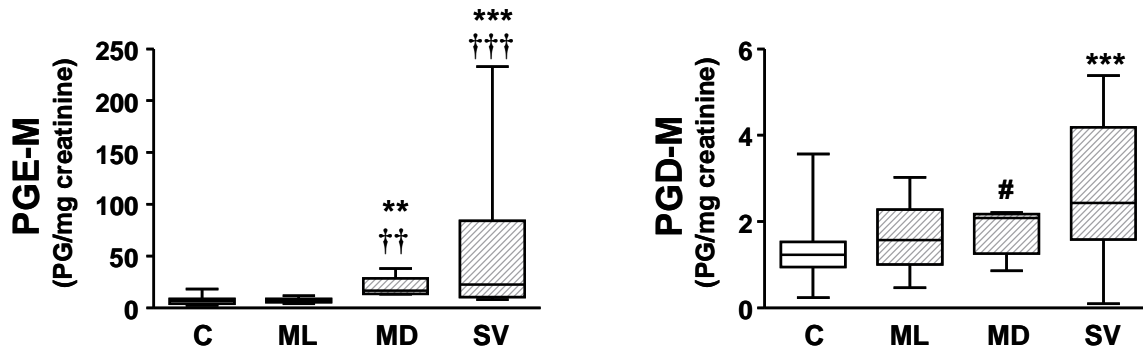
Figure III.6. Concentrations of PGE-M and PGD-M in urine of healthy controls (C) and mild (ML), moderate (MD) and severe (SV) cystic fibrosis genotypes. Results expressed as ng PG/mg creatinine. (*)P=0.065 compared to SV, **P<0.01 compared to healthy controls, ***P<0.001 compared to healthy controls. ††P<0.01 compared to ML.

5. DISCUSSION AND CONCLUSIONS

In CF, AA release is increased and this may contribute to the high levels of eicosanoids production in these patients. In contrast, concentrations of linoleic acid, a precursor of AA, are low (Strandvik, 1996, Carlstedt-Duke, 1986). The latter finding appears to be secondary to the abnormally active AA metabolisms, and if not compensated it might result in a limited AA release and reduced eicosanoid production (Strandvik, 1996). Moreover, *in vitro* and experimental animal studies have demonstrated that CFTR dysfunction induces and enhances AA metabolism, leading to an increased expression of COX-2, which results in PGE_2 overproduction (Medjane, 2005, Chen, 2012). In keeping with these observations, clinical studies reported increased levels of PGE_2 in various fluids and secretions (Roca-Ferrer, 2006, Zakrzewski, 1987, Yang, 2012, Strandvik, 1996) from CF patients.

Our study demonstrates for the first time an association between the severity of CFTR dysfunction and the production of PGE_2, in keeping with the results of experimental studies that have reported the role of CFTR in the regulation of the cyclooxygenase pathway of arachidonic acid metabolism (Medjane, 2005, Chen,

2012). In contrast with our results, in a previous study Strandvik at al (Strandvik, 1996) could not find any relationship between urinary PGE-M levels and patients' genotype severity, probably because the study included only 14 genotyped patients, almost all of them suffering from a severe form of the disease (all but one had pancreatic insufficiency).

Our study also shows a relationship between the clinical parameters of disease severity, such as pancreatic insufficiency and urinary levels of PGE-M. The six patients with conserved exocrine function suffer from a mild CFTR dysfunction – as expected, given the well-known relationship between exocrine pancreatic function and CFTR dysfunction severity (Ooi, 2011, Kristidis, 1992). In contrast, there was no correlation between tPGE-M levels and the parameters of lung function. This is not an unexpected finding, as it is generally accepted that FEV_1 has become a relatively insensitive tool for monitoring CF lung disease severity and progression, thanks to improvements in CF therapy over the last decades (Que, 2006).

Although there is *in vitro* and experimental evidence supporting the notion that alterations in CFTR can directly regulate COX-2, and therefore PGE_2 production, the potential contribution of the bacterial products and/or pro-inflammatory cytokines usually present in CF lungs should also be considered. Bacterial lipopolysaccharide can induce COX-2 in various cells (Clayton, 2005, Korhonen, 2004), and interleukin 1 (IL-1), which is increased in the airways of CF (Osika, 1999), can also contribute to the upregulation of the expression of COX-2 in lung cells (Clayton, 2005). Interestingly, bacterial products from *Pseudomonas aeruginosa*, a bacterium often found in patients with CF have been shown, in *in vitro* studies, to activate the cytoplasmic phospholipase A2 involved in AA production from membrane phospholipids and prostaglandin generation (Hurley, 2011). In our study, however, we could not find any difference in PGE-M production in patients colonized with PA compared with those who were not. This finding suggests that PA infections appear not to play a significant role in the enhanced PGE_2 production detected in CF.

PGE_2 is a lipid mediator of central importance in the regulation of inflammation. The use of COX inhibitors is broadly accepted as a therapeutic modality in inflammatory diseases. On the basis of the information available, however, it is not clear whether the inhibition of COX enzymes can be useful or deleterious in inflammatory airways diseases such as asthma (Vancheri, 2004). PGE_2 has a biphasic synthesis, firstly during onset, and secondly during resolution, so the inhibition of PGE_2 synthesis can potentially be either beneficial or harmful (Vancheri, 2004). In fact, evidence from animal studies indicates that COX enzymes and PGE_2 play protective roles in regulating airway inflammation and airway function following an allergenic stimulus (Vancheri, 2004). In contrast with asthma, treatment with COX inhibitors such as ibuprofen are able to delay the progression of CF lung disease (Konstan, 2007), a finding that supports the notion that PGs may play a role in the regulation of airway inflammation in CF patients. In these studies, however, there was no assessment of the relationship between the production of PGE_2, the severity of CFTR dysfunction and the response to ibuprofen. The mechanisms involved in the beneficial effects of ibuprofen in CF remain to be elucidated. Interestingly, in a recent study ibuprofen was shown to increase the function of F508delta CFTR in cultured human airway cells, a mechanism that may help explain the therapeutic effects of the drug (Li, 2008).

The production of PGD_2, a major COX metabolite synthesized in activated mast cells and macrophages, was also found to be elevated in CF patients, compared with healthy controls (Smith, 2000). In CF patients, however, levels of tPGD-M were much lower than those of tPGE-M and they were not associated with the severity of CFTR dysfunction. PGD_2 was first indentified as a potent mediator in asthma (Smith, 2000) but its role in CF remains to be investigated.

Our study has some methodological limitations. The classification of CF genotype severity into threes categories used in our study has a limited support in the literature. Although the three categories of genotype severity, mild,

moderate, and severe, are considered in studies aiming to determine the relationship between CFRT genotypes and their phenotypic expression, only the mild (Class IV and V) and severe phenotypes (Class I, II, III) are well defined (Ooi, 2011, Kristidis, 1992). In some studies the term moderate is applied to those genotypes mostly carrying heterozygous mutations resulting from the combination of severe class I or II and class III, IV and V mutation (Ooi, 2011, Kristidis, 1992). These combinations appear to be associated with a lower predisposition to the early development of pancreatic insufficiency (Ooi, 2011, Kristidis, 1992, Walkowiak, 2005, Ooi, 2012). However, the difference in the predisposition to develop pancreatic insufficiency between severe (homozygote Class I and/or Class II) and moderate genotypes (heterozygote Class I/ Class II and Class III, IV or V is small, particularly in comparison with the difference between severe/moderate and mild-mild (Class IV-V) genotypes (Ooi, 2011, Kristidis, 1992, Walkowiak, 2005, Ooi, 2012). We were aware of these limitations when we classified our patients into three groups. Nevertheless, we found that the production of PGE_2 appears to be at least partially associated with genotype severity, according to our classification. Our results should be interpreted with caution, however, and need to be replicated in further studies.

In addition, although our study shows a relationship between the predicted severity of CFTR dysfunction and PGE_2 production, tPGE-M urinary levels showed a large variability even in patients with the same CFTR dysfunction. This observation concurs with the variability observed in the phenotype expression of CF in patients carrying the same CFTR genotype (Zielenski, 2000). The 5-class system is useful as a conceptual framework but it has several limitations, including: (i) molecular changes in different mutations within the same class (especially Class IV and V) may have varying functional consequences, and (ii) mutations may have overlapping molecular defects which could be assigned to more than one class (Ooi, 2012, Zielenski, 2000). Moreover, it is generally accepted that environmental factors and the influence of various regulatory genes interacting with CFTR can also modify CF disease expression and progression (Zielenski, 2000). Interestingly, a recent study reported a relationship

between two polymorphisms of the COX-2 gene and parameters of lung function (Czerska, 2010). Further studies are needed to clarify whether these COX-2 polymorphisms are also involved in the variability of PGE_2 production observed in CF patients.

In summary, our study demonstrates an association between the severity of CFTR dysfunction and the production of PGE_2, a finding that supports previous experimental studies that demonstrated the key role of CFTR in the regulation of the COX pathway of AA metabolism. New therapeutic agents such as disease-modifying agents are in development to treat CF patients (Zielenski, 2000). Sensitive and accurate outcome measures are needed to test the effectiveness of these drugs in randomized clinical trials. The assay method used in this study is sensitive, precise and accurate for detecting, identifying and quantitating the PGEM and PGDM prostaglandin metabolites by LC-MS/MS. This method can detect 40 pg of both metabolites. Given the association of PGE-M urinary levels with CFTR dysfunction found in our study, we reasoned that the measurement of PGE-M in urine samples would provide a useful tool for assessing the efficacy of new therapeutic agents. Our results suggest that PGE-M and PGD-M concentrations in human urine samples may serve as potential biomarkers in inflammatory conditions and diseases particularly CF.

IV. DISCUSSION OF STUDY 1. And STUDY 2.

Arachidonic acid (AA) metabolites are among the hundreds of cellular products that are involved in the regulation of inflammatory responses. AA is formed from the cellular membrane phospholipids through the action of phospholipase A2. AA is the substrate for various enzymes that contribute to the biosynthesis of numerous products with pro and anti-inflammatory effects. The cyclooxgenase (COX) pathway has two enzymes called COX-1 and COX-2, which are involved in the biosynthesis of prostanoids such as prostaglandin E2 (PGE_2). PGE_2 plays different roles in inflammation. On the one hand, it acts as pro-inflammatory substance, while on the other hand it has anti-inflammatory actions. These opposite functions are mostly due to the different levels of cellular expression of the four PGE_2 receptors (EP1-EP4). Stimulation of EP2 and EP4 results in anti-inflammatory effects, while the activation of EP3 produces pro-inflammatory actions.

Chronic rhinosinusitis with nasal polyps (CRSwNP), asthma and cystic fibrosis (CF) are all examples of airway inflammatory diseases. CRSwNP is frequently associated with asthma and C.The origin of NP in both diseases remains to be elucidated. Whatever it may be, one intriguing finding is the different regulation of AA and biosynthesis of PGE_2 in NP from the two diseases.

In CF there is an increased release of AA from the cell membrane phospholipids which is transformed into high amounts of PGE_2 via the action of an activated COX-2. This finding fits perfectly with the accepted dogma that under conditions of inflammation the expression of COX-2 increases and results in an increased synthesis of various prostanoids, including PGE_2. In contrast with this finding, in NP associated with asthma, and especially in those with aspirin-intolerance, the COX-2 is not upregulated and thus the production of PGE_2 does not increase. The reasons that may account for these differences are still unclear.

The studies undertaken in the present thesis seek a better understanding of the origin of the differences in the regulation of AA metabolism in asthma and CF.

In the first study we investigated whether the low PGE_2 production of NP in asthma patients, with and without aspirin intolerance, is due, at least in part, to an insufficient release of AA, which is the substrate for the generation of PGE_2 via the action of COX-1 and COX-2. To do this, we studied the fatty acid composition of cultured fibroblasts derived from NP of asthma patients, with and without aspirin intolerance, and from the nasal mucosa of healthy subjects (control group).

The results of the study do not support the hypothesis because we found that the percentage of AA was higher in NP fibroblasts than in the mucosa fibroblasts of the healthy controls. Therefore, the low PGE_2 production reported in NP cannot be explained by an insufficient supply of AA, as AA metabolism in NP appears to be similar in asthma and CF patients.

Our study also found up-regulation of omega-3 fatty acids docosapentaenoic DPA (C22:5) and docosahexaenoic DHA (C22:6) release. This finding contrasts with the reported decrease in omega-3 fatty acids in the nasal mucosa of CF patients. The reasons for this difference are not known. The significance of this difference in the regulation of omega-3 metabolism in NP from asthma and CF is also unclear.

It is well known, however, that omega-3 fatty acids have the ability to competitively inhibit AA oxygenation by COX-2. We are tempted to speculate that an increased release of omega-3 in NP from asthma patients might reduce AA supply to the COX-2, which would finally result in the reduction of PGE_2 biosynthesis. This hypothesis is also substantiated by some studies in animal models of asthma, as well as others carried out in humans.

In the experimental study a diet rich in omega-3 fatty acids administered to asthmatic mice enhanced the allergen-induced inflammatory reaction, whereas in the human study carried out by our group, patients with aspirin-intolerant asthma were submitted either to a diet supplemented with omega-3 fatty acid (fish

enriched diet) or to the usual diet. The enriched omega-3 diet was associated with a clinical deterioration in aspirin-intolerant asthma patients. We speculate that in both studies the deterioration in inflammatory responses and the clinical course might result from the capacity of omega-3 to reduce the biosynthesis of PEG_2, a substance with proven anti-inflammatory and anti-asthmatic actions.

In the second study we aimed to investigate the mechanisms involved in the very high biosynthesis of PGE_2 reported in CF. Previous studies have considered that the high production of PGE_2 derives from a very active inflammatory process in the airways of CF patients. Chronic respiratory infections usually present in CF patients could contribute to the activation of the COX-2 enzyme and the subsequent increased release of PGE2. Various observations have cast doubt, however, on this explanation, including the signs of inflammation described early in the life of CF patients before respiratory infections began to appear in the natural course of the disease. Moreover, recent *in vitro* studies carried out in cells carrying the defective CFTR gene found that the genetic alteration in the protein that regulates ion channels can explain the increased expression of COX-2 and the subsequent increased PGE_2 release, without the contribution of any infection or inflammatory stimulus. Similar observations have been reported using animal models of CF. In our study we reasoned that the findings from *in vitro* and experimental animal observations should also be present *in vivo* in CF patients. We also hypothesized that since CF occurs in patients suffering from a disease that varied in severity, depending on the severity of the mutations affecting the CFTR gene, the level of PGE_2 production should reflect the severity of CFTR genetic dysfunction.

To measure PGE_2 production, a highly unstable metabolite submitted to rapid degradation, we used a recently introduced analytical method that assesses a terminal urine metabolite of PGE_2 called tetranor-PGE-M, which can be measured by liquid chromatography and mass spectrometry.

The study confirmed the hypothesis and demonstrated the correlation between the urinary levels of tetranor PGE-M and the severity of the genetic alteration. The levels of tetranor PGE-M were significantly higher in CF patients with severe mutations than in those with moderate and mild alterations. Moreover, we also observed that the levels of tetranor-PGE-M were also correlated with the severity of CF phenotype, which is usually associated with severe genotype alterations. Patients with pancreatic insufficiency, a clinical marker of CF severity, had the highest urinary tetranor PGE-M levels, while CF patients who maintained the exocrine pancereatic function, representing the mild phenotypes of the disease, showed tetranor PGE-M levels similar to those found in the urine of the healthy controls.

All in all, these findings demonstrate, for the first time *in vivo,* that the reported increase in COX-2 expression and enhanced PGE_2 production in experimental and *in vitro* studies are directly related to the genetic anomaly that causes CF disease. These findings also allow us to speculate that the quantification of tetranor PGE-M urinary levels may help to assess the efficacy of the therapeutic agents recently introduced into the treatment of CF to improve the altered function of CFTR.

V. CONCLUSIONS

FIRST STUDY

1. The results of the study do not support the hypothesis because we found that the percentage of AA was higher in NP fibroblasts than in healthy control mucosa fibroblasts. Therefore, the low PGE_2 production reported in NP cannot be explained by an insufficient supply of AA. AA metabolism appears to be similar in asthma and cystic fibrosis.

2. Incubation of fibroblasts with aspirin did not induce any change in AA release. This finding suggests that aspirin does not enhance the release of AA available to the cyclooxignease and lipooxygenase enzymes.

3. In addition to the increased production of AA, there were also higher levels of omega-3 fatty acids DPA (C22:5) and DHA (C22:6) in the fibroblast derived from NP. This increased release of omega-3 in NP from asthma patients might reduce AA supply to the COX-2 that would finally result in the reduction of PGE_2 biosynthesis.

SECOND STUDY

1. The study demonstrated that there is a correlation between PGE_2 production and the severity of the genetic dysfunction of the CFTR gene.

2. We also observed that the production of PGE_2 also correlated with the severity of CF phenotype which is usually associated with presence of pancreatic insufficiency.

3. The assay method used in this study is sensitive, precise and accurate for detecting, identifying and measuring PGEM and PGDM prostaglandin metabolites by LC-MS/MS.

4. Quantification of PGE-M urinary levels may help to assess the efficacy of the therapeutic agents recently introduced in the treatment of CF addressed to improve the altered function of CFTR.

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APPENDIX A.
RESUMEN DEL TRABAJO DE LA TESIS

INTRODUCCIÓN

Enfermedades inflamatorias de las vías aéreas

Asma Bronquial. El asma es una enfermedad muy prevalente que afecta al 10% de la población. La enfermedad es consecuencia de una reacción inflamatoria de las vías aéreas, que es responsable del desarrollo de una obstrucción bronquial oscilante y reversible y de una marcada hiperrrespuesta bronquial a estímulos inespecíficos. La gravedad del asma varia de formas leves con síntomas intermitentes a formas graves que se caracterizan por presentar síntomas diarios e intensos, que obligan a tratamientos regulares con glucocorticoides administrados por vía inhalatoria.

En el origen del asma concurren diversos factores entre ellos la alergia. En muchos pacientes asmáticos se detecta la presencia de respuestas alérgicas mediadas por la inmunoglobulina E (IgE) la cual es producida en exceso por algunos pacientes. Sin embargo, en otros pacientes, en particular en los que la enfermedad se inicia en la edad adulta, no se logra demostrar la existencia de un componente alérgico. En cualquier caso, e independientemente de la presencia o ausencia de alergia, las lesiones inflamatorias de los asmáticos alérgicos y no alérgicos son similares, destacando en la mayoría de ellos la presencia de una inflamación eosinofílica con lesiones de remodelación en forma de hipertrofia de la musculatura lisa, hiperplasia de las glándulas de secreción y engrosamiento de la membrana subepitelial ocasionada por el depósito de material colágeno.

Asma con intolerancia a los antiinflamatorios no esteroides (AINEs). En algunos pacientes asmáticos la toma de un AINE ocasiona episodios de broncoespasmo que con frecuencia son muy intensos. Al asma con estas características se lo conoce como asma con intolerancia a los AINEs. Se sabe que las crisis no tienen un origen inmunológico y que están relacionadas al

hecho de que los AINEs ejercen una función reguladora del metabolismo del ácido araquidónico (AA) inhibiendo las enzimas conocidas como ciclooxigenasas (COX) que regulan la síntesis de de prostanoides entre los que se encuentra la prostaglandina E2 (PGE$_2$). El mecanismo responsable del fenómeno parece estar relacionado con algunas anomalías en la regulación del metabolismo del AA.

Un dato clínico característico de estos pacientes es que el asma siempre se asocia a la presencia de una rinosinusitis crónica (RSC) con poliposis nasal (PN).

Rinosinusitis crónica con poliposis nasal (RSCcPN). Esta enfermedad se define como un proceso inflamatorio de la nariz y de los senos paranasales que ocasiona varios síntomas clínicos entre los que se encuentran: obstrucción/congestión, secreción, dolor facial a la presión sobre la zona de los senos, y alteración del sentido del olfato.

La RSC puede acompañarse o no de PN. La RCC asociada al asma es siempre bilateral y cuando se desarrollan NP también son bilaterales.

Los NP son formaciones edematosas originadas generalmente en le meato medio nasal. En su análisis histológico destaca la presencia de un proceso inflamatorio edematoso con abundantes eosinófilos y mastocitos activados. Además de presentarse asociados al asma, la RSCcPN también se detecta en pacientes con alteraciones en las defensas inmunológicas que favorecen las infecciones repetidas o crónicas de las vías aéreas superiores e inferiores como es el caso de la fibrosis quística (FQ). En estos casos las células inflamatorias que predominan son los neutrófilos.

Fibrosis quística (FQ). La FQ es la enfermedad genética grave mas frecuente, con una incidencia que varía entre el 1 por 2000 al 1 por 6500 niños nacidos vivos. Está ocasionada por un defecto en el gen regulador de la conductancia

transmembrna de la FQ, conocido en las siglas en inglés como CFTR (*cystic fibrosis transmembrane conductance regulator*). La proteína CFTR actúa como un canal de cloro regulador del transporte iónico a través de la membrana apical de las células epieliales. Mutaciones en el gen del CFTR ocasiona alteraciones en la regulación del paso de iones y agua a través de las células epiteliales lo cual altera la producción normal de moco, sudor, lágrimas, saliva y enzimas digestivas. Estos canales son necesarios para el funcionamiento normal de los pulmones y el tubo digestivo.

Los pacientess con FQ sufren problemas sinusales y bronquiales con infecciones repetidas y alteraciones digestivas (íleo meconial, insuficiencia pancreática), todas ellas debidas a la formación de secreciones espesas ya sean nasales, bronquiales o del páncreas que ocluyen las vías respiratorias y las vías excretoras del páncreas. La enfermedad respiratoria se caracteriza por su cronicidad y por la facilidad para desarrollar colonización crónica de las vías aéreas por gérmenes como la *pseudomanas aeurginosa*.

La FQ fue considerada una enfermedad letal hasta hace unas pocas décadas, aunque hoy en día el pronóstico de la enfermedad ha cambiado radicalmente, gracias al tratamiento precoz de las infecciones y la terapia substitutiva digestiva, para evitar las consecuencias de la malnutrición ocasionada por la insuficiencia pancreática.

La alteración genetica se hereda de forma autosómica recesiva, es decir que es necesario que los padres sean portadores de una copia mutada del gen. Han sido idenficadas más de 1000 mutaciones del gen de la FQ. El tipo de mutaciones varía dependiendo de las poblaciones estudiadas. La mutación más comun es la conocida como F508delta aunque su frecuencia varia mucho, desde prevalencias muy bajas como ocurre en Tunez (17.9%), hasta las muy elevadas de Dinamarca (90%). En España se encuentra en aproximadamente en el 50% de los casos.

Las mutaciones se pueden agrupar in diferentes clases teniendo en cuanta las alteraciones que provocan en los mecanismos moleculares y las consecuencias funcionales que tiene sobre la actividad de la proteína CFTR.

Clase I: Síntesis defectuosa de la proteína. (Efecto: ausencia de proteína en la zona apical de la membrana). Las mutaciones que pertenencen a esta categoría se asocian a la falta de biosíntesis o biosíntesis defectuosa que ocasiona la formación de diferentes proteínas anómalas. La expresión clínica (fenotipo) de estas alteraciones suelen ser graves (insuficiencia pancreática, bronquiectasias, etc).

Clase II: Alteración en el procesado o movilización de la proteína. (Resultado: ausencia de la proteína en la zona apical de la membrane). Esta variante genética fracasa en el procesado de maduración de proteina y de su traslado a la zona apical de la membrana. La ausencia de proteína en la membrana celular se asocia a formas (fenotipos) graves de la enfermedad.

Clase III: Regulación defectuosa (Efecto: Una cantidad normal pero defectuosa de una proteína no funcionante en la zona apical de la membrana). Estas mutaciones ocasionan una alteración en la activación del canal. Se asocian a formas moderadas o graves de la enfermedad.

Class IV: Disminución de la conductancia (Efecto: Una cantidad normal de proteína con una actividad residual en la zona apical de la membrana). Estas mutaciones no impiden la formación del poro del canal de intercambio iónico, pero el funcionamiento del mismo es defectuoso. Estas variantes suelen asociarse a formas leves de la enfermedad.

Clase V: Reducción de la sínteis y tráfico (Efecto: Reducción de la cantidad funinane de proteía CFTR). Las mutaciones de esta clase dan lugar a una sintesis defectuosa dela proteína o a un tráfico alterado de la misma. Estas mutaciones se asocian a formas leves de la enfermedad.

El desarrollo de técnicas para analizar la estructura de los genes, ha permitido llevar a cabo análisis detallados de las mutaciones del gen del CFTR y agrupar a los pacientes de acuerdo a su caracterización genética. Estos estudios han demostrado que el grado de correlación entre la gravedad de la alteración genética y la gravedad del fenotipo de la enfermedad, varía dependiendo del componente de la enfermedad que es analizado. Asi, por ejemplo, se ha observado una buena correlación entre gravedad genética e insuficiencia pancreática. En cambio la correlación con la enfermedad pulmonar es pobre o inexistente.

Metabolismo de los ácidos grasos y del ácido araquidónico.

Los ácidos grasos son biomoléculas formadas por una cadena hidrocarbonada que puede ser de longitud variable dependiendo del número de átomos de carbono que incorpora. Cada átomo de carbono está unido al precedente y al que le sigue por un enlace covalente sencillo o doble. En un extremo de la molécula, se encuentra un grupo carboxilo (COOH) y en el otro extremo el último átomo de carbono se une a átomos de hidrógeno por medio de los dos enlaces que le quedan libres.

Los ácidos grasos forman parte de los fosfolípidos y los glicolípidos, moléculas que forman la bicapa lipídica de las membranas celulares. La denominación de los ácidos grasos se hace de dos formas. En un caso se emplea el número de átomos de carbono seguido por dos puntos y el número de dobles enlaces. La localización de los enlaces se designa teniendo en cuenta el número del átomo en que se ecuentran contando a partir del grupo carboxilio. El ácido oleico, por jemplo, es el 18:1(9): El número 18 nos indica el número de carbonos, el número 1, el número de dobles enlaces y el 9 señala que el doble enlace comienza en el carbono número 9, empezando la cuenta desde el extremo carboxilo. En otra nomenclatura que se emplea para identificar a los ácidos grasos insaturados, la posición que ocupan los dobles enlaces se indica con respecto al último carbono de la cadena al cual se le designa la letra omega (   ), que es la última letra del

alfabeto griego y de ahí se derivan las denominaciones   -3 y   -6. Un ácido graso   -3 es el que tiene un primer doble enlace entre los carbonos 3 y 4, y un ácido graso   -6 es el que lo tiene entre los carbonos 6 y 7.

Los ácidos grasos se subclasifican en saturados, que son los que no tienen dobles enlaces en su estructura química, e insaturados que son los que contienen dobles enlaces. Los ácidos graso insaturados pueden contener uno o más dobles enlaces. Los que contienen un solo enlace se denominan monosaturados (MUFAs en la siglas en inglés). Los que contienen dos o más se denominan poliinsaturados (PUFAs en las siglas inglesas).

Algunos ácidos grasos se denominan esenciales, ya que el organismo humano no los puede sintetizar y los debe adquirir por medio de la dieta. Los ácidos linoleico, linolénico y araquidónico (AA) son ejemplos de ácidos grasos esenciales.

Los ácidos grasos tienen numerosas funciones en el organismo. Por una parte aportan energía y participan en los procesos celulares que requieren la presencia de oxígneo. También juegan un papel fundamental en la formación de estructuras. Los fosfolípidos y los esfingolipidos formas la bicapa lipídica de las membranas celulares.

Los ácidos grasos son precursores de numerosas moléculas con efectos fisiológicos reguladores. El producto mayoritario de los omega-6 es el AA, mientras que el ácido eicosapentaenoico (EPA) y el ácido (DHA) lo son de los omega-3. Los omega-3 y 6 no son interconvertibles y se pueden diferenciar por tener efectos metabólicos y funcionales diferentes que a menudo son antagónicos. Además los ácidos grasos son los precursores de los eicosanoides, incluyéndose en este grupo de metabolitos los prostanoides que incluyen las prostaglandinas, los troboxanos y la prostaciclina y los derivados de las vías de la lipoxigenasa con numerosos metabolitos entre los que se encuentran los leucotrienos.

Los PUFAs son esenciales para el crecimiento y desarrollo de los seres humanos, por ello es esencial que formen parte de la dieta en las proporciones adecuadas. Los omega-3 se encuentran de forma abundante en los aceites de pescado, aunque también se pueden adquirir ingiriendo aceites vegetales. Muchos aceites vegetales son ricos en ácido linoleico, el cual es transformado en el organismo en AA.

El papel del balance entre los omega-3 y omega-6 de la dieta ha sido motivo de numerosos estudios e hipótesis sobre sobre su importancia en el desarrollo de enfermedades en los países avanzados. Algunos estudios muestran que la razón de la ingesta de omega-6 a omega-3 ha ido cambiando desde una razón de 1 en el pasado reciente a la actual que es 10-20:1, lo que indica que las dietas actuales son deficientes en omega-3. El papel que desarrollan los omega-3 (EPA y DHA) en el organismo difiere del que tienen los omega-6 (ácido araquidónico), en los que se refiere a sus funciones metabólicas y fisiológicas. Un hecho a resaltar, es que en algunos casos los omega-3 compiten con el AA en el empleo de los fosfoliípidos que ambos utilizan como substrato, lo que lleva a la síntesis de eicosanoides con capacidades biológicas diferentes.

Eicosanoids. Los eicosanoids son una familia de metabolitos que actúan como mensajeros intercelulares y que por ello son conocidos como mensajeros lipídicos.

El primer paso en la síntesis de los eicosanoides es la liberación del AA a partir de los fosfolípidos de las membranas celulares. Los eisosanoides también pueden sintetizarse a partir de otros ácidos grasos como el EPA y el ácido dihomo- -linolenico.

La liberación del AA tiene lugar por la acción de varias fosfolipasas. Se reconocen 5 tipos de fospfolipasas (PLA en las siglas inglesas): PLA_2 citosólica ($cPLA_2$), PLA_2 secretada, PLA_2 dependiente de calcio ($iPLA_2$), factor activadotr

de la acetiltranferasa plaquearia (PAF-AH) y la PLA_2 lisosómica ($LPLA_2$) (Schaloske, 2006). La fosfolipasa citosolica es fosforilada y activada por una MAPcinasa para liberar AA a partir de los glicerofosfolípidos. Habitualmente hay poco substrato disponible para la enzima, ya que el AA se conserva en las células secuestrado como glicerofosfolípidos y triglicéridos. Las células regulan de forma estrecha la cantidad de AA que liberan mediante la activación de la CoA sintasa y la CoA transferasa. Los eicosanoides tiene una vida media muy corta ya que son rápidamente metabolizados por la acción de varias enzimas específicas.

Biosíntesis de los eicosanoids. El AA se puede metabolizar por tres vías principales para formar una serie de productos conocidos en su conjunto como eisosanoides, los cuales son liberados por las células en concentraciones muy bajas (nanomolares) que actúan de forma autocrina y paracrina sobre las células diana. Entre estas familias de productos se encuentran los denominados prostanoides que engloban las prostaglandinas, tromboxanos y prostaciclinas, las cuales se sintetizan por la acción de las enzimas denominadas ciclooxigenasas (COX). La vía de las lipoxigenasas conocidas como 5-lipoxigenasa, 12-lipoxigenasa y 15-lipoxigenasa forman diversos metabolitos entre los que destacan los leucotrienos por su importante implicación en las enfermedades inflamatorias respiratorias.

La vía de la COX cuenta con dos enzimas denominadas COX-1 y COX-2, la primera se encuentra en todas las células y tiene funciones fisiológicas reguladoras, mientras que la COX- está regulada por un gen inducible en situaciones de inflamación y proliferación. Los antiinflamatorios no esteroides (AINEs) clásicos como el ácido acetilsalicílico (aspirina), la indometacina, el ibupreno, etc inhiben tanto la COX-1 como la COX-2, mientras que los denominados coxibs, entre los que se encuentran el celeoxib, el eterocoxib y el valdexoxib, actúan mayoritariamente sobre la COX-2.

Finalmente destacar el metabolismo transcelular del AA, en el que participan dos enzimas localizadas en células diferentes, que colaboran para sintetizar las lipoxinas, sustancias a las que se les atribuye acciones antiinflamatorias. A señalar la síntesis de metabolitos similares a las lipoxinas por la acción inhibidora que ejercen los AINEs sobre la vía de la cicloxigenasa y que origina la formación de los derivados 15-epoxi lipoxinas que también desarrollan acciones antiinflamatorias.

Receptores de los eiscosanoides. Los eicosanoides ejercen sus acciones biológicas a través de su unión a receptores de membrana específicos. Se conocen 9 receptores de prostanoides, cuatro de ellos se unen a las prostaglandina E2 y se conocen como EP1, EP2, EP3 y EP4, otros dos se acoplan a la PGD_2 (DP_1 y DP_2), a ellos se unen el receptor FP de la prostaglandinaF2 alfa., el IP de la prostaciclina y el TP de los tromboxanos.

Los receptores de prostanoides pertenecen a la familia de los acoplados a proteínas G, con la excepción del DP2 (también conocido como CRTH2), el cual es miembro de la familia de los receptores de quimioatractantes.

Los receptores EP participan en la regulación de numerosas funciones celulares. La expresión de estos receptores varía dependiendo de los tipos celulares analizados y ello puede explicar en parte las variadas acciones que puede ejercer la prostaglandina E2 (PGE_2). El EP1 es el receptor que se expresa menos en las células, con la excepción de la musculatura lisa en la que muy probablemente media en su contracción. El receptor EP2 actúa como relajador e inhibidor de la activación de células efectoras, como los mastocitos y basófilos. Este receptor regula las funciones biológica ejercidas por el binomio COX-2/PGE2, los cuales se autorregulan y participan en el control de la proliferación celular, la angiogénesis y la apoptosis de varias células Varios estudios han demostrado que el EP3 está involucrado en la contracción del músculo liso y en la activación de las terminaciones nerviosas sensitivas que regulan la tos. El receptor EP4 participa en la regulación de las funciones y proliferación del

endotelio, promoviendo la angiogénesis y junto al EP3 contribuyen a la linfangiogénesis, a través de la inducción de los factores de crecimiento vascular VEGF-C y VEGF-D.

El receptor FP participa en la broncoconstrición inducida por la PGF_{2alfa}. Los IP participan en la homeostasis de las plaquetas y los vasos.Los receptores TP tienen un efecto opositor a los ejercidos por los IP en plaquetas y vasos sanguíneos.

Ácidos grasos y metabolismo del ácido araquidónico en las enfermedades inflamatorias de las vías aéreas (rinosinusitis crónica, poliposis nasal, asma y fibrosis quística

Existen pocos estudios en la literatura que investiguen el metabolismo de los ácidos grasos en las células o tejidos de los pacientes con rinosinustis crónica, poliposis y asma. Tan solo un trabajo analizó los niveles de AA en plaquetas y encontró que estaban disminuidos en relación a las plaquetas de sujetos sanos. Estos resultados deben ser analizados con precaución, ya que las plaquetas no son las células ideales para investigar el metabolismo del ácido araquidónico, debido a que son células prácticamente desprovistas de COX-2, una enzima fundamental para regular el metabolismo del AA en las enfermedades inflamatorias.

Son numerosos los estudios que investigan la regulación de las diferentes vías metabólicas del ácido AA y la síntesis de sus productos en la rinosinusitis, los pólipos y el asma, utilizando células (epiteliales, fibroblastos) y muestras de tejido, analizadas ex-vivo, procedentes de las vías respiratorias de pacientes con poliposis nasal y asma con o sin intolerancia a los AINEs. Estos tabajos han mostrado diversas alteraciones en el metabolismo del AA, alteraciones que son particularmente marcadas en los pacientes con intolerancia a los AINEs.

Se han descrito alteraciones en la via de la COX destacando la síntesis reducida de PGE_2 en las células y muestras de tejido de mucosa nasal y de pólipos nasales de pacientes asmáticos con intolerancia a los AINEs. La baja producción de PGE_2 se encontró asociada a la reducida expresión de la enzima COX-2. Las razones responsables de esta alteración son por ahora desconocidas. Se han investigado causas genéticas y epigenéticas presentes en el gen de la COX-2, sin que se haya podido demostrar la existencia de peculiaridades o alteraciones en el mismo, que puedan explicar la incapacidad del gen para regularse al alza en las situaciones de inflamación.

La producción de lipoxinas está aumentada en el asma grave, aunque en el caso del asma con intolerancia a los AINEs, el aumento es menor del esperado en relación a la gravedad de la enfermedad. Dada la dependencia que tiene la síntesis de lipoxinas de una función adecuada de la COX-2, se intepreta que esta relativa deficiencia en la síntesis de lipoxinas en el asma grave con intolerancia a los AINEs, puede estar motivada por la alteración en la regulación de esta enzima.

Al contrario del asma, en la enfermedad obstructiva crónica de las vías aéreas se ha observado el esperado aumento en la producción de PGE_2 asociado a un aumento en la expresión de la COX-2.

Como ha sido señalado el AA es el substrato metabólico a partir del cual se sintetiza la PGE_2. Lógicamente, un aumento en la síntesis de PGE_2 en situaciones de inflamación, debe estar asociado a un aumento paralelo del aporte del substrato, es decir del AA. En algunas enfermedades con una alta producción de PGE_2, como es el caso de la fibrosis quística (ver más adelante), se ha observado la existencia de una liberación aumentada y acelerada de AA con el fin de seguir proveyendo a la COX-2 del fuel necesario para aumentar la producción de PGE_2.

Dado que en el asma la síntesis de PGE_2 está reducida y dada la dependencia de este proceso del aporte de AA, se puede especular con la posibilidad de que un bajo aporte de AA a la maquinaria metabólica podría, explicar, al menos en parte, la baja producción de PGE_2 en los pacientes asmáticos, en especial en aquellos con intoleracia a los AINEs que destacan por la escas producción de PGE_2 en situación basal y cuando sus células (epiteliales, fibroblastos) son estimuladas con substancias proinflamatorias.

En claro y llamativo contraste con lo descrito en la poliposis nasal y el asma, en la FQ, una enfermedad genética caracterizada por la presencia de un proceso inflamatorio muy activo de las vías aéreas que alcanza a los senos paranasales y la vías aéreas inferiores, se caracteriza por la presencia de una marcada producción de PGE_2 que se ha podido detectar analizando sus niveles en diversos secreciones y fluidos procedentes de estos pacientes, como son el esputo, la orina, la saliva y los lavados broncoalveolares. Los pacientes con FQ tambien pueden desarrollar pólipos nasales bilaterales asociados a un rinosinustis crónica. En estos pólipos, en claro contraste con lo encontrado en los pólipos de los pacientes asmáticos, la COX-2 está marcadamente regulada al alza y lo mismo ocurre, aunque en menor grado, con la COX-1. El aumento en la COX-2 se interpretó inicialmente como la consecuencia esperada de la existencia de un proceso inflamatorio secundario a los procesos infecciosos recurrentes y a menudo crónicos de estos pacientes. Los procesos infecciosos se han atribuido, clásicamente, a las alteraciones ocasionadas por la alteración del gen regulador de la conductancia transmembrana de la FQ (CFTR), lo que ocasiona secreciones espesas difíciles de eliminar que obstruyen los senos nasales y las vías áreas y favorecen las infecciones. No obstante, diversas observaciones constataron que con anterioridad al desarrollo de las infecciones recurrentes, los pulmones de los pacientes con FQ mostraban signos de inflamación, hecho que hizo pensar que la alteración de CFTR podría estar propiciando el desarrollo, por mecanismos desconocidos, de respuestas inflamatorias. Posteriormente, se observó en modelos *in vitro* utilizando cultivos de células portadoras de la alteración del CFRT, que la producción de PGE_2

estaba aumentada, coincidiendo con un aumento en la expresión de la COX-2. Este mismo fenómeno se pudo demostrar en modelos animales. Estas observaciones han llevado a considerar que el aumento en la síntesis de PGE_2 asociado al aumento de la expresión de la COX-2, no obedece a una respuesta inflamatoria secundaria a las infecciones, sino que pueden estar directamente relacionada a la alteración genética del gen CFRT, que por razones y mecanismos aún no bien conocidos, actúa como un regulador directo del metabolismo del AA. La alta producción de PGE_2 obliga a un metabolismo acelerado del AA que es su substrato. Divesos estudios, entre ellos algunos utlizando biopsias nasales, han mostrado un aumento marcado de la liberación de AA a partir de los glicofosfolípidos de las membranas celulares en pacientes afectados de FQ.

Hipótesis y Objetivos del estudio

El estudio establece dos hipótesis que son la base de dos trabajos.

Antecedentes que justifican el planteamiento de la primera hipótesis

1. Los metabolitos del AA juegan un papel importante en la regulación de las respuestas inflamatorias, aceptándose que en las enfermedades inflamatorias existe un aumento en la actividad de las vías metabólicas entre las que se encuentra la de la ciclooxigenasa que da como resultado un aumento en la síntesis de prostanoides como la PGE_2.

2. El esperado aumento de la producción de PGE_2 asociado al incremento en la actvidad de la enzima COX-2 responsable de su síntesis, no se observa en el tejido inflamado de los pólipos nasales de pacientes con asma, en especial en los que son hipersensibles a los AINEs.

3. En contraste con la poliposis nasal asociada al asma con intolerancia a los AINEs, en los pólipos nasales de pacientes con fibrosis quística se ha observado el esperado aumento en la expresión de la COX-2 que se asocia a un incremento en la síntesis de PGE_2.

4. En la FQ coincidiendo con el aumento en la síntesis de PGE_2 se ha observado el esperado aumento en la liberación del AA que es el substrato metabólico que sustenta la síntesis de esta prostaglandina.

5. Dada la estrecha asociación entre producción de PGE_2 y la liberación de AA y en base a los datos a las observaciones señaladas se estable la hipótesis.

Primera Hipótesis

La alteración en la producción de la PGE_2 en los pólipos de pacientes con asma y en especial en los que presentan intolerancia a los AINEs, es debida, al menos en parte, a un insuficiente aporte de AA para ser metabolizada por la vía de la ciclooxigenasa.

Antecedentes que justifican el planteamiento de la segunda hipótesis

1. La fiibrosis quística es una enfermedad genética ocasionada por una regulación defectuosa del gen regulador de la conductancia transmembrana de la fibrosis quística (CFTR).

2. Las diversas mutaciones del gen se pueden clasificar en varios grupos de acuerdo a la gravedad de la disfunción de los mecanismos moleculares y a la predicción de sus efectos sobre la función reguladora de la proteína CFTR.

3. La gravedad de la disfunción de la proteína CFTR se correlaciona con la gravedad de la expresion clínica (fenotipo): a mayor disfunción molecular,

le corresponde una enfermedad más grave, habitualmente determinada por la presencia a ausencia de insuficiencia pancreática.

4. Estudios *in vitro* y con animals de experimentación han demostrado que la disfunción del CFTR induce y activa el metabolismo del AA, lo que da como resultado un aumento en la expresión de la COX-2 y de la producción de PGE_2.

5. No hay estudios que hayan analizado *in vivo* la relación entre gravedad de la disfunción genética del CFTR y la producción de PGE_2.

Segunda Hipótesis

En patients con fibrosis quística existe una relación entre la producción de PGE_2 con la gravedad de la alteración genética del CFTR y con la gravedad de la enfermedad determinada por la presencia o ausencia de insuficiencia pancreática.

Objetivos del estudio

- Objetivo General

Contribuir a clarificar el papel del metabolismo del ácido arquidónico en las enfermedades inflamatorias de las vías áreas.

- Objetivos específicos

- **Primer estudio**

Objetivo principal: Determinar y comparar la composición de ácidos grasos en fibroblastos procedentes de mucosa nasal de sujetos sanos y de pólipos nasales obtenidos de pacientes asmáticos con y sin intolerancia a los AINEs.

Objetivo secundario: Analizar y comparar el efecto de la aspirina y un estímulo inflamatorio (interleucina 1) sobre la composición de ácidos grasos en fibroblastos procedentes de mucosas nasales sanas y de pólipos nasales de pacientes asmáticos con y sin intolerancia a los AINEs.

- **Segundo estudio**

Objetivo principal: Evaluar en pacientes con fibrosis quistica la relación entre la producción de prostanoides (PGE_2 y PGD_2) con la gravedad de la disfunción genética y la gravedad de la enfermedad, esta última determinada por la alteración de la función pancreática exocrina y los datos de la exploración de la función pulmonar.

Objetivo secundario: Evaluar la utilidad de la medición de la producción de prostanoides mediante la determinación de los niveles urinarios de PGE- M y PGD-M metabolitos terminales de la PGE_2 y la PGD_2 respectivamente.

PRIMER ESTUDIO

Título: Estudio de la composición de ácidos grasos en fibroblastos cultivados procedentes de mucosa nasal sana y de pólipos nasales.

- **Sujetos y métodos**

Se cultivaron fibroblasto procedentes de mucosas sanas de pacientes (n=6) sometidos a cirugía correctora nasal sin historia de asma o enfermedades de las vías aéreas y de pólipos procedentes de resecciónes quirúrgicas en pacientes asmáticos tolerantes a los AINEs (n=6) e intolerantes a los AINEs (n=6).   Los fibroblastos fueron sometidos a la estimulación con lisinato de ácido acetil salicílico (L-ASA). La composición lipídica de los fibroblastos se analizó mediante cromatrografia de gases en situación basal y tras la estimulación con la L-ASA.

- **Resultados**

Los ácidos grasos omega-6 ácido dihomo-gamma-linolenico (C20:3) y AA (C20:4), asi como los ácidos grasos omega-3, docosapentaenoico DPA (C22:5) y docosahexaenoico DHA (C22:6) se encontraban en porcentajes significativamente más altos en los fibroblastos procedenes de pólipos nasales en comparación a los fibroblasts procedentes de mucosa nasal sana. El porcentaje de ácido palmítico (C16:0) y ácido palmitoleico (C16:1) fue signficativamente más alto en los fibroblastos procedentes de pólipos nasales de pacientes asmáticos con intolerancia a los AINEs en comaparación a los de pacientes asmáticos tolerantes a los AINEs. La incubación con aspirina no causó cambios significativos en el porcentaje de los ácidos omega-3 o omega-6.

- **Discusión**

La concentración de AA libre en células en situación de reposo suele ser bajo y aumenta en situaciones de inflamación o remodelado como ha sido descrito en la piel de pacientes con psoriasis y lesiones cutáneas hipertróficas, facilitándose con ello la síntesis de diversos metabolitos, como las prostaglandinas y lipoxinas que participan en la regulación de las respuestas inflamatorias.

En la fibrosis quística una enfermedad inflamatoria de las vías áeeas se ha observado un aumento en la biosíntesis de AA que se asocia a una elevada producción de PGE_2 asociada a un aumento de la expresión de COX-2. Los pacientes con fibrosis quística suelen presentar rinosinusitis crónica con poliposis nasal. En muestras de estos polipos se ha podido demostrar un marcado aumento dela expresión de la COX-2.

Todos estas observaciones contrastan con las reportadas en pólipos de pacientes asmáticos con intolerancia a los AINEs en los cuales, a pesar de sufrir un intenso proceso inflamatorio, la expresión de la COX-2 no está aumentada y tampoco lo esta la producción de PGE_2. Dada la estrecha interrelación entre la biosíntesis de AA y la producción de PGE_2 la hipótesis del estudio estableció

que en estos pacientes la biosíntesis escasa de AA podría contribuir a explicar la baja producción de PGE_2.

Los resultados del estudio no permiten sostener la hipótesis, ya que los fibroblastos de los pólipos lieraban más AA que los de los sujetos sanos.

Nuestro estudio además encontró que la liberación aumetada del AA, un ácido graso omega-6, se asociaba al aumento en los porcentajes de los ácidos grasos omega-3 DPA (C22:5) y DHA (C22:6).

Las membranas de la mayoría de las células contienen mayores cantidades de AA que de otros precursores potenciales de prostaglandinas como el EPA. Eso explica que el AA sea habitualmente el precursor para la biosíntesis de prostaglandinas y que los omega-3 tengan una menor contribución en la sínstesis de estos mediadores. Sin embargo, cuando los omega-3 se encuentran en las células en niveles altos, pueden ocasionar, por un mecanismo competitivo, una disminución de los niveles de AA en las membranas de las celulas inflamatorias, con lo cual puede reducirse la cantidad de AA que está disponible para producir prostanoides. Además, el EPA inhibe de forma competitiva la oxigenación del AA por la COX. Con todo ello y por medio de varios mecanismos, los ácidos grasos omega-3 pueden reducir la producción de prostanoides como la PGE_2.

Basados en estas observaciones varios estudios han evaluado los efectos de la manipulación de la dieta en el tratamiento de las enfermedades inflamatorias, entre ellas el asma. La cantidad de AA ofertable se puede modificar, aunque lentamente, aumentando la cantidad de los PUFA omega-6 presentes en la dieta. Por el contrario la cantidad de PUFA omega-3, que habitualmente están presentes en menor proporción en las células, pueden verse rápidamente aumentada mediante la suplementación de la dieta con PUFA omega-3.

Aunque algunos estudios no controlados han mostrado efectos beneficiosos de de las dietas ricas en omega-3 en el asma, estudios realizados controlados no

han podido confirmar esos supuestos efectos beneficiosos de las dietas ricas omega-3, como son las dietas ricas en pescado, en el asma.

La observación de un aumento en los omega-3 en fibroblastos procedentes de tejidos inflamados en nuestro estudio, contrasta con los datos previamente reportados en el tejido nasal de pacientes con fibrosis quística, en los que se ha observado junto al aumento de AA una proporción muy baja de DHA.

Nuestros datos permiten especular con la posibilidad de que el aumento detectado de omega-3 en los pólipos nasales, podría, a traves del mecanismo competitivo anteiormente explicado, reducir la oxigenación del AA por la COX y con ello disminuir la producción de PGE_2. Si esta hipótesis fuera cierta, cualquier intento de aumentar la cantidad de omega-3 en las membranas celulares mediante el incremento del aporte dietético de omega-3, podría tener un efecto negativo sobre la evolución del asma en pacientes con intolerancia a los AINEs. En línea con esta posibilidad, merece la pena señalar que un estudio previo realizado por nuestro grupo, mostró que una dieta rica en omaga-3 inducía una reducción en la síntesis de prostanoides, al tiempo que se asociaba a un ligero deterioro clínico de la función pulmonar. Esta observación concuerda con la de un estudio reciente que encontró que una dieta rica en omega-3, aumentaba la respuesta inflamatoria y la liberación de citocinas proinflamatorias, como la enterleucina 5 (IL-5) y la interleucina 13 (IL-13), en un modelo de asma murino.
En el asma con intolerancia a los AINEs, la exposición a un AINE ocasiona un aumento en la síntesis de leucotrienos. En nuestro estudio evaluamos si la incubación con aspirina provocaba alguna alteración en la liberación de ácidos grasos que pudieran explicar la activación de la vía de la lipoxigenasa. No se obsevaron cambios relevantes, lo que parece indicar que el mecanismo que induce un aumento en la liberación de leucotrienos, no está relacionado con cambios en la movilización del AA desde las membranas celulares.

En síntesis, el objetivo de este estudio fue investigar la posibilidad de que la baja producción de PGE_2 previamente detectada en fibroblastos de pacientes

asmáticos, alteración más acentuada en los pacientes con intolerancia a los AINEs, estuviera causada, el menos en parte, por una reducción en el aporte de AA. Los resultados han mostrado que al contrario de lo supuesto en la hipótesis, la proporción de AA en los fibroblastos de los pacientes es superior al de los sujetos controles. El estudio también encontró un aumento porcentual de omega-3 en los fibroblastos de los pacientes. La posibilidad de que la presencia aumentada de ácidos grasos omega-3, pueda contribuir a reducir la producción de PGE_2 en los fibroblastos de pólipos nasales, a través de la inhibición competitiva de la oxigenación de COX-2 y de la reducción del AA disponible para esta enzima requiere ser evaluada en futuros estudios.

SEGUNDO ESTUDIO

Título: Medición de los metabolitos urinarios principales de la PGE_2 y la PGD_2 en al fibrosis quística: Correlación con la gravedad de la enfermedad.

- **Pacientes y métodos**

Se incluyeron en el studio 24 sujetos sanos y 35 pacientes con fibrosis quística (FQ). Todos los pacientes fueron sometidos a la evaluación de la función pancreática exocrina, al estudio de la función pulmonar mediante espirometría y a un estudio genético. De acuerdo a los resultados del genotipado, los pacientes fueron clasificados en tres grupos: leve, moderado y grave. Pacientes portadores de mutaciones de la clase I y II en sus dos alelos fueron considerado graves. Los que tenían mutaciones I o II en un alelo y uno de clase III en el otro fueron etiquetados como moderados y los que eran portadores de mutaciones Class I o II asociadas a una de clase IV o V fueron considerados leves.

Todos los pacientes del grupo grave (n=22) tenían insuficiencia pancreática. Todos menos uno de los considerados moderados (n=5) también tenían insuficiencia pancreática. Finalmente, solo 3 de los 8 pacientes con una mutación leve en su genotipo tenían insuficiancia pancreática. Se recogieron muestras de orina y en ellas se evaluaron los los niveles de PGE-M y PGD-

mediante cromatografía liquida en tandem con espectrometría de masas. Los niveles de ambos metabolitos se expresaron en ng por mg de creatinina.

- **Resultados**

Los niveles de PGE-M y de PGD-M fueron significativamente más altos en la orina de los pacientes en comparación a los controles sanos. De la misma manera, los niveles de PGD-M también fueron signiificativamente más elevados en los pacientes que en los controles.

Los niveles de PGE-M y PGD-M se correlacionaban de forma significativa tanto en los sujetos sanos como en los pacientes. No hubo diferencias significativas en los niveles de creatinina entre los controles (1.10; 0.80-1.45 mg/ml) y los pacientes (0.97; 0.55-1.31 mg/ml). Todos los valores se muestran en forma de mediana y percentiles 25 y 75.

Correlaciones con los parámetros de gravedad

Los valores del FEV_1 disminuyeron en paralelo con la gravedad. En los pacientes portadores del genotipo grave el FEV_1 fue del 93% del valor teórico (intérvalo 44-120), del 98% (intérvalo de 90-109) en pacientes con el genotipo moderado y del 102% (intérvalo 80-117) en los portadores de un genotipo leve, pero las diferencias eran pequeñas y no alcanzaron valor estadístico significativo.

Tampoco se encontraron correlaciones entre los niveles urinarios de PGE-M y PGD-M con los resultados de la espirometría (FEV_1, FVC). Los pacientes con insuficiencia pancreática (n=29) tenían valores más altos de PGE-M (19.11: 9.95-57.85 ng/mgCr) que los pacientes que tenían la función exocrina pancreática conservada (n=6) (8.64; 5.78-14.34 ng/mgCr) ($p<0.01$). Lo mismo ocurría con los niveles de PGD-M que fueron más altos en los pacientes con insuficiencia pancreática (2.19:1.58-3.88 ng/mgCr) que en aquellos que la

conservaban normal (1.59:0,73-2.68 ng/mgCr), pero en este caso las diferencias no fueron estadísticamente significativas.

Todos los pacientes que estaban colonizados por *Pseudomonas aeuriginosa* eran portadores del fenotipo más grave. Sin embargo, no hubo diferencias ni con los niveles del PGE-M, ni con los del PGD-M, entre los pacientes colonizados (n=11) (PGE-M, 13.53:8.77-38.11ng/mgCr; PGD-M1.63:1.39-3.65ng/mgCr) con los que no lo estaban (n=24) (PGE-M:16.89:9.17-50.80ng/mgCr; PGD-M: 2.21:1.59-3.69ng/mgCr).

Correlaciones entre la producción de prostanoides con las mutaciones del CFTR

La comparación de los niveles urinarios de PGE-M entre los controles y los pacientes mostró que no había diferencias entre los controles y los pacientes portadores de las mutaciones leves. Si que había diferencias significativas entre los moderados (p<0.01) y los graves (p<0.001) con los controles sanos. También se observaron diferencias estadísticamente significativas entre los genotipos moderado (p<0.01) y grave (p<0.001), con los leves y también entre los genotipos moderado y leve (p<0.01).

Se encontraron diferencias significativas (p<0.05) en los niveles de PGD-M entre los portadores de un genotipo grave con los controles sanos, pero no entre controles y los genotipos moderados (cerca de la significación <p0.06) y leves. No hubo diferencias estadísticamente significativas entre los grupos según gravedad.

- **Discusión Y Conclusiones**

En la FQ la liberación de AA está aumentada lo cual puede contribuir a la producción de grandes cantidades de prostanoides. Además, varios estudios realizados con células *in vitro* y con animals de experimentación han demostrado que la disfunción del CFTR induce y estimula el metabolismo del AA, lo que lleva a un aumento en la expresión de la COX-2, que da como resultado final el

aumento en la producción de PGE_2. En línea con estos resulados, numerosas observaciones clínicas han demostrado niveles elevados de PGE_2 en diversos fluidos y secreciones procedentes de pacientes con FQ (orina, saliva, esputo, lavado broncoalveolar).

En este estudio hipotetizamos que, dado que en la FQ existen alteraciones genéticas con diferentes grado de gravedad, según el tipo de mutaciones presentes en los pacientes, los niveles de producción de prostanoides deberán guardar relación con la gravedad de la disfución del CFTR. Los resultados de nuestro estudio confirman la hipótesis, ya que observamos que: a mayor gravedad en la disfunción genética, mayor es la producción de PGE_2.

Nuestro estudio también encontró una relación entre la gravedad de la expresión fenotípica de la enfermedad, determinada mediante el estudio de la función exocrina pancreática, el parámetro más sensible para graduar la gravedad clínica del proceso, con los niveles urinarios de PGE-M. Los pacientes que tenían la function pancreática conservada, eran portadores de la disfunción leve del CFTR y presentaban niveles de PGE-M similares a los controles sanos. Sin embargo, no hubo correlación entre los parámetros de función pulmonar (FEV_1 y FVC) con los niveles urinarios de PGE-M. Hasta cierto punto este resultado era esperado, ya que se conoce que la medición del FEV_1 es un parámetro relativamente insensible para valorar la gravedad y progresión de la FQ.

Aunque parece existir una relación directa entre la alteración genética del CFTR y la producción de PGE_2, no se puede descartar que estímulos infecciosos o proinflamatorios, habitualmente presentes en las vías aéreas de los pacientes con FQ, puedan contribuir a aumentar la expresión de la COX-2 y con ello aumentar la síntesis de PGE_2. Lipopolisacáridos bacterianos o citocinas como la IL-1 , que están presentes en el pulmón de los pacientes con FQ, pueden contribuir a aumentar la producción de PGE_2. Por ejemplo, productos procedentes de *Pseudomonas aeruginosa*, son capaces de activar la fosfolipasa A2 y con ello potenciar la síntesis de prostanoides en estudios *in vitro.* Sin

embargo, en nuestro estudio no observamos ninguna diferencia en la producción de PGE-M entre los pacientes colonizados y los no colonizados por esta bacteria.

Los antiinflamatorios no esteroides como el ibuprofeno, se han empleado en el tratamiento de la FQ, habiéndose observado algunos efectos beneficiosos sobre la función pulmonar. Estas observaciones que parecen demostrar que los prostanoides juegan un papel en la regulación de la respuesta inflamatoria de la FQ, aunque los mecanismos potencialmente responsables de los efectos beneficiosos del ibuprofeno son desconocidos.

La producción de PGD-M originado a partir de la PGD_2, que es un metabolito de la COX sintetizado especialmente por mastocitos y macrófagos, también la encontramos elevada en los pacientes FQ, aunque en contraste con la PGE-M sus niveles fuerom mucho más bajos y no se encontraron correlaciones significativas con la gravedad de la disfunción genética del CFTR. El papel de la PGD_2 en la FQ es por ahora desconocido.

Nuestro estudio tiene algunas limitaciones entre las que se incluye la clasificación empleada, la cual tiene poco soporte en al literatura, ya que aunque las tres categorias de gravedad del genotipo: leve, moderada y grave, se citan en estudios dirigidos a establecer la relación ente los genotipos del CFTR y su expresión fenotípica, solo los fenotipos leves (Clases IV y V) y los graves (Clases I, II, III) están bien definidos. En algunos estudios el término moderado se aplica a aquellos genotipos que contienen mutaciones heterocigóticas resultado de combinaciones de clase I y II con clases III, IV y V mutation. Estas combinaciones estan asociadas a una menor predisposición a desarrollar insuficiencia pancreática. No obstante, la diferencia en la predisposición entre graves (homocigotos de Clase I o Class II) y los moderados (heterocigotos de Clase I/ Clase II y de Clase III, IV o V) es pequeña, sobre todo en comparación con la diferencia que existe entre los genotipos severo/moderado con los leve/leve (Clases IV-V). Éramos conscientes de estas limitaciones cuando

clasificamos nuestros pacientes. A pesar de todos estos inconvenientes encontramos que la producción de PGE$_2$ está, al menos en parte, asociada a la gravedad del genotipo.

Los valores de PGE-M urinario aunque están relacionados con la gravedad de la alteración genotípica, mostraron una gran variabilidad, lo cual sugiere que, además de la alteración genética, otros factores ambientales y otros genes que regulan las respuestas inflamatorias pueden estar involucrados en la producción de eicosanoides en la FQ.

Las futuras terapias de la FQ están encaminadas a mejorar el funcionamiento defectuoso del CFTR. Dada la relación existente entre el grado de afectación del CFTR con los niveles urinarios de PGE-M, cabe la posibilidad de que esta metodología podría aplicarse para valorar la eficacia de los nuevos agentes terapeúticos.

DISCUSIÓN CONJUNTA DE LOS DOS TRABAJOS (STUDY 1 & STUDY 2)

Entre los centenares de productos metabólicos celulares implicados en la regulación de las respuestas inflamatorias se encuentran los metabolitos del AA. El ácido AA se forma a partir de fosfolípidos de la membrana celular por la acción de la fosfolipasa A. A su vez el AA es el sustrato a partir del cual diversas vías enzimáticas producen numerosos productos con acciones proinflamatorias y antiinflamatorias. A través de la vía de la COX y con la intervención de sus dos enzimas: COX-1 y COX-2 se generan los prostanoides entre los cuales se encuentra la PGE$_2$. El papel de la PGE$_2$ en la inflamación es diverso, por una lado se le han atribuido funciones proinflamatorias, pero por el otro se la considera una molécula antiinflamatoria. Se interpreta que esas funciones contrapuestas están en su mayor parte reguladas por la expresión diferenciada

de los 4 receptores de la PGE_2 en las células. La estimulación de los receptores EP2 y EP4 tiene efectos antiinflamatorios mientras que las del EP3 inducen lo contrario.

En la rinosinusits crónica con poliposis, el asma y la FQ son ejemplos de enfermedades inflamatorias. La rinosinustis crónica con poliposis nasal se encuentra asociada tanto al asma como a la FQ. El origen de la poliposis nasal en ambos procesos es desconocido, pero un hecho sorprendente es el comportamiento diferente del metabolismo del AA y de la biosíntesis de PGE_2 en los pólipos de ambas enfermedades.

En el caso la FQ se ha comprobado que en la mucosa nasal existe un aumento en la liberación de AA a partir de los fosfolípidos de las membranas celulares y que este AA es trasformado en grandes cantidades de PGE_2 gracias al aumento marcado de la actividad de la COX-2. Esto hallazgos casan perfectamente con el dogma que establece que en situaciones de inflamación, se produce un aumento en la expresión de la COX-2, que da como resultado el aumento en la sintesis de diversos prostanoides, entre los cuales se encuentra la PGE_2. En contraste con este hallazgo y al contrario de lo esperado, en los pólipos de pacientes con rinusinusits crónica asociados al asma, y en particular en los pacientes con intolerancia a los AINEs, la COX-2 no está regulada al alza y por ello la biosíntesis de PGE_2 no está aumentada. Las razones por las que ocurren estas diferencias son deconodidas.

Los trabajos de esta tesis han estado dirigidos a conocer mejor el origen del comportamamiento diferente del metabolismo del AA en el asma y la FQ. En un primer estudio se ha investigado si la baja producción de PGE_2 en los pólipos de pacientes con asma asociada o no intolerancia a los AINEs, estaba motivada por un aporte insuficiente de AA, que es el substrato a partir del cual se genera la PGE_2. Para ello se procedió a estudiar la distribución de los ácidos grasos en fibroblastos procedentes de pólipos de pacientes asmáticos con y sin intolerancia a los AINEs. Los resultados del estudio van en contra de la

hipótesis, ya que se pudo comprobar que el porcentaje de AA presente en los pólipos de pacientes asmáticos, era superior al encontrado en los fibroblastos obtenido de mucosas sanas que actuaban como controles. Por lo tanto la síntesis deficiente de PGE_2 no puede ser atribuida a una falta de aporte del substrato. En este aspecto los pólipos de los pacientes asmáticos parecen comportarse igual al los de la FQ. El estudio demostró que, además del AA, los ácidos grasos omega-3, docosapentaenoico DPA (C22:5) y docosahexaenoico DHA (C22:6) también se encontraban en porcentajes significativamente más altos en los fibroblastos procedenes de pólipos nasales, en comparación a los fibroblastos procedentes de mucosa nasal sana. Este hecho contrasta con lo observado en la FQ, en la que se ha encontrado una disminución en la síntesis nasal de omega-3. El origen y significado de estos hallazgos es desconocido, aunque el hecho de que los omega-3 pueden inhibir de forma competitiva la oxigenación del AA por la COX, nos ha permitido especular con la posibilidad de que un mayor aporte de omega-3 en los pólipos podría, por un mecanismo competitivo, reducir el aporte de AA hacia la COX-2 y con ello afectar la producción de PGE_2. Esta hipóteis está sustentada por estudios en animales de experimentació e incluso en humanos. En el primer caso se pudo comprobar que la administración de una dieta rica en omega-3 aumentaba la respuesta inflamatoria en ratones asmáticos y en el segundo caso se trata de un ensayo clínico realizado por nuestro grupo que demostró que una dieta rica en omega-3 (dieta rica en pescado) ocasionaba el deterioro clínico de enfermos asmáticos intolerantes a los AINEs.

En el segundo trabajo nos propusimos investigar los mecanismos implicados en la producción elevada de PGE_2 en la FQ. Estudios iniciales consideraron que la producción elevada de PGE_2 era atribuible a la presencia de un proceso inflamatorio crónico en las vías aéreas de estos pacientes, en buena parte debido a los episodios infecciosos crónicos que sufren. Sin embargo, observaciones recientes realizadas en células in vitro y en animales de experimentación, han demostrado que la alteración genética del CFTR es capaz por si sola y sin el concurso de un proceos inflamatorio, de inducir la expresión

de la COX-2 y con ello aumentar la síntesis de PGE_2. En nuestro estudio razonamos que el mismo fenómeno debería existir *in vivo* en los pacientes afectados de FQ. Dado que la FQ puede mostrarse con diferentes grados de gravedad dependiendo de la importancia de las mutaciones del gen de la CFTR, el objetivo del estudio fue evaluar la relación de la gravedad de la afectación genética con la producción de PGE_2.

Para medir la producción de PGE_2, un metabolito muy inestable debido a su rápida degradación, empleamos un método recientemente introducido, que emplea la valoración de la biosíntesis de PGE_2 mediante la cuantificación de un metabolito terminal de esta prostaglandina denominado tetranor PGE-M, el cual se puede medir en orina mediante cromatografía líquida y espectrometría de masas. El estudio permitió confirmar la hipótesis, ya que se pudo comprobar que los niveles de PGE-M estaban relacionados con la gravedad de la alteración genética ya que los niveles urinarios más elevados se encontraron en las formas graves, los niveles más bajos en las formas leves y los valores intermedios en los casos moderados. Además, se pudo comprobar que la traducción fenotípica de la gravedad de las mutaciones genéticas, habitualmente detectable por la presencia de insuficiencia pancreática en las formas graves, se asociaba a niveles urinarios de PGE-M mucho más altos que en los pacientes que portaban mutaciones más leves y que conservaban la función pancreática.

Estos datos permiten especular con la posibilidad de usar la determinación de los niveles urinarios de PGE-M en la valoración de la eficacia terapéutica de los nuevos tratamientos dirigidos a mejorar la función alterada del CFTR.

CONCLUSIONES

PRIMER ESTUDIO

1. Los resultados del estudio desmienten la hipótesis ya que la proporción de AA presente en los pólipos de los pacientes asmáticos fue más alto del

encontrado en las mucosas sanas. Por lo tanto la baja producción de PGE$_2$ observada en los pólipos no puede ser explicada por un aporte insuficiente de AA. El metabolismo del AA en el asma parece ser similar al de la FQ.

2. La incubación de los fibroblastos con aspirina no indujo ningún cambio significativo en la liberación de AA. Esta observación sugiere que la aspirina no incrementa la movilización del AA hacia las vías metabólicas de la ciclooxigenasa y lipoxigenasa.

3. El aumento en la liberación de AA se asocia al de los ácidos grasos omega-3 DPA (C22:5) y DHA (C22:6) in los fibroblastos procedentes de pólipos nasales. Este aumento en omega-3 en los pacienes asmáticos podría reducir el aporte de AA a la COX-2 lo que podría finalmente contribuir a reducir la biosíntesis de PGE$_2$.

SEGUNDO ESTUDIO

1. El estudio demuestra que existe una correlación entre la producción de PGE$_2$ y la gravedad de la disfunción genética del gen del CFTR.

2. La producción de PGE$_2$ también está correlacionada con la gravedad del fenotipo habitualmente asociada a la presencia de insuficiencia pancreática.

3. El método de análisis empleado de cromatografia líquida y espectrometria de masas es sensible, preciso y exacto para detectar, identificar y medir los niveles de PGE-M y PGD-M, metabolitos de las prostaglandinas PGE$_2$ y PGD$_2$ respectivamente.

4. La medición de los niveles urinarios de PGE-M puede ser un método útil para evaluar la eficacia de los nuevos tratamientos de la CF dirigidos a mejorar la función alterada del CFTR.

APPENDIX B.
ARTICLES I PRESENTACIONS A CONGRESSOS FETS AMB ELS TREBALLS DE LA TESI

Publicacions en revistes (articles originals)

1. **Jabr S,** Roca-Ferrer J, Picado C. Fatty acid composition of cultured fibroblasts derived from healthy nasal mucosa and nasal polyps. **Prostaglandins, Leukotrienes and Fatty Acids** (en avaluació).

2. **Jabr S**, Gartner S, Milne G, Roca-Ferrer J, Moreno A, Gelpi E, Picado C. Quantification of Major Urinary Metabolites of PGE$_2$ and PGD$_2$ in Cystic Fibrosis: Correlation with Disease Severity. **Prostaglandins, Leukotrienes and Fatty Acids** 2013: june (on line)

Publicacions en revistes (abstracts de congressos)

1. **Jabr S**, Roca-Ferrer J, Cofan M, Picado C. Variation in fatty acid composition of cultured nasal fibroblast derived from nasal mucosa and nasal polyps : Role of culture passages. **C**hemistry and Laboratory Medicine 2011 vol.:49 Pàg.:S747 -S74

Presentacions en Congressos

1. **Fourth European Workshop on Lipid Mediators. Psteur Institut. Paris. 2012. Jabr S**, Roca-Ferrer J, Cofan M, Picado C. Variation in fatty acid composition of cultured nasal fibroblast derived from nasal mucosa and nasal polyps : Role of culture passages

2. **European Cystic Fibrsois Society. Lisbon. 2013.** Gartner S, **Jabr S**, Milne G, Roca-Ferrer J, Moreno A, Gelpi E, Picado C. Quantification of Major Urinary Metabolites of PGE$_2$ and PGD$_2$ in Cystic Fibrosis: Correlation with Disease Severity

APPENDIX C.
BEQUES I FINANÇAMENT

Finançament dels estudis

La Doctoranda **Suha Jabr** ha gaudit d'una beca del Ministerio Español de Asuntos Exteriores y Cooperación (3 anys).

Els estudis han estat subvencionats per beques de:

1. Fundació Marató TV3
2. Fondo de Investigaciones Sanitarias
3. Fundació Catalana de Pneumologia (FUCAP)
4. Fundació Española de Neumología y Cirugía Torácica (SEPAR)

Aprovació Comité Ètic

Tots els estudis han estat aprovats per el Comité Ètic de l'Hospital Clínic de Barcelona.

Finançament dels estudis

Suha Jabr

CONTRIBUTION TO THE STUDY OF THE ROLE OF ARACHIDONIC ACID METABOLISM IN AIRWAY INFLAMMATORY DISEASES (Nasal polyps, Asthma and Cystic Fibrosis)

Fibroblasts from nasal polyps of asthma patients have reduced expression of cyclooxygenase-2 and production of prostaglandin (PG) E_2. It is hypothesized that the reported alterations are due to alterations in the availability of AA. So we aimed to determine the fatty acid composition of airway fibroblasts from healthy subjects and from asthma patients with and without aspirin intolerance.

In patients with cystic fibrosis there is a relationship between prostanoid production and cystic fibrosis (CF) genotype severity, and also with the severity of the phenotype expression determined by the presence or absence of pancreatic insufficiency. We aimed to assess the relationship in patients with cystic fibrosis between prostanoid production and lung function values, pancreatic function as a measure of CF severity, and genotype severity. And to assess the relationship between PGE-M and PGD-M urinary metabolites of PGE_2 and PGD_2 and CF severity.