Effect of Omega-3 Polyunsaturated Fatty Acids on Inflammatory Biomarkers in Chronic Obstructive Pulmonary Disease

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DECLARATION

I certify that this thesis, which I submit to the University of Salford as partial fulfilment of the requirements for a Degree of Doctor of Philosophy, is a presentation of my own research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions.

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ACKNOWLEDGMENTS

Contribution to this thesis	
Medicines Evaluation Unit (MEU) (Staff/ Clinicians)	Subjects recruitment Subjects demographic and respiratory functions data Blood collection
University of Sterling (Lab technicians)	Erythrocyte membrane fatty acid analysis
University of Salford (PhD candidate)	Blood transporting and processing Demographic and respiratory functions data interpretation Biomarker analysis/ ELISA Assay PBMCs tissue cultures and Biomarker analysis / ELISA Assay Erythrocyte membrane fatty acids data interpretation Statistical analysis

LIST OF ABBREVIATIONS

ALA	α-linolenic Acid
ARA	Arachidonic Acid
ATP	Adenosine Triphosphate
ATS	American Thoracic Society
BAL	Bronchial-alveolar Lavage
BMI	Body mass index
BODE	BMI, airflow obstruction, dyspnea and exercise capacity
BSA	Bovin Serum Albumin
CAT	COPD Assessment Test
CBQC	COPD Biomarker Qualification Consortium
CC16	Clara cell protein-16
CCL2	Monocyte Chemoattractant Protein 1
CCL18	Chemokine (C-C motif) ligand 18
CCR2	CC-chemokine Receptor 2
CD31	Cluster of Differentiation 31

ConA	Concanavalin A
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CRP	C-Reactive Protein
CTGF	Connective Tissue Growth Factor
CVD	Cardiovascular Disease
DAMPs	Damage-associated molecular patterns
DHA	Docosahexaenoic Acid
DLCO	Diffusing Capacity of Carbon Monoxide
DMSO	Dimethyl Sulfoxide
DPA	Docosapentaenoic Acid
ECM	Extracellular Matrix
EGFRs	Epidermal Growth Factor Receptors
ELISA	Enzyme-linked Immunosorbant Assays
EPA	Eicosapentaenoic Acid
ERS	The European Respiratory Society

FAME	Fatty Acid Methyl Esters
FEV1	Forced Expiratory Volume 1
FGF	Transforming Growth Factor
FVC	Forced Vital Capacity
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	Streptavidin-Horseradish Peroxidase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GRO-a	Growth-Related Oncogene-a
ICS	Inhaled Corticosteroids
IFN-γ	Interferon-γ
IL-6	Interleukin-6
IL-8	Interleukin-8
IMT	Intima Media Thickness
IP10	IFN-inducible protein 10lio
LA	Linoleic Acid

LABA	Long-acting Bronchodilators
LAMA	Long-acting Muscarinic Antagonists
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTB4R	Leukotriene B4 Receptor
LTB4	Leukotriene B4
MAPKs	Mitogen-activated Protein Kinases
MDA	Malondialdehyde
MIA	Multiplex Immunoassays
MIG	Monokine induced by Gamma interferon
MIP-1α	Macrophage Inflammatory Protein 1-alpha
MIP-4	Macrophage Inflammatory protein-4
MMP	Matrix Metalloproteinase
mMRC	Modified Medical Research Council
MPs	Microparticles
NE	Neutrophil Elastase

NF-κB	Nuclear Factor Kappa Beta
NHS	National Health Service
NK	Natural Killer
NLRs	Nucleotide-binding oligomerization domain-like receptors
ODE	Organic Dust Exposure
PAMPs	Pathogen Associated Molecular Patterns
PARC	Pulmonary and Activation-regulated Chemokine
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PECAM	Platelet Endothelial Cell Adhesion Molecule
PHA	Phytohemagglutinin
PPAR-γ	Peroxisome Proliferator Activated Receptor γ
PRRs	Pattern Recognition Receptors
PUFAs	Polyunsaturated Fatty Acids
PYH	Pack Year History
RBC	Red Blood Cell

ROS	Reactive Oxygen Species
RPMI 1640	Roswell Park Memorial Institute 1640
SAA	Serum Amyloid A
SABA	Short-acting Bronchodilators
SP-D	Surfactant Protein-D
sRAGE	Soluble Receptor for Advanced Glycation End Products
Tc	T Cytotoxic
Th	T Helper
TGF-β	Transforming Growth Factor-β
TLC	Total Lung Capacity
TLRs	Toll-like Receptors
TMB	Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor-α
Treg	Regulatory T cell
USFDA	US Food and Drug Administration
WHO	World Health Organization

Abstract

Background Rationale

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality worldwide. It is a chronic inflammatory disease characterised by a not fully reversible airflow limitation. Several pro-inflammatory biomarkers are thought to be involved in the pathogenesis of COPD, but the association of particular biomarkers with COPD is still unclear. Omega-3 polyunsaturated fatty acids (omega-3 PUFAs) may be effective therapeutically in reducing airway inflammation in individuals exposed to smoke or other irritants. The results of previous studies have indicated that omega-3 PUFAs may have beneficial effects on COPD.

Aim

The aim of the present work is to determine systemic and pulmonary inflammatory biomarkers level in patients with COPD, smokers without COPD and healthy controls. Further, to determine *in vitro* the inflammatory mediator release from inflammatory immune cells particularly peripheral blood mononuclear cells (PBMCs). To investigate the effect of omega-3 PUFAs, Eicosapentaenoic acid, docosahexaenoic acid, on biomarkers release in PBMCs, and the relationships between these biomarkers activity, fatty acid analysis and COPD.

Methods

The current study data is from case-control study of 42 subjects; 15 patients with COPD (COPD), 15 healthy smokers (HS), and 12 healthy non-smoking controls (HNS), recruited from University Hospital South Manchester. Blood samples were processed to separate plasma, PBMCs and RBCs fractions. IL-6, TNF-α, IL-8, IP10, CRP, fibrinogen, SPD, CC16, CD31, CCL18, and RAGE level were measured in plasma by ELISA. Additionally, IL6, IL8, TNFα, CD31, IP10, and RAGE release were measured in PBMCs, with and without EPA and DHA

treatment, by ELISA. Individual PUFAs profile was carried out on RBCs. Comparisons between data of the three groups were made.

Result

Highly significant differences were observed in data (mean ± standard deviation) of patients' respiratory functions when comparing COPD to HS and HNS groups. The preFEV1% of COPD (52 ± 22) and FEV_1/FVC ratio (41.5 ± 12) are highly considered significantly higher than those from HS (99.5 ± 10.2), (69.2 ± 19.3) and HNS (99.6 ± 13), (76 ± 5.38) groups ($p < 0.001$). There was a significant correlation between FEV1% and age (P=0.0009) as well as BMI (P=0.024) of entire study subjects. Similarly, a significant correlation was seen between FEV1/FVC ratio and age (P<0.0001), BMI (P=0.0009) as well as weight of entire study subjects (P=0.022).

The levels of CRP, Fibrinogen were all significantly high in plasma of COPD group (4.48±28 mg/L) and (409±181mg/dl) when compared to those in HNS groups (2.27±2 mg/L) (p =0.007) and (137±49 mg/dl) (p <0.0001) respectively. The plasma levels of CC16 significantly were lower in plasma of COPD group (2.75±1.19 ng/ml) compared to HNS groups (5.77±2.66 ng/ml) (p =0.014). The release of IL-6, IL-8, and TNF-α, from PBMCs was significantly higher in COPD (4.07±0.46 ng/ml), (16.3 ±0.22 ng/ml), (219±11.9 pg/ml) when compared to HNS groups (0.16 ng/ml), (6.6±0.1.07 ng/ml), and (0 ng/ml) (p<0.001). PBMCs treated with 100μmol EPA or DHA showed decrease of IL6, IL8, and TNFα release.

Discussion

This study showed significant associations of higher level of several biomarkers that were detected in COPD group with the disease inflammation. The results also suggest that PBMC are already activated in the circulation in COPD patients, which confirms further that COPD is a multicomponent disease, involving both airway and systemic inflammation. The statistically significant correlation between the levels of CRP in plasma of patients with COPD and the percentage of Pre FEV_1/FVC ratio could be beneficial for patient treatment. CRP, fibrinogen and

CC16 in particular have emerged as potentially useful biomarkers and require further investigation. Additionally, omega-3 fatty acids; EPA and DHA have the ability to reduce cytokines release from PBMCs.

Keywords: COPD, Inflammation, biomarker, PBMCs, omega-3 PUFAs, EPA, DHA

Chapter 1

1. Introduction

1.1 Inflammation

Inflammation is the body's natural immediate response to infection or injury. This response triggers the immunological process of eliminating foreign pathogens, irritants and toxins and repairing damaged tissue in order to maintain tissue homeostasis. Inflammatory activation involves several biological functions such as leukocyte migration, proliferation of T-cells, interferon response, nuclear factor kappa beta (NF-κB) signaling, and regulation of cell death (Koniarevic *et al.,* 2014). It is initiated when an injury to cells or tissue occurs and is mediated by a complex network of pro-inflammatory mediators, inflammatory cells and signalling pathways. The innate immune system can recognize either microbial derived constituents known as pathogen associated molecular patterns (PAMPs), released during infection or endogenous damage-associated molecular patterns (DAMPs). These molecules are identified by pattern recognition receptors (PRRs), which can be found either attached to the cell membrane on toll-like receptors (TLRs) or within the cytoplasm on nucleotide-binding oligomerization domain-like receptors (NLRs). After the recognition of the pathogen or other irritant, the innate immune system responds with an inflammatory response by stimulating innate immune cells such as macrophages and neutrophils which secret pro-inflammatory factors to promote inflammation (Hosseinian et al., 2015; Lee *et al.,* 2016).

Pro-inflammatory mediators include; cytokine proteins termed interleukins (IL), which regulate differentiation, function and inflammation communication and response with immune cells; chemokines, which drive chemotaxis; and interferons, which react against viral invasion. These molecules are involved in both innate and adaptive immunity. The chemokines and adhesion molecules expressed by endothelial cells initiate interactions among circulating immune cells. Cells of the innate immune system, including monocyte-derived macrophages, dendritic cells, and granulocytes; neutrophils, basophils, and eosinophils, and the acquired immune system, including T and B lymphocytes, are involved in the inflammation process (Profumo *et al.,* 2012). For example, granulocytes and macrophages eliminate pathogens and

clear away cellular waste and damaged tissues. Helper T lymphocytes regulate the activities of macrophages, natural killer cells and B lymphocytes, while cytotoxic T lymphocytes and B lymphocytes are involved in eliminating pathogens (Calder, 2002) (Figure 1.1, next page).

It is suggested that inflammasomes, specialised inflammatory signaling pathway, regulate the maturation and secretion of pro-inflammatory cytokines, via the regulation of caspase-1–dependent proteolytic processing. Cytokines are extracellular signalling small proteins <80 kDa in mass and produced by different cell types. Cytokines are involved in cell-to-cell interactions and their effect may be influenced by other cytokines released from the same cell or from target cells following activation by the cytokine (Chung 2001). Inflammation is intended to be a protective process and should be controlled to eliminate the initial cause of the injury; however any defect within the normal resolution process may convert acute inflammation to chronic inflammation. Uncontrolled inflammation response also my result in severe inflammation that can cause tissue damage, impair tissue healing, and fibrosis (Lee *et al.,* 2016). In addition, deregulation of cytokines and chemokines can enhance the progression of pathologies related to chronic inflammatory and autoimmune diseases. Therefore, investigation of various systemic cytokines and chemokines may increase understanding of the immunological changes in patients suffering from autoimmunity and chronic inflammation (Giudetti and Cagnazzo, 2012; Kleiner *et al.,* 2013).
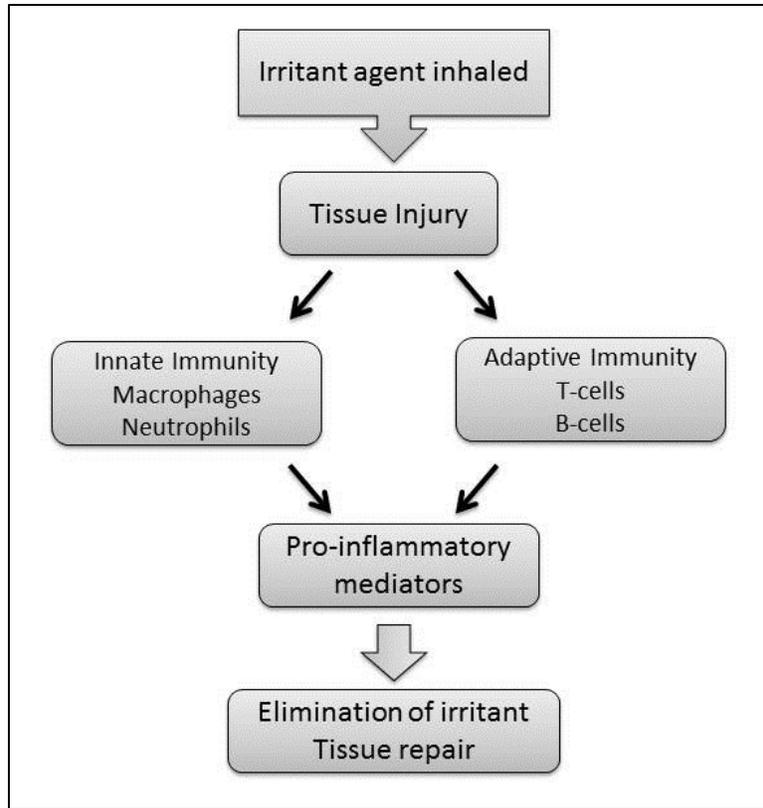
Figure 1.1: The interplay between component of the innate and acquired immune responses. *Immune cells involved in COPD including; macrophages, neutrophils and T-cells, are recruited to the injury tissue via the actions of various chemokines. Once activated, they contribute in irritant and pathogen elimination and tissue resolving and repair.*

1.2 Chronic Obstructive Pulmonary Disease (COPD)

The lung is a vital organ and it main function is to uptake and transfer of oxygen which is essential for metabolism. The lung is highly susceptible to inhaled foreign materials, irritant particles and different pathogens such as dusts, fuels, and microorganisms, which can lead to infection, inflammation and tissue injury. The first defense mechanism to protect the respiratory system from infectious agents and irritants is played by the lining innate immune system. The lung tissue consists of different cell types, including epithelial, endothelial, secretory, and inflammatory cells. The lung tissue and epithelial airway with innate and adaptive immune cells are able to trigger the inflammatory events in response to endogenous and exogenous irritants. As previously mentioned, an exaggerated inflammatory response might result in chronic

inflammation which can lead to the development of chronic obstructive pulmonary disease (COPD) (Lee *et al.,* 2016; Colarusso *et al.,* 2017).

COPD is a multicomponent chronic lung disease that decreases quality of life due to shortness of breath and chronic cough. Different pathogenic processes, inflammatory cells and their mediators are involved during disease progression and tissue damage, including; inflammation, alteration of immune responses, protease and anti-protease imbalance, cell proliferation and apoptotic cell death (Toraldo *et al.,* 2013). Many inflammatory mediators have been implicated in COPD, including lipids, free radicals, cytokines, chemokines and growth factors. These mediators are derived from inflammatory and structural cells in both the lung airways and the circulation, to interact with each other in the site of tissue injury.

Alterations in the lungs are characterized by several complicated respiratory and pathological features including, destruction of the lung parenchyma and alveoli, inflammation of the central airways with chronic bronchitis, and inflammation of the small airways with chronic bronchiolitis (Tuder and Petrache, 2012; Caramori *et al.,* 2014). This result in dyspnoea, progressive airflow obstruction that is not fully reversible, productive cough, fatigue, wheeze, weight loss, exacerbations and repeated respiratory infections (Fulton et al., 2012). Airway obstruction is caused by inflammation of the bronchi, which leads to increased mucus secretion with goblet cell and submucosal gland hyperplasia (Chung *et al.,* 2001). Lung parenchyma becomes infiltrated by neutrophils, macrophages and other inflammatory immune cells causing a loss of surface area for gas exchange. The destruction of lung parenchyma leads to distal airway compression, known as emphysema. All these events lead to irreversible decline of lung function (Fulton *et al.,* 2012; Matsuyama *et al.,* 2005).

In 1998, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) was established to direct global attention toward COPD. GOLD defined COPD as 'a disease state characterized by airflow limitation that is not fully reversible' (Korani *et al.,* 2016). In 2001, GOLD divided patients with COPD into four grades: GOLD A (mild); GOLD B (moderate); GOLD C (severe); and GOLD D (very severe) in addition to grade 0, which included people with normal spirometry that had a history of smoking and respiratory symptoms. In 2011 GOLD guidelines have removed stage 0 and COPD grades are determined by a COPD assessment of

symptoms, severity of airflow obstruction, and history of exacerbations and presence of comorbidities. Airflow limitation is determined by spirometry using a fixed-ratio of a forced expiratory volume in the first one second to the forced vital capacity (FEV1/FVC) <0.70 after bronchodilator which considered diagnostic of COPD. FEV1is the amounts of air that the patient can exhale in the first second, while FVC is the total volume of air that the patient can exhale in one breathe. It is also a measure of the amount of air the lungs hold. (Table 1.1) (Bailey *et al*., 2012)

Table 1.1: COPD severity according to 2011 GOLD grades A–D.

Grade	Risk	Symptoms	Airflow obstruction	Exacerbation history
GOLD A	Low	Low symptom burden	FEV1 of 50% or greater	Low exacerbation rate (0–1/year)
GOLD B	Low	Higher symptom burden	FEV1 of 50% or greater	Low exacerbation rate (0–1/year).
GOLD C	High	Low symptom burden	FEV1 of 50%	and/or high exacerbation rate (≥2/year).
GOLD D	High	Higher symptom burden	FEV1 of 50%	and/or high exacerbation rate (≥/year).

Symptom burden was measured by either the modified Medical Research Council (mMRC) questionnaire or the COPD assessment test (CAT) (Meng et al., 2016).

The GOLD guidelines suggest that the presence of FEV1 (% predicted) < 80% of the predicted value in combination with a FEV1/FVC < 70% confirms the diagnosis of COPD (Queiroz *et al.,* 2016). The percent predicted FEV1 is used as marker of disease severity and progression. While the post bronchodilator FEV1 is used to determine severity stage. However, FEV1 is an unreliable marker of disease activity, as it correlates poorly with both symptoms and disease progression (Vestbo *et al.,* 2013). The degree of emphysema and airway wall thickness can be measured by chest computed tomography (CT) scan (Lee *et al.,* 2018).

The inflammatory process is not limited to the pulmonary system, and its impact can extend to extra-pulmonary organs resulting in complications involving endocrine, metabolic and cardiovascular systems (Heidari 2012). Lung inflammation can pass through the systemic circulation causing systemic inflammations and comorbidities, which are frequently found in COPD patients, including cardiovascular disease (CVD), lung cancer, skeletal muscle dysfunction, diabetes, osteoporosis, and cachexia. CVD include four separate entities: ischemic heart disease, heart failure, atrial fibrillation, and hypertension. Psychiatric conditions, such as depression and anxiety, are also comorbidities of COPD (Lee *et al.,* 2018). These COPD comorbidities can occur at any COPD stage. Lung cancer and CVD are the most frequent comorbidity in COPD. It has been found that lung cancer is the most frequent cause of death in patients with mild to moderate COPD. Osteoporosis is often associated with decreased body mass index and low fat-free mass (Vestbo *et al.,* 2013). Skeletal muscle dysfunction is one of the main systemic effects of COPD and is often accompanied by loss of muscle mass. The mechanisms involved in loss of skeletal muscle mass and strength are likely caused by interaction between local and systemic factors to modify, the phenotype and function of any specific muscle. Systemic factors including inflammation and oxidative stress are thought to interact with local factors such as muscle inactivity. These result in functional, metabolic, and structural changes that weaken muscle work (Barnes and Celli, 2009).

It is unclear whether systemic inflammation is pathological or results from an overspill of inflammatory mediators from the lung to the circulation (Fulton *et al.,* 2012). Systemic inflammation and inflammation of non-pulmonary organs are known to adversely affect a patient's quality of life, which lead to increase the risk of hospitalization and mortality as well as costs (Tuder and Petrache, 2012; Calder, 2012; Toraldo *et al.,* 2013; Caramori *et al.,* 2014). CVD and lung cancer are the most common causes of death in COPD patients. In addition, at least 50% of COPD patients have bacterial infection of the lower airways that can increase both acute exacerbations and the risk of community acquired pneumonia (Barnes, 2010). Furthermore, respiratory viral infections including influenza and respiratory syncytial virus account for 64 % of COPD exacerbations (Wu *et al.,* 2016).

1.2.1 Prevalence

Most data available on COPD come from developed countries. There is a lack of studies in Africa, South East Asia and the Eastern Mediterranean region (Adeloye *et al.,* 2015). The prevalence of COPD is 0.2% in Japan, and 37% in USA, while India and China account for 66% worldwide COPD mortality. About 90% of COPD deaths occur in low and middle income countries (Rajkumar *et al.,* 2016). It has been estimated that 4–15% of the world's adult population suffers from COPD (Novgorodtseva *et al.,* 2013). It is the fourth leading cause of chronic morbidity and mortality. However, the World Health Organization (WHO) has predicted that by 2030, COPD will be the third leading cause of death worldwide (from sixth in 1990).

In 2005, 3 million people died of COPD, representing 5% of all worldwide deaths, and the number is estimated to increase to 8.6% in 2030 (Rajkumar *et al.,* 2016). The WHO estimated that about 65 million people worldwide have moderate-to-severe COPD. The mortality rate is higher in men than in women, and in the next 40 years the risk of developing COPD in healthy population over the age of 40 will be 12.7% for men and 8.3% for women. However, the prevalence in women has increased because of the rising number of those older than 50 years who smoke. In general, women smoke less than men, suggesting that they may be more susceptible to developing COPD in response to cigarette smoke exposure than men. During 2000, similar numbers of men and women died from COPD in the USA (Decramer *et al.,* 2012; Fulton *et al.,* 2012; Toraldo *et al.,* 2013; Nurwidya *et al.,* 2016). In the US, the annual cost of COPD has been estimated at about $50 billion, most of it related to hospitalization (Caramori *et al.,* 2014). In Europe about 23 million people have COPD, with an estimated annual cost of €141 billion including health care cost (Müller *et al.,* 2016).

The UK National Health Service (NHS) has suggested that more than 3 million people in the UK have COPD, and more than 1.5 million adults have been diagnosed with it (Dixon *et al.,* 2016). It has been estimated that COPD causes about 25,000 deaths in the UK annually. In England, a study was done to estimate the prevalence, healthcare costs, and mortality rate among people with COPD for the period 2011–2030; such a study could help governments to plan their healthcare budgets and financial resources. The study findings showed that in England, the number of people with COPD is increasing particularly in females by 54% comparing to 24% in males. These increases were driven by more women developing COPD. The annual healthcare

costs of COPD will increase from £1.50 billion in 2011 to £2.32 billion in 2030, while the number of deaths will increase from 99,200 in 2011, to 129,400 by 2030 (Figures 1.2, 1.3, and 1.4) (McLean *et al.,* 2016). The priority for the NHS regarding COPD management is to improve the health quality of life for patients with COPD, to reduce premature death, avoid unnecessary admissions and time in hospital (Dixon *et al.,* 2016).
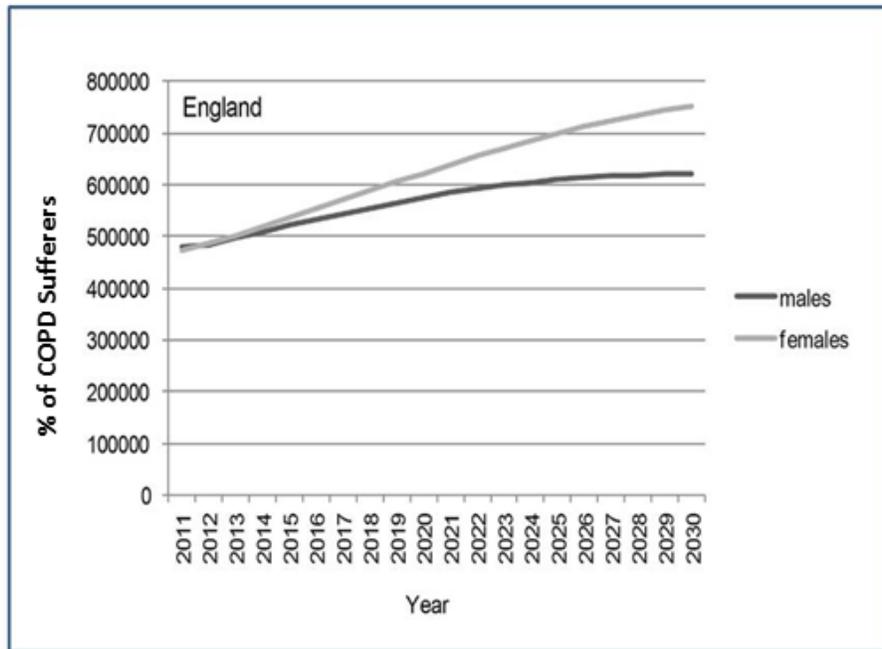
Figure 1.2: Projected number of people with COPD in England (McLean *et al.,* 2016)
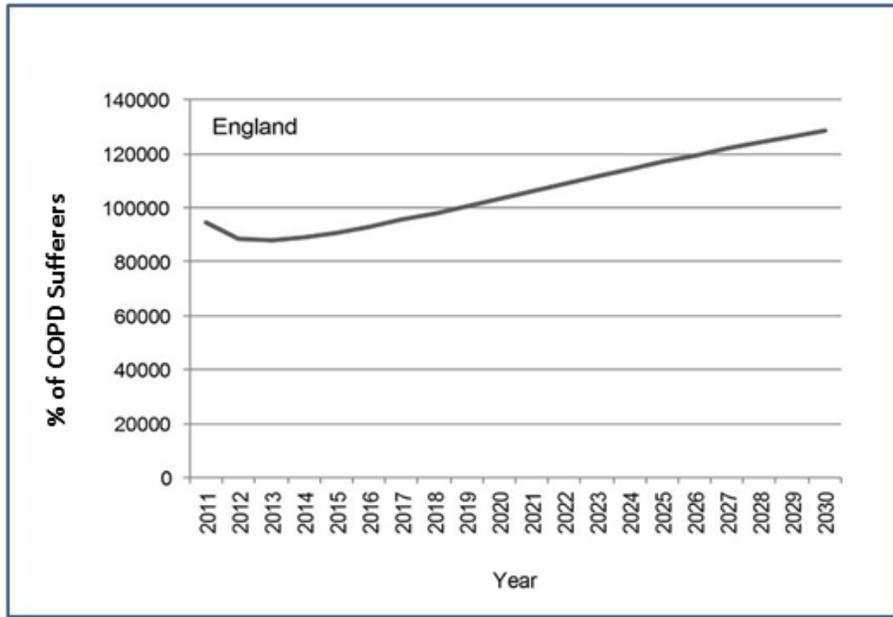
Figure 1.3: Projected number of deaths among people with COPD in England (McLean *et al.*, 2016).
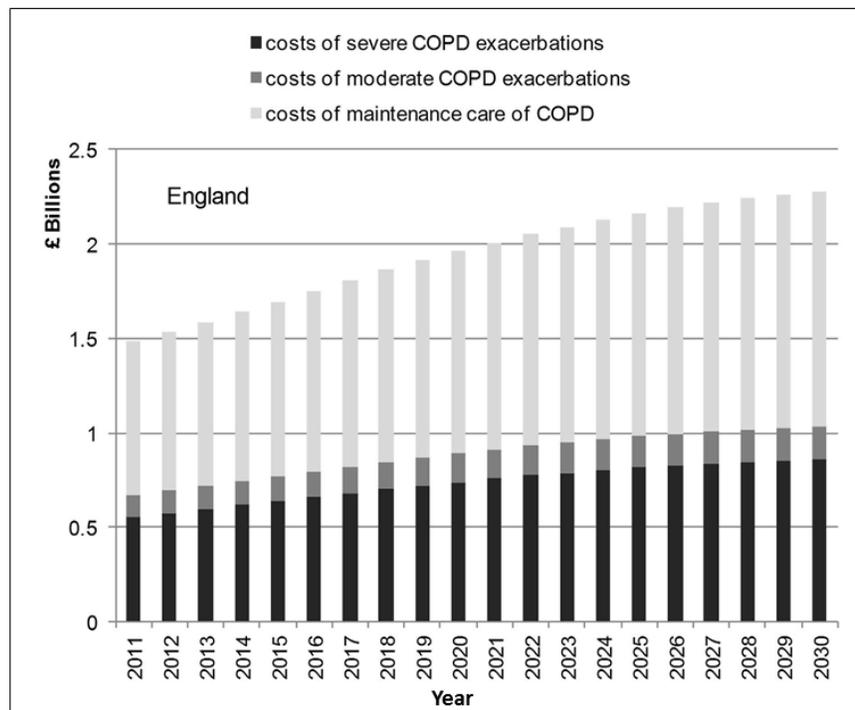
Figure 1.4: Projected direct healthcare costs of COPD in England (McLean *et al.*, 2016)

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1.2.2 Risk factors

Smoking is a major risk factor for various lung diseases and is the fifth leading cause of deaths worldwide (Hsiao *et al.,* 2013). However, in 2012 smoking was the third most common cause of death (WHO, Titz *et al.,* 2016). The WHO estimated that in 2005, 5.4 million people died due to smoking, and that the number will increase to 8.3 million per year by 2030. The main risk factor for COPD is cigarette smoking, which accounts for more than 90% of COPD cases in western countries. Smoking is considered a pro-inflammatory factor and it has been associated with development of chronic respiratory and airflow-obstruction diseases. The prevalence of COPD in women has increased because the number of smoking women has increased (Decramer *et al.,* 2012). In addition to nicotine, cigarette smoke contains other injurious agents, including heavy metals and carcinogens. It is believed that oxidative stress induced by cigarette smoke exposure is also involved in the pathophysiology of COPD. Smokers are exposed to various oxidants, including free oxygen radicals such as peroxide (O_2^-), which cause lipid peroxidation; α- and β-unsaturated aldehydes; acrolein (C_3H_4O) and crotonaldehyde ($CH_3CH=CHCHO$), which cause protein carbonylation; and superoxide (O^{-2}); hydroxyl radical (OH^-), hydrogen peroxide ($H_2O_{2)}$ (Tuder and Petrache, 2012).

However, 25–45% of COPD patients have never smoked, and only 20 % of smokers develop COPD and smokers who do not have COPD have a lesser degree of inflammation than those who have COPD. Such statistic suggests that genetic and environmental factors other than smoking may be involved. Individual genetic predisposition to develop COPD and exposure to environmental pollution and biomass smoke may have an impact on disease predisposition, and may explain why some patients who develop COPD are never smoke (Eickmeier *et al.,* 2010; Wald *et al.,* 2014; Varraso *et al.,* 2015). The combination of these risk factors alters lung development and immunity and results in the predisposition for COPD (Decramer *et al.,* 2012) (Figure 1.5).
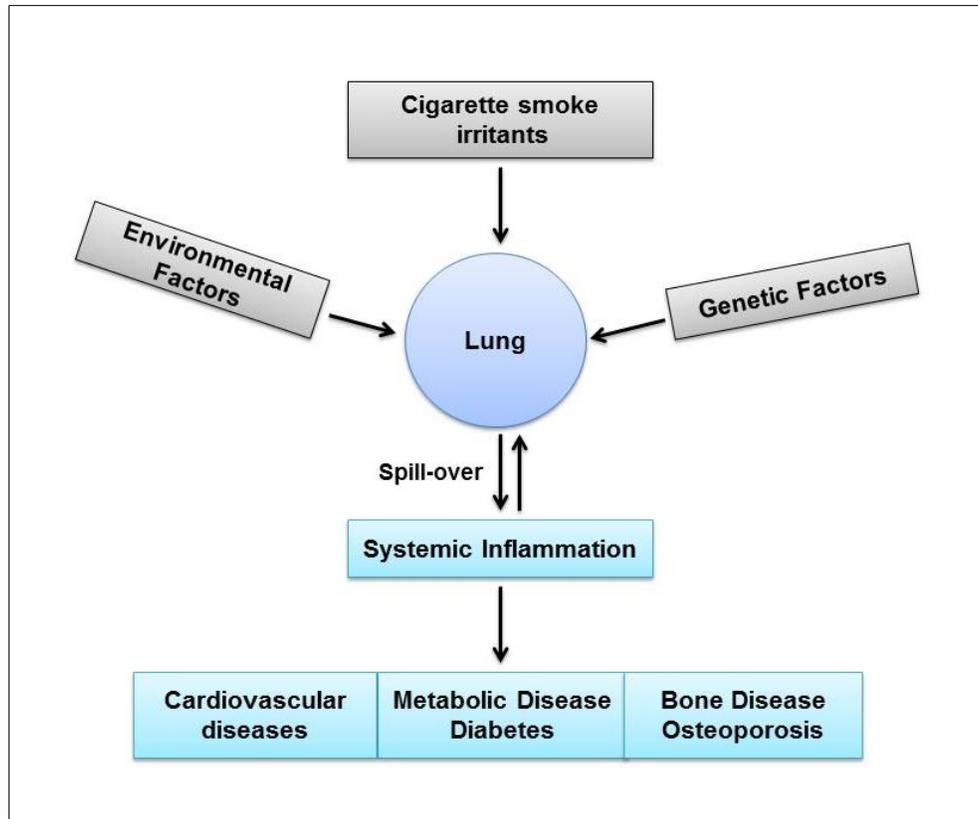
Figure 1.5: Systemic inflammation and comorbidities in COPD. *Patients with COPD have lung inflammation that may spill over into the systemic circulation, leading to increase the propensity to cardiovascular, metabolic, and bone diseases. There might be a genetic predisposition to developing COPD in smokers in addition to outdoor irritants factors.*

Other risk factors include maternal smoking, respiratory infections, poor nutrition, and exposure to indoor and outdoor air pollution, noxious particles, gases, occupational chemicals and dust at the workplace (Decramer *et al.,* 2012; Tuder and Petrache, 2012). About 15–20 % of COPD cases are due to occupational exposures to pollutants, and about 50 % of COPD death rate found in developing countries as a result of the exposure to biomass smoke during lifetime (Terzikhan *et al.,* 2016). Other chronic respiratory disease such as asthma and post-tuberculosis are important predisposing conditions for progression of COPD (Rajkumar *et al.,* 2016).

1.2.3 Pathophysiology of COPD

In COPD the pathogenesis of inflammation, airway remodeling, and alveolar destruction is complex and not completely understood (Toraldo, 2013). Lung inflammation is regulated by a complex mechanism that involves various types of cells and pro-inflammatory mediators. (Berg and Wright 2016).The exposure of cigarette smoke, microorganisms and other noxious inhaled substances cause chronic airway inflammation and tissue destruction by activating structural and inflammatory cells, including airway epithelial cells and alveolar macrophages, within the lungs. These cells stimulate the release of early cytokines and chemotactic mediators, which attract immune system inflammatory cells, including neutrophils, monocytes and lymphocytes, into the lung, which in turn causes chronic inflammation (Rovina *et al.,* 2013). Cigarette smoking also causes structural and functional changes in the airway ciliary epithelium, and immune cells such as alveolar macrophages, neutrophils, and lymphocytes (Nurwidya *et al.,* 2016). According to Lapperre *et al* (2006) it is possible that inflammation which caused by smoking occurs in two stages; an early stage, during which neutrophils and macrophages are present in pulmonary epithelial and submucosal tissue, and a late phase with the additional participation of lymphocytes and eosinophils from circulation.

Epithelial changes occurred mostly in the large airways than the small airways, with an increase in goblet and basal cell hyperplasia, and squamous cell metaplasia. The small airways is the major site of airway obstruction in subjects with COPD (Hogg *et al.,* 2013).Unlike large airways, small airways lack cartilage, and have a greater mass of smooth muscles and fewer goblet cells in the epithelial layer (Stewart and Criner, 2013). In the airway lumen, more mucus can be found, which is produced by the goblet cells and enlarged mucus glands. The inflammatory cells infiltrate into the mucosa, submucosa, and glandular layers of lung tissue resulting in structural changes in small airways, thickening of their walls and emphysema, loss of tissue elasticity, mucus hypersecretion and defects in the tissue-repair process. The increased thickness of the airway walls occurs as a result of increases in smooth muscle mass, mucus gland activity and extracellular matrix (ECM) deposition (Brandsma *et al.,* 2017) (Figure 1.6).

Vascular changes constitute another histopathological feature in patients with COPD. Vascular changes are caused by the pathophysiological changes in the normal biochemical function of the endothelium. The main vascular changes can be seen in thickened arteriole walls, reduced lumen, increased contraction, and reduced ability to vasodilate, together causing pulmonary hypertension, which is a major cause of morbidity in COPD. The increased thickness of the intima in the arterial vasculature caused by smooth muscle proliferation, with increased deposition of both elastin and collagen (Berg and Wright, 2016).The distance between the leading edge of the lumen intima echo and the leading edge of the media-adventitia layer is known as intima media Thickness (IMT), and consider as a measure of atherosclerosis that independently predicts CVD. The normal IMT of healthy subjects is 0.74 ± 0.14 mm. Some studies reported that IMT <0.8 mm is associated with normal healthy individuals and a value >1 mm is associated with atherosclerosis and increased CVD risk. A previous study found that IMT of healthy subject was 0.75 ± 0.011 mm (Paul *et al.,* 2012). Increased IMT in patients with COPD has been found to be associated with deaths from CVD. IMT was significantly higher in COPD patients than in control group (1.07 mm vs 0.86 mm) (Alpaydin *et al.,* 2013). In the same line, IMT was also significantly higher in patients with COPD than in those without COPD (1.40 mm and 1.24mm, respectively). Another study found that IMT increased more in patients with COPD than in patients without COPD, and such an increase was higher in patients with both COPD and CVD than in patients with CVD or COPD only (Köseoğlu *et al.,* 2016).
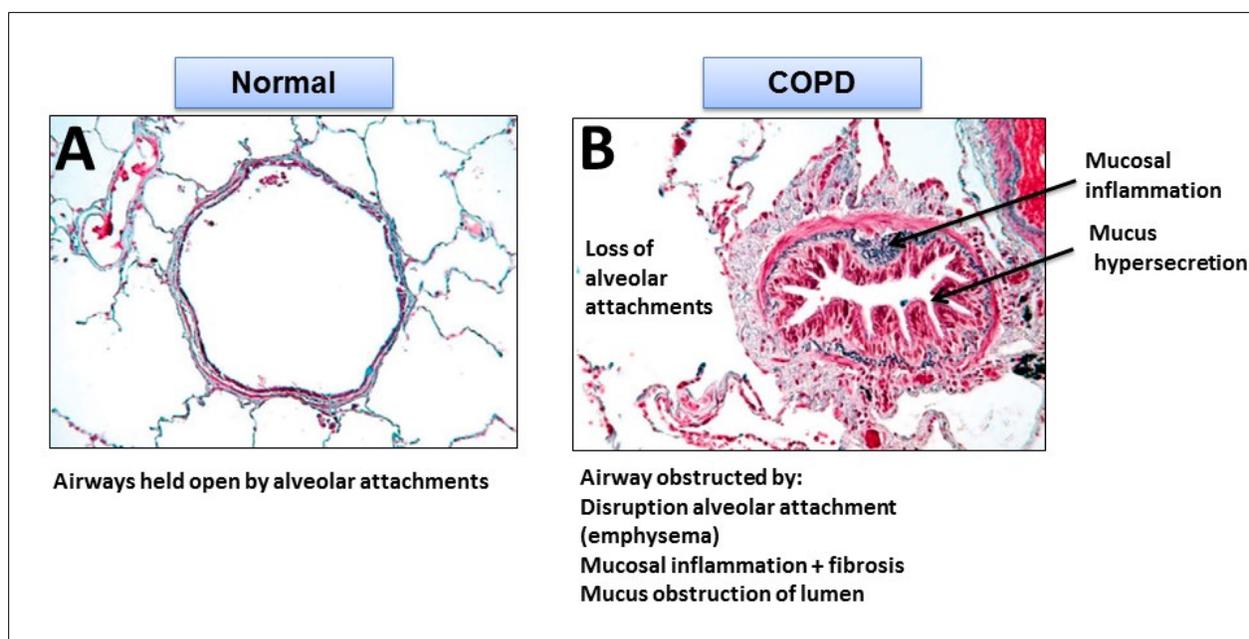
Figure 1.6: Comparison of airway in a healthy individual and in-patient with COPD. *(A) In normal subjects the airway is distended by alveolar attachments which allowing alveolar emptying on expiration and lung deflation. (B) In COPD airways are narrowed by infiltration of inflammatory cells, mucosal, alveolar attachments are disrupted because of emphysema, thus contributing to airway closure, trapping gas in the alveoli and resulting in hyperinflation (Barnes 2004; Decramer et al, 2012).*

As COPD progresses, aggregations of lymphoid tissues develop around the small airways, causing alveolar enlargement. Cigarette smoke dysregulates the alveolar repair processes by stimulating airway epithelial cells to release transforming growth factor-β (TGF-β), which induces fibrotic tissue remodeling. In healthy subjects, to maintain lung homoeostasis cellular apoptosis and matrix destruction are countered by cellular renewal and matrix repair, controlling the inflammation process. As previously mentioned, an uncontrolled inflammatory response might result in chronic lung inflammation which can progress to COPD. In COPD cells are metabolically active, but do not divide, which leads to increased inflammation, reduced cell regeneration and carcinogenesis (Decramer *et al.,* 2012).

Oxidative Stress is reported to play an important role in the pathophysiology of COPD. An imbalance between oxidants and antioxidants has been proposed as a critical event in the pathogenesis of COPD (Ilieva *et al.,* 2014).When ROS are produced in excess of the antioxidant defense mechanisms, oxidative stress occurs causing damage to lipids, proteins and DNA. Lipid

peroxidation is the major consequence of oxidative stress which leads to cellular damage PUFAs react with oxygen radicals and oxidised to produce several lipid end-products like hydroperoxides and aldehydes. For examples, Malondialdehyde (MDA) aldehyde is considered as potential biomarker to assess oxidative stress status in COPD patients (Waseem *et al.,* 2011; Zinellu *et al.,* 2016). Various Studies have reported that the plasma levels of MDA are increased in COPD patients compared to healthy controls. Waseem *et al*, 2001, reported an increase in MDA levels in COPD patients, as compared to healthy Controls, and it level negatively correlated with lung functions. Another Study conducted by Ilieva *et al.,* 2014, showed that MDA levels are significantly higher in patient with COPD than in healthy controls. Increased oxidative stress in patients with COPD, demonstrated by the increased of MDA in the plasma of these patients. Another study found that oxidative marker MDA is higher in COPD patients and its level correlate with lung functions and disease severity (Bajpai *et al.,* 2017).

1.2.4   Inflammatory cells involved in COPD

The inflammation seen in COPD patients involves inflammatory cells of the innate (macrophages, neutrophils, eosinophils, mast cells, natural killer cells, innate lymphoid cells and dendritic cells), and adaptive (T and B lymphocytes) immune system, as well as structural cells; airway epithelial cells, endothelial cells, fibroblasts, and smooth-muscle cells. These cells secrete various pro-inflammatory mediators, including cytokines, chemokines, growth factors and lipid mediators, which are implicated in COPD pathogenesis (Barnes 2016) (Figure 1.7).
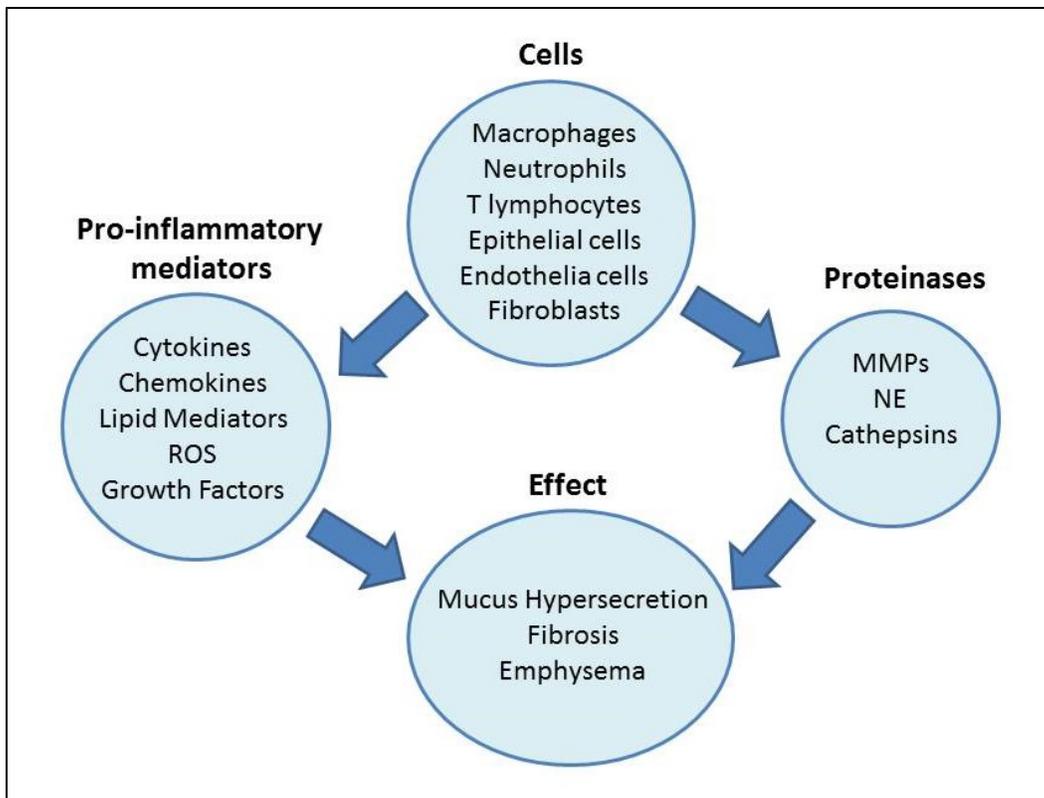
Figure 1.7: The complexity of inflammation in COPD. *Different activated inflammatory immune and lung cells release several mediators; chemokines, ROS, which prolongs neutrophils' survival; cytokines that amplifies inflammation. Macrophages secrete many inflammatory proteins that trigger the inflammatory process in patients with COPD including several chemokines that attract Neutrophils and lymphocytes. Proteinases including, NE, proteinase C (cathepsins), and MMPs cause elastolysis, and contribute to emphysema together with T lymphocytes. NE also causes mucus hypersecretion. Release of growth factors can induce fibrosis of the small airways. This combination of mediators that attract and activate inflammatory cells and proteinases results in the typical pathophysiology of COPD. Abbreviations: NE, neutrophils elastase; MMPs, matrix metalloproteinases; ROS, reactive oxygen species (adapted and updated from Barnes 2004).*

1.2.4.1   Structural Cells involved in COPD

Airway epithelial cells can secrete cytokines that either initiate local inflammatory effects or amplify inflammatory events previously initiated by inflammatory cells. Airway epithelial cells activated by cigarette smoke and other noxious agents, produce inflammatory mediators, including tumour necrosis factor (TNF-α), interleukin-1β (IL-1β), granulocyte-macrophage colony-stimulating factor (GM-CSF) and TGF-β1, which activate myofibroblasts and airway smooth-muscle cells, causing cell proliferation and fibrosis. In addition, epithelial cells produce several chemokines that activate neutrophil recruitment in COPD, including leukotriene B4

(LTB4), IL-1 (CXCL1), IL-8 (CXCL8), CXCL9 or monokine induced by gamma interferon (MIG), CXCL10 (IFN-inducible protein 10) (IP-10) or small-inducible cytokine B10, and growth-related oncogene-a (GRO-a). IL-8 also mediates the chemotaxis of neutrophils, eosinophils and T-cells (Rovina *et al.,* 2013).

In patients with COPD, expression of CXCL10 by bronchial epithelial cells is increased, which may lead to accumulation of CD8 Tc1 and CD4 Th1 cells in the lung tissue. Airway epithelial cells secrete mucus from goblet cells, antioxidants and anti-proteases, all of which are involved in the defence system of the airways. However, cigarette smoke and other irritants affect this defence mechanism, causing mucus hyperplasia, which is common feature in COPD patients (Barnes 2016). Airway epithelial cells can modulate the production of histamine by basophil, and inhibit the production of the IL-4-mediated B-lymphocytes IgE production. Other structural cells include; Fibroblasts, myofibroblasts and smooth-muscle cells are thought to be involved in airway remodelling via production of collagen and proteoglycans (Giudetti and Cagnazzo 2012).

1.2.4.2   Innate Immunity Cells

The lungs of a COPD patient are infiltrated with increased numbers of macrophages, neutrophils, T lymphocytes, dendritic cells and B lymphocytes. Macrophages and neutrophils play a role in the pathogenesis of COPD by releasing proteinases that degrade the ECM and damage the lung parenchyma, leading to emphysema (Tubby *et al.,* 2011).

Monocytes recruited from the blood enter the lung tissues, where they differentiate into macrophages in addition to the tissue resident macrophages. A study reported the presence of macrophages with different phenotypes in lungs tissue of healthy subjects. Those cells either originate locally from pulmonary dendritic cells and alveolar macrophages or derived from blood monocytes (Desch *et al.,* 2016). Macrophages are found in an increased number in the lungs of patients with COPD and are involved in host defence, airway remodeling and parenchymal destruction. It has been suggested that increased lung macrophage numbers are due to increased recruitment of blood monocytes. Further, cigarette smoke, which stimulates the expression of anti-apoptotic genes in macrophages, may delay apoptosis causing accumulation of macrophage in lung tissue and airways of patients with COPD (Ravi *et al.,* 2017). Several studies have

reported that the increased numbers of macrophages, in the airways, lung tissue, Bronchial-alveolar Lavage (BAL) fluid and sputum of COPD patients is correlated with the severity of emphysema. Data indicate that in COPD patients, alveolar and monocyte-derived macrophages are more activated and release higher levels of inflammatory mediators, includes chemokines, cytokines and proteases, while their phagocytic ability and apoptotic activity is reduced (Barnes, 2016).

When stimulated by cigarette smoke, activated neutrophils and macrophages release several inflammatory mediators, including, IL-6, IL-10, TNF-α, CXCL1, IL8, monocyte chemoattractant protein 1 (CCL2), LTB4 and (reactive oxygen species) ROS, all of which cause membrane lipid peroxidation and alveolar epithelial injury. Macrophages also secrete CXCL9, CXCL10 and CXCL11, which are chemotactic for Tc1 and Th1 cells. Alveolar macrophages release proteinases, including the matrix metalloproteinases (MMPs) MMP-2, MMP-9 and MMP-12; cathepsins K, L and S; and NE (Barnes 2016). Macrophages also secrete IL-8 and LTB4, to stimulate neutrophils to release IL-8 and LTB4 to attract further neutrophils to the site of inflammation (Toraldo *et al.,* 2013).

Increased numbers of neutrophils have been found in the sputum and BAL fluid of COPD patients and correlate with the severity of the disease. The migration of neutrophils from circulation into the respiratory tract involved adhesion molecules, cytokines, and chemotactic mediators include, LTB4, CXCL1, CXCL2, CXCL3, CXCL5 and IL-8, released by epithelial cells, alveolar macrophages and T-cells. The levels of these chemotactic factors have been found to be elevated in the airways of COPD patients (Blidberg *et al.,* 2013; Caramori *et al.,* 2014). Like macrophages, neutrophils secrete serine proteases, including NE, cathepsin G, proteinase-3, MMP-8 and MMP-9, all of which may contribute to alveolar destruction.

It is likely that airway neutrophilia is linked to mucus hypersecretion in chronic bronchitis, because neutrophil elastase (NE), cathepsin G, and proteinase-3 are potent stimulants of mucus secretion from submucosal glands and goblet cells. It is suggested that mucus hyperplasia is induced by epidermal growth factor receptors (EGFRs), which are activated by NE secreted by neutrophils, causing release of transforming growth factor alpha (TGF-α) (a ligand of EGFRs) (Barnes 2004; Rovina *et al.,* 2013). Andelid *et al.* (2015) found that concentrations of

neutrophil and NE protein were higher in both smokers with COPD and in healthy smokers than in healthy non-smoker subjects.

Increased eosinophil numbers have been observed in the airways and BAL fluid of patients with COPD. However, others studies have not reported the same observation in airway biopsy specimens, BAL fluid or sputum. Dendritic cells can activate various inflammatory immune cells, including macrophages, neutrophils and T and B-lymphocytes, and may play an important role in the lungs inflammation response to cigarette smoke and irritants. Dendritic cells are located near the surface in airways and lungs, and so can inspect the entry of inhaled substances. Studies have shown increased numbers of dendritic cells in the lungs of COPD patients (Barnes 2016).

1.2.4.3   Adaptive Immunity Cells

B-cells have been observed in the airways organized into small primary or secondary follicles, and also found scattered in the parenchyma. These follicles are notable in patients with severe COPD but less in healthy smokers (Brandsma *et al.,* 2017). Increased numbers of neutrophils and B lymphocytes are usually associated with severe COPD (Caramori *et al.,* 2014). Several studies have examined the phenotype of T lymphocyte in the alveolar wall as different populations of lymphocytes involved in alveolar enlargement and lung destruction in COPD (Borchers *et al.,* 2007).

T-cells progenitors originate in the bone marrow and develop in the thymus. The major families of T-cells express either CD4 or CD8 on their surface. Different immunologic stimuli trigger differentiation of naive CD4 T-cells via different developmental pathways give arise to different T-cell phenotypes subsets such as; T-helper 1 (Th1) and T-helper 2 (Th2) cells, T-helper 17 (Th17) cells and regulatory T-cells (Tregs). All these phenotypes are involved in host defence in different pathological or disease states. Other subsets of T-cells include cytotoxic T (Tc) CD8 cells are involved in pathogen elimination (George, 2008; Lane *et al.,* 2010; Calder 2015). Th1 cells are involved in host defence against intracellular pathogens by secreting IL-12, IFN-γ, macrophage inflammatory protein (MIP)-1α, and MIP-1β which mainly activate macrophages during inflammation. Th2 cells are involved in clearance of extracellular pathogens, eosinophilic inflammation, and secret IL-10, IL-5, IL-4, IL-9, IL-13, and IgE in

allergic reactions, and enhance antibody production by B-cells. Treg cells have anti-inflammatory, immune-regulatory and suppressive effects and are involved in adaptive immune responses. Tregs are able to suppress CD4 and CD8 T-cells and can be further subdivided into natural Tregs, inducible Tregs, Th3 cells that produce TGF-β and type I regulatory cells.

Another T-cell, the γδ T-cell, can suppress the proliferation of and cytokine production by activated effector T-cells (George, 2008; Lane *et al.,* 2010; Profumo *et al.,* 2012). Other subtypes, the Th17 and Th9 cells, are involved in inflammation and pathogenesis in autoimmune diseases (Jager and Kuchroo, 2010; Profumo *et al.,* 2012). IL-17 (IL-17A), which is produced mainly by Th17 CD4 and Tc17 CD8 cells, activates the release of CXCL1, CXCL8 and GM-CSF from airway epithelial cells and smooth-muscle cells (Caramori *et al.,* 2014). One study has suggested that Th17 cytokines are involved in the pathogenesis of COPD, as the levels of IL-17A, IL-17F and IL-17R were increased in epithelial cells and infiltrated cells in the sub-epithelium of both the small and large airways of COPD patients than in healthy controls (Montalbano *et al.,* 2015**).**

Cigarette smoke can stimulate adaptive immune responses in the lungs of COPD patients, through the activation of Tc, Th1 and Th17 cells, alongside B-cell antibody production. CD8 T-cells, that clear viral infections, have been shown to be present in increased numbers in the lungs of patients with COPD. However, they fail to protect against respiratory viral infection. A study by McKendry *et al* (2016) confirmed a dysregulation of CD8 T-cells responses to viral infection in the lungs of COPD patients, which may explain both their susceptibility to viral infection and exacerbations. CD8 T-cells activate and recruit macrophages via producing different chemokines and cytokines such as IFN-γ. Further, CD8 T-cells are able to induce cytolysis and apoptosis of alveolar epithelial cells through release of proteolytic enzymes such as perforins, granzyme-B, and TNF-α causing cell death (Majo *et al.,* 2001). Some studies documented that the number of CD8 T-cells is increased in lung parenchyma and airways of patients with COPD with late stages of airflow limitation and emphysema. These findings may suggest a possible role of CD8 T-cells in the pathogenesis of emphysema in patients with COPD (Finkelstein *et al.,* 1995; Saetta *et al.,* 1999; Majo *et al,* 2001). Domagała-Kulawik *et al* (2011) also showed that the proportions of CD8 T-cells in the blood of COPD patients were significantly higher than in healthy subjects. CD8 T-cells belong to regulatory cells family and this observation adds another argument to the

hypothesis that the autoimmune reaction plays a role in the pathogenesis of COPD. In contrast to the previous result, another study found that the number of CD4 T-cells was lower in COPD compared to controls, while there was no difference between COPD and control in CD8 T-cell and monocyte number (Roberts *et al.,* 2015).

It is thought that CD8 T-cells in COPD mainly protect lung epithelial tissue after exposure to smoke or other irritants. While different cell types and tissue continue to accumulate this affect T-cells function causing an increase of inflammation. Motz and colleagues reported for the first time that cigarette smoke exposure leads to the generation of pathogenic T-cells capable of inducing COPD-like disease in mice. Transfer of CD3 T-cells from the lungs of cigarette smoke-exposed mice into Rag2$^{-/-}$ recipients induce pulmonary changes pathognomonic of COPD included; monocyte/macrophage and neutrophil accumulation, increased expression of cytokines and chemokines, activation of proteases, apoptosis of alveolar epithelial cells, ECM degradation, and emphysema (Motz *et al.,* 2010). The numbers of Th17 cells, which secrete IL-17A and IL-22, are also increased in the airways of patients with COPD and might involve in neutrophilic inflammation (Barnes 2016). Natural killer (NK) T-cells, also found in increased numbers in COPD lungs, may drive alveolar cell apoptosis via lung epithelial cell NKGD2 ligands (Borchers *et al.,* 2009). The interaction between inflammatory immune and structural cells involved in inflammatory mechanisms of COPD is simplified in (Figure 1.8).
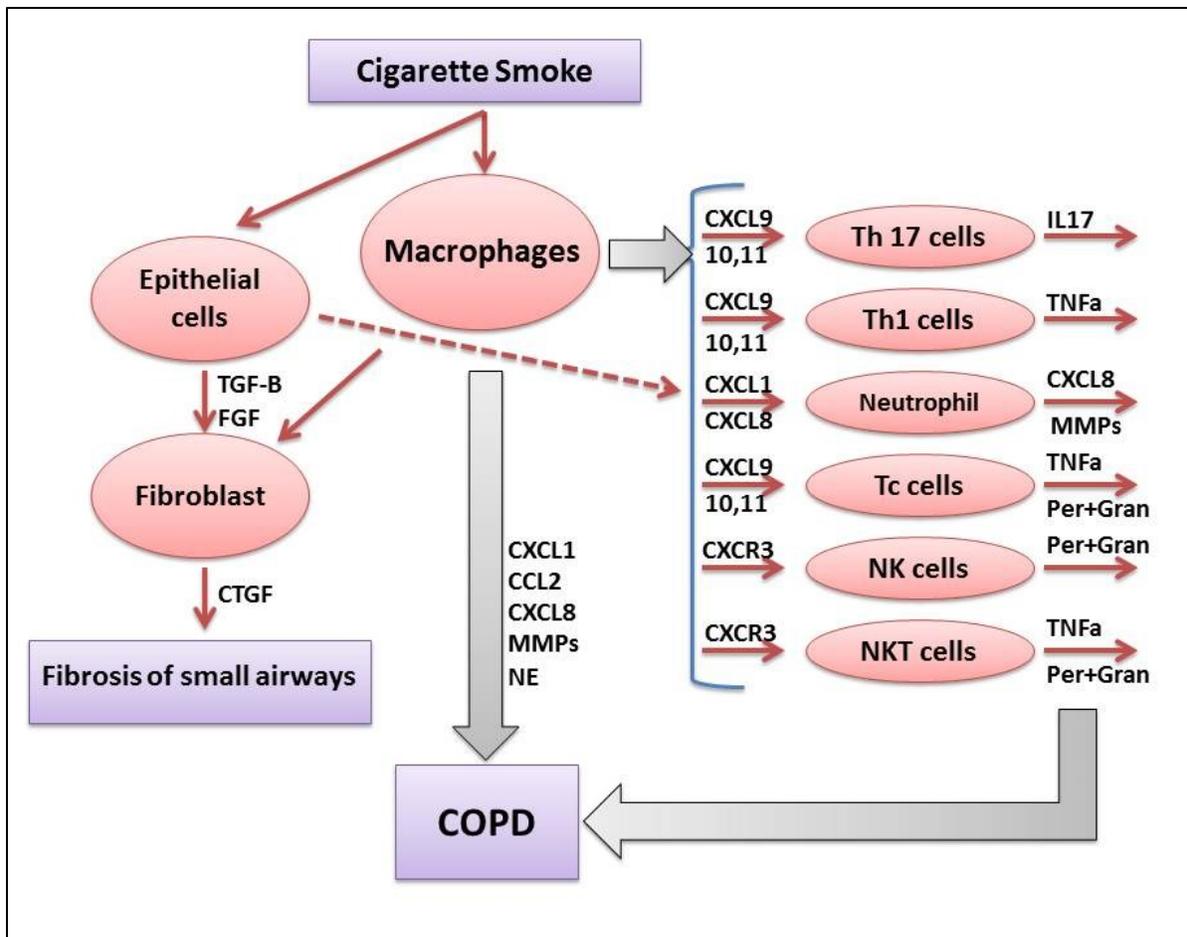
Figure 1.8: Inflammatory and immune cells involved in inflammatory mechanisms of COPD. *Inhaled substances such as cigarette smoke and other irritants activate alveolar macrophages (derived from circulating monocytes and differentiate within the lung) and epithelial cells in the respiratory tract to secret growth factors such as TGF-β and FGFs, which stimulate fibroblast proliferation, resulting in fibrosis in the small airways. These cells also release the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, all of which amplify inflammation, and several chemotactic factors that attract inflammatory cells to the lungs, including CC-chemokine ligand 2 (CCL2), which acts on CC-chemokine receptor 2 (CCR2) to attract monocytes, CXC chemokine ligand 1 (CXCL1) and CXCL8, which attract neutrophils and monocytes (which differentiate into macrophages in the lungs) and CXCL9, CXCL10 and CXCL11, which act on CXCR3 to attract T helper 1 (Th1) cells and cytotoxic T-cells (Tc cells). These inflammatory cells together with macrophages and epithelial cells release proteases, such as matrix metalloproteinase 9 (MMP9), which cause elastin degradation and emphysema and stimulate mucus hypersecretion. Abbreviations: Gran, granzymes; Per, perforin; neutrophils elastase, NE; (TGF)-β, transforming growth factor; FGF, fibroblast growth factors, CTGF, connective tissue growth factor; MMPs, matrix metalloproteinases; NKT, natural killer T cells; NK, natural killer cells (adapted and updated from Barnes 2010; Tubby et al., 2011).*

1.2.5 Pro-inflammatory Biomarkers in COPD

As previously mentioned, various immune cells and lung structural cells secrete a variety of pro-inflammatory mediators, including cytokines, chemokines, growth factors and lipid mediators, which play an important role in COPD pathogenesis and inflammation. Studies of the pathogenic mechanisms of COPD and newer technologies have identified diagnostic biomarkers of disease activity, which can be considered therapeutic targets of COPD. A biomarker may be defined as an indicator of measurable characteristics of normal biologic processes, the progression or severity of a disease or response to treatment. Hence, biomarkers could help physicians design targeted therapies based on patient biomarker levels. However, to evaluate the clinical utility of biomarkers in treating COPD patient, further research is required (Khan and Daga, 2015; Chen *et al.,* 2016). Several biomarkers involved in the pathophysiology and inflammatory process of COPD have been identified and measured, but little is known about their relationship to COPD development, progression and severity (Korani *et al.,* 2016).

It has been documented that various pulmonary and systemic inflammatory biomarkers including, TNF-α, IL-6, IL-8, IL-18, fibrinogen, acute phase proteins, including C-reactive protein (CRP), serum amyloid A (SAA) and surfactant protein (SP-D), are elevated in patients with COPD when compared with healthy subjects (Barnes 2010, Toraldo *et al.,* 2013, Williams *et al.,* 2016). Previous studies have identified specific biomarkers that may distinguish individuals with COPD from healthy subjects. These biomarkers include, lung-derived Clara cell protein-16 (CC16), SP-D, extracellular matrix breakdown markers; MMP-8 and -9, and systemic inflammatory biomarkers include; fibrinogen, CRP, and cytokines; IL-6 and IL-8, CCL-18 (Pinto-Plata *et al.,* 2007). Markers that have been investigated as potential indicators for COPD can be determined from different samples include sputum, exhaled breath condensate, BAL fluid, lung biopsies and blood (Comes *et al.,* 2016).

A three-year cohort study of 1,843 COPD patients investigated the associations between mortality and the levels of several biomarkers, including SP-D, IL-6, IL-8, CC16, TNF-α, fibrinogen and CRP. Elevated plasma fibrinogen levels were found to be associated with increased risk of exacerbations in COPD patients. The results suggested that using a panel of specific biomarkers rather than individual biomarkers helped to establish clinical variables to predict COPD mortality (Celli *et al.,* 2012). Furthermore, Chen *et al.* (2016) conducted a

systematic review to evaluate biomarkers that might be involved in COPD. The review included 59 studies, in which the most commonly studied biomarkers were CRP, IL-6 and TNF-α. The results showed that CRP levels were higher in COPD patients than in control subjects, while IL-6 and TNF-α had variable results. The use of CRP as a biomarker was investigated in 28 of the 59 screened studies, and 26 of those studies reported a significant increase in CRP concentration. In the same line, another study reported that serum levels of circulating CRP were significantly higher in COPD patients than in healthy subjects. CRP is an acute-phase protein synthesized mainly by the hepatocytes in response to tissue damage or inflammation (Korani *et al.,* 2016). Also a study by Kleniewska *et al.* (2016) concluded that CRP and fibrinogen are important systemic biomarkers and that IL1-β, IL-6, TNF-α and MMP-9 could be considered local biomarkers of inflammation in the airways of COPD patients.

Fibrinogen could be the most promising blood biomarker of COPD and has been considered for qualification as a drug-development tool by the US Food and Drug Administration (USFDA) and the European Medicines Agency (Duvoix *et al.,* 2012). Fibrinogen, an acute-phase plasma glycoprotein, is synthesized in the liver and converted by thrombin into fibrin during blood coagulation. The normal range of blood fibrinogen level is 1.5–3.5 g/L but can increase three folds when stimulated by increased IL-6 production (Khan and Daga, 2015). Several studies have investigated the association between fibrinogen and the risk of developing COPD, its progression, severity and related mortality. A higher level of blood fibrinogen was reported in COPD patients when compared with healthy subjects (Mannino *et al.,* 2015). Duvoix *et al.* (2012) reviewed the association between fibrinogen and COPD and concluded that fibrinogen is associated with COPD severity but does not predict the decline of lung function. In addition, fibrinogen may help to identify COPD patients at high risk of mortality and those at risk of future exacerbations. Furthermore, a review of data from five COPD studies reported high fibrinogen levels in 44.7% of COPD patients. It has been suggested that fibrinogen level higher than 350 mg/dL may increase the risk of exacerbation and mortality (Mannino *et al.,* 2015).

The level of pulmonary biomarkers has been shown to be different in patients with COPD when compared with healthy control. Lower levels of CC16 secreted by Clara cells have been observed in the serum of COPD patients. Clara cells are epithelial cells located in terminal bronchioles and are involved in airway regeneration and immune responses following injury. In addition, the expression of SP-D decreases in the lungs of COPD patients, while the level increases in the plasma. SP-D, which is located in the endoplasmic reticulum of type II pneumocytes and the secretory granules of Clara cells, is thought to be implicated in COPD pathogenesis, including ROS production and inflammatory responses in alveolar macrophages (Khan and Daga, 2015).

Cytokines are small extra-cellular signalling glyco-proteins that regulate the growth, differentiation and function of cells related to immune system response and inflammation (Arican *et al.,* 2005). TNF-α is secreted by various structural and immune cells, including epithelial cells, macrophages, T lymphocytes and airway smooth-muscle cells, in response to pathogenic infection and during autoimmune response (Himmerich *et al.,* 2006). TNF-α is involved in the pathogenesis of COPD by stimulating and releasing various pro-inflammatory mediators that cause tissue damage and remodelling. Serum levels of TNF-α is significantly higher in COPD patients than in healthy subjects (Korani *et al.,* 2016). Furthermore, Selvarajah *et al*. (2016) found increased levels of TNF-α and IL-1β in the serum of COPD patients (GOLD stages A–C) and in some healthy smokers and healthy non-smokers. The levels of IL-1β are elevated in the sputum, BAL and serum of patients with stable COPD (Caramori *et al.,* 2014).

Another study confirmed that COPD patients had higher sputum concentrations of IL-6 and IL-1β. IL-6 strongly triggers CRP production in the liver, and increased IL-6 plasma levels are associated with increased CRP levels in COPD patients. IL-6 levels in both the plasma and sputum of patients with stable COPD are higher than in controls (Caramori *et al.,* 2014).

Several CXC chemokines involved in recruiting inflammatory cells from the circulation to the lungs are elevated in COPD patients. IL-8 levels were found to be elevated in the induced sputum of patients with COPD, and this elevation was correlated with disease severity and increased further during exacerbations. In line with this observation, CXCL1 levels were found to be highly elevated in the sputum and BAL fluid of COPD patients, while CXCL5 expression was found to be increased in the airway epithelial cells during COPD exacerbations (Caramori *et*

al., 2014). Inflammasomes are multimeric cytoplasmic protein signalling complexes that regulate the expression of the pro-inflammatory cytokines IL-1β, IL-18 and IL-33 in response to various irritants, causing neutrophilic inflammation. NLRP3 inflammasomes have been found to be activated in patients with inflammatory lung diseases, especially during acute exacerbations, in response to invading pathogens, oxidative stress and ATP (Barnes, 2016). However, no increases in NLRP3 inflammasomes were found in patients with stable COPD, and only small increases were found in patients with severe COPD (Caramori *et al.,* 2014).

Human plasma and serum samples are widely used in clinical and biological studies. Plasma sample is the predominant biological sample to assess health and disease in clinical settings and in most of clinical trials (Yu *et al.,* 2011). Serum is obtained from blood that has coagulated. During coagulation Fibrin clot formation removes proteins (e.g., fibrinogen) from the blood sample along with blood cells and related coagulation factors, which are separated from serum by centrifugation after blood collection. During this process, activated platelets release proteins include; proinflammatory cytokines and various metabolites into the serum, which can alter the biomarkers levels relative to plasma (Yu *et al,* 2011; O'Neal *et al.,* 2014).

Previous studies investigated the advantage of using plasma sample over serum. For examples, the concentrations analysis data of 163 metabolites in plasma and serum samples collected from 377 fasting individuals indicating good reproducibility in both matrices, and significantly better stability of plasma compared to serum (Yu *et al.,* 2011). In the same line, a pilot study was performed in 24 COPD patients comparing serum, Ethylenediaminetetraacetic acid (EDTA) plasma, and EDTA plasma with proteinase inhibitors (P100™), to determine the most reliable sample type for measuring blood biomarkers in COPD cohort. Several biomarkers candidate involved COPD pathogenesis has different values in plasma and serum (e.g., fibrinogen, MMPs, and cytokines).The study results concluded that using serum and plasma were similar for most measured markers, with few exceptions include fibrinogen and MMP2.There was no difference in mean markers levels between EDTA plasma and P100 plasma. These results suggesting that platelet activation alters concentrations of many biomarkers which in turn increase serum values, relative to plasma, for factors that are released from platelets or leukocytes during clotting. On the other hand, serum values decrease, relative to plasma, for factors that localized in clots (O'Neal *et al.,* 2014). In the current study plasma

sample was preferred over serum because it can be separated immediately after blood collection, thus minimizing potential changes that may occur *ex vivo*. In addition, coagulation process consumes all blood clotting factors, and release biomarkers from platelets or leukocytes which makes serum unsuitable in measuring many biomarkers (Dakappagari *et al.,* 2017). The variations of different biomarkers levels and age between patients with COPD and healthy controls from previous studies are summarised in (Table 1.2). In most of those studies COPD patients' age is matching the healthy controls age. Thus variability in biomarkers level between patients and healthy subjects is not related to age.

Table 1.2: Variations of biomarkers levels between patients with COPD and healthy controls from previous studies.

Marker	Proposed Pathophysiological role	Sample	Concentration in COPD Age	Concentration in control Age	Reference
IL-6 pg/ml	Inflammatory cytokine	Serum	1.5 [0.8-3.1] 40–75 yrs	0.4 [0.2-0.9] 40–75 yrs	Agusti et *al., 2012*
			14.016 ± 3.018 [6.6-17.8] 21- 77 yrs	3.030 ± 1.076 [1.9-5] 18 - 68 yrs	El-Shimy *et al.,* 2014
			39.21±27.34 73.1±13.0 yrs	23.41±12.86 74.5±7.9 yrs	Wang *et al.,* 2014
			1.4 [0.42-2.1] 66 (57-73) yrs	0	Queiroz *et al.,* 2016

IL-8 pg/ml	Inflammatory chemokine	Serum	6.9 [3.2-13.3] 40–75 yrs	4.3 [2.3-7.2] 40–75 yrs	Agusti *et al.,* 2012
			47.12±30.00 73.1±13.0 yrs	21.70±18.61 74.5±7.9 yrs	Wang *et al.,* 2014
			10.57±0.55 72 ± 4.7 yrs	NA	Liu *et al.,* 2014
			146.43 ± 28.52 [64.8–188] 21- 77 yrs	36.2 ± 6.33 [25–44] 18 - 68 yrs	El-Shimy *et al.,* 2014
			6.0 70.8±8.1 yrs	5.5 59.0±10.2 yrs	Ishikawa *et al.,* 2015
			[0-57.5] 66 (57-73) yrs	0	Queiroz *et al.,* 2016
Fibrinogen mg/dl	Blood coagulation factor	Plasma	448.0 [388.-517] 40–75 yrs	369.0 [326-432] 40–75 yrs	Agusti *et al.,* 2012
g/L			5.09±1.86 62.29± 7 yrs	2.07±0.57 62.29± 7 yrs	Fattouh and AlKady 2014

Fibrinogen mg/dl	Blood coagulation factor	plasma	445 [387-512] 63 (7) yrs	NA	Müllerova *et al.,* 2015
			300.0 70.8±8.1 yrs	289.0 59.0±10.2 yrs	Ishikawa *et al.,* 2015
µg/ml		Plasma	2,992 [2,550-3,965] 62 (50-71) yrs	3,135 [2,730-3,597] 55 (51-64) yrs	Akiki *et al.,* 2016
mg/dl			250.87	162.7	Thomas and Yuvarajan 2016
CRP mg/L	Acute phase reactants	Serum	4.82 ± 1.97 54 ± 7.58 yrs	0.88 ±0.48 53 ± 8.68 yrs	Agarwal *et al.,* 2013
			39.46 54 ± 7.58 yrs	4.2 53± 8.68 yrs	Fattouh and Al-kady 2014
			7.50±5.46 73.1±13.0 yrs	3.86±3.75 74.5±7.9 yrs	Wang *et al.,* 2014
		Plasma	3.4 [1.7-8.5] 63 ± 8 yrs	0.6 [0.1-0.9] 58 ± 5.9 yrs	Gopal *et al.,* 2014
			3.2 [1.6-7.1] 63 (7) yrs	NA	Müllerova *et al.,* 2015

CRP ng/ml		Serum	594.0 70±8.1 yrs	327.0 59±10.2 yrs	Ishikawa *et al*., 2015
			8.41 [3.48-14.3] 62 (50-71) yrs	9.72 [4.37-15.5] 55 (51-64) yrs	Akiki *et al.,* 2016
µg/ml			3.74± 0.20 53.9± 5.95 yrs	1.30 ±0.14 49.37±14.37 yrs	Korani *et al.,* 2016
TNF-α pg/ml	Inflammatory cytokine	Serum	2.35 [2.35-7.80] 40–75 yrs	2.35 [2.35-2.35] 40–75 yrs	Agusti *et al.,* 2012
			112.7±80.20 73.1±13.0 yrs	73.46±50.44 74.5±7.9 yrs	Wang *et al.,* 2014
			3.16±0.77 72 ± 4.7 yrs	NA	Liu *et al.,* 2014
			43.833 ± 10.53 [30.22–74.4] 21- 77 yrs	18.52 ± 4.028 [12.2–24.2] 18 - 68 yrs	El-Shimy *et al.,* 2014
			35.88± 5.97 53.9± 5.95 yrs	8.79 ±0.57 49.37±14.37 yrs	Korani *et al.,* 2016

TNF-α pg/ml		Serum	2.9 [0.95-6.03] 66 (57-73) yrs	0.35 [0-1.9]	Queiroz et al.,2016
CCL18/ PARC ng/ml	Lung-inflammatory protein Chemokine/lymphocytes	Serum	105 63 (7) yrs	80 54 (9) yrs	Sin et al., 2011
		Plasma	105 [82-136] 63 (7) yrs	NA	Müllerova et al., 2015
IP10/ CXCL-10	Inflammatory chemokine	Serum	149.4 [103-215] 72.6 yrs	111.7 [82-178] 67.4 yrs	Quint et al., 2010
CC16 ng/ml	Lung inflammatory protein	Serum	4.9 64.5 (6.0) yrs	6.4 59.7 (8.8) yrs	Lomas et al., 2008
			8.5 70.8±8.1 yrs	5.6 59.0±10.2 yrs	Ishikawa et al., 2015
			7.29 71.7 (8.23) yrs	NA	Labonté et al., 2016

SP-D	Pulmonary protein involved in innate immunity	Serum	121.1 [84.9–174.2] 63.4±7.2 yrs	82.2 [56.1–117.7] 53.2±8.6 yrs	Lomas *et al.,* 2009
ng/ml			138.07 40–75 yrs	NA	Agusti *et al.,* 2012
		Sputum Serum	45.46±37.78 173.23±186.93 72 ± 4.7 yrs	31.68±12.04 89.59±70.29 44 ± 8.5 yrs	Liu *et al.,* 2014
		Sputum Serum	59.6 36.7 70±8.1 yrs	45.5 21.1 59±10.2 yrs	Ishikawa *et al.,* 2015
			155.175	NA	Doubková *et al.,* 2015
			1,510 [986-2,174] 62 (50-71) yrs	1,269 [664-1884] 55 (51-64) yrs	Akiki *et al.,* 2016

sRAGE pg/ml	Pro-inflammatory recognition receptor of cell surface	Plasma	1351 63.6 ± 6.9 yrs	1797 53.8 ± 8.9 yrs	Cheng *et al.,* 2013
			512 63 ± 8 yrs	2531 58 ± 5.9 yrs	Gopal *et al.,* 2014
		Sputum	500 [300-900] 5400 [2800–5450] 42.73 ± 6.26 yrs	400 [300-800] 3630 [2530–4550] 37.29±4.46 yrs	Hassanein *et al.,* 2016

Data expressed as mean, mean ± standard deviation or mean [range]. Definition of abbreviations: IL-6, Interleukin-6; IL-8, Interleukin-8; TNF-α,Tumor necrosis factor; CRP, creative reactive protein; CC16, clara cells 16; CCL18, Chemokine (C-C motif) ligand 18; CD31, cluster of differentiation 31; SP-D, Surfactant protein D; IP10, Interferon gamma-induced protein 10; sRAGE, Soluble receptor for advanced glycation end products.

Findings of elevated levels of a cytokine or chemokine in COPD do not guarantee that blocking their actions will be effective as an anti-inflammatory therapy. Furthermore, although anti-cytokine trials were effective in animal models of the disease, inhibiting these mediators was not effective in clinical trials in human subjects with COPD. Some studies have reported that using antibodies to block IL-1β and IL-8 were not successful in treating COPD. In line with that, anti-TNF therapies were also ineffective in treating COPD and may increase the risk of infection and many side effects. For example, treatment with infliximab for six months showed no clinical benefit, with increased risk of lung cancer and pneumonia being observed (Caramori *et al.,* 2014).

Cytokines are not disease-specific biomarkers, as their levels may increase for various reasons, including inflammation, infection and autoimmunity. Therefore, cytokine levels may also be elevated in some healthy non-smokers as well as smokers and COPD patients, making it difficult to identify cytokines that are COPD-specific biomarkers. In summary, biomarkers could be used as factors predictive of the severity of inflammation in COPD patients, as their increase may indicate inflammation. However, many of these biomarkers are variable in stable COPD and are not appropriate to use when diagnosing or predicting the disease. In addition, some circulating cytokines have been found to be increased in combination but not individually (Selvarajah *et al.,* 2016). The challenge is to determine whether all these biomarkers individually or in combination are useful in clinical practice. In addition, a clinically useful biomarker must be reproducible in stable disease, as are fibrinogen, SP-D and CC-16. Lack of reproducibility may limit the use of other biomarkers, including CCL18, CRP, IL-6 and IL-8 (Duvoix *et al.,* 2012). Therefore, the search continues for a single biomarker or combination of them that is viable for use in individual patients.

In COPD patients, various growth factors enhance the differentiation and survival of inflammatory cells and structural cells, causing airway remodelling. For example, GM-CSF, which is secreted by macrophages, epithelial cells and T-cells in response to inflammation, stimulates the differentiation and survival of macrophages and neutrophils. In addition, TGF-β and fibroblast growth factors (FGFs) induce proliferation of fibroblasts and airway smooth-muscle cells, causing fibrosis of the small airways (Barnes, 2009). There is an increase in pro-inflammatory lipid mediator prostaglandins, PGE2 and PGF2α, and LTB4 levels in exhaled breath condensates of patients with COPD. LTB4 concentration is also increased in induced sputum (Barnes 2016).

1.2.6 Treatment

Early diagnosis of COPD is recommended as lack of awareness and knowledge from patients with COPD leads to delayed diagnosis and medical treatment, especially in non-smoking individuals (Novgorodtseva *et al.,* 2013; Dixon *et al.,* 2016). To improve the health of COPD patients, smoking must be stopped and exposure to environmental and occupational risk factors must be reduced. In addition, yearly influenza vaccinations are recommended. Current treatments available for COPD include bronchodilator medications, corticosteroids, long-term oxygen therapy and pulmonary rehabilitation. The main corticosteroids and inhaled bronchodilators include improved long-acting β2-adrenergic receptor agonists, long-acting anticholinergics, theophylline and a combination of one or more of these. In addition, the phosphodiesterase-4 inhibitor roflumilast decreases exacerbations in patients with severe airflow obstruction and chronic bronchitis. Novel therapies targeting systemic inflammation and oxidative stress include combination of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, antioxidants and omega-3 PUFAs still under investigation (Williams *et al.,* 2016). In a few severe cases of COPD, surgery, including lung transplantation and lung-volume reduction, may be recommended.

Current treatments may decrease the frequency and severity of exacerbations and increase exercise capacity but are expensive and associated with side effects. While the global therapies cost for COPD exceeds $44 billion per year, these therapies still do not completely prevent symptoms, stop disease progression or systemic inflammation, reduce mortality or slow the long-term decline in lung function. Therefore, an effective, safe, affordable, therapy is needed to manage the progression of COPD and prevent exacerbations, thereby providing significant health and economic benefits (Meng *et al.,* 2016; Kunnumakkara *et al.,* 2018). Therefore, it is necessary to target specific inflammatory mediators or cytokines to block the inflammatory and remodelling processes. However, developing such COPD therapies continues to be a challenge because of the heterogeneity of the disease, as the symptoms not reflect specifically the biological process of the disease phenotype, and the lack of disease-specific biomarkers. (Decramer *et al,* 2012; Caramori *et al.,* 2014; Roberts *et al.,* 2015; Sin *et al.,* 2015; Williams *et al.,* 2016).

There is a need for a novel and accurate diagnostic test that would lead to early diagnosis to differentiate COPD from other respiratory diseases, to monitor disease progression, to predict exacerbation, to establish correct treatment to improve symptoms, quality of life and to avoid unnecessary hospital admissions (Comes *et al.,* 2016, Dixon *et al.,* 2016). Several studies have focused on improving dietary intake, which could slow COPD progression and improve management. In addition, many observational and experimental studies have evaluated dietary intake of individual nutrients throughout disease stages to clarify their role in the disease (Berthon and Wood, 2015). The next section focuses on the role of omega-3 PUFAs in COPD.

1.3 Polyunsaturated Fatty Acids

1.3.1 Biological Effects and Metabolic Functions

PUFAs are the structural components of the phospholipids of all biological cells membranes and the substrates for eicosanoid synthesis (Liu *et al.,* 2014). The length and degree of unsaturated fatty acids in membrane phospholipids are significant (Ristić-medić *et al.,* 2013). Any changes in the fatty acids composition of the cell membranes may alter membrane fluidity, activities, including formation of lipid rafts, the permeability and transportation of membrane-bound enzymes and the function of receptors. In addition, changes in composition affect cell-signalling pathways, which may alter the activity of transcription factors, gene expression and most of the functions of immune system cells, including secretion, chemotaxis and sensitivity to microorganisms (Calder 2012; Novgorodtseva *et al.,* 2013; Wald *et al.,* 2014). PUFAs play an important role in regulating inflammatory processes and responses, and the balance between omega-3 and -6 PUFAs may play an important role in the progression and severity of inflammatory responses (Calder 2007). It suggested that chronic respiratory disease development is associated with disruption of the fatty acid composition in erythrocyte membranes and in the ratio between precursors of pro- and anti-inflammatory eicosanoids (Novgorodtseva *et al,* 2013).

Omega-6 fatty acid, linoleic acid (LA), and omega-3, α-linolenic acid (ALA), are essential fatty acids that the human body cannot synthesize and must obtain from the diet (Ristić-medić *et al.,* 2013). These essential fatty acids are absorbed by intestinal cells and then transported to the liver, to be metabolized via desaturation and/or elongation reactions to generate the long chains of the omega-3 and -6 PUFA families (Giudetti and Cagnazzo, 2012). Omega-3 and -6 PUFAs are recognised depending on the position of the first double bond from the methyl group end (Matsuyama *et al.,* 2005). The omega-6 PUFAs include arachidonic acid (ARA), and the omega-3 PUFAs (also known as n-3 or ω-3) include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), a-linolenic acid (ALA), and docosapentaenoic acid (DPA) (Khorsan *et al.,* 2014). Omega-3 fatty acids are an important family of PUFAs and involved in normal growth and development of various human tissues. EPA has 20 carbon atoms and 5 double bonds (20:5n-3). DHA has a chain with 22 carbon atoms and 6 double bounds (22:6n-3) (Figure 1.9) (Calder 2015).
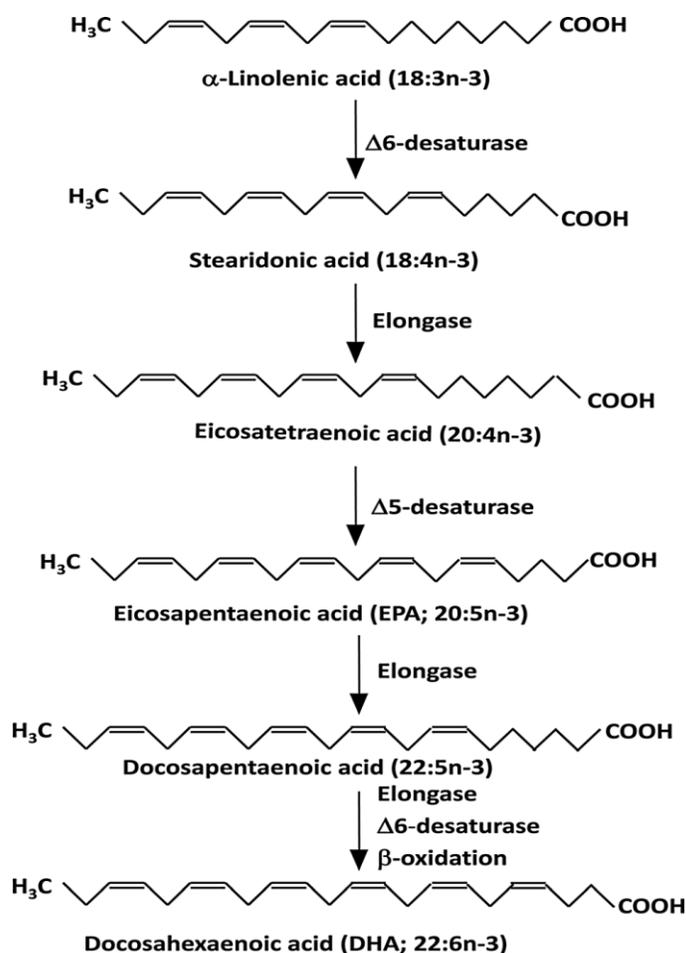
Figure 1.9: Outline of the metabolic pathway of biosynthesis omega-3 fatty acids. *DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid (Calder 2015).*

LA can be found in green leaves, various seeds and vegetable oils, including corn, sunflower, soybean, grapeseed and walnut. Omega-3 PUFAs like DHA and EPA are present in lean fish (cod), oily fish (salmon, tuna, mackerel, herring and sardine), other seafood, range-fed poultry, eggs and farm animals. Lean fish store lipids in their livers, while oily fish store lipids in their flesh. Other less biologically potent FAs include ALA found in green plant tissues, vegetable oils, including soybean, rapeseed, walnuts, canola oil, and flaxseed and linseed (Calder, 2012; Khorsan *et al.,* 2014).

Several studies investigated the significance of the omega-6 to omega-3 fatty acid ratio in chronic inflammatory diseases. The ratio of omega-6 PUFA in the western diet has increased significantly compared to previous decades. The current western diet has a ratio of omega-6 to -3 PUFAs that ranges from 20–25/1, up from 1/1 (Mansara *et al.,* 2015). Current studies recommended that lower intake of omega-6 compared to greater intake of omega-3 fatty acids may reduce the risk of many chronic inflammatory diseases; include CVD, inflammatory bowel disease, cancer, and rheumatoid arthritis. A lower omega-6 to omega-3 PUFAs ratio diet has been recommended in order to reduce the formation of pro-inflammatory eicosanoids from omega-6 PUFAs and to increase the production of anti-inflammatory mediators from omega-3 PUFAs. The PUFA profiles in patients could be changed through consumption. A diet supplemented with omega-3 PUFAs partially replace omega-6 PUFAs in the cell membranes of erythrocytes, platelets, monocytes, lymphocytes and granulocytes, and colon, and hepatic cells (Ristić-medić *et al.,* 2013; Khorsan *et al.,* 2014). Thus, the fatty acid composition of human inflammatory cells influences the function of those cells (Calder, 2015).

1.3.2 The role of PUFAs in Inflammation

In Western diets the human cell membrane is typically rich in Omega-6 PUFA; ARA, which is the major substrate for the biosynthesis of the eicosanoid, a class of pro-inflammatory mediators, including, prostaglandin, thromboxanes, and leukotrienes. Eicosanoids are generated from ARA via the action of cyclooxygenase (prostaglandins, thromboxanes), lipoxygenase (leukotrienes) and/or cytochrome P450 pathways. These pro-inflammatory mediators are involved in regulation of inflammation, immunity, platelet aggregation, and smooth muscle contraction. However, excess production of eicosanoids from ARA may play a crucial role in the development of COPD, particularly in the recruitment of inflammatory cells in the airways, the regulation of vascular and bronchial tone and the development of oxidative stress which result in lipid peroxidation (Calder, 2014; Antus and Kardos, 2015).

ARA can be replaced through oral administration of the omega-3 PUFAs; EPA and DHA, causing decreased production of eicosanoid pro-inflammatory mediators, and an increase in proportions of EPA and DHA in inflammatory cells. The effect of omega-3 fatty acids on

inflammatory cell responses, and on inflammatory processes, involves various mechanisms include modified cell membrane phospholipid fatty acid composition, alteration of lipid rafts, inhibition of activation of the pro-inflammatory transcription factor NF-κB so reducing expression of inflammatory genes, and activation of the anti-inflammatory transcription factor NR1C3 include; peroxisome proliferator activated receptor ɣ (PPAR-γ) (Calder, 2012; Calder, 2015).

In healthy subjects, dietary supplementation with omega-3 PUFAs has been associated with reduced levels of IL-1, thromboxane 2 and prostaglandin E2 and down-regulated expression of TGF-β (Ristić-medić *et al.,* 2013). Furthermore, omega-3 PUFAs attenuated the ability of immune system inflammatory cells to produce several inflammatory cytokines; IL-1, IL-6, IL-8 and TNF-α in healthy subject and rheumatoid arthritis patients (Matsuyama *et al.,* 2005). The decrease of the inflammatory cytokines production may occur indirectly by altering the transcription-factor activation of various inflammatory genes. Thus, omega-3 PUFAs were investigated in many studies as therapeutic options in various chronic inflammatory diseases (Calder, 2015).

1.3.3   Epidemiological and Clinical Studies of Omega-3 PUFAs in COPD

The omega-3 PUFAs are involved in several human metabolic processes and considered as important components of cell membranes (Scaglia *et al.,* 2016). Diets enriched with high amounts of omega-3 PUFAs have been shown to be beneficial in treating autoimmune diseases and inflammatory diseases, including rheumatoid arthritis (Miles and Calder 2012), CVD (Calder, 2017), neurological diseases, Crohn's disease, and may benefit COPD patients (Calder, 2012). Omega-3 PUFAs are beneficial in several inflammatory lung conditions including asthma (Miyata and Arita 2015), and COPD (Liu *et al.,* 2014; Williams *et al.,* 2016; Calder, 2018). In patients with COPD, a low omega-3 PUFA intake or conversion might be considered as a risk factor for chronic inflammation, and lung functional decline, through a variety of mechanisms, including cell surface and intracellular receptors, that control inflammatory cell signalling and gene expression (Atlantis and Cochrane 2016). There is support for this notion from a number of epidemiological and observational studies that have investigated the effects of omega-3 PUFAs on lung inflammation and COPD (Wood, 2015). In addition, experimental studies have shown

that higher intake of omega-3 PUFAs improves the immune system and may play a protective role in preventing and managing COPD (Toraldo *et al.,* 2013; Wald *et al.,* 2014).

Fan *et al.* (2003) published the first study describing the correlation between dietary omega-3 PUFAs and T-cells. Their findings indicated that omega-3 PUFAs modulated the fatty acids composition of T-cell membranes and thus may influence the signalling pathway and modulate T-cells activation *in vivo*. Matsuyama *et al.* (2005) examined the effect of omega-3 PUFAs on COPD patients. Their study investigated the level of inflammatory mediators in 64 COPD patients over two years. Patients were divided into two groups, each of which received a supplement. The first group received omega-3 PUFAs, and the second group received omega-6 PUFAs. The results showed that in those receiving the supplement rich in omega-3 PUFAs, LTB4 levels in the serum and sputum decreased significantly, as did TNF-α and IL-8 levels in the sputum. There was no significant change in the omega-6 PUFA-rich supplement group. This result indicated that diets supplemented with omega-3 PUFAs significantly decreased selected cytokine levels in COPD patients. Further, a longitudinal study published by Varraso *et al.* (2015) examined the effect of omega-3 PUFA intake from 1984–2000 on the risk of developing COPD. The study included 120,175 women and men. The results indicated that increased fish intake was inversely associated with the risk of COPD. Nordgren *et al.* (2014) examined whether dietary DHA supplementation could reduce the airway inflammation caused by organic dust exposure (ODE). They used DHA-treated human bronchial epithelial cells, lung fibroblasts, monocyte cell cultures, murine lung slices and an animal model in an *in-vivo* study. The results showed that DHA pre-treatment decreased production of ODE-induced inflammatory cytokines (IL-6 and TNF-α) dose-dependently. Collectively, these data demonstrate that DHA affects several types of lung cells to reduce airway inflammation and may be considered a therapeutic strategy for doing so.

Omegas-3 PUFAs down-regulate the production of pro-inflammatory cytokines from monocytes and macrophages, decrease the expression of cellular adhesion molecules on monocytes and endothelial cells and reduce production of ROS in neutrophils (Berthon and Wood, 2015). It has been suggested that DHA and EPA are able to inhibit the activation of transcription factor NF-κB, consequently reducing the transcription of a number of inflammatory cytokines and chemokines. NFκB is involved in inducing the expression of inflammatory genes

encoding several cytokines, adhesion molecules, MMPs, COX-2, TNF-α, IL-1β, IL-6 and inducible NO synthase in response to inflammatory stimuli. In addition, EPA and DHA have been reported to decrease the production of the pro-inflammatory lipid mediators, prostaglandin E2 and LTB4 (Calder, 2012). EPA and DHA significantly decreased MMP-9 production and activity in PBMCs from healthy controls (Shinto *et al.,* 2011). Yan *et al.* (2013) reported that the omega-3 PUFAs DHA and EPA exerted an anti-inflammatory effect by inhibiting activation of the NLRP3 inflammasomes, which prevented subsequent caspase-1 activation and IL-1β secretion. Another study reported increased levels of IL-10, an anti-inflammatory cytokine that inhibits the synthesis of many pro-inflammatory cytokines, when monocytes were treated with EPA and DHA. However, treatment did not affect levels of TNF-α and IL-6 (Jaudszus *et al.,* 2013). From all these data, it may be concluded that omega-3 PUFAs have anti-inflammatory effects and can decrease production of inflammatory markers associated with COPD progression.

As mentioned earlier, cigarette smoke activates the ROS-sensitive MAPKs/NFκB signalling pathway, resulting in lung inflammation. Liu *et al.* (2014) confirmed that EPA possesses anti-inflammatory and anti-oxidant properties. They used a murine model and primary human bronchial epithelial cells to determine the effect of EPA. Their *in-vivo* and *in-vitro* observations confirmed that events induced by cigarette smoke were inhibited by either pre-treatment or daily supplementation with EPA. These data confirmed that dietary antioxidant therapies targeting oxidative stress should help to improve lung inflammation induced by cigarette smoke. Another study investigated the effect of a novel anti-inflammatory intervention that included antioxidants, statins, omega-3 PUFAs; DHA and EPA, and lycopene on airway and systemic inflammation in COPD. The results confirmed that statins, omega-3 PUFAs and lycopene may have some anti-inflammatory effects systemically and that omega-3 PUFAs decreased expression of the leukotriene B4 receptor (LTB4R) gene and systemically increased levels of CXCL10. However, one limitation of that study is that it does not confirm whether the anti-inflammatory effects seen are from the combined use of rosuvastatin, lycopene and, omega-3 PUFAs or whether they are an effect of long term statin use or just an effect of lycopene and, omega-3 PUFAs supplementation (Williams et al., 2016).

Several recent studies have linked higher blood levels of omega-3 PUFA and/or diet enriched with omega-3 PUFAs with higher longevity. For instant, Japanese are among the longest lived populations, have very high omega-3 PUFA levels. In 2004, the level of EPA and DHA in red blood cell (RBC) membrane was defined as the omega-3 index. It reflects the sum of the percentages of EPA and DHA measured in RBC cell membrane to total fatty acids content. The omega-3 index is considered a marker of PUFA status. The recommended value of 8% or more of the total PUFAs probably prevents CVD in western populations. However, if the index value is lower than 4 %, there is an increased risk of mortality due to cardiovascular events. The question of whether increased intakes of EPA and DHA will prolong life remains unclear, as observational studies cannot confirm whether higher Omega-3 index will lower risk for death. Unfortunately, omega-3 index role has not been studied in other diseases (Scaglia *et al.,* 2016; Harris *et al.,* 2017).

1.3.4   Omega-3 PUFAs Metabolites

Resolution of the inflammation process is mediated by several endogenous pro-resolving lipid metabolites, and the entire system is balanced between pro-inflammatory and pro-resolving pathways. Several studies have reported that lipid mediators in the lungs may be involved in resolving lung inflammation. These pro-resolving lipid mediators are generated by the metabolism of PUFAs via several enzymatic reactions involving the cyclo-oxygenase and lipoxygenase and/or cytochrome P450 pathways. The anti-inflammatory and pro-resolving effects of omega-3 lipid metabolites, including resolvins, protectins and maresin, have been observed in a variety of pre-clinical model studies, including those in cells, tissues and animal models (Hsiao *et al.,* 2013). In contrast, lipid metabolites that derive from omega-6 PUFAs, including prostaglandins and leukotrienes, act as pro-inflammatory mediators (Figure 1.10 and 1.11) (Miyata and Arita, 2015). These metabolites often termed oxylipins to cover several metabolites originating PUFAs (Schunck *et al.,* 2018). Omega-3 oxylipins display cardioprotective, anti-inflammatory, and anti-allergic properties that contribute to the beneficial effects of omega-3 PUFAs in different disease conditions. Include renal fibrosis (Sharma *et al.,* 2016), asthma and airway inflammatory diseases (Mochimaru *et al.,* 2018).
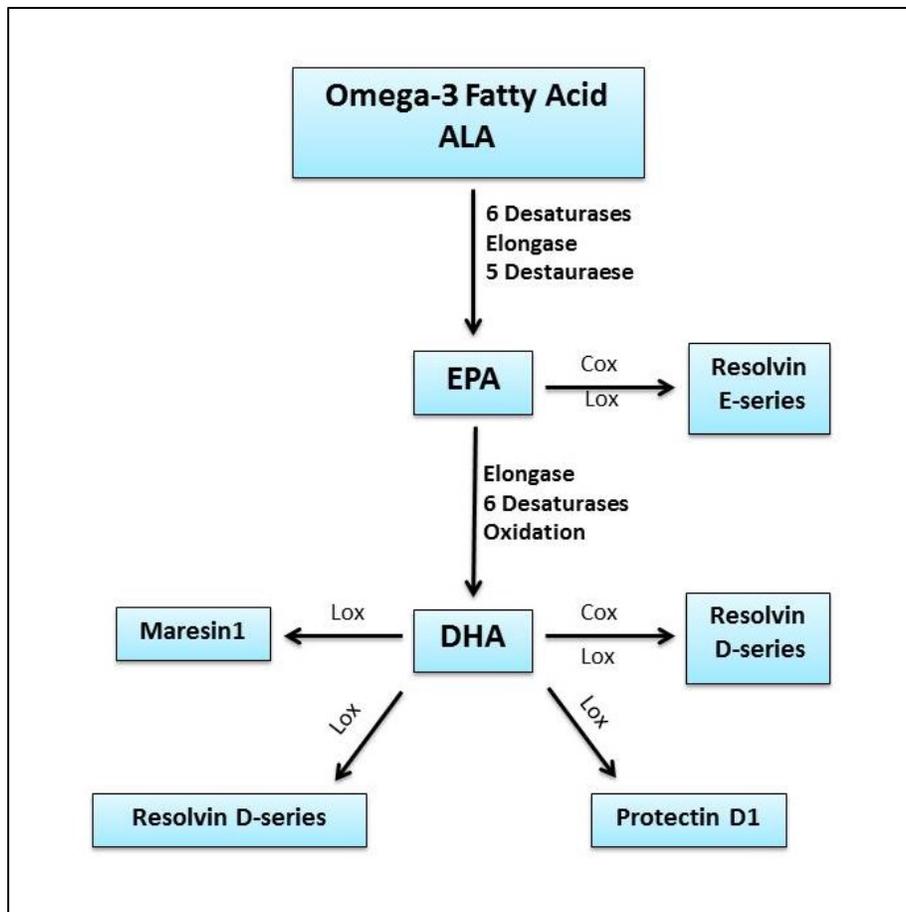
Figure 1.10: Outline of the pathway of Omega-3 fatty acids and bioactive metabolites biosynthesis from ALA. *Metabolism of eicosapentaenoic acid (EPA, C20:5, n-3) by COX-2 in combination with aspirin generates resolvins of the E series, which have pro-resolving activity. EPA conversion to DHA occur in multiple steps involving elongation, 6 desaturation, and -oxidation. DHA metabolism by LOX and COX in combination with aspirin generates resolvins and protectins of the D series. Abbreviations: ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, Eicosapentaenoic Acid; COX, cyclooxygenase; LOX, lipoxygenase (adapted and updated from Giudetti and Cagnazzo 2012).*
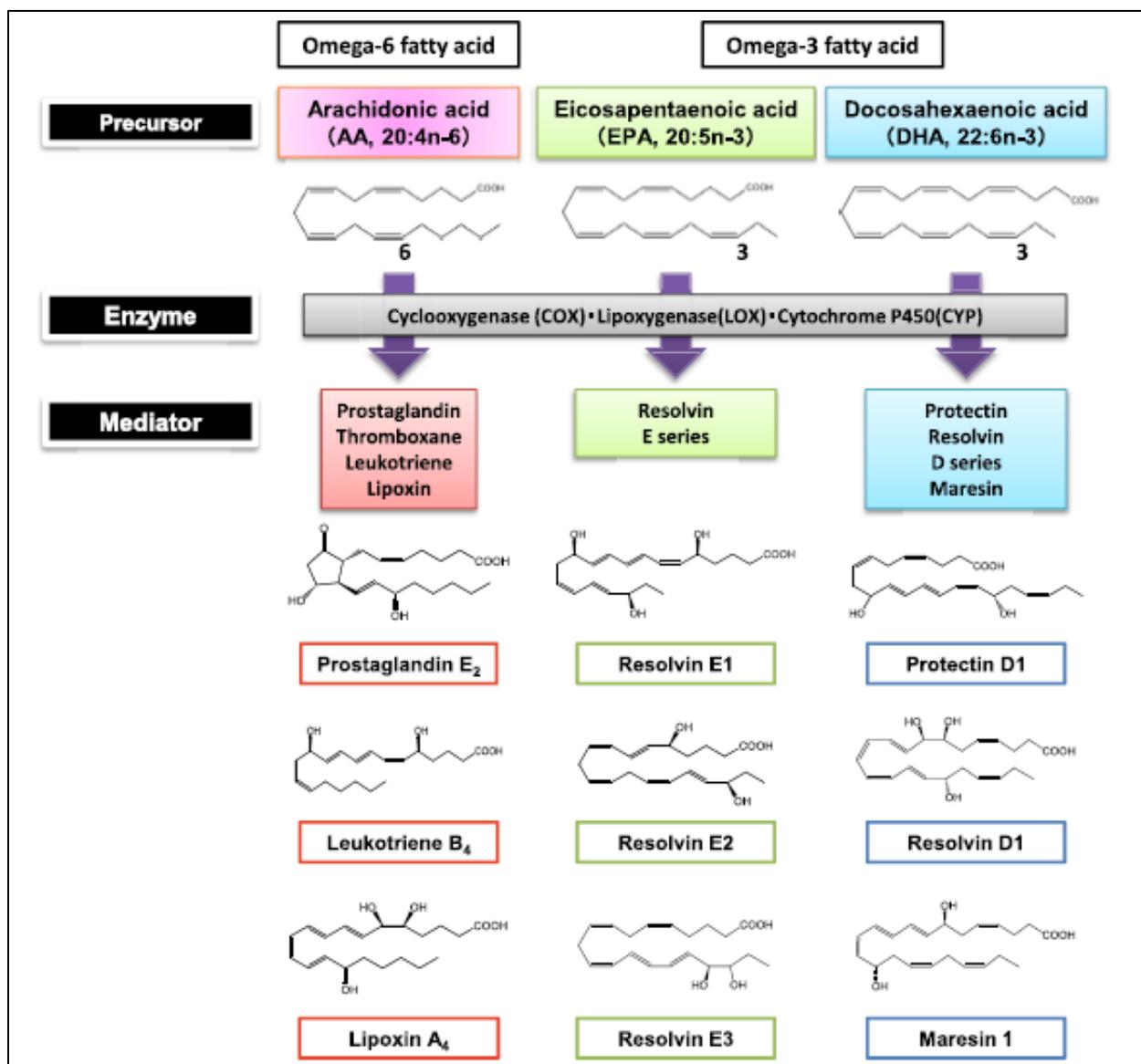
Figure 1.11: The biosynthesis of pro-resolving lipid metabolites. *Omega-6 FAs arachidonic acid is a metabolic precursor to eicosanoids (prostaglandins and leukotrienes), that act as pro-inflammatory mediators. Omega-3 FAs are converted to resolvins and protectins with anti-inflammatory and pro-resolving Properties (Miyata and Arita, 2015)*.

Several *in-vivo* studies have reported that resolvin E1, resolvin D1 and protectin D1 inhibit the trans-endothelial migration of neutrophils, thereby preventing neutrophilic influx to the site of inflammation, and also enhance neutrophil removal by macrophage efferocytosis, increase phagocytic capacities, promote neutrophil clearance across the lung mucosa, and enhance wound healing. Defective macrophage efferocytosis in COPD patients may prevent the resolution of inflammation. In addition, these lipid mediators can increase lung resistance to infection during injury and decrease production of inflammatory cytokines induced by cigarette smoke (Nordgren *et al.,* 2013; Barnes, 2016). It have been reported that the biosynthesis of anti-inflammatory lipid mediators, lipoxin A4 or protectin D1, are dysregulated in severe asthma, suggesting that chronic inflammation in the lung might result from an imbalance between pro- and anti-inflammatory molecule (Miyata and Arita, 2015).

The intravenous administration of protectin D1 in a murine model decreased the number of inflammatory cells in mucus and BAL fluid and inhibited airway hyper-responsiveness. Protectin D1also inhibited TNF-α and IL-1β production and appeared to be involved in protecting tissue from damage (Serhan *et al.,* 2008, Serhan *et al.,* 2010). Resolvin D1 also has shown anti-inflammatory effects. Its intravenous administration decreased IL-5 production, inhibited IL-1β and mucus production and eosinophil accumulation in the airways. In addition, resolvin D1 enhanced phagocytosis *in vitro* and clearance of inflammatory apoptotic cells in the airway (Miyata and Arita, 2015). Furthermore, it promoted resolution of airway inflammation in mice caused by either acute lung injury or cigarette smoke (Hsiao *et al.,* 2013). Resolvin E1 reduced the levels of IL-6 and IL-1β released in lung tissue following acute lung injury (Seki *et al.,* 2010). It also reduced IL-13 production, lymphocyte recruitment, and airway hyper-responsiveness in a murine model of asthma (Aoki *et al.,* 2008). Maresin-1 is the most potent pro-resolving mediator that activates macrophage efferocytosis. Therefore, a stable structural analogue of this mediator might be useful to COPD patients. Nordgren *et al.* (2013) showed that maresin-1 reduced the pro-inflammatory response of bronchial epithelial cells induced by exposure to organic dust. Thus, endogenous mediators or stable analogues of these mediators that activate the same receptors may promote resolution of inflammation in COPD patients (Barnes, 2016).

1.4 Aims and Objectives

Aims

There are several studies on COPD-related biomarkers. Recent studies indicated that several biomarkers might be involved in airway inflammation and immunity, but the available results on the association of some biomarkers with COPD are not consistent. There is a need for biological biomarkers for better evaluation of patients with COPD. This study aims to test the hypothesis that levels of inflammatory biomarkers are increased in circulation and from inflammatory cells in patients with COPD comparing to healthy subjects. Identification of plasma, pulmonary, and inflammatory immune cell biomarkers may facilitate improved diagnosis and prognosis in COPD.

Chapter 3

Hypothesis

In the current study it was proposed that with the progression of pulmonary inflammation, the respiratory function will be reduced in COPD patients. Further a number of subjects' demographics features (e.g. age, weight, BMI) could be correlated with pulmonary function data of the studied groups.

Objectives

1. Studying the baseline demographics, clinical characteristics and lung function tests of the study cohort.

Chapter 4

Hypothesis

It was proposed that with the progression of inflammation the level of systemic and pulmonary inflammatory biomarkers will increase too. Such a relationship could be utilized as a disease prognostic tool. Based on an analysis of the existing literature a number of potential biomarkers was selected for evaluation in the studied groups.

Objectives

1. Measure and compare the basal level of selected inflammatory markers in plasma samples of the study groups.
2. Investigate the relationship between plasma level of inflammatory biomarkers and pulmonary function parameters in subject with COPD.

Chapter 5

Hypothesis

It was proposed that the release of inflammatory biomarkers will increase from stimulated and unstimulated PBMCs from COPD patients when compared with PBMCs from healthy subjects. This will indicate that immune cell is already activated in COPD patients. Based on an analysis of the existing literature a number of inflammatory biomarkers was selected for evaluation in PBMCs of the study cohort. Additionally, it was hypothesized that omega-3 PUFAs, EPA and DHA could reduce the cytokine responses in PBMCs as these immune cells involved in adaptive and innate immunity.

Objectives

1. Measure and compare the inflammatory biomarkers basal release from PBMCs in the studies groups
2. Investigation the immunomodulatory effect of omega-3 PUFA on *in vitro* PBMCs inflammatory mediators release in the three studied groups

Chapter 6

Hypothesis

It was proposed that fatty acids profile in RBCs from subjects with COPD is modified when compared to healthy subject; such alteration could be utilized as a disease marker.

Objectives

1. Investigating baseline fatty acid profiles of erythrocyte (RBCs) membrane in the study cohort and the association with COPD

2. Chapter 2

2.1 Materials and Methods

2.1.1 Ethical considerations

The study was approved by the college ethics panel at Salford University. All subjects were recruited after informed consent by the Medicines Evaluation Unit (MEU), based at University Hospital South Manchester (Wythenshawe). MEU have ethical approval for the recruitment of subjects and provision of blood samples for these studies under the terms of the approval allows researchers to utilize and store clinical materials at third party (University of Salford) laboratories (see appendix A for ethics documents).

2.1.2 The Characteristics of the Study Population

2.1.2.1 Patient Population

The study population was divided into three groups: (1) Group included 15 patients with COPD (COPD). (2) Group included 15 healthy smokers (HS). (3) Group included 12 participants who were healthy, nonsmoking volunteers (HNS). Individual involved in collecting study cohort demographic features and pulmonary functions are staffs from MEU working in the research department. All participants were subjected to the following; through history taking, full clinical examination, routine laboratory investigations, body mass index (BMI), pulmonary function tests; FEV1, FVC, and FEV1%. The diagnosis of COPD and the assessment of its severity were defined and classified according to the criteria reported by GOLD guidelines for COPD management with the inclusion and exclusion criteria as stated below (Korani *et al.,* 2016; *Meng et al., 2016;* Queiroz *et al.,* 2016).

The subjects included in this project were required to meet the following criteria; patients with COPD post BD FEV1/FVC ratio <0.70, pack year >10. Smokers with normal lung function, pre BD FEV1/FVC ratio >0.70, FEV1 >80% predicted, pack year >10. Healthy never smoked subject, pre BD FEV1/FVC ratio >0.70, FEV1 >80% predicted, pack year <1.

Exclusion criteria include**:**

All healthy subjects with history of a chronic respiratory disorder
All volunteers with the following; History of acute respiratory disease within 6 weeks prior to visit
History of other non-respiratory inflammatory disease
Blood safety review: Donated 1 pint (500mls) of blood in the last 16 weeks
Got a chesty cough, sore throat or active cold sore
Pregnant or a woman with a baby less than 9 months old
Taking antibiotics or finished taking them within the last 7 days
Had hepatitis or jaundice in the last 12 months
Had acupuncture, ear piercing, and body piercing, tattooing or semi-permanent make-up in the last 4 months
HIV positive or thinks they may be HIV positive
Ever injected or been injected with drugs including body building drugs
Been in contact with an infectious disease or been given immunisations in the last 8 weeks
Any known Haemophilia or a related blood clotting disorder who has received clotting factor concentrates or had a history of anaemia

2.1.3   Collection and Processing of Blood Samples

Different types of samples can be utilised to identify COPD biomarkers, those samples can be obtained either from the lung or from the system. Samples from the lungs could be sputum, exhaled breath, BAL fluid and biopsies. However, Sputum requires expertise and time. Exhaled breath has poor reproducibility and low protein content. Additionally, BAL fluid and biopsies samples techniques are high risk, invasive, and expensive even it provide with more data on whole lung sections. Samples obtained from system could be plasma or serum. Although systemic samples are easier to obtain, with low risk technique, high reproducibility data,

however the blood may have a smaller biomarker signal compared to those obtained directly from the lung samples (Bradford *et al.,* 2017).

Blood have been used as a suitable source of inflammatory cells in many studies investigating disease pathogenesis and evaluating the effects of candidate novel therapies. The advantage of blood is ease of collection samples, non-invasiveness, high reproducibility, and the ability to standardise measurements for most assays (Chen *et al.,* 2016; Bradford *et al.,* 2017). In the current study, blood sample was selected for biomarker investigation and also because from blood sample other fractions, that are required in the current study, can be recovered includes PBMCs and RBCs. Those fractions will be required in the current study for circulating biomarkers investigation and fatty acid analysis.

Patient recruitment and samples collection and started on February 2016 and continued until March 2017. Whole blood samples 20 ml were collected into vacutainer tube, containing EDTA as an anticoagulant from each patient of the studied groups. Individuals involved in sample collect are technicians and staff from MEU working in the research department and with experience in research subjects' recruitment and have knowledge of the study protocol. Blood samples were then transported and processed by the academic team at University of Salford. Samples were processed and stored according to guidelines at the University of Salford. Samples were collected and processed on the same day. However, only few samples were collected on the next day. Each sample was provided with patient number, date of collection and study group. Assessment of outcome was blinded to exposure status and no patient identifiable data was used in the analysis or publication of the results.

2.1.3.1   *Sample Processing*

Each blood sample 20ml was mixed with Roswell Park Memorial Institute 1640 media (RPMI 1640) (Bioser, Labtech International Ltd, Sussex, UK) without supplement in a 1:1 ratio then diluted blood layered onto Biosera Ficoll lymphocyte separation media (sterile, filter, Labtech International Ltd, Sussex, UK) in a ratio 1:1 in a slow stream to maintain the gradient and centrifuged (400g, brake setting at 9, accuspin 400centrifuge, Fisher Scientific, Leicestershire, UK) for 30 mins in room temperature. The plasma top layer was collect in five 1ml aliquots and stored at -80°C for later analysis. The blood bottom layer (RBCs) was collect

in five 0.5ml aliquots and stored at − 80 C for later analysis. Samples were labelled with 3-line code using the Brady BMP-21 label printer as follow:

CPXXXX-YY

ZZ/ZZ/ZZ

01

Abbreviation definition:

CP=study code (COPD PUFA)

X=Patient identifier (MEUXXXX)

Y=sample type (PL=plasma; PB=PBMCs; RB=RBCs)

Z=date (format 26/03/16, for 26th March 2016)

01=aliquot number

2.1.3.2   *Purification of PBMCs by Ficoll Density Gradient Protocol*

The interface layer of enriched PBMCs was removed carefully using a sterile Pasteur pipette to a new tube, washed with RPMI (no supplement) to comprise a 40 ml total volume and centrifuged (400g, brake setting at 0, accuspin 400centrifuge, Fisher Scientific, Leicestershire, UK), for 10 mins at room temperature. The top layer was aspirated and cell pellet containing PBMC was resuspended in RPMI 1640 medium supplemented with 1% (v/v) L-glutamine (Lonza, Verviers, Belgium), 10% (v/v) fetal bovine serum (FBS) (Biosera, Labtech International Ltd, Sussex, UK), 1% (v/v) penicillin (100 U/ml), and streptomycin (100 mg/ml) (Biosera antibiotic-antimycotic, Labtech International Ltd, Sussex, UK).

A total cell count was performed using the Marienfeld Neubauer improved hemocytometer (Lauda-Königshofen, Germany) and viability was evaluated by the trypan blue exclusion method, where a volume of 10 µl of cell suspension was diluted in 10 µl Trypan blue solution (0.4%) (Sigma, Ayrshire, UK) in an Eppendorf tube and a viable count was performed under light microscopy. Ten microliters of Trypan Blue-cell suspension was applied to

the hemocytometer very gently to fill both chambers underneath the coverslip, allowing the cell suspension to be drawn out by capillary action. Unstained cells in the four sets of 16 corners squares of the hemocytometer were counted using hand tally counter. The average cell count from each of the sets of 16 corner squares was multiplied y by 10,000 (10^4) and again multiplied by 5 to correct for the 1:5 dilution from the Trypan blue addition. The final value is the number of viable cells/mL in the original cell suspension

The cell count was adjusted to a concentration of 1×10^6 cells/ml and stored in frozen media mix, FBS with 10% dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, UK). During cryo-preparation of cells DMSO is act as cryo-protectant for cell membrane during the freezing process. Cryovials were placed into a Mr Frosty container (Fisher Scientific, UK), which slows the rate of cooling to approximately -1°C per minute. The Mr Frosty container was transferred to -80ºC for 24hrs, after which cryovials were then placed in liquid nitrogen storage for long term storage (Figure 2.1).
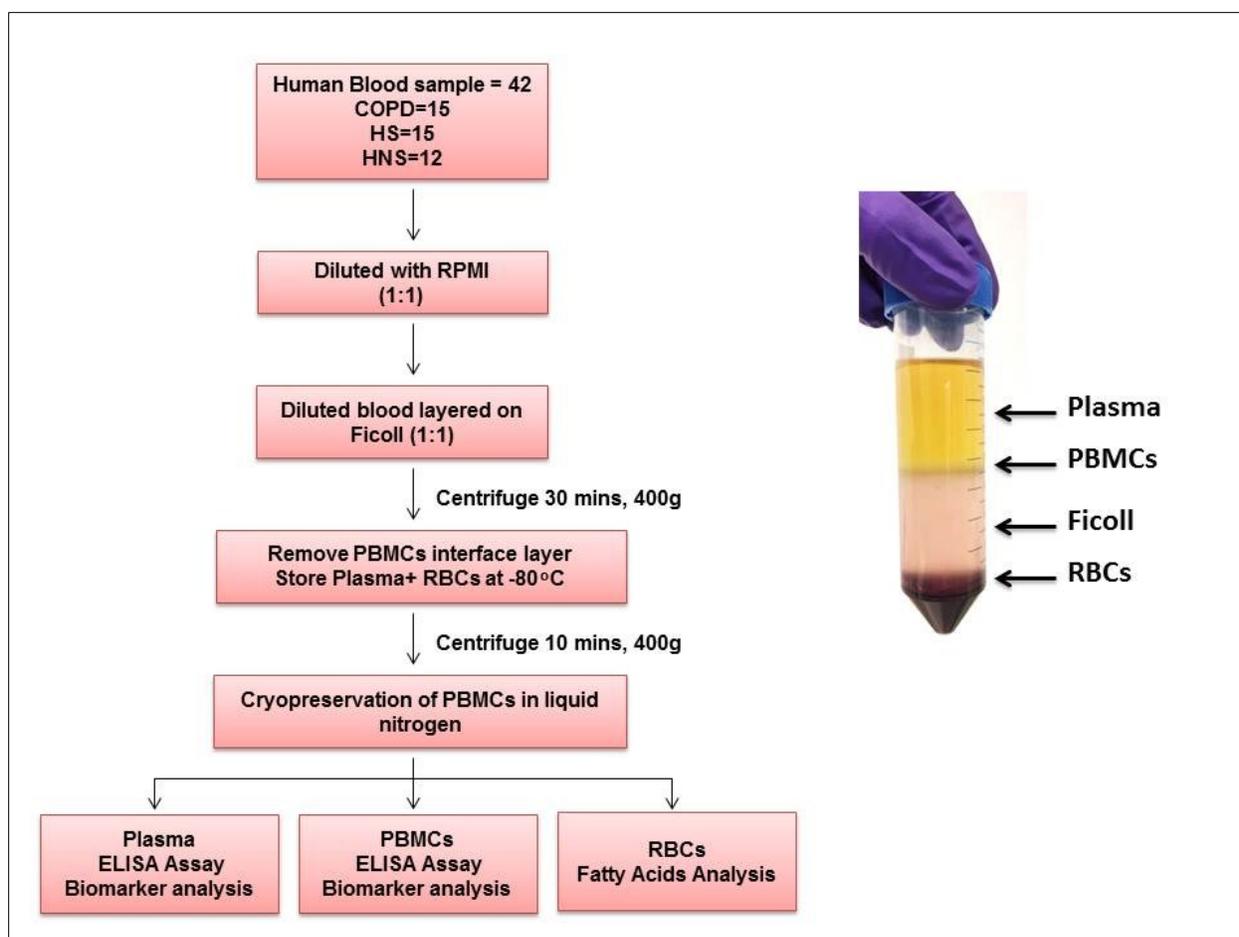
Figure 2.1: The Ficoll density gradient techniques for separation of PBMCs. *Blood is diluted with RPMI media (1:1), then layered on Ficoll separation media (1:1) and centrifuged as indicated in the text. Top layer plasma were removed and stored, PBMCs are enriched in the interphase between plasma and Ficoll layers, whereas red blood cells are pelleted.*

2.1.3.3 Recovery of PBMCs

A cryovial of PBMCs was removed from the liquid nitrogen and thawed with agitation in a 37°C water bath (Fisher Scientific, Leicestershire, UK) for one minute. The vial was then wiped with 70% ethanol (Fisher Scientific, Leicestershire, UK) before opening it in biological safety cabinet (Labgard class II biology safety cabinet, Thermoscientific, UK), and the content of the vial was transferred aseptically into a new sterile 50 ml tube containing 10 ml of RPMI 1640 media without supplement. This was centrifuged at 400g, for 10 mins (plate Eppendorf centrifuge 5804/ Heraeus™ Megafuge™ E16 Centrifuge, Fisher Scientific, Loughborough, UK).

Cells were re-suspended in 1ml RPMI media with supplement, and then a cell count was performed as described earlier (section 2.1.3.2). The cell concentration was adjusted (with RPMI media) according to the experiment protocol.

2.1.3.4 *Enzyme-linked Immunosorbant Assays (ELISA)*

Several methodologies have been developed for measuring biomarkers. ELISA is currently the most commonly used technique to measure biomarkers due to the high specificity and sensitivity. Using the same principle, more rapid, automated method like bead-based multiplex immunoassays (MIA) have been developed to detect multiple cytokines in a single sample (Jager et al., 2009). ELISA is a common laboratory technique which depends on a plate-based assay designed to measure the concentration of different substances such as proteins, antibodies and hormones in a complex biological matrix (e.g. plasma, serum, blood, tissue). In the ELISA assay, an antigen must be immobilized to a solid surface usually a polystyrene multi-well plate and then complexed with an antibody that is linked to an enzyme. Detection is achieved by conjugated enzyme activity when incubated with a substrate to produce a measureable coloured end product, which reflects the amount of target present in the original sample. ELISA assays are rapid and the simple technique permits high throughput of samples.

2.1.4   **ELISA for R&D Systems-based**

2.1.4.1   *Reagent Preparation*

ELISA duoset kits (R&D Systems, Oxford, UK) were used to measure the biomarkers release in recovered supernatants from PBMC culture and plasma samples. Detail of ELISA sensitivity ranges for each biomarker and the required reagents diluent and sample needed for each assay is summarised in chapter (4 and 5). Anti-human capture antibody was reconstituted in 0.5 ml of PBS and further dilution was made in PBS, to the working concentration. Anti-human detection antibody was reconstituted in 1.0 ml of reagent diluent 1% BSA in PBS, and further dilution was made in reagent diluent to the desire working concentration.

2.1.4.2 Standard Preparation

Each recombinant human standard vial was reconstituted with 0.5 mL of deionized H_2O or PBS according to manufacture instructions. After initial reconstitution, 1ml of high standard was prepared per plate assayed at the concentration indicated. A seven point standard curve using 2-fold serial dilutions in reagent diluent was prepared (Figure 2.2).Working dilutions should be prepared on experiment day and used immediately.
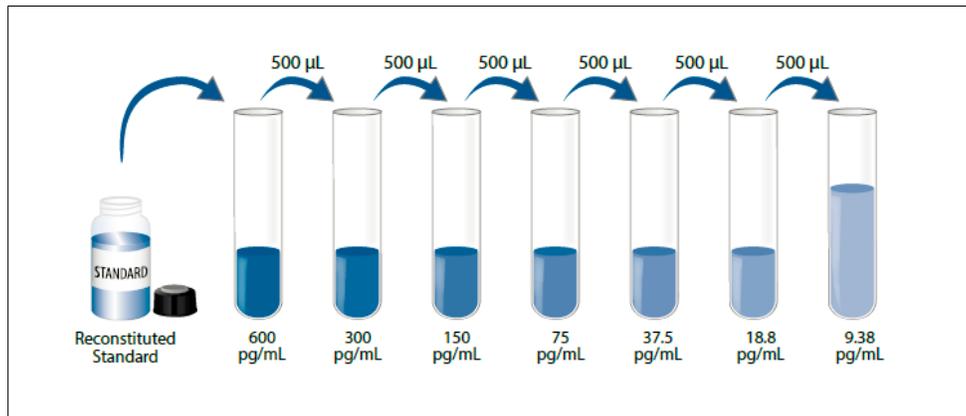
Figure 2.2: The preparation of seven point standard curve using 2-fold serial dilutions in reagent diluent (examples; recombinant human IL-6 Standard, rndsystems.com).

2.1.4.3 Sample Preparation and Cell Culture

The PBMCs were isolated from 20mL of whole blood consisting of anti-coagulant EDTA from patients with COPD, healthy smokers and healthy nonsmokers on Ficoll density gradient by centrifugation at 400 ×g at the room temperature for 30 minutes as previously mentioned. Before performing the experiment, PBMCs from frozen were washed and counted. The total cell count was calculated by using trypan blue exclusion method. Cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue, and the viable cells were counted and compared with the total amount of cells in the suspension and in all of the experiments. The cell suspension was adjusted to 1×10^6 cells/ml.

2.1.4.4 ELISA Assays Procedures

The biomarkers measurement was performed on plasma and the supernatant of the cultured PBMCs samples. The following procedure is for the R and D System Company. All assay steps are summarized in (Figure 2.3).

Day 1:

A 96-well plate was coated with 100 µl of the diluted anti-human capture antibody per well. The plate was sealed (Greiner Bio-One GmbH, Ltd, Stonehouse, Germany) and incubated overnight at room temperature.

Day 2:

The 96-well plate was washed twice with wash buffer (0.05% Tween® 20 in PBS, pH 7.2-7.4) using auto washer equipment (Mikura, West Sussex, UK). After each wash step any remaining wash buffer was removed by blotting the plate against clean paper towels. The plate was blocked by adding 300µl of reagent diluent, 1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered, to each well, and was incubated at room temperature for a minimum of 1 hour. The wash step was repeated and 100µl of sample or standards was added per well as duplicate. The plate was sealed with an adhesive strip and incubated 2 hours at room temperature. The wash step was repeated and 100µL of the diluted detection antibody was added to each well. The plate was sealed with an adhesive strip and incubated 2 hours at room temperature. The wash step was repeated and 100µl of the working dilution of Streptavidin-HRP (streptavidin conjugated to horseradish-peroxidase, 1:40) (R&D Systems, Catalog #DY 893975) was added to each well. The plate was covered and placed away from direct light for 20 minutes at room temperature. The wash step was repeated and 100µl of substrate solution, a mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine) (1:1, R&D Systems, Catalog # DY999) was added to each well and plate was incubated for 20 minutes at room temperature away from direct light, then 50µl of stop solution (2 N H_2SO_4) (R&D Systems, Catalog # DY994) was added to each well. The optical density of each well was determined using a microplate reader (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific, UK) (see appendix B for software user manual guide), set to 450 nm with a wave length correction at 570nm. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the

concentration on the x-axis and a best fit curve was drawn through the points on the graph. The concentration of biomarkers in sample was determined by interpolating the blank control subtracted absorbance values against the standard curve. Resulting value was multiplied by the appropriate sample dilution factor to obtain the concentration of biomarker in the sample. Three experiments were performed and standard error was determined from the standard deviation derived from the means of total sample number.

Figure 2.3: DouSet ELISA system Assay principles. *1) Analyte- capture antibody is bound to polystyrene 96-well microplate. Unbound capture antibody is washed away. Plates are blocked and washed.2) Samples or standards are added and any analyte present is bound by the immobilized antibody. Unbound materials are washed away. 3) streptavidin-HRP binds to the detection antibody. Unbound streptavidin-HRP is washed away. 4) TMB substrate is added to the wells and a blue color develops in proportion to the amount of target analyte present in the sample. Color development is stopped by stop solution turning the color in the wells to yellow. The absorbance of the color at 450 nm is measured (rndsystems.com).*

3. Chapter 3: Studying the baseline demographics, clinical characteristics and lung function tests of the study cohort

3.1 Background

According to GOLD accepted definition COPD is a ''disease state characterised by airflow limitation that is not fully reversible'' (Korani *et al.,* 2016). The diagnosis of COPD is based on the presence of respiratory symptoms, and the demonstration of chronic airflow obstruction. The main cause of airflow limitation is small airway remodelling and emphysema (Sileikienė *et al.,* 2017). Many COPD patients are diagnosed on the basis of clinical symptoms and without the assessment of airflow limitation with spirometry. Inadequate diagnosis of COPD can lead to insufficient treatment, increase health costs and patient's hospital readmission (Andreeva *et al.,* 2017). There are several pulmonary function tests that can be used to assess COPD diagnosis, including spirometry measurements, gas transfer (diffusing capacity), lung plethysmograph also known as total lung capacity (TLC), arterial blood gas analysis, BODE (BMI, airflow obstruction, dyspnea and exercise capacity).

In COPD patients, TLC is increased as a result of the destruction of lung tissue by emphysema. This leads to a loss of elastic recoil that allows the lungs volume to increase. Another lung function test is diffusing capacity of carbon monoxide (DLCO) which measures the ability of the lungs to transfer carbon monoxide (DLCO) from inhaled air to the hemoglobin of the red blood cells in pulmonary capillaries. The DLCO decreases with increasing severity of COPD because of parenchymal destruction and loss of pulmonary alveoli and capillary bed due to emphysema, which lead to decrease surface area available for gas transfer (Bailey 2012; Boutou *et al.,* 2013).

Spirometry measurements of FEV1% and FEV1/FVC reflect disease severity of airflow obstruction, and play a fundamental role in classifications of the COPD into different stages (FernaÂndez-Villar *et al.,* 2018). Clinical trials in COPD normally include measuring one of the main parameters of lung function which is FEV1%. It is importance because it reflects the index of airflow obstruction that measures both symptomatic relief and disease progression (Korani *et*

al., 2016). FEV1 is the volume of air that is forcibly exhaled in the first second, whereas forced vital capacity (FVC) represents the total volume of air in the lungs can be exhaled after a full inspiration. The FEV1 has been used to classify COPD patients to different stages according to disease severity and to describe progression of the disease (Cazzola, *et al.,* 2008).Whereas FVC is a parameter for lung restriction, and lung fibrotic diseases (Shibata e al., 2013). GOLD fix cut-off values of FEV1% <80% with a post-bronchodilator FEV1/FVC ratio <70% were classified as having airway limitation with a possibility of COPD. However, there was controversy about the fix cutoff value, so the GOLD guidelines and the combined American College of Physicians, American College of Chest Physicians, American Thoracic Society (ATS) and the European Respiratory Society (ERS) COPD guidelines recommend using the reference cutoff of <0.70, regardless of age and gender. Because FEV1/FVC ratio is known to decrease by age, so using this cutoff is not perfect as it may lead to an over-diagnosis of COPD in the elderly or an under-diagnosis in young patients (Bailey 2012). In 2004, the ERS and the ATS recommended the use of the lower limit of normal instead of fixed cut-off values. The lower limit of normal can be calculated from age corrected and defined by the lower fifth percentile of the reference population (Andreeva *et al.,* 2017).

Most longitudinal studies of COPD do not have post-bronchodilator lung function available. A Previous study analysed data from the Lung Health Study (randomized multicenter clinical trial) to determine whether pre- or post-bronchodilator lung function predicts mortality. Survival analysis and logistic regression predicting death and controlling for age, sex, smoking status, and measures of lung function were conducted. The study found that both pre- and post-bronchodilator lung function predicted mortality with a similar degree of accuracy (Mannino *et al.* 2011). However a study finds that post-bronchodilator spirometry may be a more accurate measure of COPD and should be used for COPD diagnosis and classification (Fortis *et al.,* 2017). Reversibility of airway obstruction is commonly used in clinical and research studies that reflect the COPD patients respond to bronchodilators. The values of FEV1and FVC before and after the bronchodilator administration are compared, and the change is calculated. Significant reversibility is identified by the ATS as an absolute changes of >12% and > 200 mL in the baseline FEV1 and/or FVC, while the ERS recommends a change of >9% of the FEV1 (Richter *et al.,* 2008; Tan *et al.,* 2012; Müller *et al.,* 2016).

In 2011, ERS published a report in order to establish clear diagnostic criteria and standardised methods to examine COPD. Their report strongly recommended measuring many different parameters of COPD patients include; spirometry measurements, respiratory symptoms, exacerbation frequency, comorbidity assessment, BMI, biological biomarkers, and chest radiography, to provide a better understanding of the disease. There are several problems regarding the diagnosis of COPD include different COPD definitions, a gap between the epidemiological and the clinical studies definition of COPD, and two proposed FEV1/FVC cut-off values for defining COPD. Further epidemiological and clinical studies are needed to improve the diagnosis of COPD (Andreeva *et al.*, 2017).

3.1.1 Objectives

- To study the comparison of baseline demographics and clinical characteristics between COPD patients, healthy smokers and healthy controls.
- To investigate the association between demographics and clinical characteristics and lung functions Parameters in COPD patients

3.2 Method

The study was conceived and forms an ongoing collaboration with the Medicines Evaluation Unit (MEU) and North West Lung Centre (NWLC) at University Hospital South Manchester (UHSM), part of Manchester University Foundation Trust. As such they are subject to NHS local research ethics committee approval (NREC), in this case South Manchester Ethics Committee. These studies are encompassed within an existing NREC approval for the use of human lung surgical resections for research into mechanisms of inflammatory lung diseases (ref: 03/SM/396). A cohort of 42 subjects enrolled in this study; 15 patients with COPD, 15 healthy smoking controls and 12 healthy nonsmoking controls was evaluated. All patients met the GOLD recommended spirometry criteria included pre-bronchodilator FEV1% (pre FEV1%) of less than 80% of the reference value with a pre-bronchodilator FEV1 to FVC ratio (pre FEV1/FVC ratio) of less than or equal to 70%. Details of the study cohort inclusion and exclusion criteria's was

mentioned earlier (Chapter 2). All patients underwent spirometry to diagnose airflow limitation including; the pre- and post-FEV1, FEV1% and FVC, as well as the FEV1/FVC ratio. Smoking data was collected by lab technicians from MEU. A form on smoking status was used to record the number of cigarettes smoked per day and the years since starting smoking. Then the number of pack year history (PYH) was determined as the average number of cigarettes smoked per day multiplied by the years since starting smoking. All subjects gave written informed consent and the study was approved by the research ethics committee at Salford University (see appendix A).

3.3 Statistical Analysis

The data were expressed and graphed as mean (standard deviation; SD). The D'Agostino–Pearson omnibus normality test was performed to test normality of the data distribution. For data normally distributed, paired t-tests or one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test was performed to determine whether the differences between the groups were statistically significant. Otherwise, if data are nonparametric comparisons were made using Kruskal–Wallis analysis followed by Dunn's multiple comparison tests. Differences were considered significant if the probability (P) value was <0.05, highly significant if P value was <0.01 and P value <0.001, and insignificant if P value was >0.05. Correlation analyses of two variables were carried out using Pearson methods, depending on the normality of the data distribution. All statistical analyses were performed using graphpad prism and InStat software, version 7.04 and 3.10.32 respectively. During the planning of the study, no power analysis was performed. However, a post hoc power analysis (Wilcoxon-Mann-Whitney test) justifies the sample size. Power and sample size analysis was calculated using G*power software version 3.

3.4 Result

The data were normally distributed and therefore multiple comparisons between groups were made using paired *t*-tests or one-way ANOVA analysis for unpaired data of variance followed by Tukeys multiple comparisons post-test to determine whether the differences between the groups were statistically significant. The baseline demographic clinical characteristics, and pulmonary function test results of all participants at recruitment, are featured in Table 3.1 and Figure 3.1.

3.4.1 Comparison of Baseline Demographics and Clinical Characteristics between Groups at Baseline

On classifying the patients according to severity of the disease, most of the patients in the current study were in the moderate category (73.4%) with a few in the mild (20%), and only 6.6% patients in the severe category of COPD as per the GOLD guidelines. On average, patients with COPD were older than the healthy smoking and healthy nonsmoking subjects. There was a significant difference in age average between COPD patients and healthy smokers (66.4 vs 56.3, P<0.05). Further, extremely significant difference in age average was observed between COPD patients and healthy nonsmokers (66.4 vs 50.1, P<0.001). Healthy nonsmoking subjects were slightly younger than healthy smoking subjects. There was a significant difference in weight between patients and healthy nonsmokers (P=0.0436). Patients were with low weight when compared with healthy nonsmoking control. The body mass index (BMI) was calculated as weight in Kg dividing by height square (m^2). At baseline, 53.3% were of normal weight (BMI 20–25 kg/m^2) and 46.6% were overweight (BMI >25 kg/m2). So, none of patients were underweight (BMI <20 kg/m^2). There were no significant differences between the three groups in terms of the body mass index (BMI) (P>0.05).

The smoking status between the smoker groups was significantly different. As expected the mean value of smoking exposure or PYH was significantly higher in COPD group when compared with healthy smokers. All patients were regularly using inhaled corticosteroids (ICS) and long-term bronchodilators medication except one patient. Patients were using short-acting bronchodilators (SABA), long-acting bronchodilators (LABA), and long-acting muscarinic antagonists (LAMA) therapies.

3.4.2 Comparison of Lung Function and Spirometry Measurements between Groups at Baseline

After spirometry testing it was found that patients with COPD had lower FEV1% predicted, and greater airflow obstruction as expected, complained of more symptoms. Healthy smoking and healthy nonsmoking subjects had normal spirometry. In general, the mean value of FEV1, FVC, FEV1%, and FEV1/FVC ratio were significantly lower in COPD group when compared with healthy smoking and healthy nonsmoking groups ($p < 0.05$). The mean value of FEV1% was statistically significantly different between COPD (52%) and healthy smoking (99.5%, $P < 0.001$), as well as healthy nonsmoking groups (99.6%, $P < 0.001$). The mean value of FEV1% is positively correlated with the disease stage. This means that patient respiratory functions are decreasing with the increasing of disease severity. The difference in the mean FEV1% measured between healthy smoking and healthy nonsmoking was statistically non-significant ($P>0.05$). All patients had an FEV1/FVC ratio $\leq 70\%$ of the predicted value and pre FEV1% <80%.

Table 3.1: Demographic, Clinical data and results of the pulmonary function test of study subjects.

Characteristic	COPD	HS	HNS
Number	15	15	12
Age, yrs	66.4(7.1)	56.3(6)	50.1(7.3)
Height	1.67	1.68	1.74
Weight, Kg	71.94 (12.3)	78.6 (10.8)	85.26 (10.7)
BMI, Kg/m^2	25.42 (3.15)	27.83 (4.13)	28.1 (3)
Pre FEV1, L	1.39 (0.57)	2.92 (0.63)	3.34(0.41)
Pre FEV1%	52.10 (22)	99.55 (10.2)	99.6(13)
Pre ratio FEV1/FVC	41.5 (11.9)	74.4 (4.26)	75.6 (5.38)
Post FEV1, L	1.52 (0.55)	2.95 (0.62)	3.44 (0.37)
Post FEV1 %	56.88 (0.18)	103.5 (20.81)	103.00 (0.13)
Post ratio FEV1/FVC	42.9 (11.6)	72.16 (20.57)	78.41 (5.26)
Reversibility, mls	136 (131.7)	32.33 (43.70)	92.50 (73.50)
Reversibility %	12.39 %	1.11%	3.22%
PYH	47 (17.2)	26 (9)	0.19 (0.66)

The data are expressed in term of mean (standard deviation) or as percentage unless otherwise stated. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. These analyses were performed using Graph Pad prism and Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; FEV1, Forced Expiratory Volume in 1 second; FVC, forced vital capacity; BMI, Body Mass Index; BD: bronchodilator; PYH, pack year history.
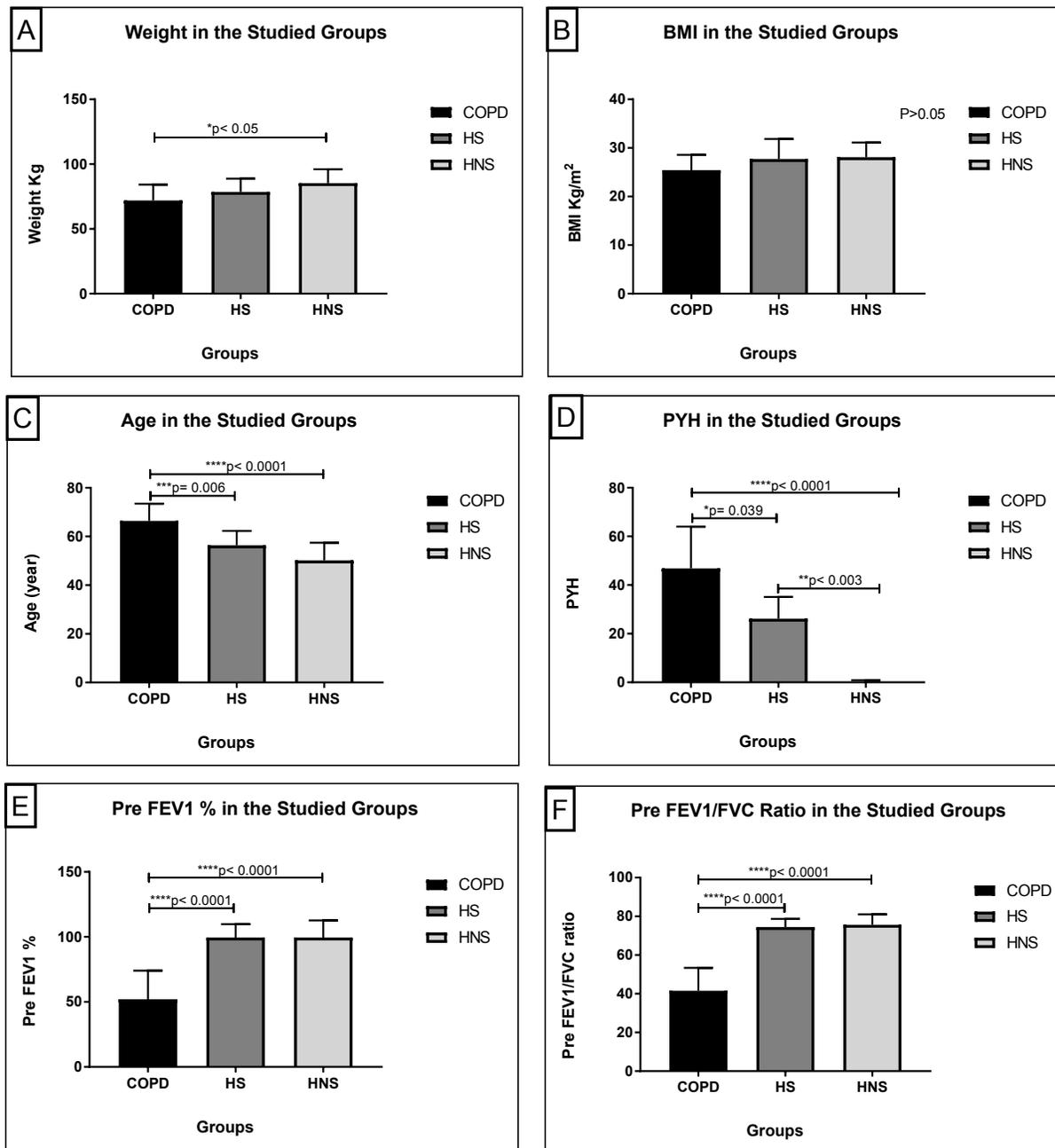
Figure 3.1: Comparison of respiratory function and parameters between the studies groups. *The mean values of weight in Kg (A), BMI Kg/m²(B), age in years (C), PYH (D), preFEV1% (E), and pre FEV1/FVC ratio (F), between the three studied groups. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. These analyses were performed using Graph Pad prism and Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; FEV1, Forced Expiratory Volume in 1 second; FVC, forced vital capacity; BMI, Body Mass Index; BD: bronchodilator; PYH, pack year history*

Linear regression analysis using Pearson methods, showed a significant correlation between FEV1% and age (P=0.0009) as well as BMI (P=0.024) but not with weight of the entire study subjects (P>0.05). Linear regression analysis also showed a significant correlation between FEV1/FVC ratio and age (P<0.0001), BMI (P=0.0009) as well as weight of entire study subjects (P=0.022) (Figure 3.2). However, no significant correlation was observed between FEV1% and FEV1/FVC ratio with age or BMI as well as weight of COPD patients (P>0.05).
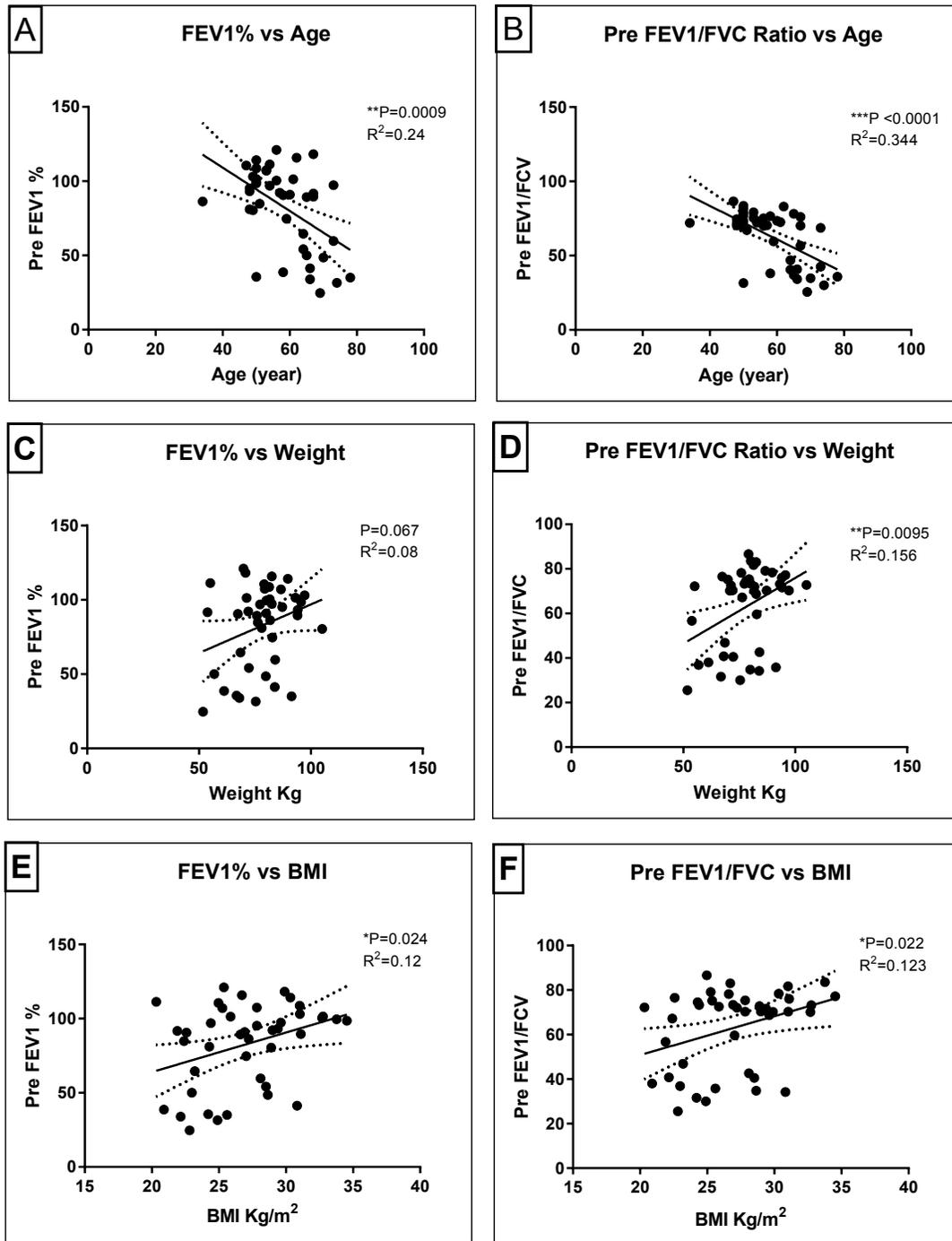
Figure 3.2: The correlation between lung severity and subject parameters. *Pre FEV1% vs age (A), FEV1/FVC vs age (B), pre FEV1% vs weight (C), FEV1/FVC vs weight (D), pre FEV1% vs BMI (E), FEV1/FVC vs BMI (F). Significance was tested using a linear regression analysis (spearman method). Dotted lines indicate the 95 % confidence intervals. These analyses were performed using Graph Pad Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; FEV1, Forced Expiratory Volume in 1 second; FVC, forced vital capacity.*

The differences between pre- and post-bronchodilator FEV1% increase in the COPD group. The mean values for pre- and post- FEV1% were 52.10% (range, 24.68%- 97.28%) and 56.88% (range, 37.00%- 96.89%), respectively, in COPD group, compared with 99.55% (range, 84.77%- 118.23%%) and 103.5% (range, 77.61%- 170.38%), respectively, in healthy smoking group, whereas they were 100% (range, 80.47-121.03%) and 103% (range, 81.39-124.72%), respectively, in the healthy nonsmoking group. The difference between the mean of pre- FEV1% and post- FEV1% was significant difference in the three groups; (COPD, P=0.002; HS, P=0.008; HNS, P=0.00009). There was a significant difference in pre- and post-FEV1 when compared the COPD patient with healthy smokers and nonsmokers (P<0.0001) (Figure 3.3).
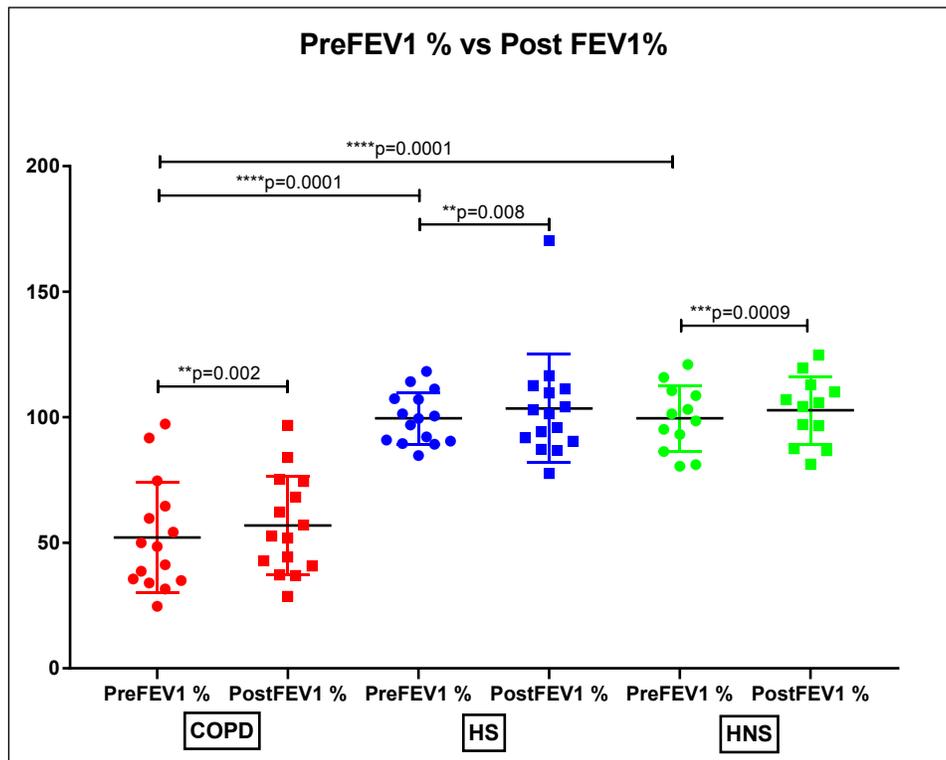
Figure 3.3: Comparison between pre FEV1% and post FEV1% in the three studied groups; COPD = 15 *(A), HS =15 (B), and HNS =12 (C). P value < 0.05 as determined by paired* t-*tests indicate a significant difference between the pre- and postFEV1, and one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the three groups. These analyses were performed using Graph Pad prism and Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; FEV1, Forced Expiratory Volume in 1 second.*

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In the current study, the bronchodilator reversibility of the study cohort was also evaluated. At baseline, 60% of patients with COPD met ATS/ERS reversibility criteria. The reversibility of patients with COPD, healthy smokers and nonsmokers were 12.39 %, 1.11% and 3.22% respectively (Figure 3.4). There was a significant difference in reversibility when compared the COPD patient with healthy smokers (P<0.0001) and nonsmokers groups (P<0.002). Interestingly, there was a negative correlations between patients' reversibility and BMI (p=0.0087), as well as weight (p=0.0143), but not with age (p=0.15) (Figure 3.5).

Figure 3.4: Comparison of the mean values of reversibility between the three studied groups. *Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. These analyses were performed using Graph Pad prism and Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers.*

Figure 3.5: The correlation between patient's characteristic and reversibility percentage. *Reversibility% vs (A) age, (B) weight, and (C) BMI. Significance was tested using a linear regression analysis (Pearson method). These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: BMI, body mass index.*

3.4.3 The Influence of Smoking Status on Lung Function Parameters

Statistically there was a significant negative correlation between smoking status and reversibility of COPD patients (p=0.0369), while no association was observed between smoking status and reversibility in healthy smokers or healthy nonsmokers groups (P>0.05) (Figure 3.6). The study cohort was split into four subgroups according to smoking status category. The total number of smoking subject is 30; COPD patient current smokers = 9, COPD patient ex-smokers =6, healthy current smokers = 11, and healthy ex-smokers =4.The mean (SD) of PYH were 38.6 (18.5) and 52.3 (14.7) for former and current smokers with COPD and 26.5 (8.24) and 26 (9.5) for former and current smokers with no COPD. There was a statically significant difference of PYH between current smokers with COPD and those with no COPD (one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test, P<0.01). The low sample size in healthy ex-smoker subgroup did not provide enough data for a statistical comparison with the COPD current smokers, as only four subjects were healthy ex-smokers There was no statically significant difference of PYH between the other subgroups (P>0.05) (Figure 3.7).
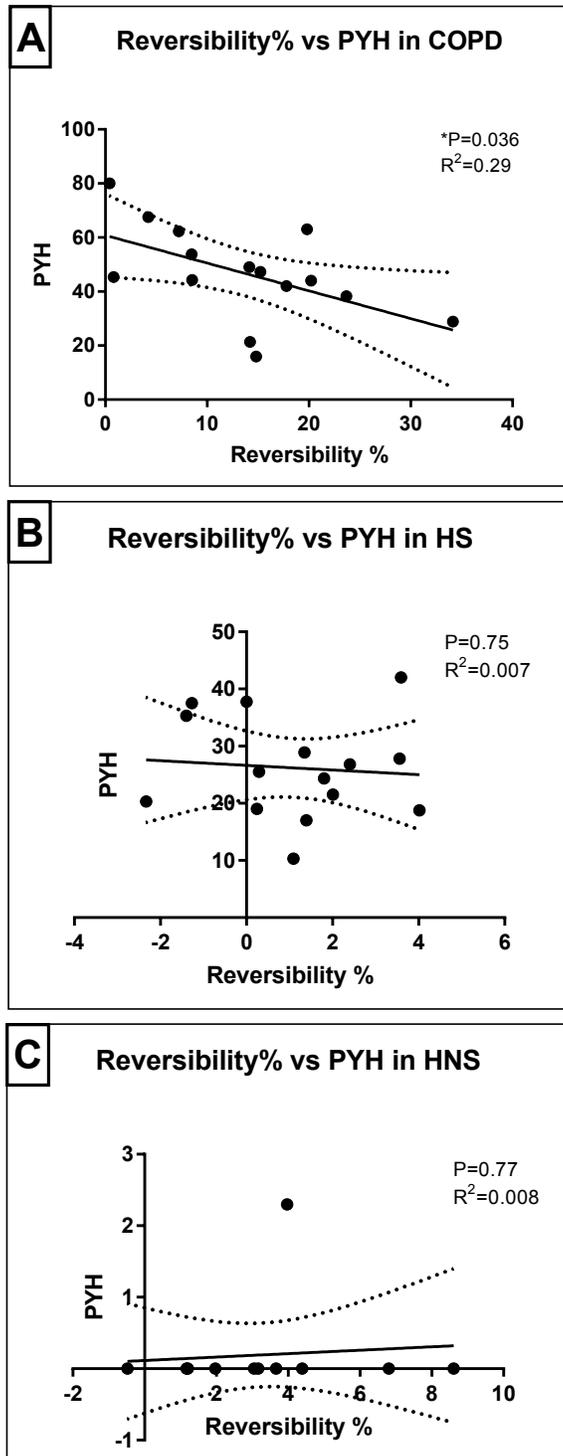
Figure 3.6: Reversibility in relation to smoking habits in the studied groups*; COPD 15 (A), HS =15 (B), and HNS =12 (C). Significance was tested using a linear regression analysis (Pearson method).These analyses were performed using Graph Pad Software. Abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; FEV1, Forced Expiratory Volume in 1 second; PYH, pack year history.*
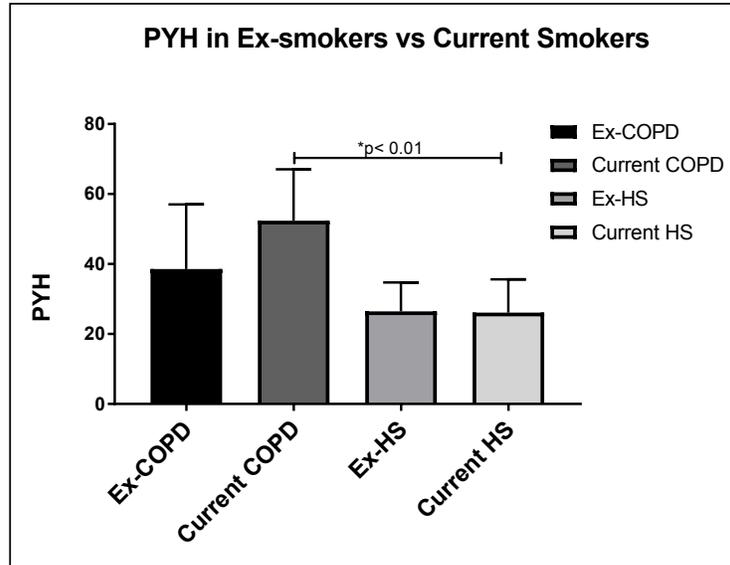
Figure 3.7: Comparison of PYH between the subgroup of the study cohort. *Total=30; ex-smokers COPD (n=6), current smoker COPD (n=9), ex-smoker HS (n=4) current smoker HS (n=11). Unpaired one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. Test indicates a significant difference between the groups. These analyses were performed using Graph Pad and Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; PYH, pack year history.*

Additionally, linear regression analysis showed no relation between smoking and FEV1% in smoker's sub-groups (P>0.05) (Figure 3.8, A-D). However, significant relation was observed between smoking and FEV1% when analysing the whole smoking group (P<0.05) (Figure 3.8-E).
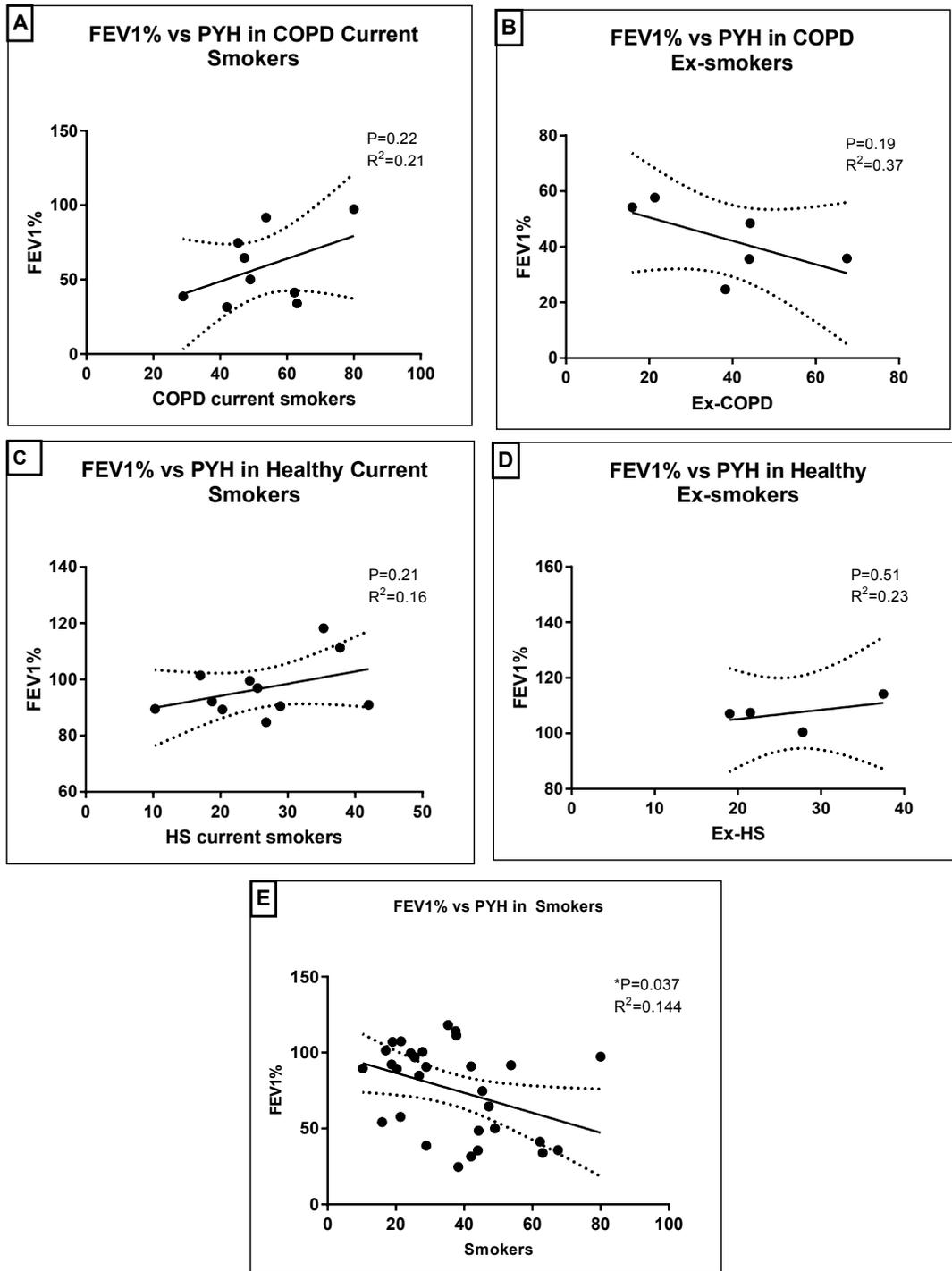
Figure 3.8: The correlation between pre FEV1% and smoking (PYH) in smokers' subgroups; *(A) COPD current smokers, (B) COPD ex-smoker (C) healthy current smokers, (D) healthy ex-smokers, and (E) all smoking subjects. Significance was tested using a linear regression analysis (Pearson method).These analyses were performed using Graph Pad Software. These analyses were performed using Graph Pad Software.* Definition of abbreviations: COPD, chronic obstructive *pulmonary disease; HS, healthy smokers; FEV1, Forced Expiratory Volume in 1 second; PYH, pack year history.*

3.4.4   Influence of ICS on COPD Severity and Smoking

The impact of ICS therapy on COPD severity, as well as smoking exposure was also investigated. There was significant positive correlation between ICS and PYH (r^2 =0.3, P = 0.032). No correlation was found between the ICS and lung function parameters; pre FEV1 (r^2 =0.022, P = 0.59), as well as pre FEV1/FVC ratio (r^2 =0.006, P = 0.78) (Figure 3.9).
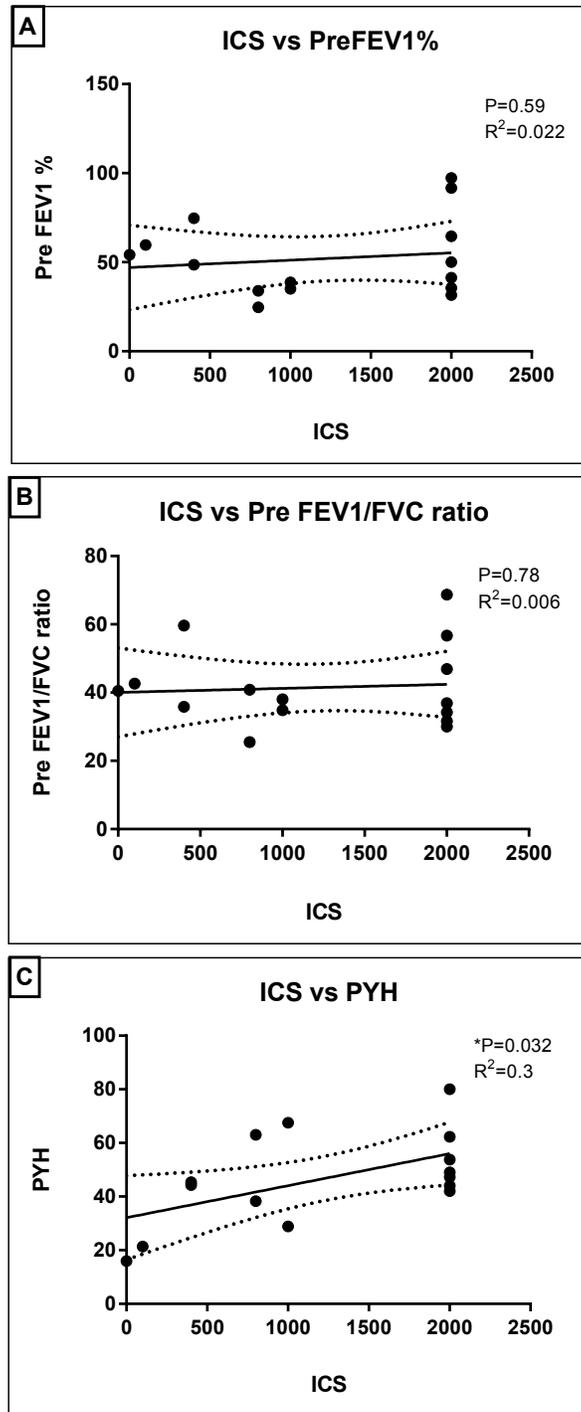
Figure 3.9: Linear regression analysis of the correlation between ICS vs disease severity and PYH. *ICS vs FEV1 %predicted (A), FEV1/FVC ratio (B) and PYH(C). Significance was tested using a linear regression analysis (Pearson method).These analyses were performed using Graph Pad Instat Software. Abbreviations: PYH, pack year history; FEV1%, percent predicted forced expiratory volume in the first second, FVC, forced vital capacity; ICS, inhaled corticosteroid.*

3.5 Discussion

COPD is a multicomponent disease characterised by several pathological changes, which include mucus hypersecretion, airway obstruction and loss of alveoli and a systemic comorbidities (Cazzola, *et al.,* 2008). Airflow limitation is the main characteristic of patients with COPD. It caused by small airway remodelling and emphysema which results from the destruction of the parenchyma induced by a chronic inflammatory response after the exposure to different toxins like cigarette smoke and environmental irritants. Subsequently resistance to airflows is increasing with goblet cell metaplasia, mucous hypersecretion and smooth muscle hypertrophy (Barnes 2016). In the present study, a significant decrease was found in the pulmonary functions of COPD patients when compared to healthy smokers and healthy controls. Patients with COPD suffered from airway restriction comparing to healthy smokers and healthy nonsmokers.   The mean value of FEV1% and pre FEV1/FVC ratio of the COPD group is 52% and 41.5% respectively which agree well with GOLD recommended spirometry criteria. GOLD reference cut-off values of FEV1% <80% with a post-bronchodilator FEV1/FVC ratio <70% were classified as having airway limitation with a possibility of COPD. Additionally, a significant decrease was found in the respiratory functions of severe COPD patients when compared with mild and moderate COPD patients. This means that patient respiratory functions are decreasing as the stage of COPD increased. The findings of the present work are consistent with those of many authors who found that pulmonary function data; pre FEV1% and pre FEV1/FVC ratio, were significantly lower in COPD patients when compared with healthy nonsmoking controls (El-shimy *et al.,* 2014, Wang *et al.,* 2014).

COPD is one of the most common chronic diseases that predominantly affect older people. Despite the increasing cases of COPD in older people, under-diagnosis and inadequate treatment in elderly patients is still a common problem. Older patients with COPD generally have a poor health status because they suffer from more chronic comorbidities, a compromised immune system, and decrease in pulmonary function. There are few studies that specifically investigate the association between COPD and age, while epidemiologic studies have reported that FEV1/FVC decreased with age. Ageing involves structural changes and decline in pulmonary functions which can cause poor ventilation and can impair the clearance of airway secretions. Thus, COPD is more prevalent in very elderly populations (Valente *et al.,* 2010). In patients with

COPD, increasing age was found to reduce quadriceps muscle strength. Increasing age was also found to be associated with an increased risk for osteoporosis in patients with COPD (Cielen *et al.,* 2014)

The controversies exist regarding the use of a fixed FEV1 /FVC ratio of 0.7 as it may lead to over-diagnosis of COPD in the elderly (Bailey 2012). In the present study, a significant difference in the age of patients and healthy subjects was observed. Patients were significantly older than those of healthy groups. The current study data is agreed with Hassanein *et al.,* (2016) study, where the COPD patient group was older than the healthy control group. It was difficult to recruit age-matched older non-smoking healthy subjects without any medical history fulfilling the inclusion and exclusion criteria. Additionally, a significant relation between FEV1% or FEV1 /FVC ratio and age was observed in the study subjects. This means that respiratory functions were decreased with age. Subsequently; the study confirmed that the proportion of COPD severity increased with age.

It known that COPD mainly is a lung disease but it is associated with other comorbidities such as cardiovascular disorders, chronic kidney disease, lung cancer, diabetes mellitus, loss of lean body mass, skeletal muscle weakness, and osteoporosis. Lung cancer and CVD are the main comorbidities found in COPD patients (Barnes, 2010). These comorbidities result in reduced patient health status, increased hospital readmission, and mortality (Decramer *et al.,* 2012).Weight loss affects 25% to 40% of COPD patients, with 25% of patients with moderate to severe disease. Patients with BMI of <20 kg/m^2 have a higher risk of acute exacerbations than those with a BMI of $\geq$ 20 kg/m^2 (Itoh *et al.,* 2013). Low body weight and BMI have been identified as a poor prognosis sign in COPD patients. Further, decreased body weight is correlated with disease severity, skeletal muscle weakness and mortality in patients with COPD. COPD patients with low weight were found to have a high prevalence of osteoporosis (57%) and muscle wasting (93%) (Cielen *et al.,* 2014). GOLD, do not provide any specific nutritional guidelines for the management of patients with COPD other than to avoid weight loss. On the other hand, obesity (BMI > 40) was associated with a significantly increased risk of death in both patients with respiratory disease and chronic lower respiratory disease (Hanson *et al.,* 2014).

Dietary supplement have shown to improve patient exercise capacity and quality of life but did not show any significant improvement in pulmonary function (Raizada *et al.,* 2014). Interestingly, in this study there was a significant difference when comparing the mean weight of COPD patients with those of healthy nonsmokers. Patients were with low body weight when comparing with healthy nonsmokers which agreed with previous studies. However, none of patients were underweight in term of BMI <20 kg/m^2. This may be because patients were shorter. Additionally, a significant association was observed between preFEV1% and BMI and between preFEV1/FVC ratio and weight as well as BMI when investigating the whole study groups. This means that subject weight or BMI may have impact on disease severity.

Smoking history and exposure, as quantified by PYH was also evaluated as one of COPD main risk factor. Statically there was a significant difference in PYH between patients and healthy smokers as well as between patients and healthy nonsmokers. This means that smoking habit is higher among patients than healthy smokers. A significant correlation was found between PYH and FEV1 in smoking subjects. However, smoking history and exposure seemed to have no impact on patient disease severity in smokers' subgroups analysis including patients' ex-smokers, patient current smokers, healthy ex-smokers, and healthy current smokers. As expected patients' current smokers were with the highest PYH rate among the other subgroups. This result is consistence with other study group finding which reported that no correlation was observed between smoking status and the percentage of predicted FEV1 (Higashimoto *et al.,* 2009). It should be noted that smokers' number was low for linear regression analysis when dividing the smokers groups into sub-groups.

Reversibility is an important diagnostic tool when investigating patients with COPD. In the current study the mean value of reversibility in the COPD group agreed with current ATS guideline cutoffs for defining a significant reversibility. The ATS/ERS guidelines recommendation of >12% and >200 ml increase from the baseline pre-bronchodilator FEV1and/or FVC in an individual subject to consider significant reversibility (Albert *et al.,* 2012; Tan *et al.,* 2012; Müller *et al.,* 2016). Interestingly, finding from the current study indicated that smoking status did influence reversibility of COPD subject, but not in healthy smokers. In addition to smoking effect, it was demonstrated that both patient weight and BMI but not age did influence the response in patients with COPD. Additionally, predictors of

reversibility in COPD weight, BMI and smoking status were also related to pre FEV1 and pre ratio. In summary, these data confirmed that smoking exposure, weight, BMI have impact on patients' reversibility. Previous study showed that high doses of ICS may affect the immune system and subsequently may associate with disease severity (Zhu et al., 2009). In the present study, all COPD patients received ICS except one. It is assumed that the study results were not influenced by the dosage of ICS, as no correlations were found between ICS doses and pre FEV1 or pre FEV1/FVC ratio. Interestingly, we found a significant association between ICS and smoking habit.

The diagnosis of COPD is based mainly on spirometry measurement and other lung functions parameters. Spirometry is easy to perform at low cost for the diagnosis and assessment of a clinical stage of COPD. However, it cannot offer much information as gas transfer or lung plethysmograph. A previous study found that gas transfer was a significant factor for prediction of survival, together with age and arterial oxygen. Applying gas transfer measurement in routine practice for patients with COPD will provide them with the best prognostic information, and future classifications of COPD instead of relying only on the severity of airflow obstruction (Boutou *et al.,* 2013). Also spirometry measurements reflect only the general pulmonary ventilation function, but cannot assess the pathologic changes and the regional morphologic and functional abnormalities. A study by Šileikienė *et al* (2017) confirms significant correlations between quantitative computed tomography (CT) measurements of emphysema and airflow obstruction, diffusion capacity, and lung volumes. Therefore, performing CT scan will enable clinicians to assess emphysema in patients even those in mild stage of airflow obstruction, which could help in prescribing personalised treatment for each patient. A recent study pointed out the role of spirometry in diagnosis for patients with acute exacerbation COPD during hospitalisation. The study finding confirmed that spirometry after several days of hospital admission due to acute exacerbation COPD is valid and reproducible in most patients and can assess in disease diagnosis and evaluation (FernaÂndez-Villar *et al.,* 2018).

COPD are heterogeneous diseases and many studies showed that individual COPD patient shows biological variability in their clinical presentation and disease severity and progression (Müller et al., 2016). Therefore, the addition of a panel of markers to lung function parameters known to predict mortality in COPD, such as age, FEV1 or hospitalizations because of

exacerbations (Cazzola, *et al.,* 2008), gas transfer, lung plethysmograph, BODE and CT measurements will improve diagnosis and prediction of mortality risk in patients with COPD.

3.6   Conclusion

In this chapter it was shown that patients pulmonary functions data were significantly different from those of healthy smokers and nonsmokers. Patients were with old age, low body weight, high in smoking habit, with greater air flow limitation than healthy smokers and healthy nonsmokers. Age, weight and BMI have an impact on subject respiratioty functions when investigatin the whole study indviduals.However these parameters have no impact on patients disease severity when invetigating the patinet group only. This may due to low size of patients sample. What is new is that patients smoking habit, weight, BMI affected bronchodilator reversibility. For better understanding of the COPD variability between patients, different parameters should be evaluted during patient diagnosis at routine clinical practices, including lung plethysmography, gas transfer, arterial oxygen, and CT, as well as multiple spirometry. This will provides with accurate diagnosis and better disease management.

4. Chapter 4: Evaluation of systemic blood biomarkers for COPD

4.1 Background

The search for circulating blood biomarkers has mainly focused on plasma or serum proteins which are known to be involved in the systemic inflammatory response to irritant or lung injury (Ongay et al., 2016). A biomarker is any molecule or material, which level or activity indicates the disease process, pathogenic processes, or pharmacologic responses to a new therapy. Currently no specific biomarkers have been identified and accepted for COPD routine clinical application. Thus there is a great interest in developing biomarkers which can assist in discovery of an effective treatment that improve health outcomes of patients with COPD. However, biomarker development is extremely challenging and expensive (Doubková *et al.,* 2015). Most of the biomarkers candidates that have been investigated still not clinically validated. COPD is heterogeneous disease in terms of the progression and response to treatment therefore it is unlikely that a single biomarker will be sufficient for COPD diagnosis, prognosis or treatment evaluation (Ongay *et al,* 2016). Several biomarkers including cytokines, chemokines, metalloproteases and acute phase reactants are implicated in inflammatory responses and are also involved in the regulation and progression of several inflammatory diseases like COPD. They are produced by a broad range of cells including monocytes and macrophages, T-lymphocytes, B-lymphocytes and mast cells, as well structural cells like endothelial cells, fibroblasts and adipocytes. Biomarkers, particularly cytokines, are associated with inflammatory diseases, with higher levels of TNF-α, IL-1β, IL-6 and IL-8 being a common feature of many inflammatory conditions (Calder 2015).

Among these biomarkers fibrinogen, an inflammatory biomarker, that has been considered as a promising biomarker in COPD. It is an acute phase soluble plasma glycoprotein, synthesized mainly in the parenchymal cell of the hepatocyte and in the megakaryocyte in bone marrow, and converted by thrombin into fibrin during blood coagulation. The normal fibrinogen levels in blood are between 1.5 and 3.5 g/L but can increase threefold during inflammation in response to increased IL-6 production (Duvoix, *et al.,* 2013; Fattouh and Alkady, 2014). Fibrinogen levels $\geq$ 350 mg/dL consider COPD patients at an increased risk of exacerbations and mortality.

Fibrinogen is elevated in COPD patients, and associated with the FEV1 (Vestbo *et al,* 2011) and disease severity (Agusti *et al.,* 2012). Higashimoto et al., (2009), found that patients with an exacerbation frequency of >2.52 exacerbations per year, were more likely to show a faster rise in plasma fibrinogen than those with a history of infrequent exacerbations during 2-year follow-up. Further, Mannino and colleagues (2015) analysed pooled data of 6376 individuals with COPD and found that plasma fibrinogen was associated with severity in moderate and severe disease. High fibrinogen levels at baseline were associated with an increased risk of hospitalized COPD exacerbations within 12 months. Additionally, high fibrinogen was associated with an increased risk of death within 36 months among all participants. Therefore, fibrinogen has been considered by the USFDA and COPD Biomarker Qualification Consortium (CBQC) for the stratification of subjects at risk for hospitalization and mortality (Duvoix *et al.,* 2012).

CRP is another acute phase inflammatory protein synthesized mainly by hepatocytes in response to tissue damage or inflammation. It contributes to the recruitment of circulating leucocytes, the uptake of low density lipoprotein cholesterol by macrophages, and destabilisation of vascular wall atheroma (Pinto-Plata *et al.,* 2006). It has the ability to bind the receptors of phagocytes which clear up apoptotic and necrotic cells. The function of CRP is regulated through IL-6 and IL-1. CRP level changes with alterations of lung function volumes, severity of disease, and development of pneumonia (Heidari 2012). It is involved in COPD pathogenesis and considered as a predictor of lung function decline and mortality in COPD (Higashimoto *et al.,* 2009). Elevated CRP level in plasma of patients with COPD was reported particularly during acute bacterial and viral exacerbations (Barnes *et al.,* 2014).Two different mechanisms have been suggested to explain the association between baseline CRP levels and COPD. The first hypothesis is linked to the effect of lung inflammation in COPD patients. It is known that prolonged exposure to cigarettes smoke leads to lung injury and inflammation causing systemic reaction. Additionally CRP level increase is secondary to other circulatory pro-inflammatory cytokines include TNF-α, IL-6, IL-8 or fibrinogen. The parallel activation of systemic inflammation maintains and increases the local airway inflammation, leading to COPD progression. The second hypothesis suggested that elevated levels of CRP are related to the occurrence of CVD rather than mortality from COPD itself. It is well known that the majority of patients with COPD died either from CVD or cancer but not from lung disease. Thus CRP could

be used as a prognostic marker in COPD, as inflammation itself may lead both to lung damage and CVD in these patients (Leuzzi et al., 2017).

Cytokines are small proteins that are released by cells, especially, but not exclusively, those that act within the inflammatory and immune systems. Cytokines act through specific receptors to affect the activity of the same releasing or other cells. Cytokines include TNF, various ILs, interferons, chemokines, and lymphokines (Calder 2015). Cytokines play key roles in many pathophysiological processes in COPD, particularly in the chronic inflammation and emphysema (Ji *et al.,* 2017). For example, IL-6 is associated with a large number of pulmonary and extra-pulmonary inflammatory manifestations in COPD (El-Shimy *et al.,* 2014; Ji *et al.,* 2017). IL-6 is a pleiotropic proinflammatory and immunomodulatory cytokine, synthesized by the airway epithelium, macrophages and other cells at the site of inflammation in response to environmental irritant or stress such as smoking. Serum IL-6 levels were significantly higher in individuals with COPD when compared with controls and increase during exacerbations (Higashimoto *et al.,* 2009; Ferrari *et al.,* 2010). IL-6 is the main regulator and activator of CRP and fibrinogen in the liver, and it is associated with CRP levels in COPD patients. It also involved in hematopoiesis, causing thrombocytosis and leukocytosis with its overexpression (Ferrari *et al.,* 2010; El-Shimy *et al.,* 2014).

TNF-α is another proinflammatory cytokine mainly produced by activated macrophages in addition to monocytes, T-cells, B-lymphocytes, fibroblasts, and endothelial cells. It is a potent cytokines that play a critical role in the pathogenesis of COPD by regulating the expression and the release of various proinflammatory mediators that cause tissue remodeling and damage (Mukhopadhyay *et al.,* 2006). The stimulation of TNF-α by Inhaled lipopolysaccharide (LPS) induce pulmonary inflammatory responses in healthy subject (Kawayama *et al.,* 2016).

In COPD, different immune inflammatory cells are implicated in lung inflammation including innate immunity; alveolar macrophages, neutrophils, dendritic cells, eosinophils, natural killer cells, and adaptive immunity; T- and B-lymphocytes. Alveolar macrophages particularly appear to regulate the inflammatory response by secreting various chemokines to attract immune cells from circulation to the injury site and to initiate T-cell immune response. Chemokines are a group of chemotactic molecules that regulate and direct the movement of leukocytes during inflammation. Chemokines are classified into four subfamilies, CXC, CC, C

and CX3C, according to the position of the conserved cysteine residues (Odler *et al.,* 2016; Muñoz-Esquerre *et al.,* 2017). The CXC chemokine IL-8, also called CXCL8, is a neutrophil and T-cells potent chemoattractant and activator, which involved in inflammation-mediated neutrophil infiltration and chemotaxis. It is secreted by monocytes, alveolar macrophages, eosinophils, pulmonary epithelium, airways smooth muscles cells, fibroblasts and endothelial cells. Bacteria and elastase released from neutrophils may also stimulate epithelial cells to produce IL-8 (Chung *et al.,* 2001). It was found *in vitro* that cigarette smoke and TNF-α can stimulate IL-8 production from macrophages (Sarir *et al.,* 2009). IL-8 levels found to be increased in BAL and sputum samples of patients with COPD (Di Stefano *et al.,* 2004).

An example of the CC-chemokine family is the CC-chemokine ligand-18 (CCL18), previously named pulmonary and activation-regulated chemokine (PARC), macrophage inflammatory protein-4 (MIP-4), is a 7-kD protein regulatory cytokine that is highly expressed in dendritic cells, monocytes and M2-macrophages in the lungs in response to pro-inflammatory Th2 cells cytokines (Sin *et al.,* 2011; Plönes *et al.,* 2012). CCL18 was shown to be a macrophage differentiation factor and has the possibility to induce an M2 macrophage phenotype, and collagen production in lung fibroblasts (Dilektasli *et al.,* 2017). CCL18 is involved in chemotaxis of naive T-cells, T-regulatory cells, Th2 cells, dendritic cells and B-cells. Because it predominantly produced in the lungs, CCL18 has been evaluated in several pulmonary diseases including; idiopathic interstitial pneumonias and systemic sclerosis (Prasse *et al.,* 2007), community-acquired pneumonia, and lung cancer (Plönes *et al.,* 2012), COPD (Sin *et al.,* 2011; Dilektasli *et al.,* 2017), where CCL18 serum levels have been found to be elevated and in some disease was associated to mortality (Spoorenberg *et al.,* 2017). Further, few studies suggest that CCL18 might consider as serum biomarker of cardiovascular hospitalization or mortality in COPD patients (Sin *et al.,* 2011; Kraaijeveld *et al.,* 2007).

Another member of the CXC chemokine family is the IFN-γ-inducible protein 10 (IP-10), also known as CXCL10, secreted by several inflammatory cells including; monocytes, lymphocytes, neutrophils, and bronchial epithelial cells in response to inflammation. The activity of this chemokine is regulated by IFN-γ and TNF-α, and mediated by binding to CXCR3 receptor which is expressed by Th1 cells and in many other cell types including macrophage and lung epithelial cells (Torvinen *et al.,* 2007; Quint *et al.,* 2010). IP-10 is potent antiviral

chemokine that able to recruit and attract activated monocytes, Th1 and Th2 lymphocytes and NK cells to the sites of infection. Because respiratory viral infections such as influenza virus, rhinoviruses, and respiratory syncytial virus are the main pathogens associated with COPD exacerbations, so it is suggested that IP-10 may also play a role in the inflammatory process of COPD (Wu *et al.,* 2016).

Several studies continue to investigate novel biomarkers which seem to show promising results that might help in the early diagnosis, progression and treatment of COPD. There has been increasing interest in using pulmonary biomarkers to understand and monitor the inflammation in the respiratory tract of patients with COPD (Doubková *et al.,* 2015). In the current study, Clara cell protein (CC16) and receptors for advances glycation end product (RAGE) were investigated as promising biomarkers of COPD. CC16 is specific pulmonary protein of the secretoglobin family with an anti-inflammatory and detoxification properties, which has been suggested to be a useful marker of airway and lung epithelial injury (Pilette *et al.,* 2001). CC16 is a small protein with molecular weight of approximately 16KDa (Doubková *et al.,* 2016). It is mainly secreted by non-ciliated, non-mucous club cells or Clara cells, which are localized in the respiratory bronchioles and by non-ciliated columnar epithelial cells present in the large and small airways (Ongay *et al.,* 2016). It can binds to phospholipids and to different cell types, inhibiting inflammation, migration and invasion. The low level of CC16 in COPD might associate with a defect in anti-inflammatory action and detoxifying effect of CC16 (Pilette *et al.,* 2001).

RAGE is a transmembrane, multi ligand belong to the immunoglobulin superfamily of cell surface molecules that is highly expressed in normal human lung. RAGE is involved in the glycation of serum lipids and proteins that alter the structure and function of endothelial and epithelial cells in COPD airways (Wu *et al.,* 2011; Cheng *et al.,* 2013; Gopal *et al.,* 2014). It is distributed and expressed at low levels in human normal tissues and cell types, but it highly expressed in the lung tissue particularly in alveolar type-I epithelial cells, bronchial smooth muscle cells, vascular endothelial cells, alveolar macrophages and alveolar type-II epithelial cells (Sambamurthy *et al.,* 2015). RAGE exists in the body in two main forms, membrane-bound RAGE (RAGE) and the circulating soluble form of RAGE (sRAGE). The latter is a product of either alternative splicing events or proteolysis of cleavage of RAGE cell surface receptor

(Cheng *et al.,* 2013). The circulating sRAGE can acts as a decoy receptor for Advanced Glycation End products (AGEs), preventing binding of AGEs to RAGE and prevents signaling at the cell surface receptor subsequently inhibit the activation of transcription factor NF-kB and inflammatory responses (Yonchuk *et al.,* 2015; Hassanein *et al.,* 2016).

NF-κB involved in regulating different aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory responses (Liu *et al* 2017). The AGEs/RAGE interaction can activate signalling pathways of NF-kB that in turn activate many proinflammatory cytokines such as IL-1, IL-6 and TNF-α. Interestingly, cytokines themselves can activate NF-kB, that increases RAGE expression and subsequent RAGE-mediated NF-kB activation result in a positive auto-regulatory loop (Wu *et al.,* 2011). Activation of RAGE signaling pathway is considered a protective mechanism to promote an inflammatory response against inhaled irritant or tissue injury. Thus, increased expression of RAGE and its ligands, and decreased sRAGE expression in the lungs suggests that RAGE signaling pathway enhances the inflammatory response in COPD (Oczypok *et al.,* 2017). Multiple studies have reported that circulating sRAGE could be a useful biomarker for COPD specifically to the presence and progression of emphysema (Yonchuk *et al.,* 2015).

Another lung specific biomarker is SP-D, a large hydrophilic glycoprotein with multimeric structure, synthesized and secreted mainly by endoplasmic reticulum of alveolar type II pneumocytes and the secretory granules of non-ciliated club cells in the bronchioles (Madsen *et al.,* 2000; Sin *et al.,* 2008; Lomas *et al.,* 2009). SP-D modulates the pulmonary immune defense; decreases the expression of pro-inflammatory cytokines, decreases oxidative stress and increases, facilitates the resolution of lung inflammation. It also enhances clearance of a wide variety of pathogens and promotes phagocytosis of apoptotic cells (Sims *et al.,* 2008). Previous studies have described associations between SP-D and COPD, in which SP-D levels were found to be elevated in COPD patients when compared to healthy smoking controls (Sin *et al*., 2008; Lomas *et al.,* 2009). It is suggested that SP-D levels may be correlated with disease progression in other pulmonary diseases, including community-acquired pneumonia and asthma (Liu at *al.,* 2014).

Microparticles (MPs) are small membrane vesicles which are released by shedding from the cell plasma membrane following tissue injury and cell apoptosis. There are different types of MPs that are defined according to specific cell membrane antigens including; Endothelial and platelet cell adhesion molecule 1 (CD31), leukocytes (CD11a), granulocytes (CD66b), and monocyte/macrophages (CD11b). Cluster of differentiation 31(CD31), also known as platelet endothelial cell adhesion molecule (PECAM), is one of the MPs which are likely to be implicated in the pathogenesis of COPD (Lacedonia *et al.* 2016). CD31 is a heavily transmembrane glycoprotein member of the immunoglobulin superfamily that is highly expressed on endothelial cells and at a lower level on platelets, macrophages, granulocytes, B-cells, some T-cells subtypes, dendritic cells and NK cells. CD31 lies outside the adherence junctions on endothelial cells, and on the surface of platelets, neutrophils, and T cells (Falati *et al.,* 2006).

CD31 is involved in vascular biology, in particular, in the regulation of platelet function, angiogenesis, thrombosis, and apoptosis, and endothelial cell permeability, trans-endothelial migration of leukocytes. Additionally, CD31 is able to enhance the activation of T- and B-cells, inhibit dendritic cell development. It is highly expressed in the vascular system of kidney, lung, and trachea while lower expressed in brain, heart, and liver (Falati *et al.,* 2006; Woodfin *et al.,* 2007). It is significant for the leukocytes transmigration across the endothelium tissue. For example, the interaction between two platelet and endothelial cell adhesion molecule CD31 expressed at the borders of the endothelial cells and on neutrophils is essential (Blidberg *et al.,* 2013). Oxidative stress induced by H_2O_2 or cigarette smoke extract stimulate the release of different types of MPs from cultured endothelial cells. An *in vitro* study by Takahashi *et al.* observed that the majority of CD31 are released from pulmonary endothelial cells in response to apoptosis when stimulated by either H_2O_2 or cigarette smoke extract. H_2O_2-induced endothelial injury and activation promote alveolar destruction in COPD lung (Takahashi *et al.,* 2014). H_2O_2 can serve as a signaling molecule at low concentrations as well as a damage agent at higher concentrations and thus has a complex cellular role. Moreover, hypochlorous acid (HOCl) generated in the presence of H_2O_2 can further lead to formation of more toxic and damaging ROS such as hydroxyl radicals ($\bullet$OH) (Boukhenouna *et al.,* 2018).

In COPD, the search for circulating blood biomarkers has mainly focused on plasma or serum proteins which are known to be involved in the systemic inflammatory response (Ongay *et al,* 2016). Several plasma biomarkers have been measured and found to differentiate COPD patients from healthy subjects. In the current study, the basal systemic levels of selected biomarkers; CRP, Fibrinogen, CC16, CD31, CCL18, IL-6, IL-8, TNFα, IP10, SP-D and RAGE, were measured and investigated for their potential utility as an inflammatory biomarkers in COPD patients and compared with their levels in healthy smokers and healthy subjects. This could facilitate early diagnosis, monitor disease progression, and evaluate treatment outcome.

4.2   Objectives

- Investigate the levels of systemic blood biomarkers in plasma samples. Data from COPD patients, healthy smokers and healthy controls will be compared.
- Study the association between the level of systemic blood biomarkers in COPD patients and disease severity.

4.3   Method

4.3.1   Prioritized COPD Protein Biomarkers

Numerous observational cohort studies have investigated different inflammatory biomarkers candidates that were selected according to their studies proposed criteria to investigate the effect of systemic inflammation, and its association with comorbidities in patients with COPD. A systematic study of previous literature and clinical studies by Ongay *et al.* (2016) provided a panel of biomarker candidates that have the potential of becoming valid and clinically useful COPD biomarkers in the near future. Based on the Ongay review and previous published work (Pinto-Plata *et al.,* 2007; Pinto-Plata *et al* 2012; Faner *et al.,* 2013) and taking account of potential biological mechanisms believed to be relevant in the pathogenesis of COPD, a panel of 11 biomarkers were selected that previously showed some association with COPD and/or showed statistically significant differences between COPD patients and healthy controls. They were grouped as highly abundant acute phase reactants creative protein: CRP and Fibrinogen;

inflammatory biomarkers: IL-6, and TNF-α; chemoattractant biomarkers: IL-8, CCL18 and IP10; and pulmonary regulated protein CC16, SP-D, RAGE and MP-CD31 (Table 4.1). The list of potential protein biomarkers was further reduced and refined by selecting only those that show activity in the circulation of all the studied groups. This follows the recommendation to demonstrate a strong, consistent and independent association between a biomarker and COPD. In this way, 5 potential proteins remained for the final biomarker panel; fibrinogen, CRP, CCL18, CC16 and CD31.

Table 4.1: Biomarkers investigated as potential COPD biomarkers within the study cohort.

Biomarker	Abbreviation	Proposed pathophysiological role and mediator effects	Sources	Reference
Tumor necrosis factor	TNF-α	Proinflammatory cytokine Activates epithelium, endothelium, antigen-presenting-cells Activates monocytes/macrophages	Macrophages T-cells Airway Epithelial cells	Kawayama *et al.,* 2016
Interleukin-8	IL -8	Inflammatory chemokine Chemoattractant and activator for monocytes/macrophages and neutrophils	Monocytes/ macrophages Pulmonary epithelium Airways fibroblasts & endothelial cells	Chung *et al.,* 2001
Interleukin-6	IL -6	Inflammatory cytokine Monocytes/macrophages T-cell growth factor Hematopoiesis	Monocytes/ macrophages Airway epithelium	Ferrari *et al.,* 2010 El-Shimy *et al.,* 2014
C-reactive protein	CRP	Acute phase reactants	Hepatocytes /Liver	Heidari 2012

Fibrinogen	NA	Blood coagulation factor	Hepatocyte/Liver Megakaryocyte/ bone marrow	Duvoix *et al.,* 2013 Fattouh &Alkady, 2014
Soluble receptor for advanced glycation endproducts	sRAGE	Pro-inflammatory pattern recognition receptor of cell surface Glycation of serum lipids and proteins of endothelial and epithelial cells	Alveolar type-I-II epithelial cells/Bronchial smooth muscle cells Vascular endothelial cells/Alveolar macrophages	Wu *et al.,* 2011 Cheng *et al.,* 2013 Gopal *et al.,* 2014 Sambamurthy *et al.,* 2015
Surfactant protein D	SP-D	Pulmonary protein involved in innate immunity Decreases the expression of pro-inflammatory cytokines Increases the resolution of lung inflammation Promotes phagocytosis of apoptotic cells	Alveolar type II Pneumocytes Non-ciliated club cells/bronchioles	Madsen *et al.,* 2000 Sin *et al.,* 2008 Lomas *et al.,* 2009

Club cell protein 16	CC16	Lung inflammatory protein anti-inflammatory and detoxification properties Inhibit cells inflammation, migration and invasion	Clara cells/ bronchioles Epithelial cell/small airways	Pilette *et al.,* 2001 Ongay *et al.,* 2016
C-C motif chemokine 18	CCL18/ PARC	Lung-predominant inflammatory protein. Pulmonary and activation-regulated chemokine for naive T-cells, T-Regulatory cells, Th2 cells, dendritic cells and B-cells.	Dendritic cells Monocytes/ macrophage (M2)/lung	Sin *et al.,* 2011 Plönes *et al.,* 2012 Dilektasli *et al.,* 2017
Interferon gamma-induced protein 10/ C-X-C motif chemokine 10	IP10/CXCL-10	Inflammatory chemokine Chemoattractant for monocytes, Th1, Th2 lymphocytes and NK cells	Monocytes Lymphocytes Neutrophils Bronchial epithelial cells/lung	Torvinen *et al.,* 2007 Quint *et al.,* 2010
Cluster of differentiation 31/ Platelet endothelial cell adhesion molecule	CD31/PECAM	Small membrane vesicles /Immunoglobulin Trans-endothelial migration of leukocytes Endothelial cell permeability Angiogenesis/Thrombosis	Endothelial cells/lung Platelets/ B-cells Macrophages T-cells /NK cells.	Falati *et al.,* 2006 Woodfin *et al.,* 2007

4.3.2   ELISA for R&D Systems-based assays

4.3.2.1   *Reagent and Standards Preparation*

All ELISA kits were purchased from R&D Systems, except a fibrinogen kit was bought from Abcam. Details of the commercial kits composition; standard, capture, detection antibodies stock and working solution concentration, standard sensitive range, enzyme substrate reagents dilution are summarised in table 4.2 and 4.3. Anti-human capture antibody was reconstituted in 0.5 ml of Dulbecco's Phosphate Buffered Saline, (PBS), (sterile, filtered, w/o Calcium w/o Magnesium, Biosera, Labtech International Ltd, Sussex, UK and further dilution was made in PBS, to the working concentration. Anti-human detection antibody was reconstituted in 1.0 ml of reagent diluent 1% BSA, sterile, filtered in PBS, and further dilution was made in reagent diluent to the desire working concentration. For standard preparation; each standard vial was reconstituted with 0.5 mL of deionized or PBS according to manufacture instructions (R&D Systems). After initial reconstitution 1ml of high standard was prepared per plate assayed at the concentration indicated. A seven point standard curve using 2-fold serial dilutions in reagent diluent was prepared. Working dilutions should be prepared on experiment day and used immediately.

Table 4.2: Summary of ELISA assay stock and working solution concentration of capture and detection antibodies for individual biomarker.

Biomarker	Capture Antibody	Detection Antibody	Capture Antibody concentration		Detection Antibody concentration	
			Stock	Working solution	Stock	Working solution
Human IL-6 DY206	Mouse Anti-Human IL-6 Antibody	Biotinylated Goat Anti-Human IL-6 Antibody	240μg/ml	2μg/ml	3μg/ml	50ng/ml
Human IL-8/ CXCL8 DY208	Mouse Anti-Human IL-8 Capture Antibody	Biotinylated Goat Anti-Human IL-8 Detection Antibody	240μg	4μg/ml	1.2μg/ml	20ng/ml
CRP DY1707	Mouse Anti-Human CRP Capture Antibody	Biotinylated Mouse Anti-Human CRP Detection Antibody	360μg	2μg/ml	16.2μg/ml	90ng/ml
Human TNF-α DY210	Mouse Anti-Human TNF-α Antibody	Biotinylated Goat Anti-Human TNF-α Antibody	480 μg/ml	4μg/ml	24μg/ml	400ng/ml

Human CC16/Uteroglobin DY4218	Rat Anti-Human Uteroglobin Capture Antibody	Biotinylated Rat Anti-Human Uteroglobin Detection Antibody	360µg/ml	2µg/ml	360µg/ml	500ng/ml
Human CD31/ PECAM-1 DY8064-05	Mouse Anti-Human CD31 Capture Antibody	Biotinylated Sheep Anti-Human CD31 Detection Antibody	480µg/ml	4µg/ml	12µg/ml	200ng/ml
Human CCL18/PARC DY394-05	Mouse Anti-Human PARC Capture Antibody	Biotinylated Goat Anti-Human PARC Detection Antibody	360µg	2µg/ml	18µg/ml	100ng/ml
Human CXCL10/ IP-10 DY266-05	Mouse Anti-Human IP-10 Capture Antibody	Biotinylated Goat Anti-Human IP-10 Detection Antibody	240µg/ml	2µg/ml	0.750 µg/ml	12.5 ng/ml

Human SP-D DY1920	Mouse Anti-Human SP-D Capture Antibody	Biotinylated Mouse Anti-Human SP-D Detection Antibody	360μg	2μg/ml	90μg/ml	500ng/ml
Human RAGE DY1145	Mouse Anti-Human RAGE Capture Antibody	Biotinylated Goat Anti-Human RAGE Detection Antibody	180μg	1μg/ml	36μg/ml	100ng/ml

Table 4.3: Summary of ELISA assay standard stock concentration, sensitivity, and enzyme substrate dilution for each biomarker.

Biomarker	Standard Stock concentration	High standard limit	Lower standard limit	Streptavidin-HRP Dilution
Human IL-6	90 ng	600 pg/mL	9.38pg/mL	1:40
Human IL8/ CXCL8	40 ng	2000pg/mL	31.3pg/mL	1:40
CRP	50 ng	1000 pg/mL	15.6 pg/mL	1:200
Human TNF-α	67.5 ng	1000 pg/mL	15.6 pg/mL	1:40
Human CC16	75 ng	2000pg/mL	31.3pg/mL	1:200
Human CD31/ PECAM-1	190 ng	10,000 pg/mL	156 pg/mL	1:40

Human CCL18/PARC	65 ng	500 pg/mL	7.81 pg/mL	1:200
Human CXCL10/ IP-10	55 ng	2000 pg/mL	31.3pg/mL	1:40
Human SP-D	75 ng	10,000 pg/mL	156 pg/mL	1:200
Human RAGE	25 ng	4000 pg/mL	62.5 pg/mL	1:200
Fibrinogen	200ng/ml	100 ng/mL	1.56 ng/mL	NA

4.3.2.2   Assays procedures

The procedure for ELISA R&D Systems-based assays was previously mentioned in details (see section 2.1.4.4).

4.3.2.3   Plasma Dilution selection

Measuring biomarkers in plasma is a very useful parameter that gives an indication of specific diseases progression or diagnosis. However human plasma is a complex matrix contain heterophilic antibodies and may require a dilution in an appropriate buffer to overcome matrix effects, and to prevent heterophilic antibodies from binding to the capture and detection antibodies used in capture ELISA, causing false positive results. Plasma sample diluent utilised as a buffer matrix for diluting out plasma samples in antigen-capture ELISA formats, and for diluting standards in ELISA standard curve preparation. Once samples are diluted, the dilution factor should be included when calculating results. Dilution of the sample before performing the actual experiment is significant parameter for determining the sensitivity of ELISA in human samples. It assess in deciding the amount of actual concentration required from the sample to obtain a result within the range of reference standard protein. Without dilution, it is difficult to achieve highly sensitive results of protein concentration. Therefore, optimum dilution for the sample of target protein is a vital parameter for obtaining better results (Thakur *et al.,* 2015).

In the current study the plasma sample diluent was a buffered BSA protein base which provides an assay environment that minimizes nonspecific binding while maintaining proper ionic strength and pH for efficient antibody-antigen interaction. The optimization of plasma samples dilution was carried out for each biomarker by using PBS supplemented with 1% BSA, to dilute plasma samples. Two plasma samples were selected from each of three studied groups; COPD, healthy smoker and healthy nonsmoker groups, to be 10-fold diluted, 1:10, 1:100, 1:1000, and 1:10000, and then assayed in 96-well plate alongside undiluted samples. ELISA assay was carried out to detect selected biomarker level in diluted and undiluted plasma samples. A 96-well plate was coated with 100 µl of the diluted anti-human capture antibody per well. The plate was sealed (Greiner Bio-One GmbH, Ltd, Stonehouse, Germany) and incubated overnight at room temperature. After the incubation, the 96-well plate was washed twice with wash buffer (0.05% Tween®20 in PBS, pH 7.2-7.4) using auto washer equipment (Mikura, West Sussex,

UK). After each wash step any remaining wash buffer was removed by blotting the plate against clean paper towels. The plate was blocked by adding 300μl of reagent diluent, 1% BSA in PBS, pH 7.2-7.4, 0.2 μm filtered, to each well, and was incubated at room temperature for a minimum of 1 hour. The wash step was repeated and 100μl of sample or standards was added per well as duplicate. The plate was sealed with an adhesive strip and incubated 2 hours at room temperature. The wash step was repeated and 100μL of the diluted detection antibody was added to each well. The plate was sealed with an adhesive strip and incubated 2 hours at room temperature. The wash step was repeated and 100μl of the working dilution of Streptavidin-HRP (streptavidin conjugated to horseradish-peroxidase, 1:40) (R&D Systems, Catalog #DY 893975) was added to each well. The plate was covered and placed away from direct light for 20 minutes at room temperature. The wash step was repeated and 100μl of substrate solution, a mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine) (1:1, R&D Systems, Catalog # DY999) was added to each well and plate was incubated for 20 minutes at room temperature away from direct light, then 50μl of stop solution (2N H_2SO_4) (R&D Systems, Catalog # DY994) was added to each well. The optical density of each well was determined using a microplate reader (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific, UK) (see appendix B for software user manual guide), set to 450 nm with a wave length correction at 570nm. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. The concentration of biomarkers in sample was determined by interpolating the blank control subtracted absorbance values against the standard curve. Resulting value was multiplied by the appropriate sample dilution factor to obtain the concentration of biomarker in the sample. Three experiments were performed and standard error was determined from the standard deviation derived from the means of total sample number. Then the appropriate dilution factor of each biomarker was used to dilute the plasma sample when assay the entire study subject. Based on obtained values undiluted concentration of plasma samples were used when dilution not required.

4.3.3 Human Fibrinogen ELISA Assay

The human fibrinogen kit assay (ab208036, Abcam, Cambridge, UK) is based on applying an affinity tag labeled capture antibody and a reporter conjugated detection antibody which immuno-capture the sample target protein in solution. This entire complex of (capture antibody- target protein-detection antibody) is in turn immobilized via immuno-affinity of an anti-tag antibody coating the well (Figure 4.1).
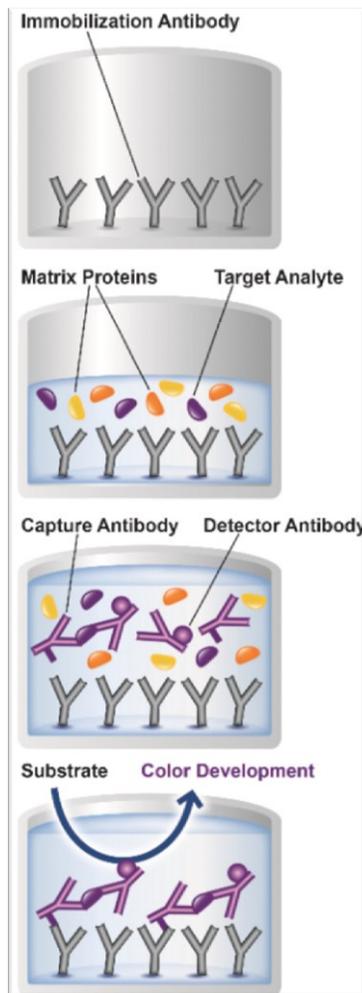
Figure 4.1: Summary of human fibrinogen simple step ELISA assay (Abcam, Cambridge, UK).

4.3.3.1 Reagent and Standard Preparation

1× Wash Buffer PT (ab206977), Proclin, was prepared by diluting 10× wash buffer PT with deionized water. Antibody mixture was prepared by diluting the capture and detector antibodies in antibody diluent CPI, (ab193969), (EDTA disodium salt, Proclin, Alizarin Red S). A total of 6 ml of the Antibody mixture was prepared by mixing 600μl 10× capture Antibody and 600μL 10× detector. A stock standard solution of 200ng/mL was prepared by reconstitute Fibrinogen human protein standard in 250μl water. The solution was mixed thoroughly and gently, and then was hold at room temperature for 10 minutes. This is the 200 ng/ml stock standard solution. A serial dilution was prepared by adding 150μl sample diluent NS, (ab193972, EDTA disodium salt and Proclin), into 8 tubes. A 150μl from the Stock Standard Solution of 200 ng/ml, (ab208036-CP164706), was added to tube number one to get100 ng/ml and the following dilution series was prepared 50, 25, 12.5, 6.25, 3.13, 1.56 ng/ml. Standard number 8 contained no protein and is considered as a Blank control. Plasma samples were diluted (1:100,000) in 1× wash buffer.

4.3.3.2 Assay Procedure

All reagents, working standards, and samples were prepared at room temperature prior to use. Fifty microliters of samples or standards was added to appropriate wells. Fifty microliters of the Antibody Cocktail was added to each well. Then the plate was sealed and incubated for 1 hour at room temperature on a plate shaker set to 400 rpm. After the incubation period each well with was washed three time with 350 μl 1× Wash Buffer PT to remove unbound material. Removal of liquid at each step is essential for good performance. After the last wash the plate was inverted and blotted against clean paper towels to remove excess liquid. Hundred microliters of TMB substrate, Hydrogen peroxide and TMB, was added to each well and the plate was incubated for 10 minutes in the dark on a plate shaker set to 400 rpm. TMB substrate was catalyzed by HRP, generating blue coloration. Then the reaction was stopped by addition of 100 μl of Stop Solution (ab208036-CP164601), phosphoric acid, to each well and was shaken on a plate shaker for 1 minute to mix, completing any color change from blue to yellow. Signal was generated proportionally to the amount of bound Fibrinogen and the optical density was measured at 450 nm was record by microplate reader. The average absorbance value for the blank control standard absorbance was calculated and subtracted from all other absorbance

values. A standard curve was created by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. A four parameter curve fit (4PL) was used to plot these values and fit a curve to the data. The concentration of the fibrinogen in the plasma sample was determined by interpolating the blank control subtracted absorbance values against the standard curve. Resulting value was multiplied by the appropriate sample dilution factor to obtain the concentration of fibrinogen in the sample.

4.3.4   Statistical Analysis

The data were expressed and graphed as mean (standard deviation; SD). The D'Agostino–Pearson omnibus normality test was performed to test normality of the data distribution. For data normally distributed one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test was performed to determine whether the differences between the groups were statistically significant. Differences were considered significant if the probability (P) value was <0.05, highly significant if P value was <0.01 and P value <0.001, and insignificant if P value was >0.05. Correlation analyses of two variables were carried out using Pearson methods, depending on the normality of the data distribution. All statistical analyses were performed using graphpad prism and InStat software, version 7.04 and 3.10.32 respectively.

4.4   Results

4.4.1   Plasma Dilution selection

The assay was carried out to estimate the proper dilution factor for plasma sample before measuring our biomarker candidates, IL-6, IL-8, TNF-α, IP-10, fibrinogen, CRP, CC-16, CD31, RAGE and CCL18, in the study cohort. Results indicated that undiluted plasma was the best option for the following biomarkers; IL-6, IL-8, TNF-α, IP-10, SP-D and RAGE. A dilution of 1:10 was suitable for CC16 and CD31, whilst 1:100 was the optimum for CCL18. A higher dilution was required for CRP of 1:10,000. For Fibrinogen the optimum dilution factor was established as 1:100,000 following supplier's recommendations. All these results are summarized in Figure 4.2.
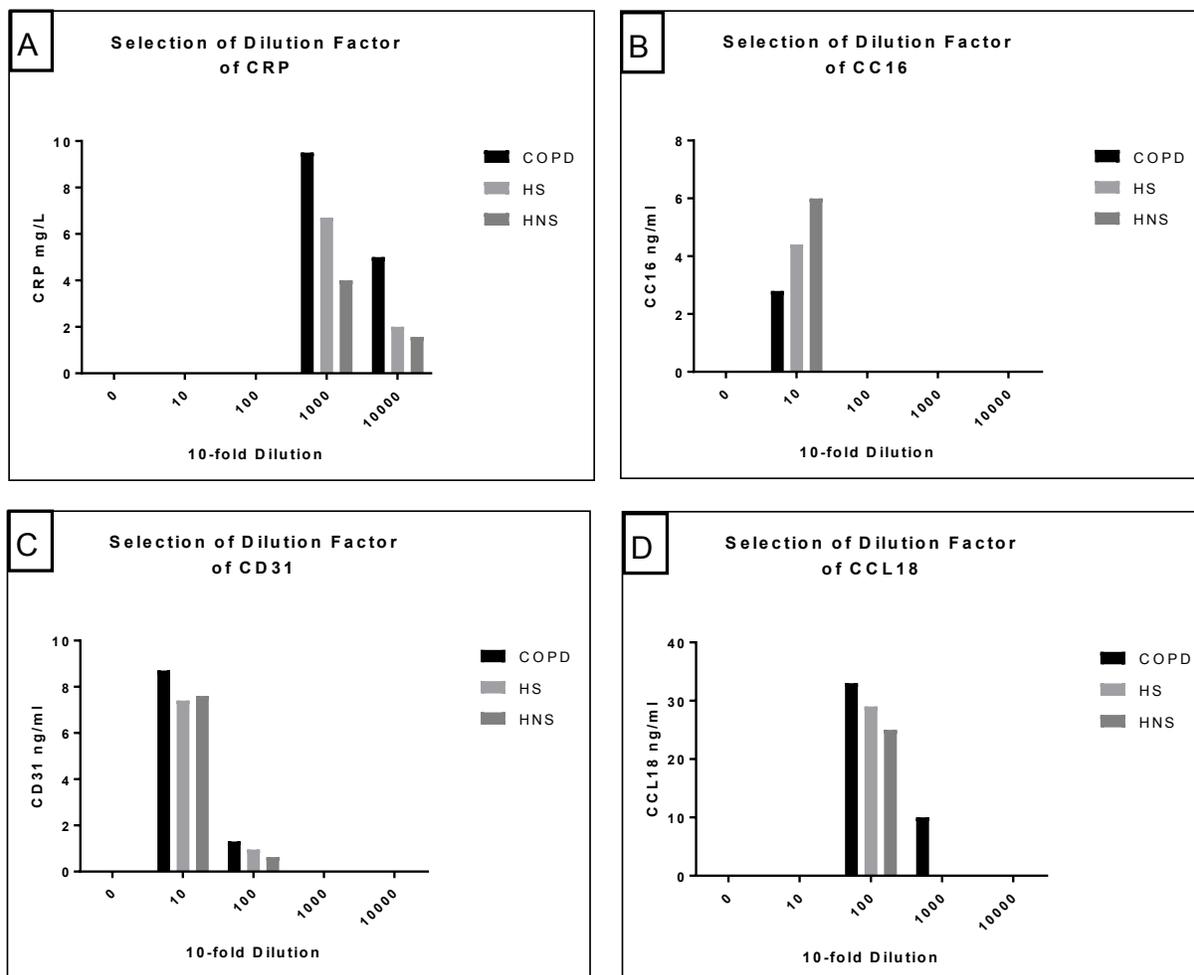
Figure 4.2: Selection of dilution factor for biomarkers in plasma samples. *The figure shows the optimum dilution factor using 10-fold dilution of plasma for each biomarker; CRP (A), CC16 (B), CD31 (C), and CCL18 (D), in COPD patients, healthy smokers and healthy controls (n=6). These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; CRP, creative reactive protein; CC16, clara cells 16; CCL18, Chemokine (C-C motif) ligand 18; CD31, cluster of differentiation 31.*

4.4.2 Prioritized COPD Protein Biomarkers

Only five biomarkers from the study candidates list showed activity in the circulation of the entire study cohort including; fibrinogen, CRP, CCL18, CC16 and CD31. In contrast, other biomarkers; IL6, IL8, TNFα, IP10, SP-D and RAGE, showed no activity in circulation of most subjects of the study groups. For example; RAGE was detectable in 26% of the entire subjects of the study group, and mostly were healthy nonsmokers. IP10 was detectable in 16% of the studied subjects whereas IL-6, TNFα, and SP-D showed activity in circulation of only 10% from the total studied subjects. However IL-8 showed no activity at all in three studied groups. There was no relation between patients' disease stage/FEV1 and the undetectable level of biomarkers. Summary of the results can be seen in Figure 4.3
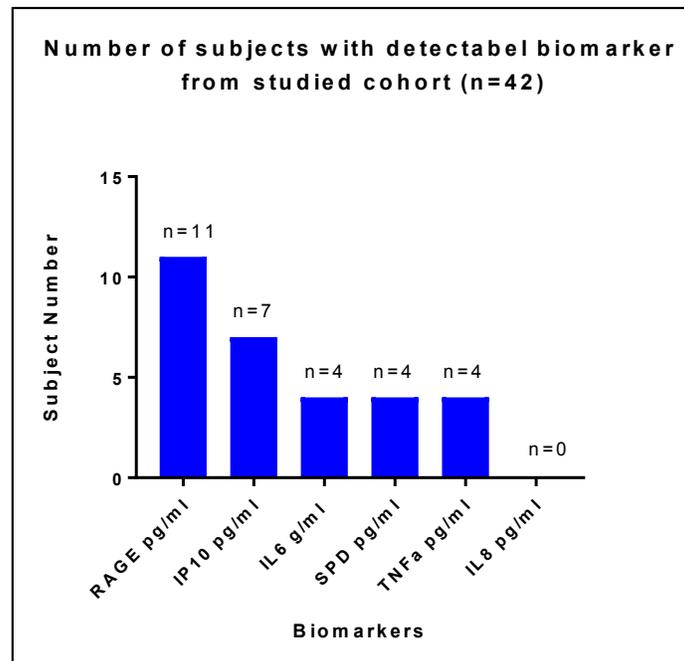
Figure 4.3: Number of subjects with detectable biomarkers compared to the entire cohort. *These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: IL6; Interleukin-6, IL8; Interleukin-8, TNFα; Tumor necrosis factor, IP-10; Interferon gamma-induced protein 10, SP-D; Surfactant protein D, and RAGE; Soluble receptor for advanced glycation end products.*

4.4.3   Measuring the level of inflammatory biomarkers in plasma samples

The plasma level of the biomarker candidates; fibrinogen, CRP, CC16, CCL18 and CD31, was measured in patients with COPD, healthy smoker and healthy nonsmokers. The mean value and SD of plasma level of CRP was significantly higher in patients with COPD (4.48 ± 28 mg/l) when compared to those of healthy nonsmoking individuals (2.27 ± 2 mg/l) ($p =0.007$). Similarly, the mean value and SD of CRP in COPD patient was higher than those in healthy smokers ($p =0.027$). Mean value and SD of total circulating fibrinogen was significantly higher in COPD patients (409 ± 181mg/dl) when comparing with healthy smokers (136 ± 66 mg/dl) ($p <0.0001$) and healthy nonsmoking controls (137 ± 49 mg/dl) ($p<0.0001$). In contrast, the mean value and SD of CC16 was significantly lower in plasma of COPD group (2.75 ± 1.19 ng/ml) compared to healthy nonsmoking group (5.77 ± 2.66 ng/ml) ($p =0.014$). The mean value and SD of CC16 in COPD patient was also lower than those in healthy smokers but differences did not reach statistical significance *(*p >0.05). However, no significant differences correlations among COPD patients, healthy smokers and healthy nonsmokers groups were observed in plasma level of CD31 (8.7, 7.4, and 7.6 ng/ml respectively) ($p > 0.05$) and CCL18 (33, 29, and 27 ng/mL respectively) ($p > 0.05$). All these results are summarized in Table 4.4 and Figures 4.4

Table 4.4: Mean value and significance of CRP, Fibrinogen, CC16, CCL18 and CD31 in the plasma of the study cohort

Biomarkers	COPD	HS	HNS	P value COPD vs HS	P value COPD vs HNS	P value HS vs HNS
CRP mg/L	4.5 (2.8)	2.4 (1.7)	1.8 (1.5)	0.027	0.007	>0.05
Fibrinogen mg/dL	409 (181)	136 (66)	137 (49)	<0.0001	<0.0001	>0.05
CC16 ng/ml	2.75 (1.2)	4.36 (3.5)	5.77 (2.7)	>0.05	0.014	>0.05
CCL18 ng/ml	33 (10)	29 (9.5)	27 (8.2)	>0.05	>0.05	>0.05
CD31 ng/ml	8.7 (6)	7.4 (3)	7.6 (6.2)	>0.05	>0.05	>0.05

Data expressed as mean (standard deviation) unless otherwise stated. The data represents the mean ± SD from three experiments. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; CRP, creative reactive protein; CC16, clara cells 16; CCL18, Chemokine (C-C motif) ligand 18; CD31, cluster of differentiation 31.
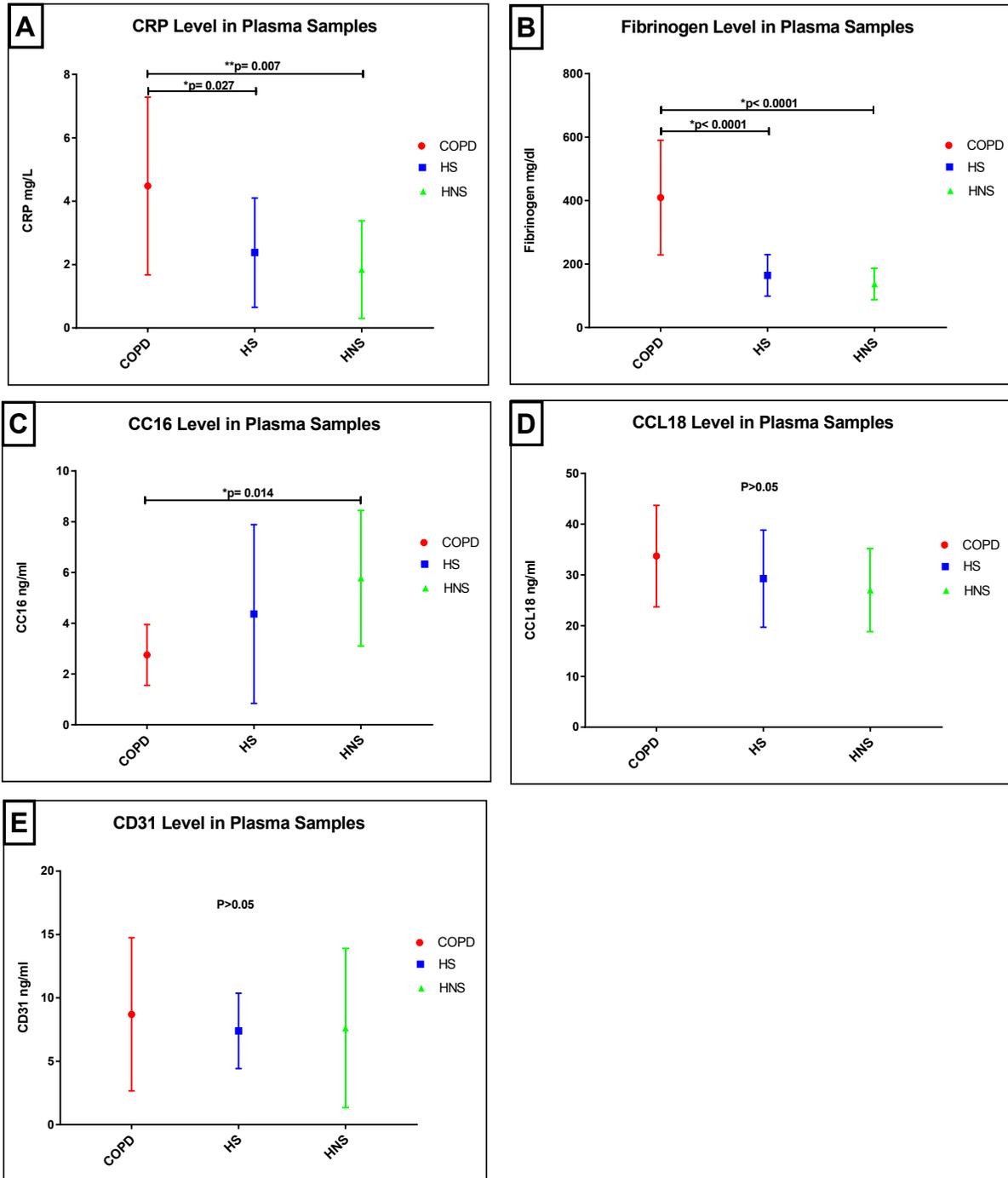
Figure 4.4: The plasma level of biomarker candidates in the studied groups. *(A) CRP (mg/l), (B) Fibrinogen (mg/dl), (C) CC16 (ng/ml), (D) CCL18 (ng/ml), and (E) CD31 (ng/ml), in plasma of COPD patients, HS and HNS. Cytokines were measured by ELISA assay (see section 2.1.4.4). P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; CRP, creative reactive protein; CC16, clara cells 16; CCL18, Chemokine (C-C motif) ligand 18; CD31, cluster of differentiation 31.*

4.4.4 The Correlation between Inflammatory Biomarkers and Disease Severity

In the current study it was shown that increased plasma level of fibrinogen, CRP, CC16, CCL18 and CD31 was positively correlate with worsen disease stage. COPD Patients in mild stage have shown lower level of circulating biomarkers that those in moderate or severe stages. A possible correlation between biomarkers plasma levels and clinical lung function data (FEV1 %predicted, preFEV1/FVC ratio) was investigated. There was a statistically significant negative correlation between the levels of CRP and the preFEV1/FVC ratio (r^2 =0.287, P = 0.039), but a non-significant correlation between the levels of CRP and the % predicted FEV1 (r^2 = 0.179, P =0.11). A moderate negative association was found between the levels of CC16 and the % predicted FEV1 (r^2 = 0.255, P =0.054). However, no association was observed between the levels of CC16 and the pre FEV1/FVC ratio (r^2 = 0.173, P =0.122). Similarly, plasma Fibrinogen, CCL18 and CD31 levels did not show correlation with the FEV1 %predicted and pre FEV1/FVC (p>0.05). So, CRP was the only inflammatory biomarker tested associated with disease severity and can be used as a predictor of lung function decline. Linear regression analysis of the relationship between inflammatory markers and COPD severity at baseline are summarised in Table 4.5 and Figure 4.5.

Table 4.5: Correlation between Plasma Biomarkers and Pulmonary Functions in COPD Cohort

Biomarkers	preFEV1 %		preFEV1/FVC ratio	
	r^2	p	r^2	p
CRP mg/L	0.179	0.115	0.287	0.039
Fibrinogen mg/dl	0.017	0.635	0.034	0.500
CC16 ng/ml	0.255	0.054	0.173	0.122
CCL18 ng/ml	0.060	0.377	0.092	0.272
CD31 ng/ml	0.009	0.913	0.005	0.934

Significance was tested using a linear regression analysis (spearman method). These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: CRP, creative reactive protein; CC16, clara cells 16; CCL18, Chemokine (C-C motif) ligand 18; CD31, cluster of differentiation 31. FEV1%, percent predicted forced expiratory volume in the first second, FVC, forced vital capacity.

A-1 Plasma CRP vs PreFEV1%
P=0.11
R^2=0.18

A-2 Plasma CRP vs Pre FEV1/FVC ratio
*P=0.039
R^2=0.28

B-1 Plasma Fibrinogen vs PreFEV1%
P=0.63
R^2=0.017

B-2 Plasma Fibrinogen vs Pre FEV1/FVC ratio
P=0.5
R^2=0.034

C-1 Plasma CC16 vs PreFEV1%
P=0.054
R^2=0.255

C-2 Plasma CC16 vs Pre FEV1/FVC ratio
P=0.122
R^2=0.173
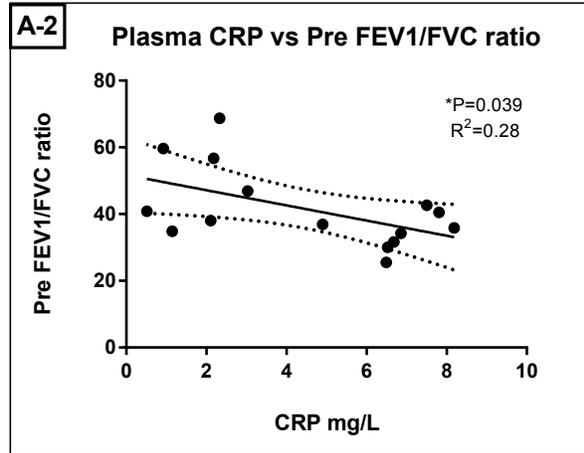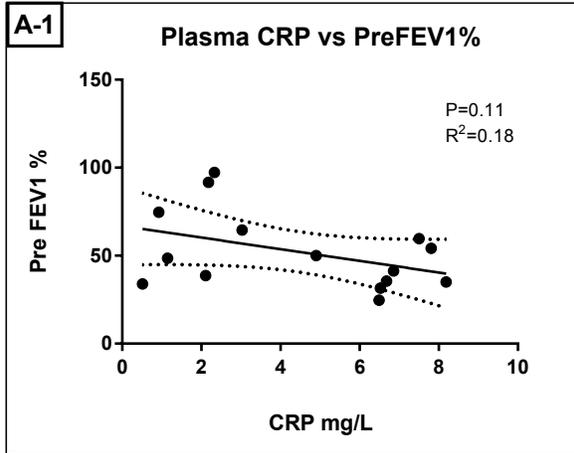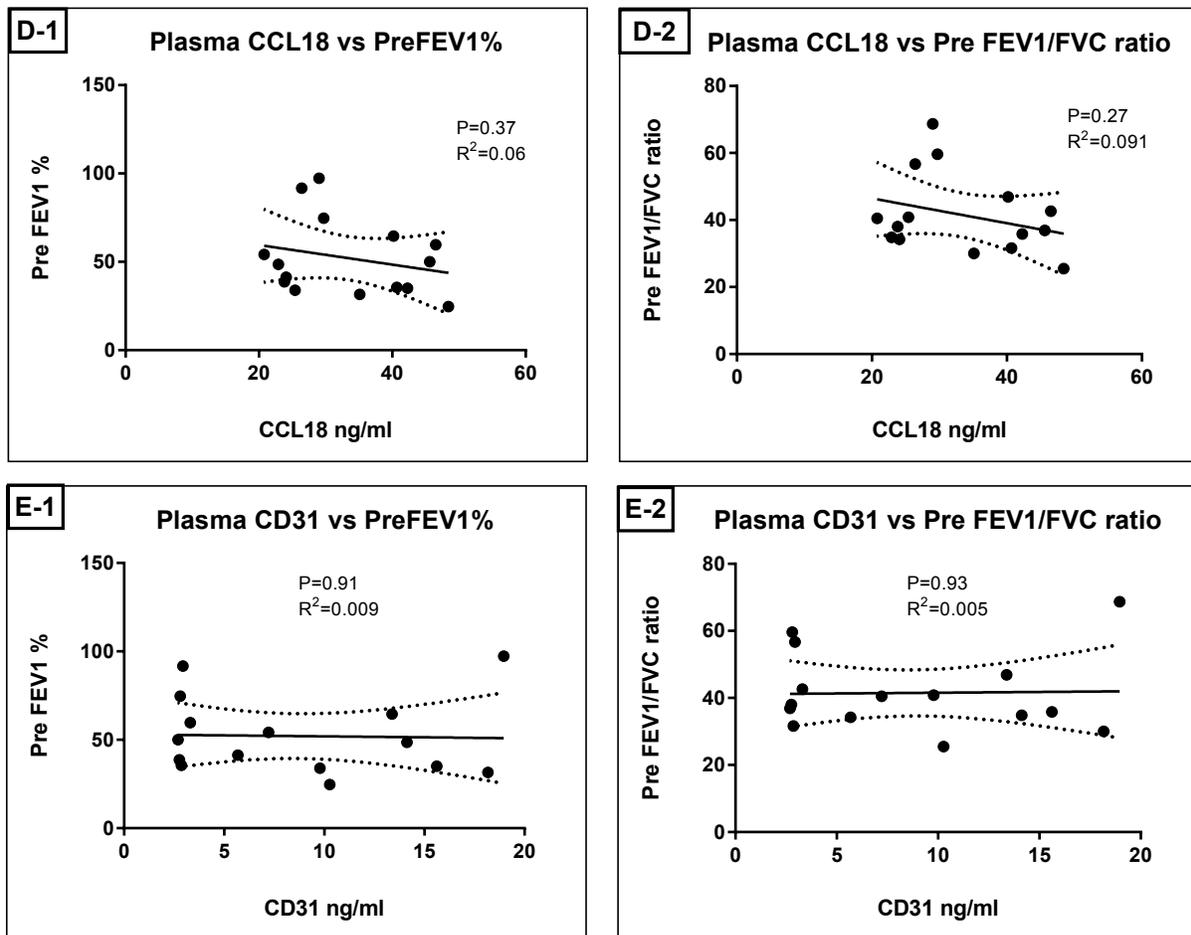
Figure 4.5: Linear regression analysis of the correlation between lung severity and plasma biomarker levels. *FEV1 % and FEV1/FVC ratio vs basal plasma level of (A1-2) CRP (mg/L), (B1-2) Fibrinogen (mg/dl), (C1-2), CC-16 (ng/ml), (D1-2) CCL-18 (ng/ml), (E1-2) CD31 (ng/ml). Significance was tested using a linear regression analysis (spearman method). Dotted lines indicate the 95 % confidence intervals. These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; CRP, creative reactive protein; CC16, clara cells 16; CCL18, Chemokin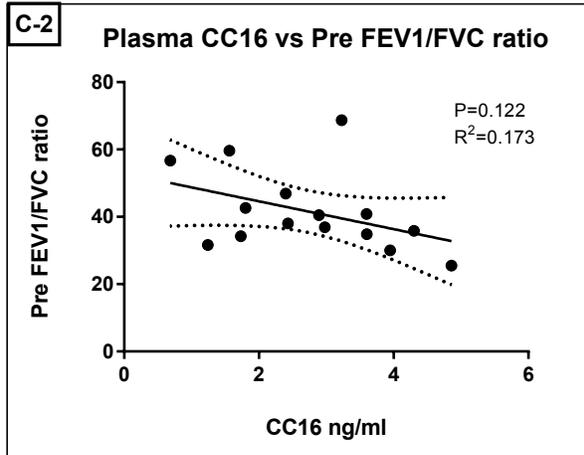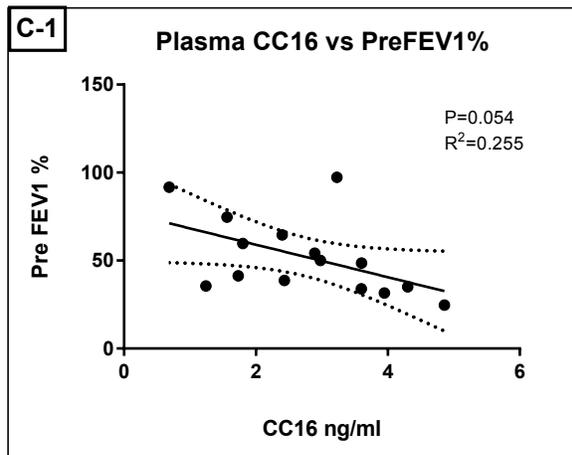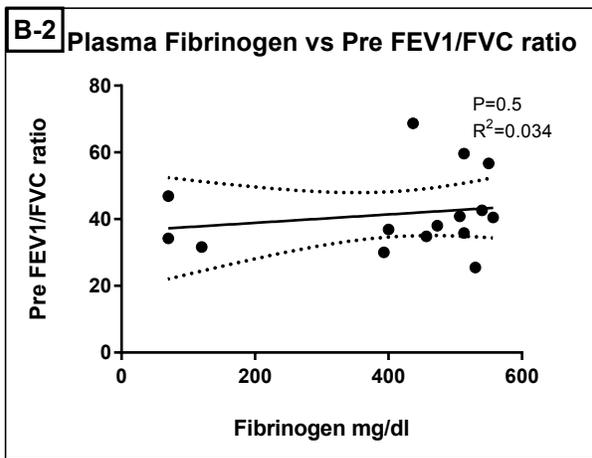e (C-C motif) ligand 18; CD31, cluster of differentiation 31; FEV1%, percent predicted forced expiratory volume in the first second, FVC, forced vital capacity.*

4.4.5   Influence of Smoking Status on Inflammatory Biomarkers

The smoking impact, quantified as PYH on biomarkers plasma levels was investigated. There was a significant negative correlation between the plasma CRP level and PYH (r^2 =0.283, P = 0.041). No correlation was found between the Plasma Fibrinogen and PYH (r^2 =0.063, P = 0.36). Similarly, plasma CC16, CCL18 and CD31 levels did not show correlation with PYH (p>0.05). In subgroup analysis of patients with COPD, no differences in biomarkers level were found between smokers and ex-smokers except with CRP. The plasma level of CRP was found to be significantly higher in COPD former smokers as compared to current smokers (6.3 vs 3.2 mg/L, p= 0.049). In summary, smoking affects the plasma level of CRP of COPD patients. Linear regression analysis of the relationship between smoking impact and inflammatory markers at baseline of COPD patients are summarised in Table 4.6 and Figure 4.6.

Table 4.6: The impact of smoking on plasma biomarkers; CRP, fibrinogen, CC16, CCL18, and CD31 in COPD cohort.

Biomarkers	PYH	
	r^2	p
CRP mg/L	0.283	0.041
Fibrinogen mg/dl	0.063	0.360
CC16 ng/ml	0.005	0.790
CCL18 ng/ml	0.066	0.350
CD31 ng/ml	0.177	0.118

Significance was tested using a linear regression analysis (spearman method) using Graph Pad Instat Software. Definition of abbreviations: CRP, creative reactive protein; CC16, clara cells 16; CCL-18, Chemokine (C-C motif) ligand 18; CD31, cluster of differentiation 31; PYH, pack year history.
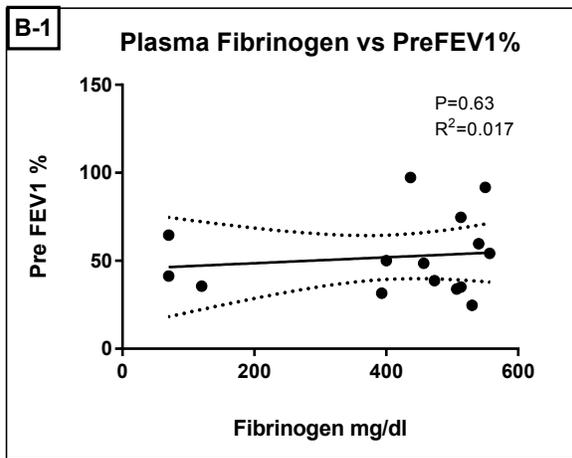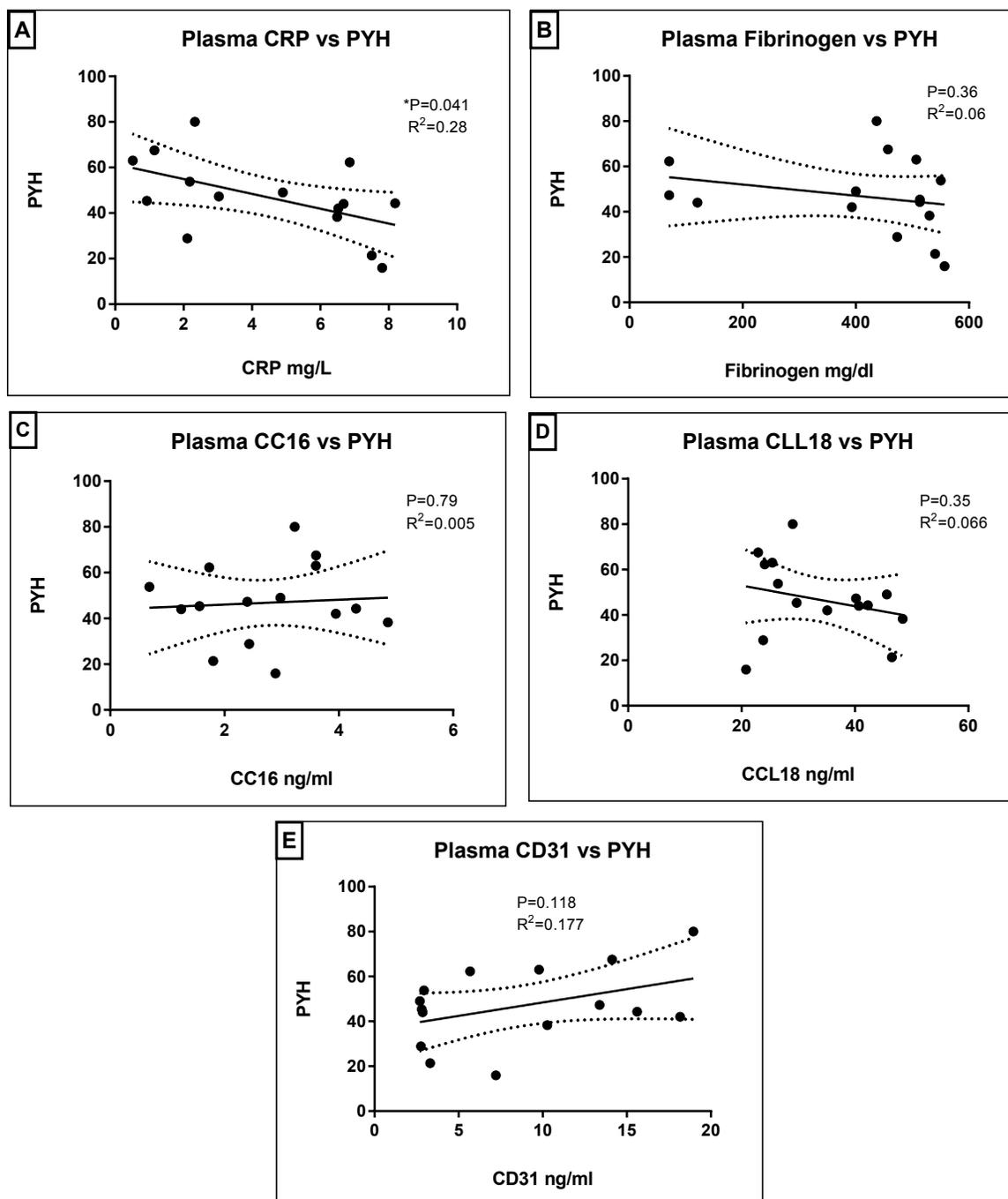
Figure 4.6: Linear regression analysis of the correlation between smoking and plasma level of biomarkers. *(A) CRP (mg/L), (B) Fibrinogen (mg/dl), (C) CC-16 (ng/ml), (D) CCL-18 (ng/ml), and (E) CD31(ng/ml). Significance was tested using a linear regression analysis (spearman method). Dotted lines indicate the 95 % confidence intervals. These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; CRP, creative reactive protein; CC16, clara cells 16; CCL18, Chemokine (C-C motif) ligand 18; CD31, cluster of differentiation 31; pack year history. FEV1%, percent predicted forced expiratory volume in the first second, FVC, forced vital capacity.*

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4.4.6   Relationship between Plasma Inflammatory Biomarkers Levels

Plasma levels of CRP and fibrinogen were determined as a measure of systemic inflammation. In order to explore the relationship between systemic biomarkers and inflammation, correlations were performed between these inflammatory biomarkers. No correlation was found between plasma levels CRP and Fibrinogen (r^2 = 0.181, p=0.63) (Figure 4.7).
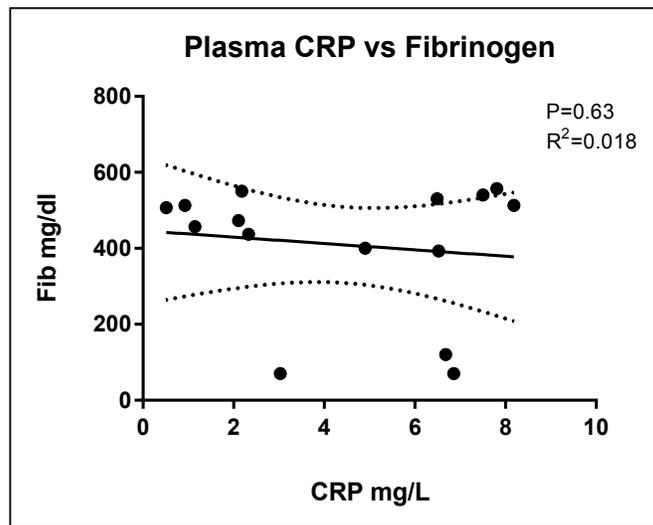
Figure 4.7: Linear regression analysis of the Correlation between CRP (mg/L) and Fibrinogen (mg/dl). *Significance was tested using a linear regression analysis (spearman method). Dotted lines indicate the 95 % confidence intervals. These analyses were performed using Graph Pad Instat Software. Abbreviation: CRP, creative reactive protein.*

4.5 Discussion

COPD is a chronic inflammatory disease that affects primarily lung and airway with systemic manifestations and exhibits significant heterogeneity. The mechanism of heterogeneity in COPD is complex, as several studies observed variations among COPD individual parameters including the clinical manifestations, frequency of acute exacerbation, disease progression and response to treatment (Sin *et al.,* 2009). Studies have been focused on COPD relevant biomarkers as these may associate with the different inflammatory phenotypes of the disease and may help to evaluate the individualized management of patients with COPD. Biomarkers can assess in evaluation of biological processes and disease pathology, the prediction of progression or response to treatment. However many published studies have produced also controversial results (Liu *et al.,* 2014).

The inflammation in COPD patients appeared to extend beyond the lungs causing systemic inflammation and COPD associated comorbidities. The biomarkers that were chosen to be investigated in the current study have previously been associated with either local or systemic inflammation of COPD. Several studies and research were focusing to determine their value as candidate COPD biomarkers. In this chapter, comparisons are reported for plasma inflammatory biomarker measurements between patients with COPD, healthy smokers and healthy nonsmoker groups using ELISA assay. ELISA is very sensitive assay which could detect the amount of protein levels in different biological tissues; plasma, serum, and cell culture recovered supernatant. Standardization of sample processing and storage, diluent type and dilution is necessary to ensure consistent results in ELISA assay. Most common processing technique involved separating plasma from the whole blood then storing plasma sample at -80°C. In the current study plasma samples were stored at -80°C until assayed. It was reported that storage and processing of samples have minor effects on plasma biomarker level measured by ELISA. There were no significant differences between measurement of plasma processed immediately or after storage at 4°C, -20°C, or -80°C (Bratcher and Gaggar 2014). In the current study 1% bovin serum albumin (BSA, sterile, filtered, Thermo Fisher Scientific, UK) in Dulbecco's phosphate buffered saline (PBS, sterile, filtered, w/o Calcium w/o Magnesium, Biosera, Labtech International Ltd, Sussex, UK) was used as a diluent to generate the serial dilutions of recombinant human standard according to manufacturer's recommendation. This diluent was

also suggested by Bratcher and Gaggar (2014). Another important factor that may affect ELISA measurement is whether the sample should be assayed neat or the diluted. Optimization of dilution of samples is highly recommended before starting the entire ELISA experiments for better and consistent measurements.

Dilution of the study plasma samples was vital before performing ELISA experiment to reduce the unspecific binding and to obtain better results. It assesses in estimation the values of detection range for antibody and target protein concentrations, and informs the amount of actual concentration required from plasma sample to start the assay (Thakur *et al.,* 2015). High BSA diluent was used to dilute plasma samples and the optimum dilution of each biomarker was used to conduct the ELISA experiment for the entire study population. To clarify further, if OD against X dilution of sample corresponds to a value fallen within the range of the standard curve, so X dilution could be an actual choice of dilution to conduct the experiment. Performing the assay with undiluted plasma sample may result in values lies beyond the standard curve range applying less or more than the optimum dilution will lead to inconclusive results.

4.5.1   Acute Phase Proteins

The main findings of the current study are that plasma CRP and fibrinogen were significantly associated with the presence of COPD. Patients with COPD were shown to have increased levels of circulating Fibrinogen and CRP when compared with healthy nonsmokers. Further, plasma fibrinogen level was also increased in COPD group when comparing to healthy smoker group.

Acute phase proteins like fibrinogen and CRP have been reported to be implicated in COPD pathogenesis. These findings are consistent with previous studies that reported elevated levels of CRP, and fibrinogen in COPD patients when comparing to healthy controls (Higashimoto *et al.,* 2009; Celli *et al.,* 2012; Agarwal *et al.*., 2013; El-Shimy *et al.,* 2014; Truedsson *et al.*, 2016; Korani *et al.,* 2016; Kleniewska *et al.,* 2016). CRP was the first biomarker to be investigated in COPD. Most studies have shown that CRP levels are frequently elevated in COPD and associated with disease severity, decline in lung function and FEV1, quality of life, exercise capacity and response to treatment (De Torres *et al.,* 2006; Tanni *et al.,* 2010; Fattouh and Alkady, *2014;* Paone *et al.,* 2016). A systematic review and meta-analysis was

performed to assess the effect of CRP level in COPD patients, found that elevated baseline CRP levels were significantly associated with higher mortality (Leuzzi *et al.,* 2017).

Several studies have shown that the plasma level of fibrinogen is increase in patient with COPD, with decline of FEV1 (Comes *et al.,* 2016). Elevated fibrinogen level in COPD also correlated with poor prognosis, an increased risk for exacerbations, hospitalization and death (Mannino *et al.,* 2015). In the ECLIPSE biomarker cohort fibrinogen was the most stable biomarker for more than 3 months (Dickens *et al.,* 2011). So fibrinogen was the only inflammatory biomarker tested associated with disease severity and can be used as a predictor of lung function decline. An increased plasma Fibrinogen and CRP level in patients with COPD may suggest a systemic inflammatory effect in these patients. But what needs to be clarified is whether systemic inflammation is a primary or secondary related to pulmonary inflammation. The plasma levels of systemic inflammatory biomarkers of fibrinogen and CRP (Higashimoto *et al.,* 2009) are good candidates as to predict rapid decline of FEV1 in COPD patients. However, little information is available on how these inflammatory biomarkers are related to lung function decline in COPD. The systemic level of CRP and fibrinogen may reflect the degree of severity of airway inflammation but they are not lung specific molecules (Akiki *et al.,* 2016).

4.5.2 Lung Specific Protein

The proteins CC16, SP-D, RAGE also were investigated in the current study. These are promising COPD biomarkers, because they are highly lung specific.

4.5.2.1 CC16

Low plasma CC16 was significantly associated with the presence of COPD, as the plasma level of CC16 in the COPD group was the lowest between the three groups. This result is in agreement with the studies which previously revealed that serum CC16 levels were decreased in patients with COPD when compared to healthy nonsmoking controls (Lomas *et al.,* 2008; Agusti *et al.,* 2012), and there was a positive correlation with disease severity and FEV1 decline over time (Vestbo *et al.,* 2011), but not with mortality (Celli *et al.,* 2012). Low expression of CC16 was reported in healthy smokers and COPD patients' airway (Laucho-Contreras *et al.,* 2015).

4.5.2.2 SP-D

In the current study lung specific protein SP-D, showed no activity in circulation of most of the study cohort. This result is inconsistent with the previous studies data who found that SP-D levels were elevated in COPD patients when compared to healthy smoking controls (Lomas *et al.,* 2009; Dickens *et al.,* 2011). In large of ECLIPSE study of 1,843 patients, the level of SP-D was also higher in COPD group than in the control group (Celli *et al.,* 2012). Moreover, a study by Akiki and collaborators found that SP-D is a more specific biomarker than CRP or fibrinogen to differentiate patients with COPD from healthy subjects. In their research the elevation of SP-D was associated with emphysema and exacerbations risk but not with COPD severity (Akiki *et al.,* 2016). SP-D plays an important role in pathogenesis of the some lung disorders. The high plasma level of lung specific protein may due to increased secretion or leakage of these proteins across the alveolar-capillary membrane into the circulation which occurs in many respiratory disorders (Madsen *et al.,* 2000, Greene *et al.,* 2002).

There are several factors influencing the measurement of SP-D by ELISA that may explain the variations of serum or plasma SP-D levels reported here and in the published literature. These factors include; processing and storage of sample, dilution factor, diluent or matrix type and anticoagulant type. Bratcher and Gaggar investigated the effect of these factors, and found that sample processing and storage as well as diluent used for the standard and sample dilution have little effects on the level of SP-D result by ELISA. However they suggested avoiding the use of EDTA as anticoagulant when assaying for SP-D by ELISA, as it gave the most inconsistent results, and values that were significantly lower than serum values. This might be a reason why SP-D could not be detected in most of the cohort samples as blood samples were collected and transported in EDTA vacutainers. Another problem affecting SP-D measurements by ELISA, is the multimeric structure of SP-D and posttranslational modifications. The degree of SP-D multimerisation has been shown to vary in the lungs during different disease. Further, posttranslational modifications of SP-D in human serum or plasma may alter or hide the epitope recognised by an ELISA kit antibody, whilst the same antibody could identify unmodified epitope of the ELISA kit standard SP-D (Kotecha *et al.,* 2013; Bratcher and Gaggar 2014). Additionally, inhaled systemic corticosteroids in conjunction with long-acting b2-adrenergic agonist therapy significantly lower serum SP-D levels in plasma of patients with COPD (Sin *et*

al., 2008). However, SP-D levels showed stability and good reproducibility over a period of 3-6 months such characteristics may validate the utility of this protein as COPD biomarker (Lomas *et al.,* 2008; Lomas *et al.,* 2009; Dickens *et al.,* 2011; Paone *et al.,* 2016).

4.5.2.3 sRAGE

Previous studies have found that RAGE and sRAGE are involved in COPD pathogenesis. However sRAGE was not detected in plasma in most of the current study cohort samples. In fact sRAGE was detectable in only 26% of the study cohort, mostly were healthy nonsmoking controls. Because of low subject number with detectable sRAGE, it was not possible to investigate the relationship between sRAGE and COPD, but could confirm that healthy nonsmoking control have shown numerically high level of sRAGE. Many researchers demonstrated that sRAGE levels in serum or plasma of COPD patients is lower compared to control subjects and is associated with GOLD stage and emphysema (Yonchuk *et al.,* 2015). Also a study that investigates plasma sRAGE in 88 patients with COPD and in 55 healthy controls, found that sRAGE levels were reduced in patients with COPD compared to healthy ex-smoking and nonsmoking controls, and its low level was positively associated with preFEV1 and preFEV1/FVC (Gopal *et al.,* 2014). Likewise, Hoonhorst and colleagues showed that plasma, sRAGE levels were significantly lower in COPD patients compared to young and old healthy controls and their value associated with decreased lung function and disease severity (Hoonhorst *et al.,* 2016). In contrast to those previous results no statistical significant difference was found among all groups regarding sRAGE level in plasma. Furthermore, no significant correlation was found between plasma sRAGE level and predicted FEV1% (Hassanein *et al.,* 2016). It can be inferred that the lower level of sRAGE, which acts as a decoy receptor to prevent inflammation may result in greater RAGE driven inflammation in COPD (Miller *et al.,* 2016).

4.5.3 Cytokines and Chemokines

The cytokines group that included TNF-α, IL-6, and chemokine; IL-8, IP10, showed no activity in circulation of most of the study cohort including patients with COPD. There was no relation between patient disease severity and the undetectable level of cytokines. As a result of that it was not possible to evaluate the association between these markers and COPD despite an association being described previously. Many authors found that the serum level of IL-6, IL-8

and TNF-α were significantly higher in COPD patients when compared with healthy nonsmokers (Heidari 2012; El-Shimy *et al.,* 2014; El Gammal *et al.,* 2015; Queiroz *et al.,* 2016; Korani *et al.,* 2016).

4.5.3.1 *TNF-α*

In this study, TNF-α was only detectable in approximately 10% of the subjects in the studied groups. The inability to detect particularly circulating TNF-α in many subjects in the current study is similar to that reported in a study by Rennard *et al.* where circulating TNF-α was detectable in approximately half of the subjects in the ECLIPSE biomarker cohort (Rennard *et al.,* 2007). In line with these results, TNF-α also was unmeasurable in most patients of ECLIPSE study (1,843 patients) had undetectable low levels of TNF-α (Celli *et al.,* 2012). Possible explanation for undetectable of TNF-α in plasma is its local and short term effects, its degradation and its short half-life, as well as its binding to receptors and renal clearance (Korani *et al.,* 2016)

4.5.3.2 *IL-6*

IL-6 was only detectable in 10% of the subjects in the studied groups. Like fibrinogen and CRP, IL-6 also belongs to the systemic inflammatory component of COPD, and is increased in COPD patients. However, the biological variability of fibrinogen was lower than for IL-6 or CRP in stable COPD subjects (Dickens *et al.,* 2011). In large of ECLIPSE study of 1,843 patients, the level of IL-6 was raised in COPD group than in the control group (Celli *et al.,* 2012). Also, Serum IL-6 was raised in patients COPD when compared to healthy nonsmokers, and there was a significant negative relationship between serum levels of IL-6 and FEV 1 and FEV1/FVC (Ardestani and Zaerin 2015). COPD patients also showed increase levels of IL-6 and IL-8 in induced sputum (Eickmeier *et al.,* 2010) and elevated levels of IL-6, IL-8, and TNF-α in serum (Tanni *et al.,* 2010; Selvarajah *et al.*, 2016) when compared to healthy controls. In contrary to the previous results, there was no significant difference between the serums levels of IL-6 and TNF-α of COPD patients and healthy subjects (Kleniewska *et al.,* 2016). Another study of two large COPD cohorts of 2123 and 1117 subjects observed that IL-6 was associated with progressive airflow limitation over 5 years and with emphysema progression over 5 years (Bradford *et al.,* 2017).

4.5.3.3 IL-8

Increased IL-8 secretion contributes to the development of COPD, and elevated IL-8 was found to be correlated with neutrophils numbers. IL-8 can chemo-attract and activate neutrophils to the site of injury, and then neutrophils can release IL-8 causing further accumulation of neutrophils in lung tissue of COPD patient. In this study, IL-8 was also one of the undetectable biomarkers in studied groups. The findings presented here are in contrary with literatures showing that IL-8 levels are elevated in COPD. The level of IL-8 was higher in COPD patients when compared with healthy controls (Zhang *et al.,* 2011). Similarly, IL-8 has been reported to be elevated in COPD patients in small study with less than 100 subjects (de-Torres *et al.,* 2013). A study by Liu *et al.* (2014), demonstrated that IL-8 concentrations were increased in serum from patients with exacerbated COPD, when compared with patients with asthma attacks. An increased serum IL-8 level in COPD patients with exacerbation suggests a systemic inflammatory effect in these patients. However, mechanisms that regulated serum IL-8 expression are not clear (Liu *et al.,* 2014). Another study of two large COPD cohorts of 2123 and 1117 subjects observed that IL-8 were associated with emphysema progression over 5 years (Bradford *et al.,* 2017). In contrast to the previous studies mentioned, Novgorodtseva *et al.* (2013) reported that the level of IL-8 in patients with COPD did not differ from that in the control group. The low or undetectable level of IL8 may be explained by the specific functions of this cytokine. IL-8 is needed to recruit and activate neutrophils. Most of the previous studies that described an association between IL8 level and COPD patient were using sputum, exhaled breath condensate and BAL fluid sample rather than plasma. Therefore, IL-8 could be considered as a local, rather than systemic, biomarker of inflammation that could be detectable in the airways rather than the plasma (Batlle *et al.,* 2012).

4.5.3.4 IP-10

Elevated levels of IP-10 have been observed in patients with COPD, and subsequently have been reported to be involved in COPD inflammation. However, in the present study no detectable basal level of IP-10 was observed in the circulation of most subjects of the study cohort. Most of plasma samples were under the limit of detection, and IP10 was only detectable in 16% of the whole study population. Torvinen *et al.* (2007) proved by *in vitro* study that the interactions between lung epithelial cells, lymphocytes and monocytes are needed for basal IP-10

secretion. IP-10 was induced when circulating monocytes, T-cells and epithelium are in close co-culture environment. Furthermore, Eickmeier group could not detect the IP-10 in many of their COPD sputum samples and found no significant differences between the COPD patients and healthy groups (Eickmeier *et al.,* 2010). The blood levels of IP-10 have not been widely investigated in patients with COPD, and most studies investigate their role in combination with other diseases where elevated levels of IP-10 were detected (Odler *et al.,* 2016). To give an example, patients with COPD have high serum IP-10 levels than controls, and it level increased from baseline to exacerbation in patients with rhinovirus induced exacerbations (Quint *et al.,* 2010). In addition, IP-10 was found significantly elevated in the circulation of systemic lupus erythematosus with pulmonary disorders involvement cohort (Odler *et al.,* 2016).

4.5.3.5 *CCL18*

Few studies have investigated CCL18 as a potential biomarker for COPD. In the current study there were no significant differences in plasma level of CCL18 of COPD patients when compared to healthy smokers and nonsmokers. This result disagrees with previous studies where CLL18 was shown to be elevated in COPD patients when compared to healthy smokers and healthy nonsmokers (Dickens *et al.,* 2011). A small study of forty eight patients with COPD has observed a significant association between serum levels of CCL18 and FEV1, dyspnea score, exercise capacity, exacerbation frequency and mortality (Pinto-Plata *et al.,* 2007). Additionally, in two large cohorts, serum CCL18 levels were found to be higher in COPD patients than in healthy smokers or healthy nonsmokers. Elevated CCL18 levels were associated with increased risk of cardiovascular hospitalization and mortality (Sin *et al.,* 2011). Another large of ECLIPSE study of 1,843 patients, found that the level of CCL18 was raised in COPD group than in the control group (Celli *et al.,* 2012).

Serum CCL18 levels were also found to be elevated in COPD patients when compared to the healthy former smokers (Dilektasli *et al.,* 2017). Both CCL18 and SP-D levels were higher in patients with more severe pneumonia which may reflect the extent of pulmonary inflammation (Spoorenberg *et al.,* 2017). In this study, it was observed that the circulating level of CCL18 was numerically higher in the COPD group but as mentioned earlier no statistically significant difference was observed in the comparisons between the three studied groups. These findings are

consistent with those of Muñoz-Esquerre *et al.* (2017), which have shown no significant difference in CCL18 level between COPD patients and healthy controls. The relatively small cohort size and high biological variability between subjects may have affected the circulation value of CCL18 that was found to be associated with mortality in two large cohorts (Sin *et al.,* 2011). Despite CCL-18 being a predominantly pulmonary marker, it may be secreted by the myeloid cells, and different organs such as liver, bone marrow. Hence, measuring CCL-18 production in BAL fluid might be more revealing. However, a close correlation between CCL-18 concentration in circulation and BAL was reported by Prasse *et al* (2007), suggesting that CCL-18 production within the lungs was reflected by the CCL-18 levels in systemic level of smokers (Dilektasli et al., 2017). CCL18 levels showed poor repeatability over time both in COPD patients and healthy controls (Dickens *et al.,* 2011). In summary, although there is some evidence of association between CCL18 and severity and mortality in COPD, further investigation is required to evaluate the utility of CCL18 as COPD biomarker (Pinto-Plata *et al.,* 2012; Ongay *et al.,* 2016).

4.5.4 CD31

Increasing evidence indicated that cardiovascular and not respiratory diseases are the main cause of death among individuals with COPD, and that endothelial injury in the pulmonary capillary vasculature leads to lung destruction. Because of that endothelial MPs such as CD31 which is involved in both the pulmonary and systemic vascular biology, are being investigated as potential biomarkers for COPD (Takahashi and Kubo, 2014). In this study the presence of CD31 was detected in plasma samples of all the three groups, and it was found that circulating levels of CD31 are higher numerically in COPD subjects compared to healthy smokers and nonsmokers groups. However this increase did not reach statistical significance due to the high biological variability between subjects and maybe this reflected the limited number of cases available for analysis. Most work that investigated the role of MPs CD31 in COPD has been limited to few studies. In these studies CD31was identified and determined by flow cytometry in platelet-free plasma or sputum according to the expression of membrane specific antigens. Thomashow and coworkers (2013) were the first to confirm that endothelial MPs including CD31 are increased in in COPD compared with control subjects and this elevation was negatively correlated FEV1 and emphysema severity.

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High plasma and sputum levels of CD31 of COPD patients negatively correlated with the severity of disease. The high levels of circulating and local CD31 could reflect a damage in small airway, while the presence of CD31 in sputum could indicate lung epithelium and vascular endothelium damage in COPD patients (Takahashi *et al.,* 2012). Further, Lacedonia *et al.* (2016) demonstrated that the level of CD31 was raised in the sputum of COPD patients, and was negatively correlated with the severity of disease. In agreement with previous studies, a recent work found that COPD patients showed significantly elevated levels of total circulating CD31 compared with healthy smokers and healthy nonsmokers (Garcia-Lucio *et al.,* 2018). In summary, the elevated levels of CD31 could be indicator of endothelial inflammation which is closely connected to the pathophysiology of COPD. Thus, CD31 could be a good candidate for the study of pulmonary endothelial damage, COPD progression, and for identifying patients who are susceptible to exacerbation (Takahashi and Kubo, 2014; Lacedonia *et al.,* 2016). The findings of the previous studies are in contrary to the current study data which suggest that CD31 are unable to different between patients with COPD and healthy control. There are several technical difficulties reported with measuring endothelial MPs. For example, there is no standard protocol for isolating and detecting circulating MPs from the plasma. In addition, differences in centrifugation protocols influence the number of MPs, and differences in flow cytometers influence the sensitivity of endothelial MPs detection (Takahashi and Kubo, 2014).

Several studies aim to identify the relationships between biomarkers and disease progression especially for treatment guiding (Comes et al., 2016). In the present study, it was noticed that the increasing plasma level of fibrinogen, CRP, CC16, CCL18 and CD31 was correlate with worsen disease stage. This means that COPD Patients in mild stage have shown lower level of circulating biomarkers that those in moderate or severe stages. These data are in line with previous studies data (Akiki *et al.,* 2016; Doubková *et al.,* 2016; Lacedonia *et al.,* 2016). Unfortunately, some of widely studied biomarkers showed poor repeatability and were unstable when measured at a three month interval which may restrict their utility as markers of disease progression or measures of the effects of drug intervention. While some other biomarkers should have a prognostic role in COPD. It is known that COPD is an inflammatory disease and CRP, fibrinogen are components of systemic inflammation, are elevated in COPD subjects compared to healthy subjects in this and in other studies (Dickens *et al.,* 2011). CRP is one of the

most widely studied biomarkers and may correlates with COPD. However, as CRP is not a pulmonary specific protein, it could be unspecific risk biomarker that reflects systemic inflammation rather than local inflammation (Liu *et al.,* 2014). Although CRP and IL-6 are both significantly raised in COPD; however their level is variable in COPD subjects over three months and may have limited utility as useful biomarkers of COPD (Dickens *et al.,* 2011).

In theory lung specific biomarkers could potentially be used as ideal biomarkers of COPD. Among lung specific proteins, SP-D and CC16 have proven to be more stable over time than CCL18 which could make them more useful COPD biomarkers. Additionally, CC16 seems to have a prognostic role in COPD patients, and the interesting results obtained here regarding CC16 should be the focus of further studies. On different note, MPs such as CD31 are known to increase in cardiovascular diseases, including myocardial infarction. Therefore, the role of other MPs in the comorbidity and the prognosis of COPD would be a great interest.

The correlation between inflammatory biomarker levels and clinical outcomes was investigated in COPD patients. A statistically negative significant correlation was observed between the levels of CRP and the predicted FEV1/FVC ratio. CRP values changed significantly with rising disease severity. So if plasma CRP level is correlated with FEV1/FVC then it may act as a surrogate marker of disease progression in individuals with COPD. This would assess clinicians to predict those who may susceptible to COPD. Other researchers have investigated the association between CRP and lung function, and shown that the mean serum CRP level was found to be significantly increased in severe cases. For example, De Torres and *et al.* (2006) indicated that when lung function worsens CRP levels increase. Similarly, Agarwal co-workers (2013) noticed that CRP level in stable COPD patients was correlated with FEV1. This might enable identification of patients at a high risk of disease progression and mortality. However, in the current study there was no association between CRP level and FEV1%. This result is consistent with a previous study finding which indicated that no association between pulmonary function test measurements (FEV1) and increase CRP levels in COPD patients (Pinto-Plata *et al.,* 2006). The lack of correlation of other biomarkers candidates (Fibrinogen, CC16, CD31 and CCL18) with patients lung function data in this study make it difficult to interpret, but may be due to the small patient sample size and that 80% of the patients had mild or moderate

obstruction. In consequence, it was not possible to provide clear evidence of an association between biomarkers levels rather than CRP and lung function parameters.

In the COPD group, smoking history and exposure seemed to have an impact on systemic level of CRP but not on fibrinogen, CC16, CCL18, and CD31. A significant correlation was observed between the levels of CRP and PYH. In subgroup analysis of patients with COPD, no differences in biomarkers level were found between smokers and ex-smokers except with CRP. The plasma level of CRP was found to be significantly higher in COPD former smokers as compared to current smokers. However, previous studies indicates that the increase of CRP levels in patients with COPD not related to smoking status (Pinto-Plata, *et al.,* 2006; Tanni *et al.,* 2010). Additionally, other researchers have shown that serum level of CRP and fibrinogen were not affected by smoking status (Higashimoto *et al.,* 2009). Cigarette smoke may enhance the inflammatory process in COPD patients, but it is not the leading cause of increased inflammatory biomarkers level (Agarwal *et al..,* 2013). In summary, it seems that a relation between smoking and level of CRP in COPD subjects requires further investigation. A possible relationship between the plasma level of CRP and fibrinogen was also evaluated. Acute phase reactants protein, CRP and fibrinogen, considered as a measure of systemic inflammation. Disappointedly, no correlation was found between plasma levels CRP and Fibrinogen.

It is known that the selection of biomarkers is incomplete, but believe this must reflect involvement of several pathways including acute phase reactant, inflammation, chemo-attraction, and pulmonary regulated proteins. The selection of the markers is based on previous studies that included stable and unstable COPD patients and smokers and non-smokers controls. COPD is heterogeneous disease and because of that the use of a single biomarker is generally considered insufficient. Using a combination of novel biomarkers with pulmonary function tests alongside other existing tools can optimise the prognosis, diagnosis, treatment of patients with COPD (Ongay *et al,* 2016).

There are some confounding factors that can affect the results. Factors include; small sample size, lack of reproducibility in an independent cohort, heterogeneity and biological variability of the patients with COPD, pathological characteristics, inflammatory profiles (Liu *et al.,* 2014; Bradford *et al.,* 2017). The number of patients was not sufficient to reach statistical significance in associations between inflammatory biomarkers and the presence of COPD.

However, the subject number in the present study was similar or larger than the number of subjects in some previous studies. For example, the number of the study cohort for Queiroz *et al.* (2016) = 30, El Gammal *et al.* (2015) = 40, Kawayama *et al.* (2016) = 37, Liu *et al.* (2014) = 19, and Hassanein *et al.* (2016)= 44.

Sample size justification is required for all clinical studies (Zheng *et al.,* 2017). Analyzing the power of a study depends on the significance level α, the sample size, and the effect size. The latter depends on the average difference and the variability of the measurement. It is recommended that sample size is estimated for an error (α) of 0.05, statistical power of 0.8 and effect size ≥ 0.5. During the planning of the study, no power analysis was performed. However, a post hoc power analysis (Wilcoxon-Mann-Whitney test) justifies the sample size. The power with the actual sample size for CRP, Fibrinogen and CC16 were 0.91, 1 and 0.98 respectively. It is possible that the lack of association between COPD and plasma CCL-18 and CD 31 concentration in the present study may be a result of inadequate statistical power rather than a true lack of association. The post hoc power analysis of CCL-18 and CD 31 data found that statistical power was insufficient to detect a significant relationship. Power and sample size analysis was calculated using G*power software version 3.

Thus, further studies with larger sample sizes from multi clinical centres and long term study may be necessary to confirm the lack of associations between these biomarkers and the presence of COPD (Lee *et al.,* 2018). Additionally, the finding of our study could be affected by the specific detection limit in the commercially ELISA kits. The very low values of some biomarkers in plasma were determined by the commercial ELISA kits, and most of the measured values fell below the validated detection limits of the relevant ELISAs. Since small variations in optical density can result in large changes in biomarker concentration and it is possible that there was some error in the measurement of low biomarker concentrations.

4.5.4   Conclusion

Blood biomarkers that are elevated in COPD subjects when compared to healthy nonsmoking controls were identified. This study showed that high level of plasma CRP, fibrinogen, and low level CC16 were associated with COPD, which suggests that CRP, fibrinogen, and CC16 might be a useful biomarker panel related to the presence of COPD. Thus,

plasma level of CRP, fibrinogen and CC16 could distinguish COPD patients from healthy controls. These results provide evidence that CRP and Fibrinogen might play a more important role in airway and systemic inflammation. Increased plasma CRP, fibrinogen can be used as biomarkers of the systemic inflammatory response in COPD patients. Several previous studies provide support for the view that CRP is a useful biomarker in COPD, particularly in defining lung function decline in COPD patients and in acting as a surrogate marker of treatment success. A significant negative correlation was detected between CRP plasma level and FEV1/FVC as well as PYH, though this study did not find an association between Fibrinogen, CC16, CD31 and CCL18 with pre FEV1% or FEV1/FVC, as well as with PYH in COPD patients. There is a need for lung-specific biomarkers derived from proteins that are either highly or only expressed in the lung tissue. For example, data presented here for CC16, support this as a promising local biomarker of an inflammatory process in the airways of COPD patients. However, further studies are required to establish the role of these biomarkers in COPD patients.

Analysis of biomarker profiles is more important that the measurement of single biomarker levels to differentiate patients with COPD from healthy subjects, and to determine the severity of lung inflammation. Monitoring the inflammatory biomarker activity is important for the assessment and management the chronic airway diseases and the decline in lung function in COPD (Pinto Plata *et al.,* 2012). Many studies have been published on the association between a specific biomarker and COPD disease, with either positive or negative results being reported. The disagreement in results can be attributed to different factors, including the heterogeneity of COPD phenotypes, low subject number, or the use of different methodologies and assays. Thus the use of advanced analytical methods like cytokine arrays, mass spectrometry, or nuclear magnetic resonance, should increase the sensitivity of the assays, and may produce important new information in the future (Liu *et al.,* 2014).

5. Chapter 5: Immunomodulatory effects of omega-3 PUFA on *ex-vivo* PBMCs

5.1 Background

Inflammation of the airways is the main pathological feature in COPD patients. Although the pathogenesis of COPD is still unclear, studies have shown that COPD pathogenesis might associate with immune system dysregulation. The initial inflammatory response to damage from smoking or irritant agents is characterized by increased number of inflammatory cells migrating from the circulation to the lung and accumulating in the airways, including neutrophils, monocytes and lymphocytes. These cells release various inflammatory mediators including IL-6, IL-8, and TNF-α, interact with each other, with structural cells of the airways, the lung parenchymal and pulmonary vasculature, result in increased COPD severity and pathogenic consequences (Duvoix *et al.,* 2013; Wu *et al.,* 2014; El-Shimy *et al.,* 2014; Quan *et al.,* 2016).

PBMCs are the immediate inflammatory cells to respond to any pathogen invasion. Many of the proinflammatory mediators produced by PBMCs different cell types play important roles in immune system monitoring relevant events and response in an inflammatory manner. PBMCs comprise the cellular part of the blood organ containing all blood cells with a mononuclear morphology. In humans the PBMC population is heterogeneous and consists from several different cell types with different functional states. Lymphocytes and monocytes are the main constituents of PBMCs and more than two thirds of the lymphocytes represented by T-cells. It includes lymphocytes (70–90%); 50–70% T-cells, 5–15% B-cells, 10–20% monocytes, 2–10% natural killer cells, dendritic cells (1–2%) and the remainder of platelets. The proportion of these populations differs between individuals. (Debey *et al.,* 2004; Haudek-Prinz *et al.,* 2012; Končarević *et al.,* 2014).

PBMCs are very important immune cells that are involved in a large number of diseases. In healthy individuals, PBMCs circulate in a quiescent cell state but are able to respond quickly and efficiently in an inflammatory manner toward any potential pathogens invasion or irritants. However, strong inflammatory responses occur in these cells when targeting any foreign particle and may also cause severe side effects. During inflammatory activation of these cells, key events include; leukocyte migration, proliferation of T-cells, NF-κB signaling and regulation of apoptosis (Haudek-Prinz *et al.,* 2012).

Several studies have evidenced that T-cells play main role in initiating and prolonging inflammation in human disease, through the production of proinflammatory mediators and via cell to cell contact interactions with different cell types through membrane receptors and their ligands. Activated T-cells are important regulators and effectors of inflammatory immune response towards tissue damage (Monaco *et al.,* 2004).The major subclasses of T-cells express either CD4 or CD8 on their surface. Different immunologic stimuli trigger differentiation of naive CD4+ T cells via different developmental pathways give arise to different T-cell phenotypes such as; Th1 and Th2 cells. Both phenotypes involved in host defence in different pathological or disease states. Other subsets including; Th17 cells and regulatory T cells (CD4+CD25+ Treg) also associated in host defence and in inflammatory disease (Calder 2015). T-cells, especially Treg cells, have been involved in the regulation of immune response and maintaining homeostasis. Previous studies showed that compared to healthy controls, the expression and the percentage of CD4+ T-cells and CD4+CD25+ Treg was significantly decreased. This suggested that the reduction in the population of Treg cells functioning suppressive immunity, which might promote occurrence and development of COPD (Domagala-Kulawik *et al.,* 2011; Quan *et al.,* 2016).

Monocyte functions comprise phagocytosis, presentation of antigens and paracrine regulation of inflammation. Monocytes act as regulator and activator of T-cell. In turn activated T-cells may further activate endothelial cells, fibroblasts, smooth muscle cells and monocytes/macrophages to produce inflammatory cytokines, chemokines, tissue factor and extracellular matrix-degrading enzymes, to promote inflammation, angiogenesis and tissue damage, and hence maintain an acute or chronic inflammatory state (Monaco et al., 2004).

During pathogen invasion, monocytes are recruited from the circulation to the tissue and differentiated in the two phenotypes M1 or M2 macrophages depending on the signals they receive from the micro-environment. The M1 macrophages involved in proinflammatory pathways and release several proinflammatory cytokines and eliminate microbial infection. The M2 macrophages have poor antigen-presenting capabilities, implicated in tissue remodeling and progression of fibrosis, and are considered to be wound-healing macrophages. The M2 macrophages secrete a specific types of chemokines including; IL-10, CCL17, CCL22, IL-1Ra, and CCL18 (Plönes *et al.,* 2012; Dilektasli *et al.,* 2017).

Macrophages are among the most abundant cells in the respiratory tract and can be classified as alveolar macrophages (AMs) that line the surface of alveoli and interstitial macrophages (IMs) that found in the interstitial space between the alveoli epithelium and blood vessels. AMs have been reported to clear inhaled irritants, bacteria, cell debris and apoptotic cells, and resolve inflammation following injury and infection. Additionally it regulates neutrophil and monocyte recruitment to the lung. Both populations display high phagocytic capacity, but AMs are larger in size and more potent phagocytes (Kapellos *et al.,* 2018). AMs constitute over 90% of the pulmonary macrophage population and thought to be originating from the bone marrow**.** In COPD macrophages have an impaired ability to clear bacteria, damaged tissue and apoptotic cells, which leads to the colonisation of the lung with pathogenic bacteria. This may be due to excessive oxidative stress that affects the phagocytic capacity of macrophages (Vlahos and Bozinovski, 2014). Different macrophage subpopulations have been identified in airways and lung tissue of COPD patients and controls using flow cytometry. Based on size and location, macrophage subsets include; Small interstitial macrophages with more pro-inflammatory function, small alveolar macrophages that had the greatest phagocytic capacity and large alveolar macrophages with low pro-inflammatory and phagocytic ability (Dewhurst *et al.,* 2017).

Dietary interventions with omega-3 PUFA have been shown to be beneficial in the treatment of inflammatory diseases such as rheumatoid arthritis (Miles and Calder 2012), breast cancer (Mansara *et al.,* 2015), and CVD (Calder 2017), the latter is the most important comorbidity in COPD. Hence, omega-3 PUFA supplementation might also be beneficial for people with COPD. Fatty acids are constituents of phospholipids and phospholipids are

components of cell membranes. The phospholipids of immune cells including; lymphocytes, monocytes, and neutrophils, isolated from the blood of healthy people consuming typical Western diets, have been reported to contain about 10 to 20% of fatty acids as; ARA, with about 0·5-1% of EPA and 1·5-3% of DHA (Miles and Calder 2012). The fatty acid composition of human blood leukocytes can be modified by increased oral intake of marine omega-3 PUFA. This results in increased proportions of EPA and DHA in cell membrane of blood monocytes, neutrophils and PBMCs (Faber *et al.,* 2011).

Several studies have previously reported on the ability of omega-3 PUFA (EPA and DHA) to decrease pro-inflammatory cytokine production from stimulated PBMC, in different chronic diseases including multiple sclerosis (Shinto *et al.,* 2011) and rheumatoid arthritis (Miles and Calder 2012) . However, most of the studies investigating the role of omega-3 fatty acids on immunomodulation have focused on the effects of orally administered EPA and DHA on immune cells reflected by cytokine production of T-cells or monocytes. Unfortunately the available measurements and outcome data of these studies are highly inconsistent (Jaudszus *et al.,* 2013). Further, the numbers of the studies that investigated the immunomodulation effect of omega-3 PUFA on human PBMC without COPD patients' omega-3 PUFA supplementation are limited.

PBMCs have been considered as marker of several diseases, including rheumatoid arthritis, and malignant diseases (Edwards *et al.,* 2007). The features of PBMC were also found to be altered in COPD (Aggarwal et al., 2016). In Patients with COPD altered PBMCs may fail to react to infection, stimuli or hypoxia (Wu *et al.,* 2014). For example, PBMCs from COPD patients fail to induce hypoxia-inducible factor1 and VEGF under hypoxic condition because of a reduction in histone deacetylase 7 (To *et al.,* 2012).

PBMC samples are suitable for analysing immune-mediated or associated diseases and evaluating therapy response (Debey *et al.,* 2004). PBMCs can be obtained easily from routinely collected blood samples, and widely used in *in vitro* studies, which is in contrast to airway tissue or lavage samples. Different methods are available for both *in vitro* and *ex vivo* investigations of PBMCs to measure activation of specific cells and production of proinflammatory mediators (Končarević *et al.,* 2014). The present study employed PBMC as an experimental model to study mitogen-specific responses. Mitogen-induced proliferation and cytokine production is often a

preferable assay to examine any immuno-toxic therapy, because it reflects the status of immune cells. The functions of immune cell are mediated by several cytokines that are stimulated by different factors including: bacterial or viral infections, drugs and/or environmental irritants. Thus, it is necessary to select a panel of biomarkers that reflects the effects of a given therapy (Mandarapu *et al.,* 2014). Data reported in Chapter 4 measured the plasma level of inflammatory biomarkers in patients with COPD, and compared these against levels in healthy smokers and nonsmoking controls. This chapter will compare release of cellular inflammatory biomarkers before and after stimulation of PBMCs recovered from the study groups.

To induce proliferation PBMCs should be triggered by using several stimulators, including different mitogens. The most common used mitogens; Concanavalin A (ConA), LPS and Phytohemagglutinin (PHA), are able to stimulate PBMCs (Molaee *et al.,* 2017). LPS which is the main structure component of gram-negative bacterial cell wall can stimulate the release of IL-8 and IL-6, from human PBMCs, and other pro-inflammatory cytokines towards bacterial invasion (Ngkelo *et al.*, 2012). PHA; lectin extracted from red kidney bean (*Phaseolus vulgaris*), is one of the potent mitogens used in cell proliferation experiments *in vitro*. It binds to cell membranes surface receptors and stimulates cell division and metabolic activity (Haudek-Prinz *et al.,* 2012).

Based on the previous findings, it is hypothesized that omega-3 PUFAs may help to reduce COPD outcomes induced by cigarette smoke and other irritants. PBMCs samples were chosen for the investigation, because they can be easily collected, and are important for the discovery of biomarkers for diagnosis and therapeutic management of COPD. The aim of this chapter is to investigate the potential effect of the two main omega-3 PUFA; EPA and DHA, on functional properties of human PBMC, evaluated through proliferation and cytokine production, in order to examine the contribution of these immune cells which are involved in innate and adaptive immunity. For this purpose, an *ex vivo* mitogen-induced PBMC proliferation model was used. PHA mitogen was used as a stimulant to induce proliferation in PBMCs which mainly consists of a mixture of lymphocytes and monocytes (Haudek-Prinz *et al.,* 2012; Končarević *et al.,* 2014, Molaee *et al.,* 2017). PBMCs were treated with different concentration of EPA or DHA, then were incubated with PHA for 24hrs (37˚C, 5% CO_2 and 95% humidity).

5.2 Objectives

- To establish basal release of inflammatory biomarkers from *ex-vivo* PBMCs
- To evaluate the effect of different concentrations of EPA and DHA on biomarkers secreted from PHA-stimulated and unstimulated *ex-vivo* PBMCs

5.3 Methods

5.3.1 Prioritized COPD Biomarkers in PBMCs

A limited number of studies have investigated PBMCs profile in COPD through cytokines production. In the current study a panel of biomarker candidates was selected based on a previous study (Kawayama *et al.,* 2016). It is suggested that the production of these biomarker from PBMCs may increase during COPD inflammation. These biomarkers include; inflammatory biomarkers: IL-6, and TNF-α; chemoattractant biomarkers: IL8, IP10 in addition to RAGE and CD31. The list of potential biomarkers was further reduced by selecting only those could be measured in PBMCs culture samples of the studied groups. In this way, four potential biomarkers remained includes; IL-6, TNF-α, IL-8 and CD31.

5.3.2 Duoset ELISA Assay

5.3.2.1 Reagent Preparation

ELISA duoset kits (R&D Systems, Oxford, UK) were used to measure the biomarkers release in recovered supernatants from PBMC culture samples. Detail of ELISA sensitivity ranges for each biomarker and the required reagents diluent and sample needed for each assay is summarised in Table (5.1, 5.2) Anti-human capture antibody was reconstituted in 0.5 ml of PBS and further dilution was made in PBS, to the working concentration. Anti-human detection antibody was reconstituted in 1.0 ml of reagent diluent 1% BSA in PBS, and further dilution was made in reagent diluent to the desire working concentration.

5.3.2.2 *Sample and Standards Preparation*

The PBMCs were isolated from 20mL of whole blood consisting of anti-coagulant EDTA from patients with COPD, healthy smokers and healthy nonsmokers on Ficoll density gradient by centrifugation at 400 ×g at the room temperature for 30 minutes as previously mentioned (chapter 2). Before performing the experiment, cryopreserved PBMCs were recovered, washed and counted. The total cell count was calculated by using trypan blue exclusion method. Cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue, and the viable cells were counted and compared with the total amount of cells in the suspension and in all of the experiments. The cell suspension was adjusted to 1×10^6 cells/ml. For standard preparation; each standard vial was reconstituted with 0.5 mL of deionized or PBS according to manufacture instructions (R&D Systems). After initial reconstitution 1ml of high standard was prepared per plate assayed at the concentration indicated. Seven point standard curves using 2-fold serial dilutions were prepared. Working dilutions were prepared on experiment day and used immediately.

Table 5.1: Summary of stock concertation and sensitivity range of standards used in ELISA assay

Biomarker	Standard Stock Concentration	High standard limit	Lower standard limit
Human IL-6	90 ng	600 pg/mL	9.38 pg/mL
Human IL8/CXCL8	40 ng	2000 pg/mL	31.3 pg/mL
Human TNF-α	67.5 ng	1000 pg/mL	15.6 pg/mL
Human CD31/ PECAM-1	190 ng	10,000 pg/mL	156 pg/mL

Table 5.2: Summary of dilutions capture and detection antibody for each biomarkers used in ELISA assay

Biomarker	Capture Antibody	Detection Antibody	Capture Antibody concentration		Detection Antibody concentration	
			Stock	**Working solution**	**Stock**	**Working solution**
Human IL-6 DY206	Mouse Anti-Human IL-6	Biotinylated Goat Anti-Human IL-6 Antibody	240 µg/ml	2µg/ml	3µg/ml	50g/ml
Human IL8/CXCL8 DY208	Mouse Anti-Human IL-8 Antibody	Biotinylated Goat Anti-Human IL-8 Antibody	240µg	4µg/ml	1.2µg/ml	20ng/ml
Human TNF-α DY210	Mouse Anti-Human TNF-α Antibody	Biotinylated Goat Anti-Human TNF-α Antibody	480 µg/ml	4 µg/ml	24 µg/ml	400 ng/ml
Human CD31/ PECAM-1 DY8064-05	Mouse Anti-Human CD31 Antibody	Biotinylated Sheep Anti-Human CD31 Antibody	480µg/ml	4µg/ml	12µg/ml	200ng/ml

5.3.3 ELISA for R&D Systems-based assays procedure

The procedure for ELISA R&D Systems-based assays was previously mentioned (see section 2.1.4.4).

5.3.3.1 Initial Optimizations

5.3.3.1.1 Supernatant Diluent Selection

PBMC-recovered supernatants may require a dilution in an appropriate buffer to avoid false negative or positive results. In the current study PBS and RPMI media were examined to dilute PBMCs-culture supernatant, to determine if PBS or RPMI could provide an assay environment that minimizes nonspecific binding, while maintaining proper ionic strength and pH for efficient antibody-antigen interaction. PBMCs-culture samples from healthy smokers were diluted with PBS or RPMI (1:1, 1:5, and 1:10) and assayed in 96-well plate alongside undiluted samples. ELISA assay was carried out as mention in section (2.1.4.4) to detect and compare the IL-6 production from PBS- or RPMI-diluted and undiluted samples. The experiments were carried out three times. A comparison between the outcomes from using two diluents was made and the appropriate diluent was chosen to dilute PBMCs-culture samples for the study experiments.

5.3.3.1.2 Supernatant Dilution Factor Selection

In the current study two PBMCs culture samples were selected from each of three studied groups; COPD, healthy smoker and healthy nonsmoker groups, to be 10-fold diluted (1:10, 1:100, and 1:1000), in RPMI media, and then assayed in 96-well plate alongside undiluted samples. ELISA assay was carried out as mention in section (2.1.4.4) to detect selected biomarker level in undiluted and diluted samples. Once samples are diluted, the dilution factor was included when calculating results. Then the dilution factor was included when assay all samples. Otherwise undiluted sample was utilised when dilution not required. Dilution of the sample before performing the actual experiment is significant parameter for determining the sensitivity of ELISA in human samples. It helps in deciding the amount of actual concentration required from the sample to obtain a result within the range of reference standard protein. Without dilution, it is difficult to achieve highly sensitive results of protein concentration.

Therefore, optimum dilution for the sample of target protein is a vital parameter for obtaining better results (Thakur *et al.,* 2015).

5.3.3.2 Cytotoxicity Assessment

5.3.3.2.1 Preparation of Fatty Acids

Omega-3 PUFA; EPA (826mM) and DHA (761mM) (Cambridge Bioscience Ltd, Cambridge, UK) stock solutions were aliquoted and stored in ethanol at -20°C. On the experiment day stock EPA and DHA solutions were diluted in RPMI media with supplements to give a 5mM intermediate stock solution. Then further dilutions were prepared from the intermediate stock solution, so that final desired concentrations are achieved in the test wells (10, 50, 100, and 500 µM). In order to minimize oxidation effects, the fatty acids were freshly prepared and were not reused again, see Table 5.3.

Table 5.3: The preparation of EPA and DHA final Concentrations

Final Concentration 1X µM	Volume 5mM stock µl	Volume of RPMI media µl
10	2.5	997.5
50	12.5	987.5
100	25	975
500	125	875

5.3.3.2.2   Assay Procedures

Preliminary cytotoxicity studies were   performed in order to address the impact of high dose EPA and DHA, on PBMC biomarkers release. Five PBMCs samples from five COPD patients (10^6 cells/well, 96-well plate), were incubated without or with 10, 50, 100, and 500μM of EPA or DHA for 24hrs in a 5% CO_2 humidified atmosphere at 37°C. The PBMCs in RPMI medium were considered as a control. Then, culture supernatants from each well were harvested (centrifuged at 400g for 10 minutes) and supernatants were collected, and ELISA assay was carried out as mentioned in section (2.1.4.4) to detect the impact of high dose of fatty acids on the biomarkers production from EPA- or DHA-treated and untreated cells. The experiments were carried out three times.

5.3.3.3   *PHA Concentration Selection*

A total amount of 20mg of PHA (Roche Diagnostics GmbH, Mannheim, Germany) was reconstituted in sterile double-distilled water, aliquoted into small micro-tubes and stored at -20°C. On PBMCs activation experiment day 1mg/ml aliquoted of PHA was further diluted with RPMI media with supplements to obtain a working solution range of 1-20 μg/ml. To find the optimal concentration of PHA mitogen that induce cell proliferation, PBMCs (10^6 cells/well, 96-well plate) were stimulated with various concentrations of PHA, including 1, 5, 10, and 20 μg/ml, and were incubated for 24hrs at 37˚C under 5% CO2 and 95% humidity. The PBMCs in RPMI medium were considered as a control for each group. Then, culture supernatants from each well were harvested (centrifuged at 400g for 10 minutes) and supernatants were collected. ELISA assay was carried out as mention in section (2.1.4.4) to detect IL-6, IL-8, TNF-α, and CD31 production from PHA-stimulated and unstimulated cells and the best concentration of PHA which induced the highest biomarker production from cells was used to activate PBMCs of the actual experiments. The experiments were carried out three times using five PBMCs samples from each group; COPD, healthy smokers and nonsmokers groups.

5.3.3.4 Biomarkers Detection from Unstimulated PBMC

Basal release of IL6, IL-8, TNF-α, and CD31 was investigated from unstimulated *ex-vivo* PBMCs from the study groups. PBMCs (10^6 cells/well, 96-well plate) were incubated for 24hrs in a 5% CO_2 humidified atmosphere at 37°C.Then, culture supernatants from each well were harvested (centrifuged at 400g for 10 minutes) and supernatants were collected. ELISA assay was carried out as mentioned in section (2.1.4.4) to detect the IL-6, TNF-α, IL-8, and CD31 production from unstimulated cells. The experiments were carried out three times using five PBMCs samples from each group; COPD, healthy smokers and healthy nonsmokers groups.

5.3.3.5 Biomarker Detection from Stimulated PBMC

Production of biomarkers; IL-6, TNF-α, IL-8 and CD31, was evaluated following stimulation of PBMCs to mimic an inflammatory *in vivo* environment. The effects on biomarkers can be analysed via direct detection of the relative concentration of biomarkers in the culture supernatant following experimental treatment in activated PBMCs (Ai *et al.,* 2013). The experiments were carried out three times using five PBMCs samples from five subjects from each studied group to assess the cellular cytokine response to an inflammatory stimulus (PHA) in the presence or absence of omega-3 PUFA (EPA or DHA). The five selected samples represent each group, as much as possible, with the least possible error and without substitution or incompleteness. Five random patient samples were selected represent the different disease stage and smoking status. Five samples selected from the smokers group represent smokers and ex-smokers, while five samples selected from the controls group were share a common character. This allows obtaining an effect size from each sub-group separately. Therefore, the differences between groups become apparent, and it allows obtaining samples from minority or under-represented populations. PBMCs samples were adjusted to 1×10^6 cells/ml in RPMI supplemented media, and 100 μL of sample was added to each well in a 96-well round bottom plates (Sarstedt AG and Co, Numbrecht, Germany). PBMC samples were cultured in either RPMI1640 culture media with media supplements with EPA or DHA doses (10, 50, 100, and 500 μM final concentration) or to RPMI medium alone as a negative control for 1 hour before the addition of PHA (5μg/ml) mitogen or medium alone only as a control. The cells were then incubated at 37∘C under 5% CO2 and 95% humidity for 24hr. Then, culture supernatants from

each well were harvested (centrifuged at 400g for 10 minutes) and supernatants were collected. ELISA assay was carried out as mentioned in section (2.1.4.4) to detect the IL-6, TNF-α, IL-8, and CD31 production from EPA or DHA treated-PBMCs.

5.3.4   Statistical Analysis

Laboratory data were expressed as standard deviation of the mean (SD). Shapiro-Wilk test was performed to test normality of the data distribution. All data were expressed and graphed as mean ± standard deviation. For data normally distributed, paired *t*-tests or one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test was performed to determine whether the differences between the groups were statistically significant. Differences were considered significant if the probability (P) value was <0.05, highly significant if P value was <0.01 and P value <0.001, and insignificant if P value was >0.05. Correlation analyses of two variables were carried out using Pearson or Spearman methods, depending on the normality of the data distribution. All statistical analyses were performed using graphPad Prism and graphPad InStat software, version 7.04 and 3.10.32 respectively.

5.4   Results

5.4.1   Prioritized COPD Biomarkers in PBMCs

Four candidate biomarkers were detectable in PBMCs culture supernatant samples including; IL-6, TNF-α, IL-8 and CD31. In contrast, other biomarkers; IP10 and RAGE were undetectable in PBMCs supernatant samples.

5.4.2 Initial Optimizations

5.4.2.1 Supernatant Diluent Selection

The assay was carried out to address the appropriate diluent to dilute PBMCs culture samples before starting out actual experiments. It was found that there was no difference between the IL-6 release from PBMC culture sample diluted with RPMI media or PBS (1:5 and 1:10, $P > 0.05$). However, a difference was detected between the IL-6 releases from PBMC culture sample diluted with RPMI media or PBS (1:1, $P<0.05$) (Figure 5.1). Based on these data, all PBMCs culture samples were diluted in RPMI medium in all subsequent experiments.
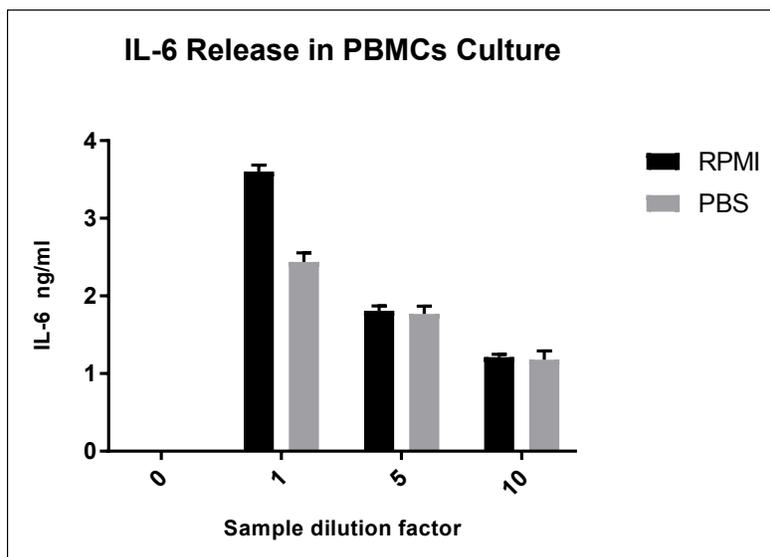
Figure 5.1: Dilution medium selection for PBMCs culture samples. *ELISA assay bar graph comparing IL6 level released from PBMCs when diluting culture samples with PBS or RPMI 1640 medium. PBMCs separated from whole blood of COPD patients, seeded in a 96-well plate and incubated for 24hrs, 5% CO 2 at 37°C. Following the incubation period cell supernatant were recovered and assayed as triplicate with dilution (1:1, 1:5, 1:10) with RPMI media or PBS alongside undiluted sample. The levels of IL-6 was quantified by ELISA assay (see section 2.1.4.4).Value are expressed as mean (n=3) (error bar represent 1 SD). P value < 0.05 as determined by paired t-tests indicates a significant difference between the two diluents. These analyses were performed using Graph Pad and Instat Software. Abbreviations: IL-6, interleukin-6; PBS, phosphate buffer saline; RPMI, Roswell Park Memorial Institute 1640.*

5.4.2.2 Supernatant Dilution Factor Selection

The assay was carried out to estimate the proper dilution factor for PBMC cuture samples before starting out actual experiments. Undiluted PBMC culture supernatants were the best option for TNF-α and CD31, while a dilution of 1:10 was appropriate for IL-6 and IL-8 (Figure 5.2).
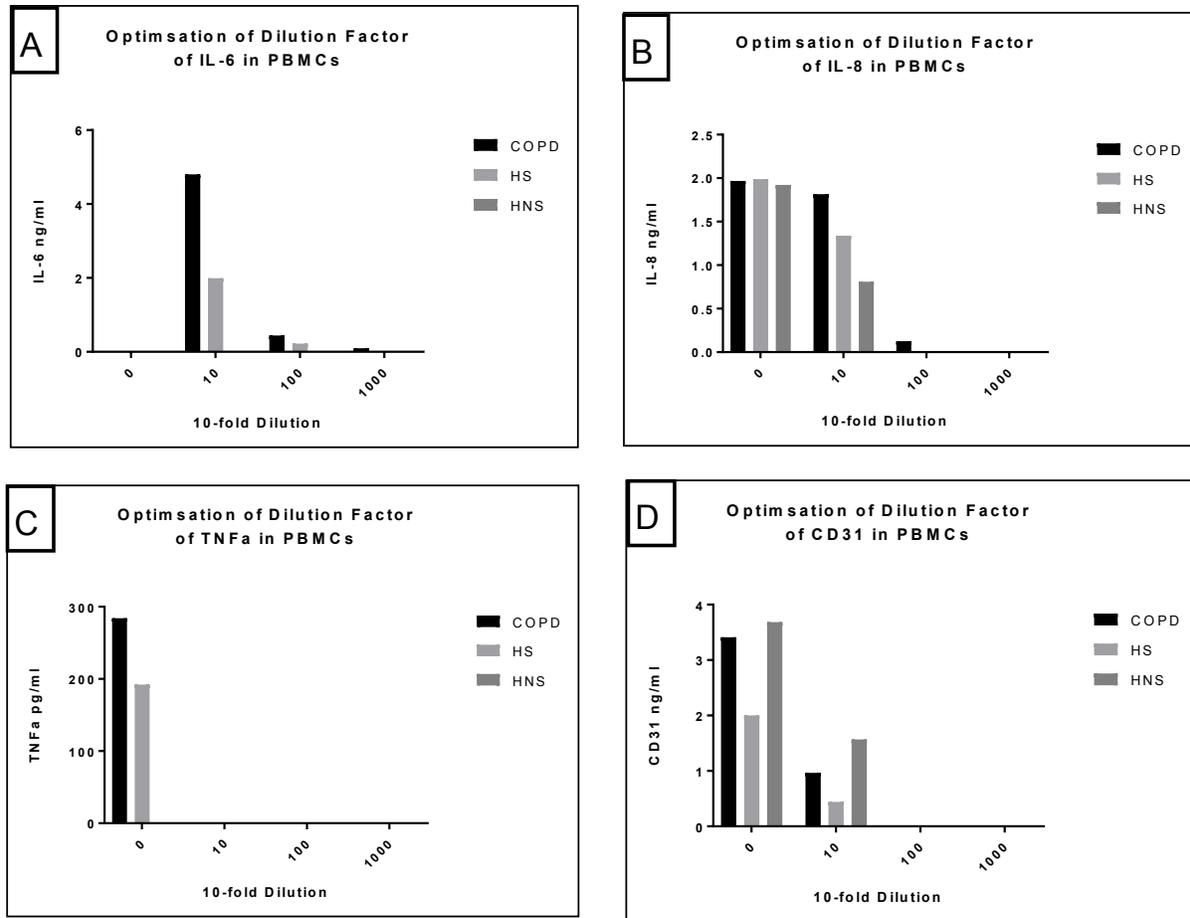
Figure 5.2: Selection of PBMC culture sample dilution. *The figure shows the optimum dilution factor using 10-fold dilution of PBMC culture sample for biomarker candidate; IL-6 (A), IL-8 (B), TNF-α (C), and CD31 (D). PBMCs separated from whole blood of COPD patients, seeded in a 96-well plate and incubated for 24hrs, 5% CO 2 at 37°C. Following the incubation period cell supernatant were recovered and assayed as triplicate with dilution (1:10, 1:100, 1:1000) with RPMI media or PBS alongside undiluted sample. The level of biomarkers was quantified by ELISA assay (see section 2.1.4.4). Value are expressed as mean (n=3, each group).These analyses were performed using Graph Pad and Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrotic factor alpha; CD31, cluster of differentiation 31.*

5.4.3 Anti-inflammatory actions of EPA and DHA on cytokine release from PBMCs

A range of concentrations of EPA or DHA were tested for any cytotoxic impact on *ex-vivo* PBMCs from COPD patients over a period of 24 hrs. The range selected encompasses the typical concentrations employed in many previous studies. Compared with the control, neither EPA nor DHA showed any cytotoxic effect at 100μM (Figure 5.3). The release of IL-6 and TNF-α from EPA-treated PBMCs was markedly decreased at a concentration of 100μM ($p<0.005$), while release of both cytokines became undetectable level at concentrations of 500μM in PBMCs treated with DHA. IL-8 release was markedly decreased at a concentration of 500μM EPA ($p<0.001$), and was undetectable in PBMC treated with 500μM DHA. However, there was no difference in CD31 release from PBMC treated with either concentration of EPA or DHA ($p>0.05$). Based on these data, a maximum fatty acid concentration of 500μM was used in all subsequent experiments

A — IL-6 Release in PBMCs-COPD Treated with EPA

B — IL-6 Release in PBMCs-COPD Treated with DHA

C — TNF-a Release in PBMCs-COPD Treated with EPA

D — TNF-a Release in PBMCs-COPD Treated with DHA
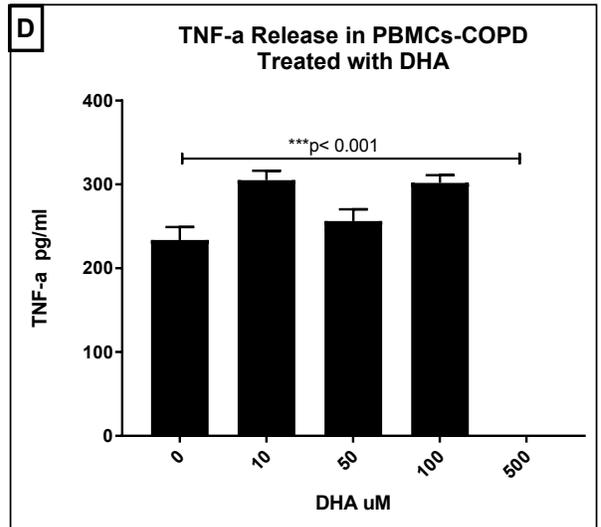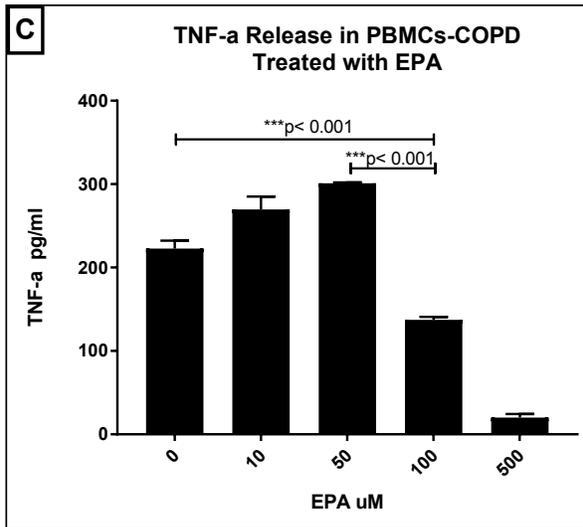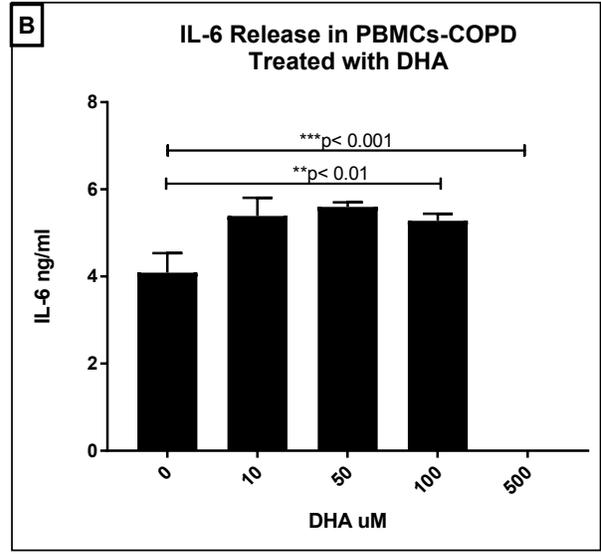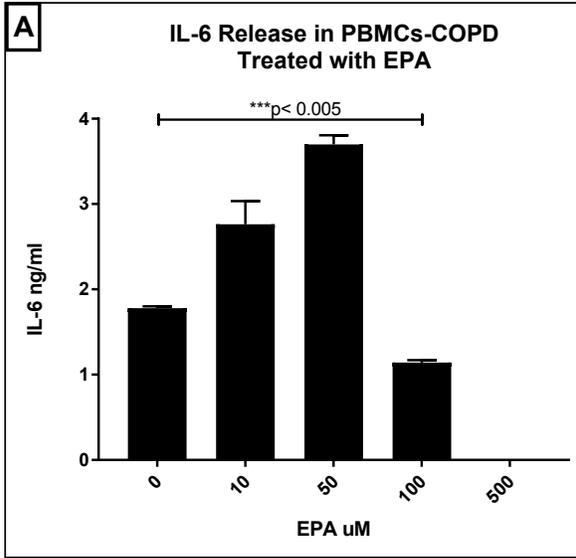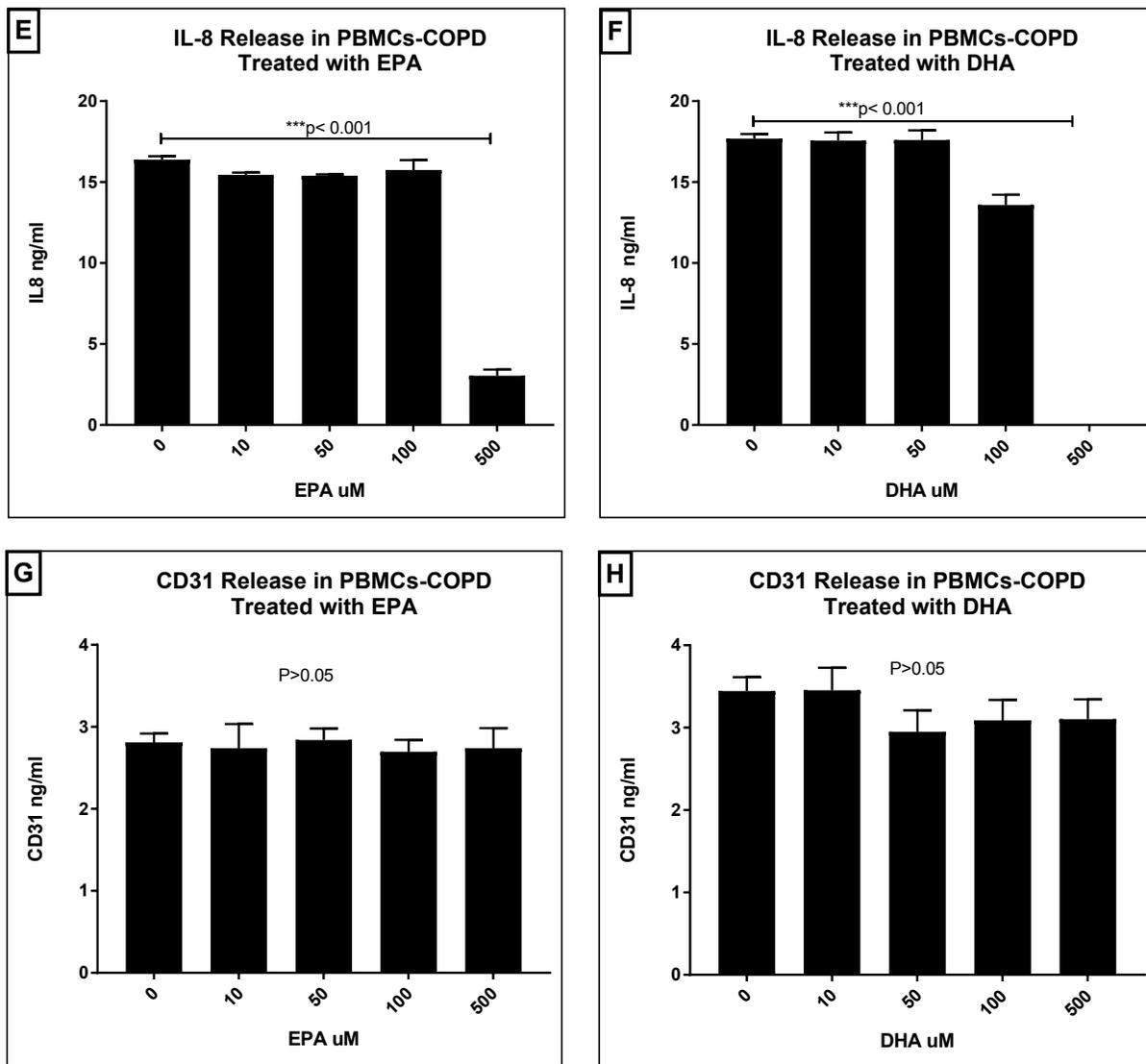
Figure 5.3: Biomarkers release from PBMCs culture samples. *ELISA assay bar graph showing the influence of omega-3 FA; EPA on IL-6 (A), DHA on IL-6 (B), EPA on TNF-α (C), and DHA on TNF-α (D), EPA on IL-8 (E), and DHA on IL-8 (F), EPA on CD31 (G), and DHA on CD31 (H), release from PBMCs-COPD. Cells were seeded in a 96-well plate and treated with or without increasing concentrations of one of the following; DHA and EPA (10, 50, 100, and 500μM) then incubated for 24 hrs, 5% CO 2 at 37°C. Following the incubation period cell supernatant were recovered and assayed as triplicate through ELISA assay to determine the level of biomarkers release (see section 2.1.4.4).Value are expressed as mean (n=5) (error bar represent 1 SD). P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between different concentrations. Definition of abbreviations: PBMCs, peripheral blood mononuclear cells; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrotic factor alpha; CD31, cluster of differentiation 31; COPD, chronic obstructive pulmonary disease; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid.*

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5.4.4 PHA Optimal Concentration Selection

Activation of PBMCs can effectively produce a large amount of biomarkers. The study aimed to find an optimal concentration of PHA mitogen which can promote a maximum amount of biomarker production from human PBMCs. The effect of PHA at different concentrations (1, 5, 10, and 20µg/ml) on biomarker production was compared to the control, measured after 24hrs from PBMCs, as seen in Figure 5.4. The results showed that the maximum production of IL-6 and TNF-α was obtained by 5µg/ml PHA-stimulated-PBMCs from the COPD group, compared to untreated cells ($p < 0.001$). No significant differences were seen in IL-6, TNF-α when stimulated with PHA at different concentrations in PBMCs from healthy smokers ($p > 0.05$). A similar trend was seen in IL-8 release from PBMCs of COPD patients. In contrast, there was a significant increase in IL-8 release at more than one concentration (1, 5, 10µg/ml) of PHA-stimulated-PBMCs from healthy smoker controls ($p < 0.001$). Additionally, there was a significant increase in IL-8 release in 5 and 10µg/ml PHA-stimulated-PBMCs of healthy nonsmoker controls ($p < 0.001$). However, IL-6 and TNF-α were undetectable in PBMCs from healthy nonsmokers with PHA at any tested concentration. Surprisingly, there was a tendency in all three subject groups for decreased CD31 release with the increasing PHA concentrations particularly with 20µg/ml PHA ($p < 0.001$). Based on these data, a 5µg/ml PHA concentration was used for PBMCs activation in all subsequent experiments

A **IL-6 Release from PHA-Stimulated PBMCs**

B **TNF-a Release from PHA-Stimulated PBMCs**
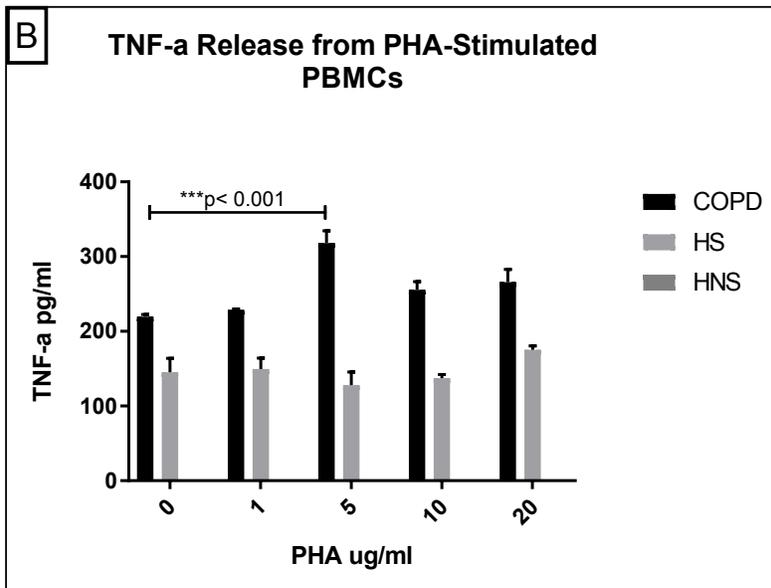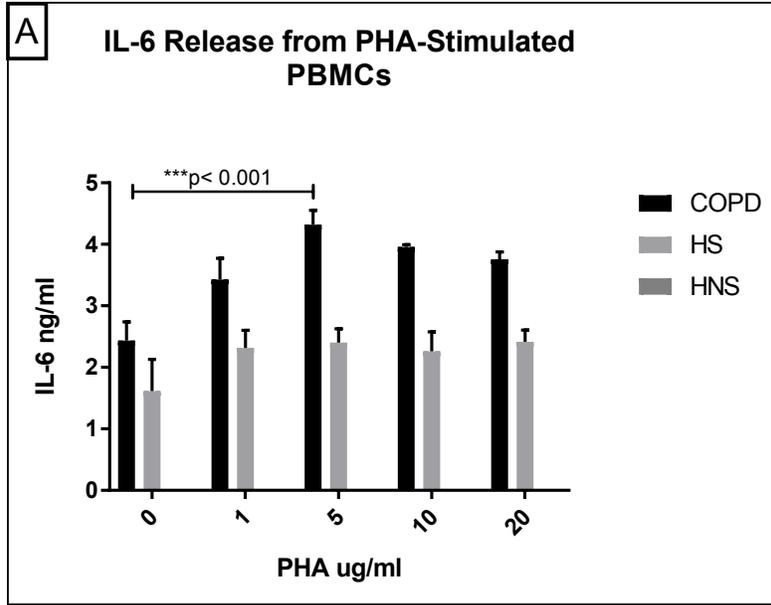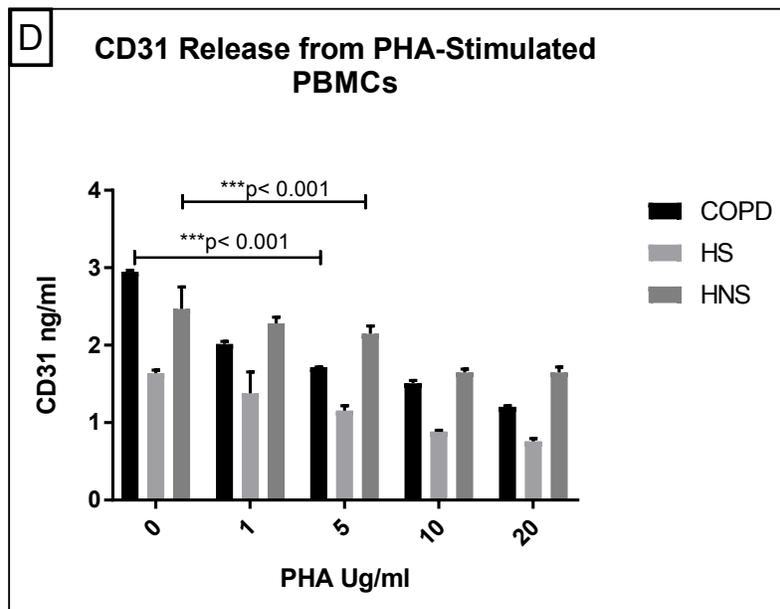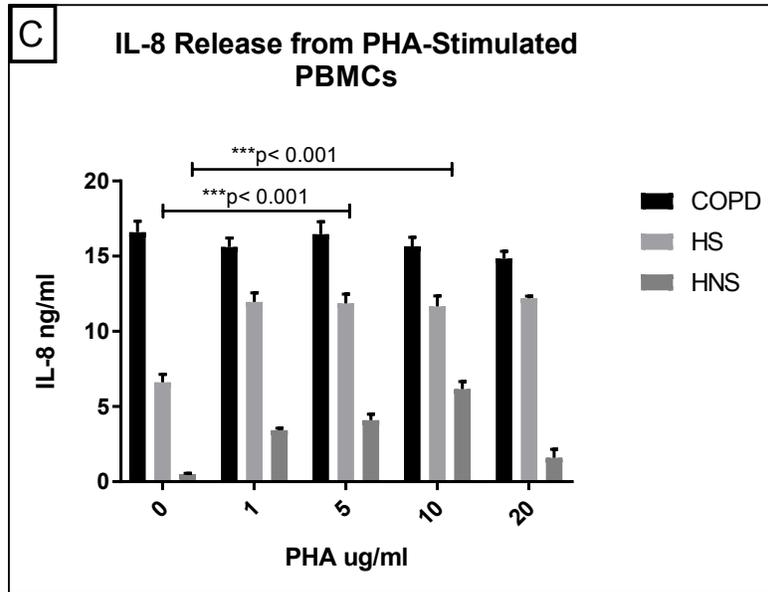
Figure 5.4: Biomarkers production from PHA-stimulated PBMCs. *ELISA assay bar graph showing effect OF PHA on IL-6 (A), TNF-α (B), IL-8 (C), CD31 (D) release of PBMCs from three studied groups. Cells were seeded in a 96-well plate, stimulated with or without increasing concentrations of PHA (1, 5, 10, and 20μg/ml) and incubated for 24hrs, 5% CO_2 at 37°C. Following the incubation period cell supernatant were recovered and assayed as triplicate through ELISA assay to determine the level of IL6, IL-8, TNF-α, and CD31 (see section 2.1.4.4).Value are expressed as mean; n=5 in each group, (error bar represent 1 SD). P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrotic factor alpha; CD31, cluster of differentiation 31; PHA, phytohaemagglutinin.*

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5.4.5 Biomarker release from unstimulated ex-vivo PBMCs

ELISA assays were performed on PBMCs to monitor secretion of pro-inflammatory biomarkers. The basal release of IL-6, IL-8, TNF-α, and CD31 was measured in PBMCs from patients with COPD, healthy smokers and healthy nonsmokers. PBMCs obtained from COPD patients, released greater amounts of IL-6 (4±0.46 ng/ml), IL-8 (16.3±0.22 ng/ml), and TNF-α (219±12 pg/ml), compared to those obtained from healthy nonsmokers (p<0.001). Similarly, the mean value and SD of IL-6, IL-8 and TNF-α in COPD patient was higher than those in healthy smokers (p <0.05). Similarly, no significant difference was observed in CD31 release in culture media from PBMCs of COPD and healthy subjects (p >0.05). In contrast, the mean value and SD of CD31 was significantly higher in healthy nonsmoking controls (3.66±0.05 ng/ml) when comparing with patients with COPD (2.96±0.11 ng/ml) and healthy smokers (1.82±0.05 ng/ml) (p <0.001). All these results are summarized in (Table 5.4) and (Figures 5.5).

Table 5.4: The mean value of biomarkers basal release from PBMCs in the studied groups.

Biomarkers	COPD	HS	HNS	P value		
				COPD vs HS	COPD vs HNS	HN vs HNS
IL6 ng/ml	4 (0.46)	2.5 (0.14)	0.16 (0.05)	<0.05	<0.001	<0.001
IL8 ng/ml	16.3 (0.22)	13.7 (0.3)	6.6 (1.07)	<0.05	<0.001	<0.05
TNFα pg/ml	219 (12)	175 (14.2)	0	<0.01	<0.001	<0.001
CD31 (PECAM) ng/ml	2.96 (0.11)	1.82 (0.05)	3.66 (0.05)	<0.001	>0.05	<0.001

Data expressed as mean (standard deviation) or as percentage unless otherwise stated. The data represents the mean ± SD from 3 experiments. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; IL-6, interleukin-6; IL-8, interleukin-8; TNFα, tumour necrosis factor; CD31, cluster of differentiation 31.
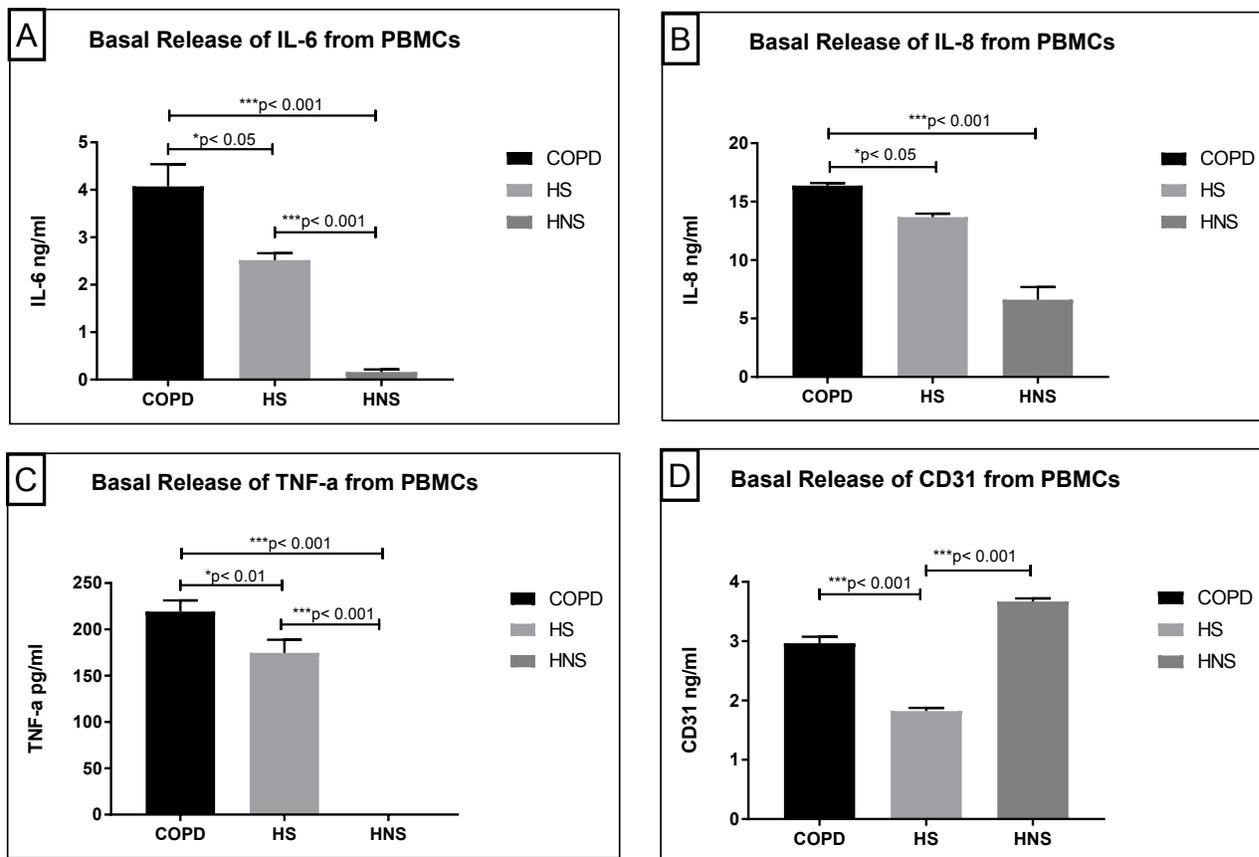
Figure 5.5: Basal releases of Biomarkers from PBMCs culture samples. *ELISA assay bar graph showing basal release IL-6 (A), IL-8 (B), TNF-α (C), CD31 (D) from PBMCs in patients with COPD, healthy smoker, and healthy nonsmokers. Cells were seeded in a 96-well plate and incubated for 24hrs, 5% CO_2 at 37°C. Then cell supernatant were recovered and assayed as triplicate through ELISA assay to determine the level of IL6, IL-8, TNF-α, and CD31 (see section 2.1.4.4). P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. Value are expressed as mean ± SD; n=5 in each group (error bar represent 1 SD). These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrotic factor alpha; CD31, cluster of differentiation 31.*

5.4.6 Effect of EPA/DHA on Inflammatory Biomarker Production in PBMCs

Production of candidate biomarkers; IL-6, TNF-α, IL-8 and CD31, from stimulated PBMCs was measured to mimic their *in vivo* natural environment. Possible anti-inflammatory properties of EPA and DHA on cellular biomarker release from PHA-stimulated and unstimulated human PBMCs were also evaluated.

5.4.6.1 IL-6

Both EPA and DHA at 100μM reduced IL-6 release in stimulated-PBMCs and unstimulated PBMCs of COPD patients and to lesser extent in healthy smokers groups in a dose-response manner. At the highest dose of EPA and DHA (500μM) IL-6 release in stimulated and stimulated PBMCs-COPD was neglible or undetectable. Stimulated PBMCs from COPD patients produced significantly higher levels of IL-6 compared to PBMCs from healthy nonsmoker group (P <0.001). In COPD patients the production of IL-6 was higher in stimulated PBMCs vs. unstimulated cells although significance was not reached (P >0.05). There was no difference in IL-6 release in stimulated PBMCs compared to unstimulated cells in healthy smokers group. IL-6 release was decrease at EPA (100μM), and was undetectable at 500μM in unstimulated cells of healthy smokers group. DHA at 500μM and 100μM reduced IL-6 release in unstimulated- and stimulated-PBMCs respectively of healthy smokers groups. The release of IL-6 from stimutlated and unstimulated EPA/DHA-PBMCs of healthy nonsmokers were udetectable (Figure 5.6).

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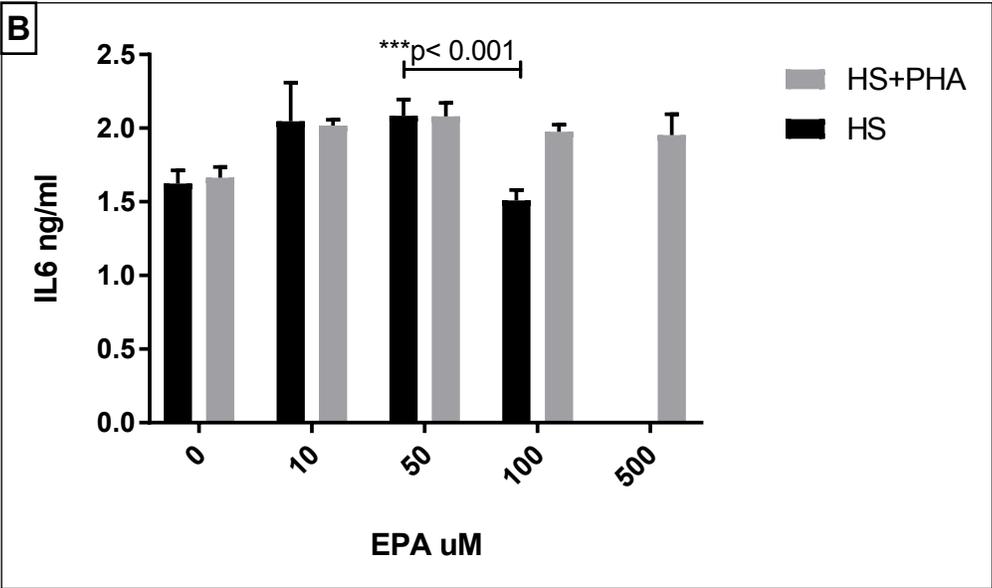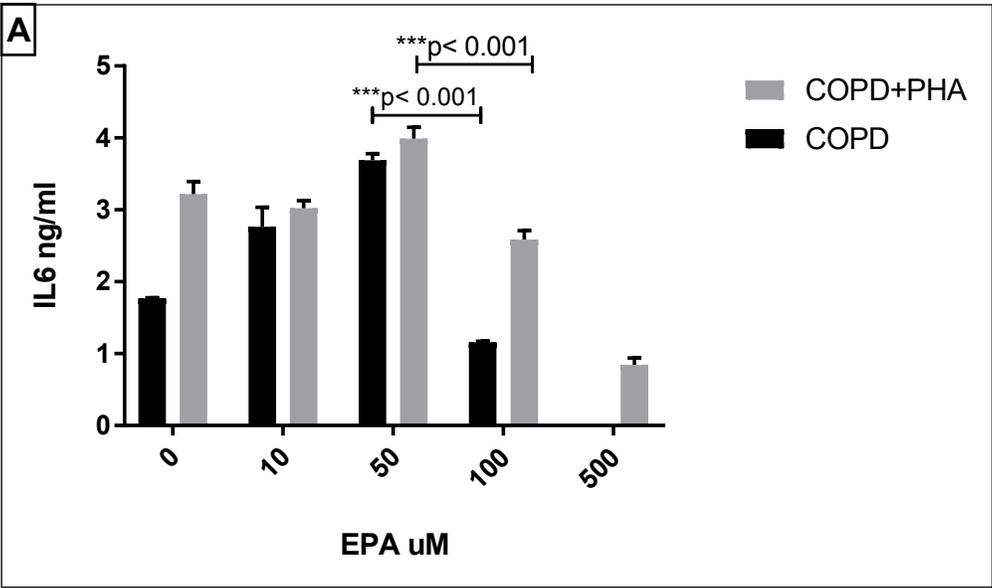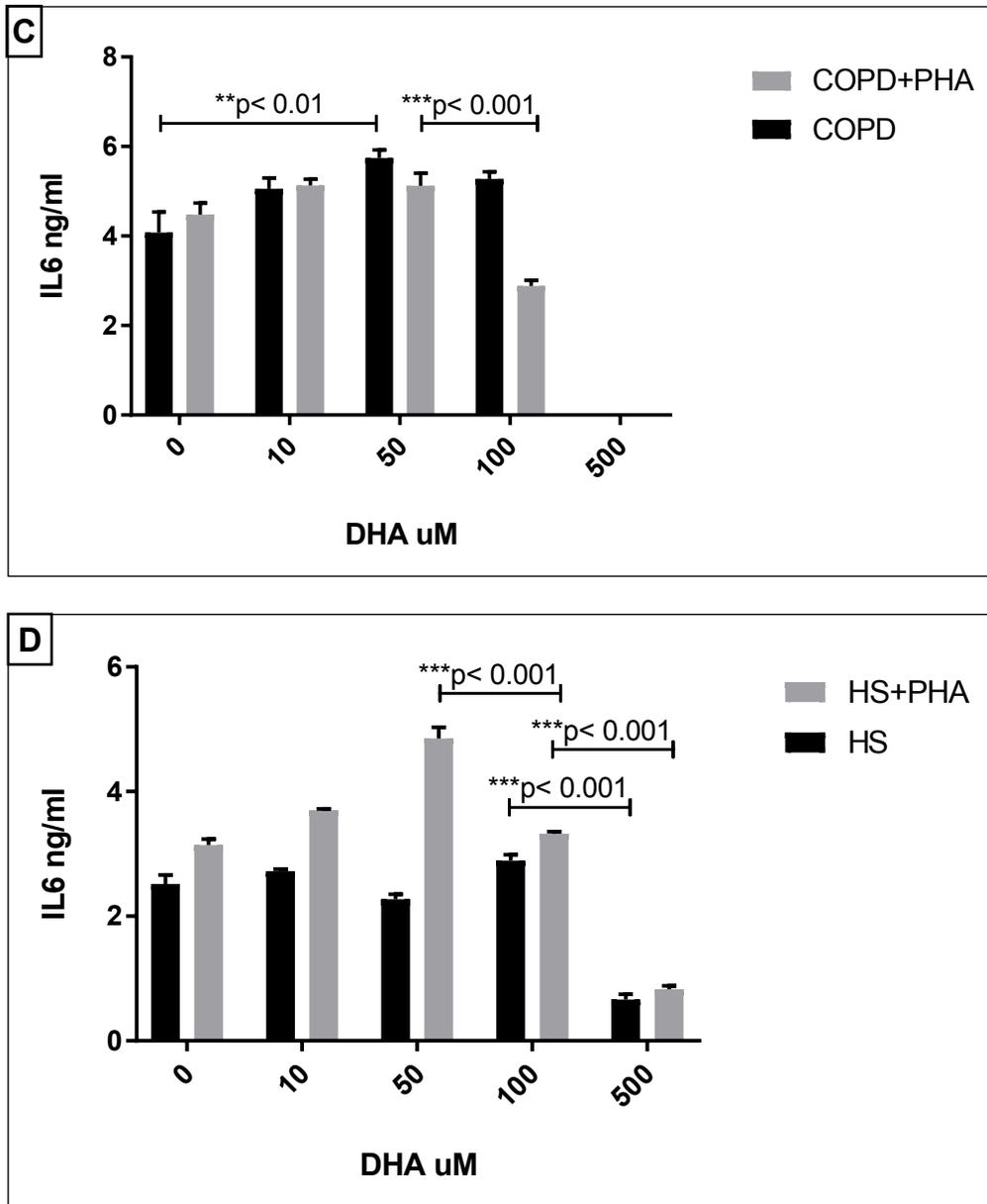
Figure 5.6:Histograms represent the Effect of EPA (A-B) or DHA(C-D) on IL-6 release in activated PBMCs. *Cells from COPD, HS, and HNS groups were stimulated or not with PHA (5μg/ml) pretreatment of EPA/DHA (0, 10, 50, 100, and 500μM) for 24hr, 5% CO$_2$ at 37°C; for each condition IL-6 release from the cell culture media were assayed as triplicate through ELISA assay (see section 2.1.4.4). Value are expressed as mean; n=5 in each group. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between concentrations. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; IL-6, interleukin-6.*

5.4.6.2 TNF-α

Both EPA and DHA at 100µM reduced TNF-α release in stimulated-PBMCs and unstimulated PBMCs of COPD patients and to lesser extent in healthy smokers groups in a dose-response manner. At the highest dose of EPA and DHA (500µM) TNF-α release in stimulated and stimulated PBMCs- COPD was neglible or undetectable. In COPD, the production of TNF-α was higher in stimulated PBMCs vs. unstimulated cells although significance was reached with ≥ 50µM EPA or DHA (P<0.05). The release of TNF-α was decreased at 100µM EPA and 500µM DHA from stimulated and unstimulated PBMCs of healthy smokers. The release of TNF-α from stimutlated and unstimulated EPA/DHA-PBMCs of healthy nonsmokers were udetectable (Figure 5.7).

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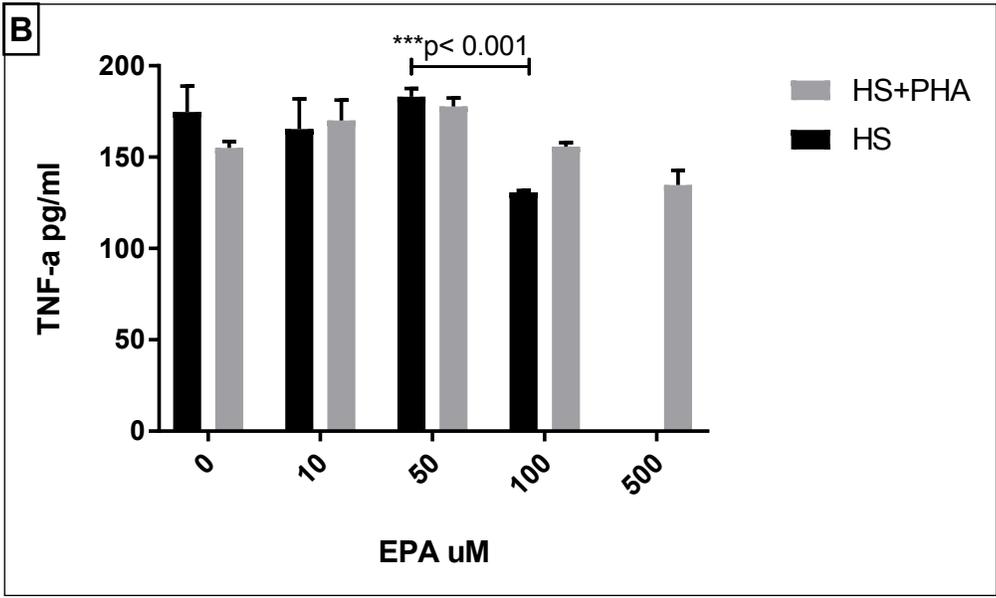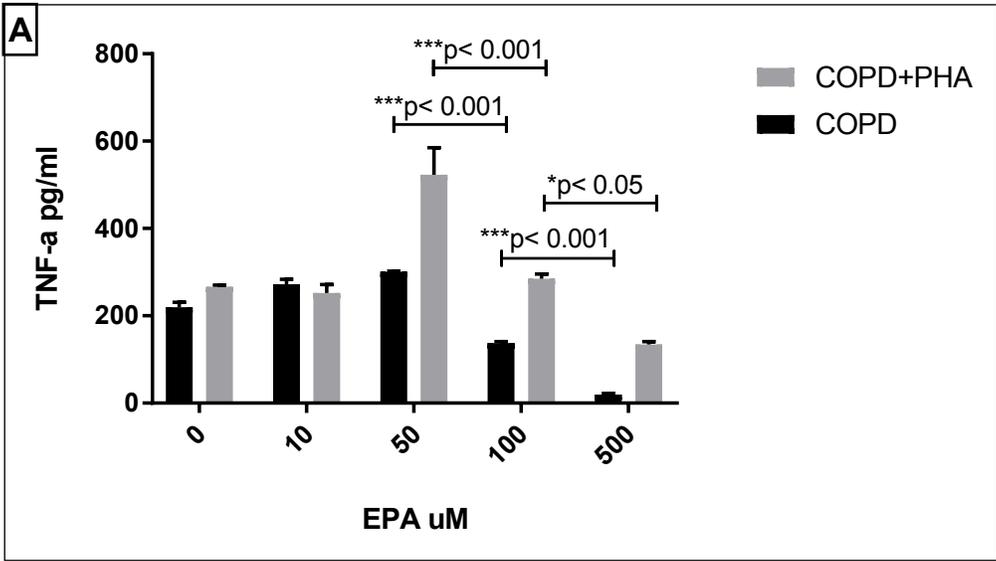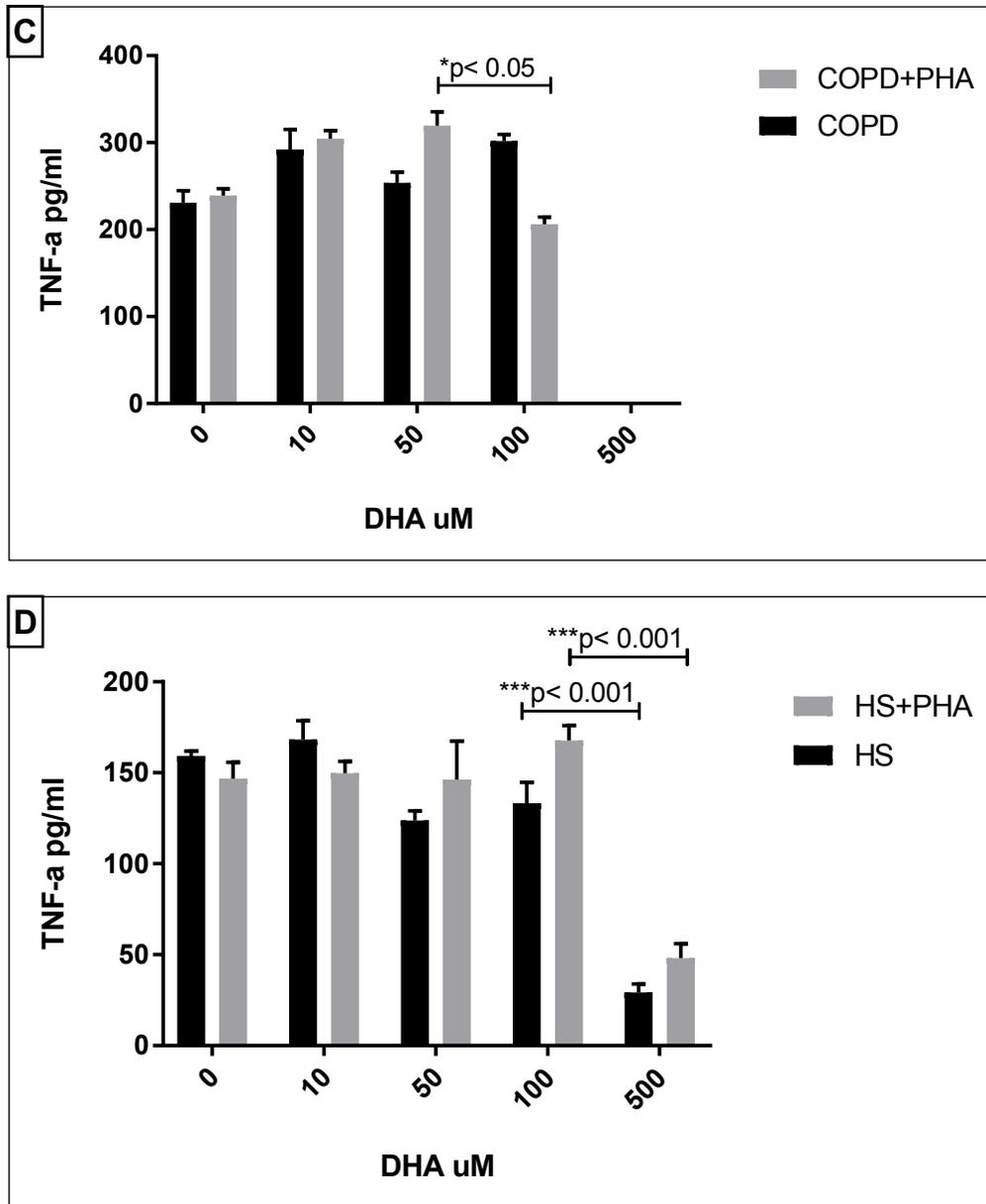
Figure 5.7: Histograms represent the Effect of EPA (A-B) or DHA (C-D) on TNF-α release in activated PBMCs. *Cells from COPD, HS, and HNS groups were stimulated in the presence or absence of PHA (5µg/ml) plus pretreatment of EPA/DHA (0, 10, 50, 100, and 500µM) for 24hr, 5% CO_2 at 37°C; for each condition TNF-α release from the cell culture media were assayed by ELISA(see section 2.1.4.4). Value are expressed as mean of three independent experiments; n=5 in each group. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between concentrations. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; TNF-α, tumor necrotic factor alpha.*

5.4.6.3   IL8

EPA at 100µM reduced IL-8 release in unstimulated PBMCs of COPD and healthy smokers groups. However, EPA treatment has no imapct on IL-8 release from PHA-stimulated PBMCs in the COPD and healthy smokers groups. The release of IL-8 from stimulated and unstimulated PBMCs of COPD group remained unaltered with DHA treatment. While DHA 500µM treatmnet reduced IL-8 release from stimulated and unstimulated PBMCs of healthy smokers. Surprisingly, DHA treatment had no effect on IL-8 production from PBMCs in the healthy nonsmokers group. The release of IL-8 from stimutlated and unstimulated PBMCs of healthy nonsmokers were changeable in the presence of EPA/DHA different concentrations (Figure 5.8).
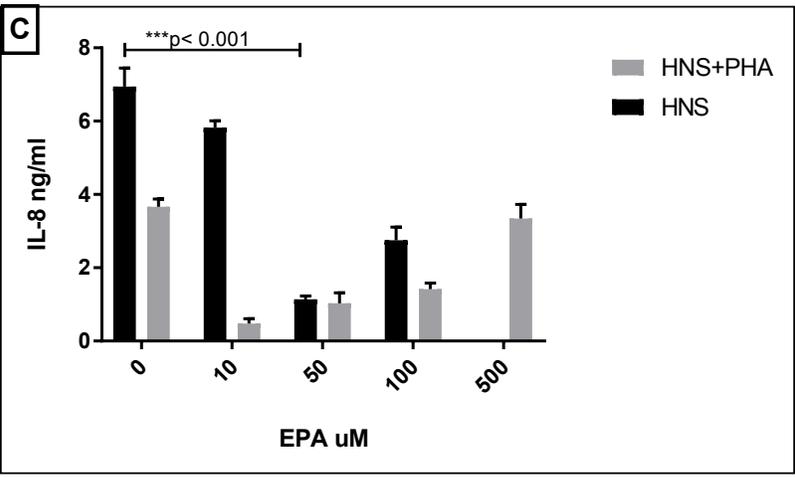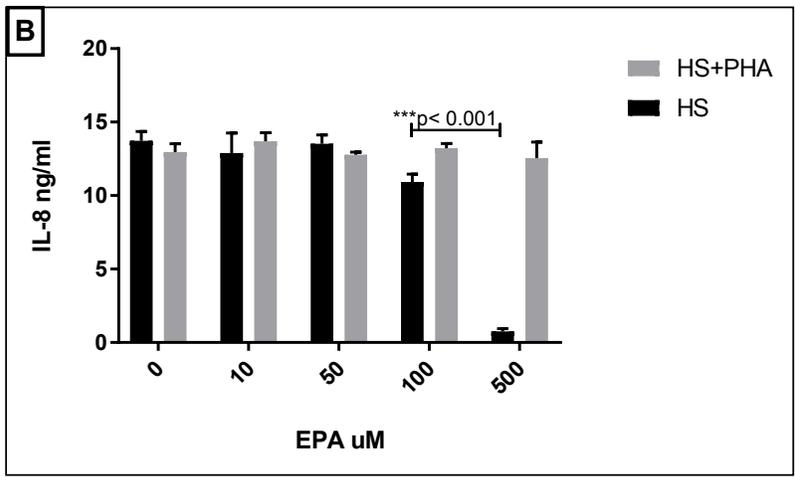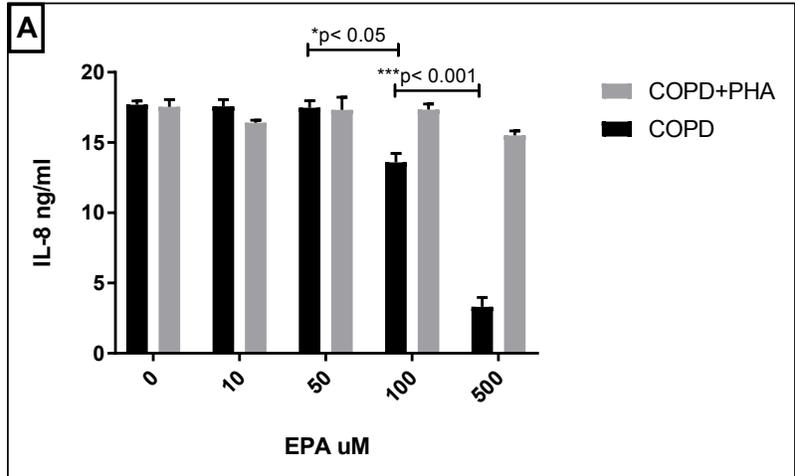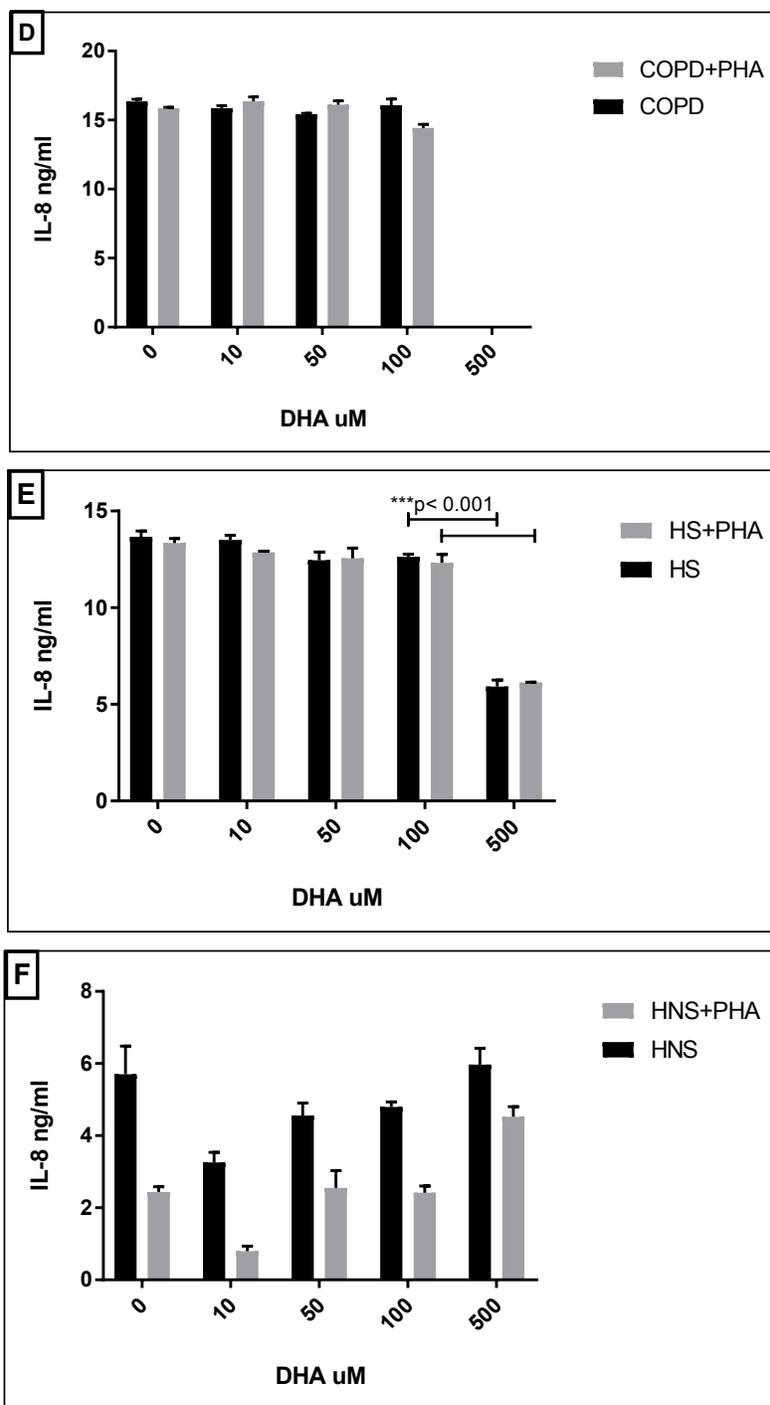
Figure 5.8: Histograms represent the Effect of EPA (A-C) or DHA (D-F) on IL-8 release in activated PBMCs. *IL-8 release were estimated by ELISA (see section 2.1.4.4). Value are expressed as mean of three independent experiments; n=5 in each group. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between concentrations. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; IL-8, interleukin-8.*

5.4.6.4 CD31

It was observed that maximal CD31 release tended to be from stimulated and unstimulated PBMCs from the healthy nonsmoking controls, compared with healthy smokers ($p<0.05$). The production of CD31 from PBMCs remained unaffected after treating the cells with either EPA or DHA (1, 5, 10, 50, 100, and 500μM) in the three study groups. However, the release of CD31 from PBMCs from healthy controls was dose-dependently suppressed by EPA although significance was not reached. There were inconsistent findings pre- and post-stimulation with CD31. Surprisingly, the production of CD31 from unstimulated-PBMCs was higher when comparing to stimulated cells treated with EPA or DHA in all the three groups (Figure 5.9).
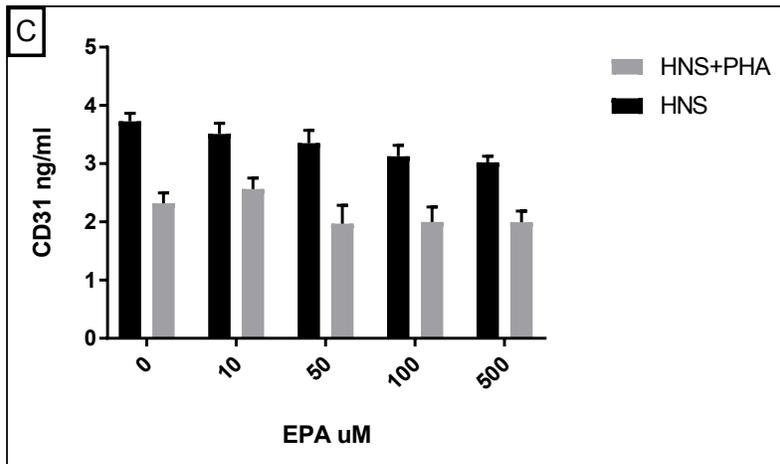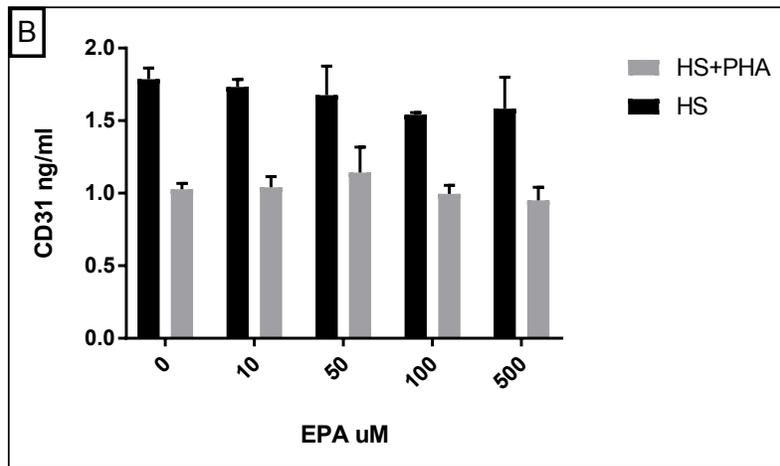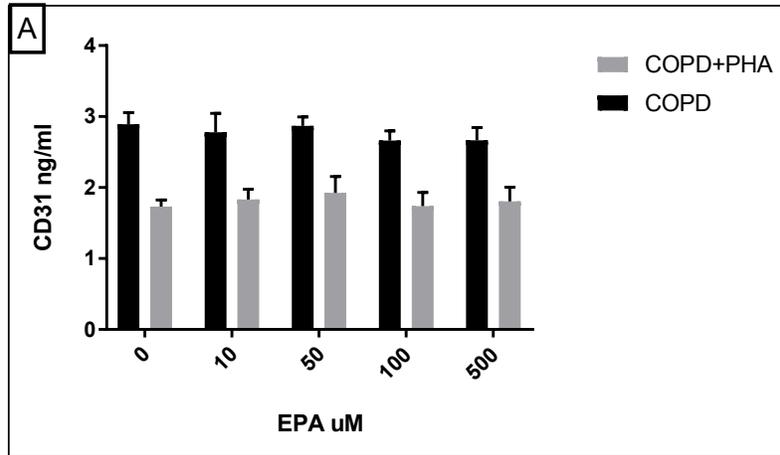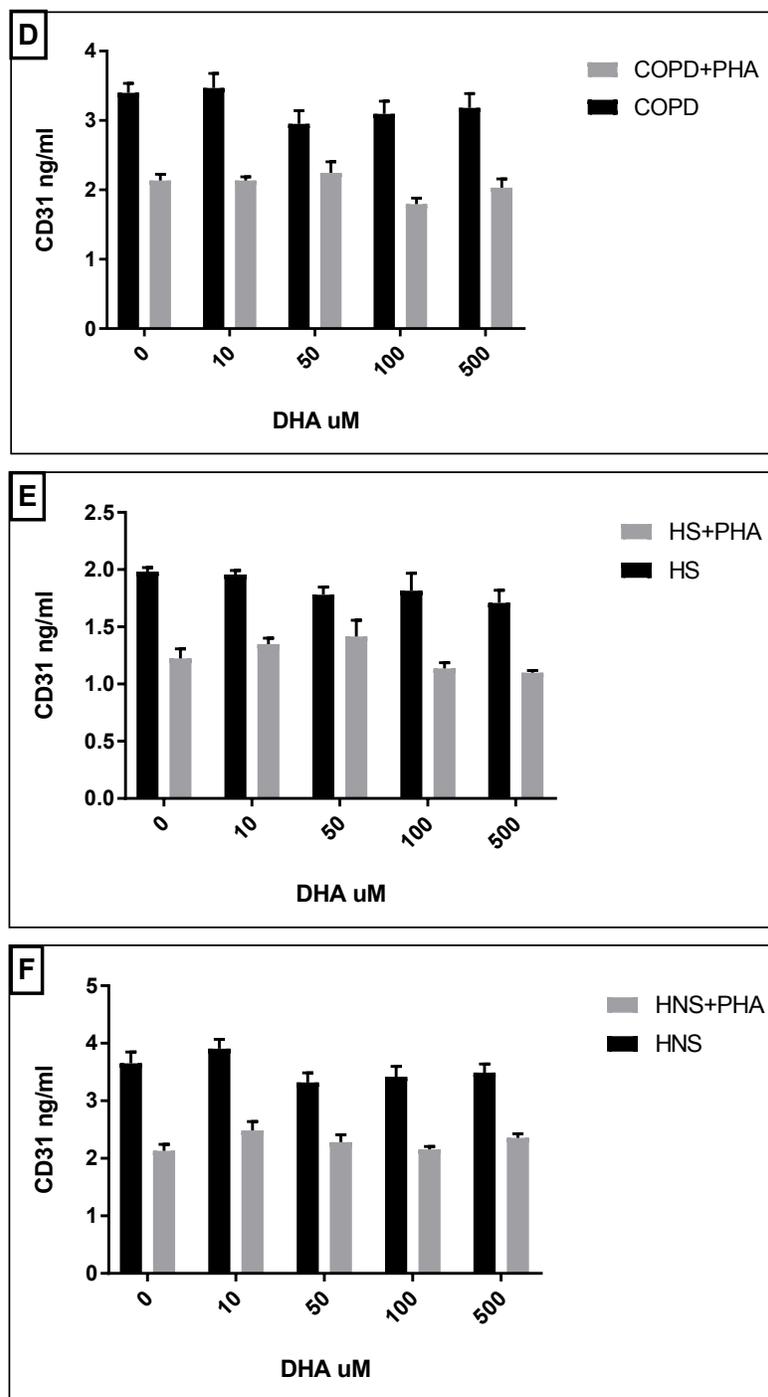
Figure 5.9: Histograms represent the Effect of EPA (A-C) or DHA (D-F) on CD31 release in activated PBMCs. *CD31 release was estimated by ELISA (see section 2.1.4.4). Value are expressed as mean from three independent experiments; n=5 in each group. P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between concentrations. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; CD31, cluster of differentiation 31.*

5.5 Discussion

Evidence from research work conducted on immune function of COPD patient indicated a close correlation between development of COPD and changes in immune function (Quan *et al* 2016). The initial inflammatory response to damage from smoking or irritant agent is characterized by neutrophils, macrophages, T-lymphocytes infiltration, and increased cytokine production such as IL-6, IL-8, and TNF-α (El-Shimy *et al.,* 2014; Korani *et al.,* 2016). Mitogen-stimulated PBMCs in culture were used as a simple *ex vivo* natural environment model to reflect inflammation in COPD. Experiments were performed with PHA as a mitogenic stimulator, since it was previously reported that PHA stimulated human peripheral blood leukocytes. It binds to cell membranes surface receptors and stimulates cell division and metabolic activity (Conti *et al.,* 2001). The selection of PHA concertation range in the current study was based on previous studies. Different studies used different PHA concentration which varied from 0.04μg/ml to 25μg/ml to trigger biomarker production from activated PBMCs (Hou *et al.,* 2010). A previous study determined that the best concentration of PHA was 20μg/ml PHA (Conti *et al.,* 2001). Moreover, Haller *et al* (2000) showed that 10μg/ml PHA was essential for the maximal PBMCs stimulation. In this study PHA various concentrations (1, 5, 10, and 20 μg/ml) were tested to find the optimal concentration of PHA mitogen which can promote a maximum amount of biomarker production from human PBMCs. It was observed that the optimum biomarkers release was seen in 5μg/ml concentration of PHA. This finding agrees with the previous observation which proved that the best concentration of PHA for the maximum production of TNF-α in the PBMCs was 5μg/ml (Molaee *et al.,* 2017). Furthermore, in a previous proteome study, PBMCs were inflammatory activated with 5μg/ml PHA (Haudek-Prinz *et al.,* 2012). Thus, 5μg/ml concentration of PHA was selected, in each case, as the optimal concentration for the human PBMCs proliferation and biomarker release.

Of the note, there were no IL-6 and TNF-α release from the control healthy subjects. This may due some cytokines local and short term effects, its degradation and its short half-life, as well as its binding to receptors and renal clearance (Korani *et al.,* 2016). Furthermore, PBMCs in healthy subject are circulating in a quiescent cell state monitoring potential immune-relevant events (Haudek-Prinz *et al.,* 2012). Hence, the production of inflammatory cytokines in PBMCs of healthy subject will be undetectable or lower than those in healthy smokers and patients.

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Candidate biomarkers production was then measured in stimulated PBMCs. The effects on biomarkers were analysed via direct detection of the relative concentration of biomarkers in the supernatant, mimicking the circulation, following experimental treatment in activated PBMCs (Ai *et al.,* 2013). In this study, only four biomarkers from a long candidate list; IL-6, TNF-α, IL-8 and CD31, were detectable by ELISA assays in PBMCs supernatant samples of the study cohort. It was hypothesized that the analysis of these different cytokines and chemokines may assist in understanding the inflammatory patterns that underlying the disease. The potential for an immunomodulatory effect of the PUFAs EPA and DHA on inflammatory biomarker release has therapeutic implications and therefore was also studied using the model.

Many studies have investigated the role of the immune inflammatory cells in the lungs in COPD (Barnes 2016). PBMCs act as a critical component in the immune system to fight infection and play an important role in the development of COPD (Wu *et al.,* 2014). It was previously reported that circulating T-cells of COPD patients are abnormally activated and stimulate pro-inflammatory cytokines production that is involved in COPD pathogenesis. This may due to the migration of lymphocytes between inflammatory sites includes lung and systemic circulation via lymph nodes. Also peripheral T-cells functions correlate with the severity of COPD. Hence, impaired lymphocyte function in peripheral blood of COPD patients may have a pathological impact on the lung (Zhu *et al.,* 2009). Inflammation in COPD characterized by increased numbers of CD8+ T lymphocytes, neutrophils and macrophages in small and large airways and in lung parenchyma and pulmonary vascular system. The increased numbers of T-cells that present in the alveolar walls are correlated with COPD severity (Paats *et al.,* 2012). Alveolar macrophages release of inflammatory cytokines, such as IL-6, that attract neutrophils into the airways. IL-6 regulates many pathways that could contribute to its effect on inflammatory disease progression. During CD4 T-cell differentiation, IL-6 promotes IL-17 and IL-21 production, and suppresses regulatory T-cell function (Ferrari *et al.,* 2013).

It was important to compare the release of inflammatory mediators from unstimulated PBMCs to investigate if PBMCs in COPD show an increased release compared with healthy smokers and healthy nonsmokers. Basal levels of TNF-α and IL-6, IL-8, were selected for measurement as these cytokines are the most important produced by PBMCs, and are implicated in several inflammatory conditions. Results show that there was a significant difference in basal

biomarkers release from PBMCs between the COPD and healthy nonsmokers groups. PBMCs obtained from COPD patients, released greater amounts of IL-6, TNF-α, and IL-8 compared to those obtained from healthy nonsmokers. Together, these observations suggest that PBMC may be pre-activated in the circulation in COPD patients, which agrees with findings from several previous studies, and supports the notion of systemic inflammation being important in COPD (Koethe *et al.,* 2000; Erik-Jan *et al*., 2006; Kawayama *et al.,* 2016). Pro-inflammatory cytokine IL-6 is involve in the homeostasis of immune response, regulate the immune response, and prevent inflammatory and autoimmune pathologies (Aldonyte *et al.,* 2003). Furthermore, IL-6 initiates the acute phase response, while IL-6 trans-signaling controls macrophage recruitment during acute inflammation (Ravi *et al.,* 2014).

TNF-α is a potent inflammatory cytokine that can induce inflammatory effects in COPD resulting in the activation of various immune cells, including neutrophils and monocytes (Barnes 2016). TNF-α is involved in neutrophil chemotaxis and migration by inducing the expression of IL-8, which is essential for neutrophil recruitment in COPD. TNF-α is secreted by LPS-stimulated macrophages, monocytes, fibroblasts, T-cells and endothelial cells. Further, overexpression of TNF-α in animal models study induces pathological changes similar to those of pulmonary emphysema and fibrosis (Mukhopadhyay, *et al.,* 2006). Elevated level of TNF-α in serum and sputum of COPD patients suggest an important role for TNF-α in COPD (Queiroz *et al.,* 2016; Korani et al., 2016). However, studies on TNF-α release by PBMCs of COPD patients remain limited. The current study finding of increased TNF-α release by PBMCs in COPD patients when compared with healthy controls is supported by Paats *et al* (2012). Likewise, Increased TNF-α and IL-8 release from PBMCs of COPD patients has been also reported in COPD patients when compared to healthy controls (Lee *et al.,* 2012; Meng *et al.,* 2016).

These baseline differences in the release of pro-inflammatory biomarkers might be important parameters in the chronic inflammatory processes in COPD. Systemic inflammation is highly associated with COPD severity. Dysregulation of cytokine production is notable in PBMCs from COPD patients. Meng *et al*. (2016) also demonstrated that normal cells could be stimulated to become pro-inflammatory by exposing them to the serum of COPD patients, whereas PBMCs from COPD patients were pro-inflammatory and activated even in the absence of serum.

Cigarette smoking is a major risk factor for COPD and can cause airway inflammation characterized by neutrophil, macrophage, and activated T lymphocyte infiltration result in increased cytokine concentrations, including TNF-α and IL-8 (Tanni *et al.,* 2010). A previous *in vitro* study reported that cigarette smoke enhanced the production of IL-8 from monocytes in a time- and concentration-dependent manner (Sarir *et al.,* 2007). Smoking also associated with excessive oxidative stress of lung structural cells, and breakdown of connective tissue leading to impaired gas exchange and elastic recoil (Robers *et al.,* 2015). In this study, it was observed that the release of inflammatory mediators from PBMCs of healthy smokers was also increased, compared with healthy nonsmoker, but this increase was not enough to reach significance. This suggests that circulating immune cells like PBMCs may be activated by smoking, with or without COPD, and that smoking may directly or indirectly increase systemic inflammation. Our results show that inflammation is present in the blood of smokers and patients with COPD (Kawayama *et al.*, 2016).

In contrast, CD31 release was maximal in the healthy nonsmokers rather than in COPD or healthy smokers. This glycoprotein membrane is highly expressed on vascular endothelial cells and to different degree on monocytes, neutrophils, B lymphocytes, some T lymphocyte subsets (Falati *et al.,* 2006). Leukocyte transmigration is a main event during tissue inflammation and is a process that is mediated and controlled by different mediators and adhesion pathways. CD31 mediate leukocyte trans-endothelial migration and inflammation via its ability to inhibit the activation of circulating platelets and leukocytes. It regulates migration of leukocytes from the vascular lumen into inflammation site which is essential for both innate and adaptive immunity, to enable tissue to respond to the inflammatory stimuli. It was reported that the expression of CD31 in *in vivo* animal model studies was found to be reduced on emigrated leukocytes (Woodfin 2007). This might explain the reduced release of CD31 in the COPD group as increased number of leukocytes including PBMCs migrated from circulation to the lung. However, evidence that could confirm this is currently lacking. On the other hand, Higgins *et al* (2008) reported that delayed processing of peripheral blood sample or PBMCs isolation and cryopreservation can lead to the detection of higher levels of CD31 expression on naive CD4 T cells in both HIV[+] and HIV[-] subjects. This could also account for the reported findings.

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Components of both innate and acquired immunity are known to be affected by omega-3 PUFA. EPA and DHA omega-3 PUFA are modulators of lymphocyte functions, and T-cells are the main lymphocytes involved in cell-mediated immunity. The serum level and diet content of EPA and DHA may affect lymphocyte proliferation, chemotaxis, adhesion molecule expression, leucocyte-endothelial adhesive interactions, cytokine production, and other immune cells functions (Calder 2012). Also it can affect the ability of inflammatory immune cells to produce eicosanoids, such as prostaglandin E2, which are involved in immuno-regulation. Both fatty acids are incorporated into the membranes of inflammatory cells from where they may influence cell signaling, transcription factor activation, and gene expression (Calder 2015). This study investigated the immunomodulatory effect of EPA or DHA on *ex-vivo* mitogen-stimulated PBMCs. Data from the current study show that inflammatory markers production from PBMCs is influenced by omega-3 PUFAs. It was found that 100μM EPA and DHA alone, decreased IL-6, TNF-α, and IL-8 secretion in both unstimulated and activated PBMCs from COPD patients. These results echo those reported by Jaudszus *et al.* (2013). Those authors speculated that treating PBMCs with EPA or DHA do not suppress immune cells function but rather promote resolution by stimulating the production of pro-resolving cytokines. This was not tested in the current study. To test that, anti-inflammatory cytokines such as IL-10 which known to have immuno-regulatory and suppressive function on T-cells, could be measured. Further, Jaudszuz *et al.*, found both EPA and DHA at 100μM increased anti-inflammatory cytokines IL-10 in monocytes without affecting TNF-α and IL-6 production. While EPA and to lesser extent DHA reduced TNF-α release in T-cells. In contrast, CD31 production remained unaffected by EPA or DHA in the three study groups. However, it was observed that the release of CD31 from healthy control PBMC was dose-dependently suppressed by EPA, without reaching significance. Thus, both EPA and DHA did show an immunomodulatory activity against activated PBMC, suggesting they may have therapeutic value. In the current study, there were no IL-6 and TNF-α release from the control healthy subjects. Previous works failed to report strong dose–response effects of EPA and/or DHA on the outcomes measured and suggest that healthy subjects are relatively insensitive to immunomodulation with omega-3 PUFA. While in patients with inflammatory conditions cytokine concentrations or production are influenced by the immunomodulatory effects of EPA and/or DHA supplementation. This difference could be due to depletion of the buffering capacity present in healthy controls, as a result of a higher turnover

rate of immune cells in an inflammatory disease and of the fatty acids in immune cell phospholipids composition for use as substrate for eicosanoid synthesis or as ligands for transcription factors (Sijben and Calder 2007).

Moreover, studies that analysed the effect of EPA and/or DHA on inflammatory cytokines in stimulated-PBMCs, in relation to clinical status in COPD patients are extremely rare (Jaudszus et al., 2013). Previous studies with PBMC from normal subjects have used oral supplementation via fish oil capsules, purified EPA or DHA supplements, fish oil-enriched diets or omega-3 PUFA enriched foods and generally did not find an effect. Reasons for this are not clear but may include: a low omega-3 PUFA therapeutic dose, since actual blood levels were not measured, or known before treatment; small sample size; short study duration; and the large variability between individual outcomes. The presence of an inflammatory disease like COPD might increase the sensitivity to the immunomodulatory effects of EPA and DHA (Sijben and Calder et al., 2007; Miles and Calder 2012).

Only a limited number of previous investigations have specifically examined the characteristics and functions of circulating PBMCs among COPD patients, and findings from these studies are often inconsistent. This may due to the differences in experimental methodologies, small sample size and variability in disease severities (Zhu et al., 2009). The data reported here support what *in vitro* previous studies have shown using human PBMC to assess agent-induced changes in endpoints like cells proliferation and cytokine production, which will certainly contribute to a better understanding of the effects of omega-3 PUFA on immune cells function. Blood is a good source for biomarker discovery; however it is difficult to understand the contributions of individual cells in whole blood (Roberts *et al.,* 2015). There was a limitation in the current study which worth mentioning. There was a lack of investigating the effect of EPA or DHA concertation values lies between 200-400 µM, as the release of some cytokines from cells treated with 500 µM EPA/DHA was undetectable. This may due to the effect of high concentration on PBMCs viability which was not tested in the current study.

A more comprehensive study using additional stimuli such as cigarette extract followed by novel inflammatory mediators' investigation in PBMCs sub-population or proteomics analysis might provide further understanding of the complex interplay between stimuli and cellular response. EPA and DHA gave rise to a variety of monohydroxylated derivatives of highly anti-

inflammatory and protective properties, such as resolvins and their precursors (Jaudszus *et al.,* 2013). Recent study by Mochimaru et al. (2018) revealed that 12-OH-17,18-EpETE, an EPA-derived endogenous metabolite, may overcome limitations of dietary EPA and DHA supplementation and provide novel options for the treatment of asthma and airway inflammatory diseases. There would be merit in investigating these bioactive lipid metabolites for their anti-inflammatory effect on PBMCs in COPD.

5.6   Conclusion

The findings presented here indicate that circulating PBMCs isolated from COPD patients show a profile of pro-inflammatory biomarkers distinct from control PBMCs. At the basal level PBMCs from patients produce significantly more inflammatory cytokines; IL-6, TNF-α, and IL-8, when compared to healthy controls. An impact from smoking can be seen in the increased release of inflammatory cytokines in PBMCs from healthy smokers when compared with healthy controls. The data confirmed that EPA and DHA individually exerted potential immunomodulatory effects characterized by significant reductions in PHA-induced cytokines release. The results here agreed with those *in vivo* previous findings and indicated that the EPA and DHA affected immune cells. Additionally, these observation may contribute to understanding of the regulatory action of EPA and DHA in immune cells and, therefore, to increasing knowledge in terms of fatty acid assessment from an immunological point of view. The findings suggest pre-activation of circulating inflammatory cells such as PBMC exists in patients. COPD is a complex disease that can be influenced by multiple factors such as genetic predisposition, cigarette smoking, and bacterial or viral infection (Sandford and Silverman 2002), and these results support the assumption that COPD is a multicomponent disease which involves systemic inflammation (Kawayama *et al.,* 2016). Importantly, the results suggest that DHA and EPA omega-3 PUFA may have an immunomodulatory impact on activated immune cells during inflammation and that further investigation of these actions in COPD are justified.

6. Chapter 6: Investigating baseline fatty acid profiles from erythrocytes (RBC) in the study cohort and the association with COPD

6.1 Background

Poly unsaturated fatty acids (PUFA) specifically omega-3 PUFAs, also known as ω-3 fatty acids or n-3 fatty acids, are important substrates for energy production and storage, and also involved in structural and functional properties of body cells. They are essential components of cell membranes and affect biological membrane structure and functions, including membrane fluidity, and subsequently organ functions. For example, different immune cells functions, such as secretion, chemotaxis, and sensitivity to microorganisms depend on the fluidity of the membrane. Fatty acids also mediate cellular metabolism and signalling pathways and contribute to the development and maintenance of body tissues (Allaire *et al.,* 2017; Schober *et al.,* 2017; (Antus and Kardos, 2015). Alteration in the PUFA composition of the cell membranes may affect the activity of membrane-bound enzymes and the expression of receptors, the membrane permeability and transport Properties. PUFA are substrates for synthesis of lipid inflammatory mediator eicosanoids which are involved in the inflammation progression in pulmonary diseases. Eicosanoids like; prostaglandins, leukotrienes, thromboxanes, are also the final products of the metabolism of PUFAs (Barnes 2008; Celli *et al.,* 2012; Novgorodtseva *et al.,* 2013).

Concentrations of all major fatty acids can be measured in the RBC membrane, whole blood and plasma. The major PUFA include EPA (20: 5n-3), DHA (22: 6n-3), and ARA (20: 4n-6). Whereas omega-3 PUFA, DHA, is essential for vision, brain neurons, and cell signaling, EPA is involved in vascular blood flow and down-regulates the production of ARA metabolites to maintain homeostasis. Several studies suggest that high intake of omega-3 fatty acid to low ratio of omega-6 fatty acid is reducing the risk of some of the chronic diseases that are associated with inflammation. It is known that diet enriched with omega-3 PUFAs tend to be positively associated with a range of health benefits especially for CVD. However, the mechanisms by which these PUFAs are involved in delivering health benefits are still unknown (Calder 2008; Bell *et al.,* 2011). It is known that western diets contained higher omega-6 ratio than omega-3 PUFA. However the balance between omega-6 and omega-3 PUFA is important for brain

development and function, and in decreasing the risk for chronic diseases. A recommended ratio of 1:1 to 2:1 omega-6/omega-3 PUFA should be the target ratio for health benefits (Simopoulos 2010).

Omega-3 PUFA, DHA and EPA are known to be anti-inflammatory mediators that are involved in several mechanisms including altering cell membrane phospholipid fatty acid composition, and stimulating anti-inflammatory and inhibiting pro-inflammatory transcription factors. Further, both have been shown to be effective in improving different inflammatory conditions including COPD (Calder *et al.,* 2018). Multiple studies suggesting that using a mixture of EPA and DHA in different forms and proportions may associate with a positive impact on inflammatory biomarkers. However, the specific effects of EPA and DHA on inflammation pathways in COPD is less understood (Allaire *et al.,* 2017).

An Omega-3 Index is considered as a parameter that reflects the dietary intake of omega-3 PUFA. It is the measurement of the amount of EPA and DHA present in RBCs membranes and is expressed as a percentage to the total fatty acids contents (Harris 2008). A high omega-3 Index (8–12%) has been associated with a lower risk of CVD and mortality in epidemiological studies. Supplementation with EPA and DHA is recommended by several health agencies including the American Heart Association for secondary coronary heart disease prevention or management of plasma triglycerides. Long-term supplementation with EPA and DHA would increase omega-3 Index value. Data from randomized double-blind study shows increase in the omega-3 Index is greater with high dose DHA, (10 weeks with 2.7 g/d), supplementation than with high dose EPA (Allaire *et al.,* 2017). Unlike CVD, there is not much evidence in the literatures that analysed the effect omega-3 index in COPD patients. To our knowledge there is no estimated omega-3 index value for COPD.

It is well known that weight loss and muscle wasting in patients with COPD affect respiratory and muscle function, exercise capacity, and health status. Few studies suggest that nutrition supplemented with high doses of PUFAs may improve body composition and exercise capacity and reduce inflammatory activity (Sugawara *et al.,* 2010). In an 8-week randomized, controlled trial, daily supplementation with EPA, DHA, and ALA in 80 patients with COPD significantly improved exercise capacity (Broekhuizen *et al.,* 2005). In another randomized, controlled trial, daily supplementation with omega-3 PUFAs with low intensity exercise for 12

weeks improve the outcomes of exercise capacity and health quality of life in malnourished patients with COPD (Sugawara *et al.,* 2010). Cachexia is a condition frequently present in malnourished patients with COPD, heart failure and cancer, can cause weight loss, muscle weakness, fatigue, and reduced mobility. In 12-week double-blind, randomized, controlled trial, a targeted medical nutrition containing high dose omega-3 fatty acids, vitamin D, and high quality protein has positive effects on blood pressure, blood lipids and on exercise-induced fatigue and dyspnea. Therefore, such medical nutrition could be clinically beneficial for pre-cachectic and cachectic patients with COPD (Calder *et al.,* 2018).

Research studies are lacking in information about the qualitative and quantitative composition of fatty acids of RBCs membranes in COPD patients. In addition, the few available studies are significantly differing in their findings, which may be related to the different samples and methods applied for fatty acids analysis and variability between patients disease severity (Novgorodtseva *et al.* 2013). There is very limited information on the effect of omega-3 PUFA on lung function and inflammation in COPD.

To begin to address this, the present study compares data from RBC fatty acid analysis including the following parameters; ARA/EPA ratio, the percentage of omega-3 PUFA/Total PUFA ratio, and Omega-3 Index between the three groups; patients with COPD, healthy smokers and healthy nonsmokers. Analysis were conducted to elucidate the potential relationship between measurements of RBC fatty acid analysis with chronic inflammation, pulmonary function outcomes (preFEV1% and pre FEV1/FVC ratio) and the impact of smoking on blood lipid profiles in patients with COPD.

6.2 Objectives

- To analyse the association between fatty acid profiles in RBC and the risk of COPD. Data from the three subject groups will be compared.
- To identify the association of omega-3 and omega-6 PUFA, with chronic inflammation and respiratory function outcomes in patients with COPD

6.3 Methods

6.3.1 Determination of RBC Fatty Acid Profile of Participants

The most reported biological sample used in analysis of fatty acid composition that reflect nutritional status is RBCs. Analysis of erythrocyte fatty acid has been used to measure the status of the PUFA, especially EPA and DHA. A previous study showed good correlations, between erythrocyte and whole blood in fatty acids concentrations (Bell *et al.,* 2011). Using RBC membrane for fatty acids determination is better than plasma, for reflecting long term dietary PUFA intake and body status with low individual biological variability. While plasma fatty acids analysis is more sensitive to recent food intake (Sun *et al.,* 2007; Harris and Thomas 2010). The profile of fatty acids was measured in RBC fractions separated by Ficoll density gradient protocol from 42 EDTA-anticoagulated blood samples of the entire study cohort, and was stored at -80°C for analysis (See chapter 2 for details). RBCs aliquots samples contain 0.5ml were sent to the nutrition group laboratories at the University of Stirling for fatty acid.

The lipid extraction of erythrocytes was performed by lab technicians in University of Stirling according to Bell *et al* (2011). It is non-invasive, economical, and accurate assay for of PUFA quantification. A single RBCs drop onto Whatman collector cards was air-dried. Then the RBCs sample was detached from the collection device using forceps and placed into a screw cap vial containing 1ml of a methylating solution (1.25 M-methanol–HCl). The vials were sealed and incubated in a hot block at 70°C for 1h, and then cooled to room temperature. Later, 2ml of distilled water and 2ml of saturated HCl solution were added. Fatty acid methylesters (FAME) were then extracted by adding 2×2ml of isohexane. FAME were separated and quantified by GLC using a 60m × 0·32mm × 0·25mm film thickness capillary column. H_2 was used as a carrier gas at a flow rate of 4ml/min and the temperature range was from 50 to 150°C at 40°C/min then to 195°C at 2°C/min, and finally to 215°C at 0·5°C/min. Individual FAME was identified by comparison with well-characterised in-house standards as well as commercial FAME mixtures. FAME composition was reported as weight percent of total identified FAMEs. All samples were analyzed together, and all samples from each subject were analyzed in the same batch to minimize analytical variability. The precision of the RBCs fatty acid analysis was conducted by measuring triplicate samples over four successive days to provide mean and

standard deviation and CV%. The ratio of ARA: EPA as well as the percentage of omega-3 PUFA/total PUFA, and Omega-3 index has been measured. This assay provides a reliable method of PUFA quantification with the percentage of omega-3 PUFA value providing a potential blood biomarker for large-scale nutritional trials.

However, specific and sensitive, less time consuming and cost-effective methods are required to measure body fatty acid profiles and to monitor their changes during clinical trials supplementation. A new method for fatty acid analysis was developed by Schober *et al* (2017), where they combined chemical ionization and tandem mass spectrometry after gas chromatography separation for measurement of fatty acid. This method is highly specific, sensitive, and rapid to analysis of fatty acid in biological specimens such as erythrocyte membranes.

6.3.2 Statistical Analysis

Laboratory data were expressed as standard deviation of the mean (SD). The D'Agostino–Pearson omnibus normality test was performed to test normality of the data distribution. All data were expressed and graphed as mean ± standard deviation. For data normally distributed, one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test was performed to determine whether the differences between the groups were statistically significant. Differences were considered significant if the probability (P) value was <0.05, highly significant if P value was <0.01 and P value <0.001, and insignificant if P value was >0.05. Correlation analyses of two variables were carried out using Pearson or Spearman methods, depending on the normality of the data distribution. All statistical analyses were performed using graphPad Prism and graphPad InStat software, version 7.04 and 3.10.32 respectively.

6.4 Results

6.4.1 Comparison of Fatty Acids between the Studied Groups

RBC membrane PUFA composition was analysed at baseline and values were expressed as mean (SD). The data for of ARA: EPA ratio as well as the percentage of omega-3 PUFA/total PUFA, and Omega 3 index of the studied groups measured in this study is listed in Table 6.1. The mean (SD) value of RBCs membrane levels of omega-3 index were 5.6 (1.7), 5.3(1.4) and 5.6 (1.3) for COPD, healthy smokers and healthy nonsmokers groups, respectively. The mean (SD) value of the percentage of omega-3 PUFA/total PUFA% were 27.2 (5), 25.7 (4) and 27.3 (3) for COPD, healthy smokers and healthy nonsmokers groups, respectively. The mean (SD) of ARA/EPA ratio were; 20 (8.5) for COPD, 20.8 (6.5) for healthy smokers and 20.2 (5.5) for healthy nonsmokers groups (Table 6.1). There were no significant differences in fatty acids measurements (ARA/EPA, omega-3 PUFA/total PUFA% and Omega 3 index) between the COPD group, healthy smokers and healthy nonsmokers groups. Although the percentage of omega-3 PUFA/Total PUFA was lower numerically in healthy smokers group compared to patients and healthy nonsmokers, it did not reach statistical significance (Figure 6.1).

Table 6.1 Comparison of RBC fatty acids composition between the studied groups

Studied Group	COPD	HS	HNS
%Omega-3 PUFA/Total PUFA	27.2 (5)	25.7 (4)	27.3 (3)
ARA/EPA	20 (8.5)	20.8 (6.5)	20.2 (5.5)
Omega-3 Index	5.6 (1.7)	5.3 (1.4)	5.6 (1.3)

The data expressed in term of mean (standard deviation) unless otherwise stated. These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers; PUFA; poly unsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid.
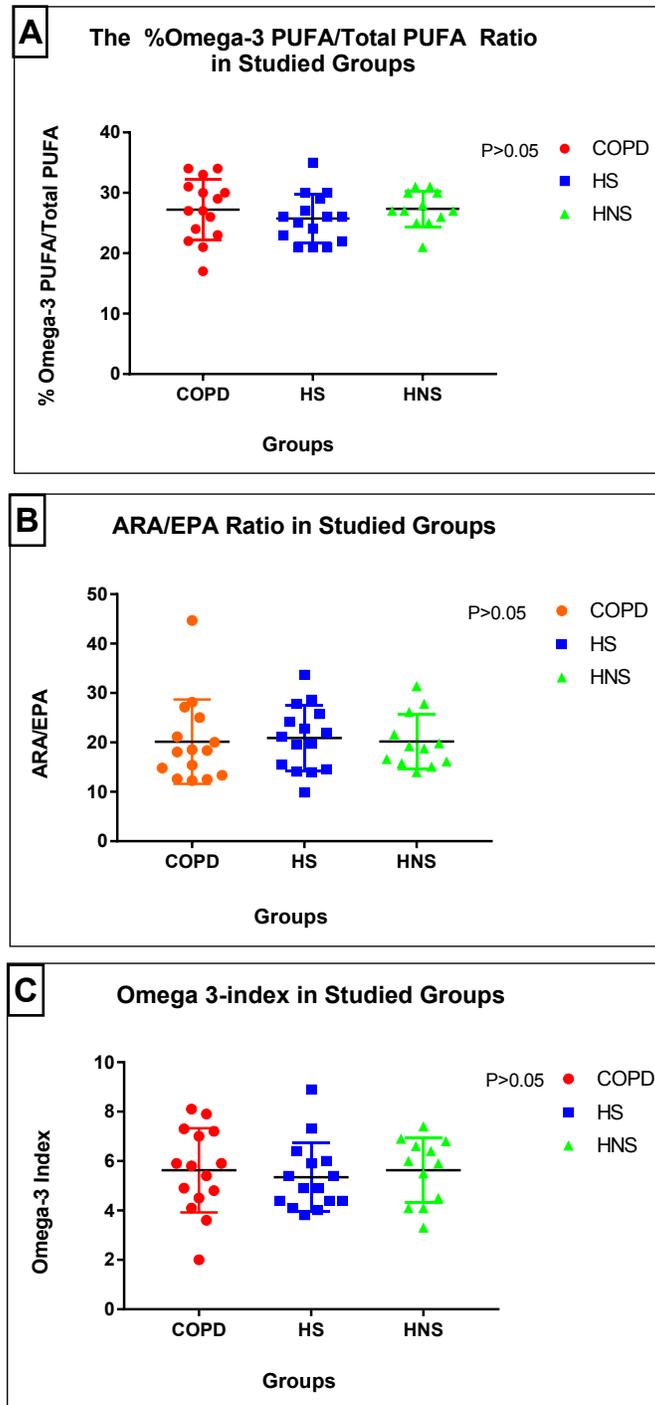
Figure 6.1: Comparison of (A) omega-3 PUFA/total PUFA, (B) ARA: EPA ratio, (C) omega-3 index. *P value < 0.05 as determined by one-way ANOVA analysis of variance followed by Tukeys multiple comparison post-test indicate a significant difference between the groups. These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; HS, healthy smokers; HNS, healthy nonsmokers PUFA; poly unsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid.*

6.4.2 Associations between Membrane Fatty Acids and Smoking in COPD

The effect of smoking on RBCs fatty acid composition of COPD patients was investigated. There was no statistically significant relation between smoking habits and the percentage of omega-3 PUFA/total PUFA, using PYH as a marker ($r^2 = 0.06$; P = 0.34). Likewise, the relationship between smoking and ARA: EPA ratio or and omega-3 index was also not statistically significant. The correlation coefficient was $r^2 = 0.16$; P = 0.13 and $r^2 = 0.13$; P = 0.17 respectively (Figure 6.2). COPD patients were further split according to smoking status into former and current smokers' sub-groups. However, there was a weak to moderate negative correlations between omega-3 index and PYH in current smokers with COPD ($r^2 = 0.36$; P = 0.08) (Figure 6.3).
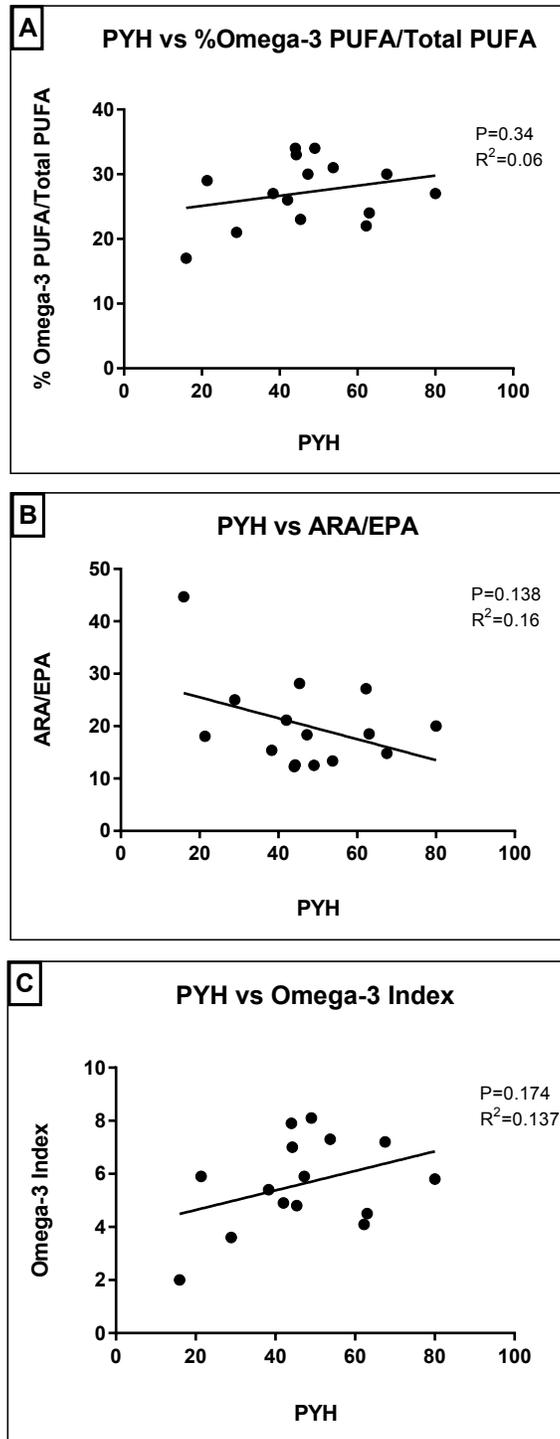
Figure 6.2: Smoking impact (PYH) in relation to fatty acid measurement, (A) percentage of omega-3 PUFA/total PUFA, (B) ARA/EPA ratio, and (C) omega-3 index in patients group. *Significance was tested using a linear regression analysis (spearman method). These analyses were performed using Graph Pad Instat Software. Abbreviations: PUFA; poly unsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid.; PYH, pack year history.*
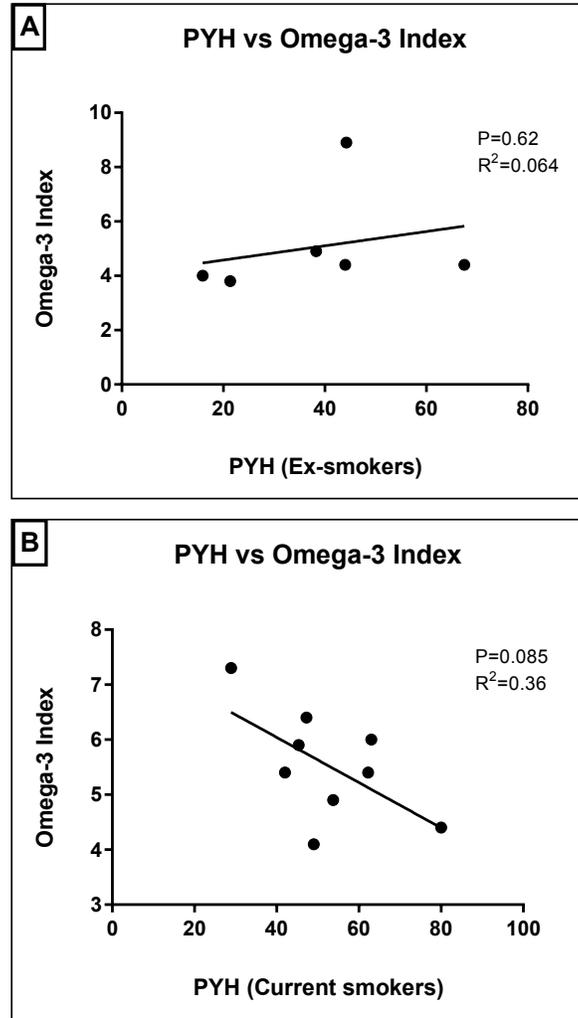
Figure 6.3: Smoking impact (PYH) in relation to omega-3 index in smoker sub-groups. *(A) Former smokers with COPD (n=6), (B) current smokers with COPD (n=9). Significance was tested using a linear regression analysis (spearman method). These analyses were performed using Graph Pad Instat Software. Abbreviations: PYH, pack year history.*

6.4.3 Associations between RBC Fatty Acids and Pulmonary Function in COPD

The relationship between fatty acid measurements and patients' pulmonary function outcome were investigated. Linear regression analysis using Pearson method showed no significant relationship between FEV1 and the percentage of omega-3 PUFA/total PUFA (r^2 = 0.001; P = 0.88), ARA: EPA ratio (r^2 = 0.006; P = 0.77), as well as Omega 3 index (r^2 = 0.016; P = 0.65). Likewise, the relationship between pre FEV1/FCV ratio and the percentage of omega-3 PUFA/total PUFA or ARA: EPA ratio, as well as omega-3 index was also not statistically significant. The correlation coefficient was r^2 = 0.006; P = 0.77, r^2 = 0.018; P = 0.63 and r^2 = 0.0005; P = 0.99 respectively (Figure 6.4). Linear regression analysis also showed no significant relationship between FEV1 and fatty acid measurements for the entire study cohort.
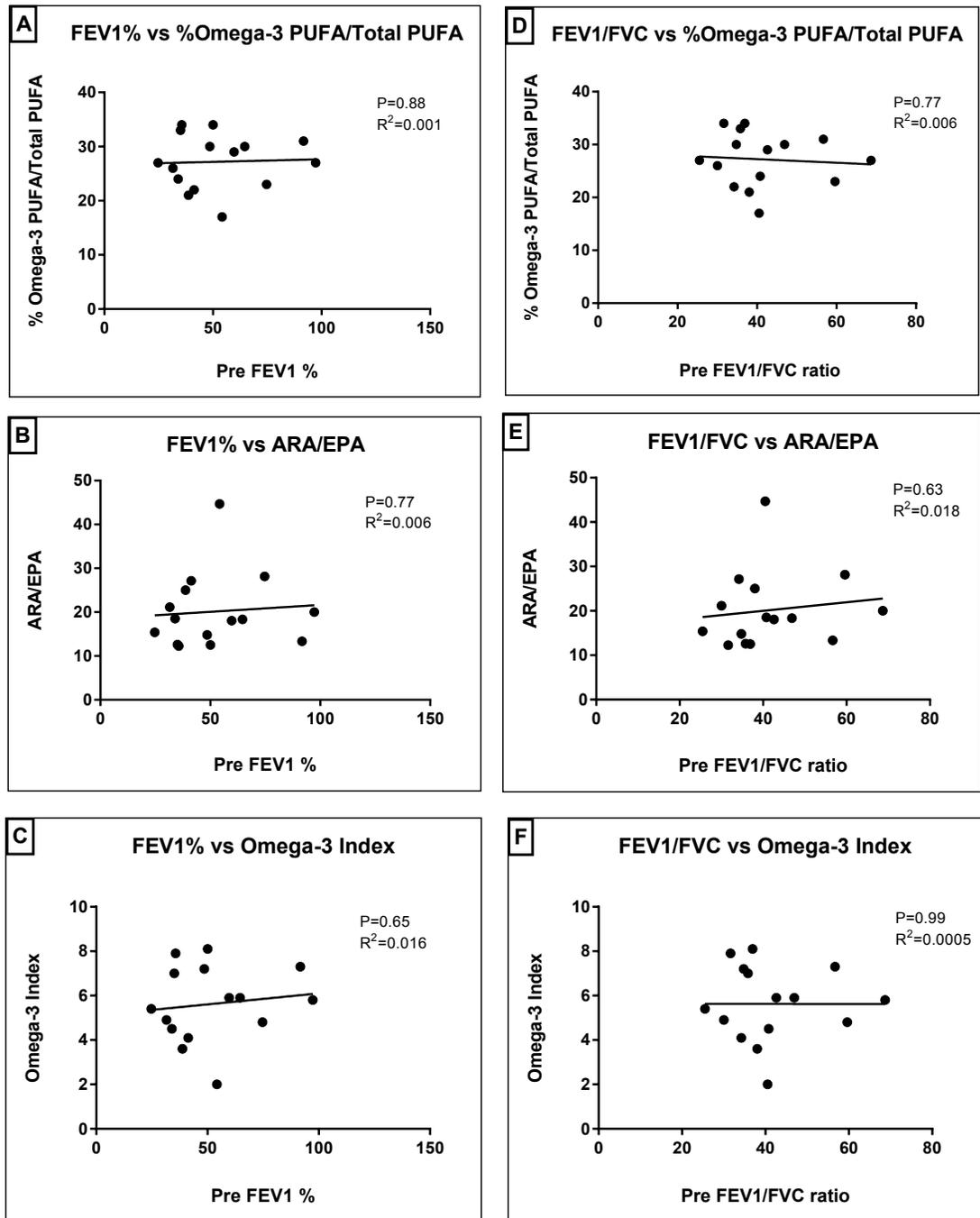
Figure 6.4: The correlation between patient's fatty acid measurement and lung severity*; (1) pre FEV1% with (A) percentage of omega-3 PUFA/total PUFA, (B) ARA/EPA ratio, (C) omega-3 index, (2) pre FEV1/FVC ratio with (D) % of omega-3 PUFA/total PUFA, (E) ARA/EPA ratio (F) omega-3 index. Significance was tested using a linear regression analysis (spearman method). These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; FEV1, Forced Expiratory Volume in 1 second; FVC, forced vital capacity, PUFA; poly unsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid.*

6.4.4 Associations between Membrane Fatty Acids and Inflammatory biomarkers in COPD

A possible relationship between fatty acid measurements and the plasma level of inflammatory biomarkers of patients were investigated. There was no statically significant relationship between plasma CRP level of patients with COPD and RBCs fatty acid measurement including; the percentage of omega-3 PUFA/total PUFA ($r^2 = 0.006$; P = 0.77), ARA: EPA ratio ($r^2 = 0.013$; P = 0.68), as well as omega-3 index ($r^2 = 0.002$; P = 0.86). Likewise, no statically significant relation was found between patients' plasma fibrinogen level and RBCs fatty acid measurements. The correlation coefficient of the percentage of omega-3 PUFA/total PUFA, ARA: EPA ratio and omega-3 index was $r^2 = 0.03$; P = 0.49, $r^2 = 0.012$; P = 0.69 and $r^2 = 0.02$; P = 0.58 respectively (Figure 6.5).
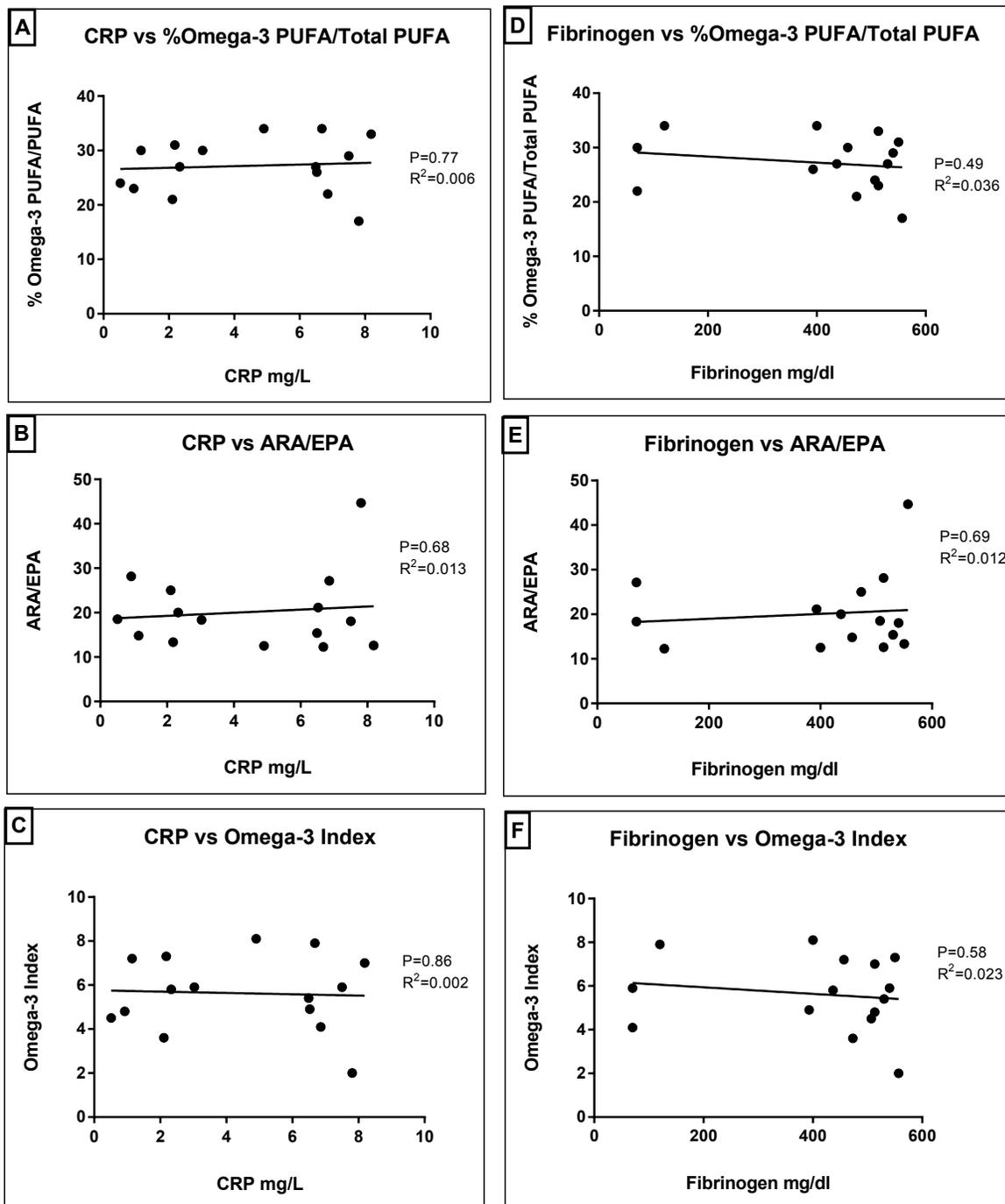
Figure 6.5: The correlation between patients' fatty acid measurements and inflammation biomarkers level. *CRP with (A) omega-3 PUFA/total PUFA (B) ARA/EPA ratio (C) omega-3 index, Fibrinogen with (D) omega-3 PUFA/total PUFA (E) ARA/EPA ratio (F) omega-3 index. Significance was tested using a linear regression analysis (spearman method). These analyses were performed using Graph Pad Instat Software. Definition of abbreviations: COPD, chronic obstructive pulmonary disease; FEV1, Forced Expiratory Volume in 1 second; FVC, forced vital capacity, : PUFA; poly unsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid; CRP, C-creative protein.*

6.5   Discussion

Fatty acids control the structure and function of biological membrane including membrane fluidity. Thus modification of the fatty acid composition in the membranes of the RBCs in patients with COPD may result in disruption of the cell structure and function and immune response (Novgorodtseva et al., 2013). For example, increased omega-6 PUFAs stimulates the formation of inflammatory mediators that involve in platelet aggregation, immune system functions, and bronchospasm. Early detection of the fatty acid alteration in patients with respiratory diseases can prevent the progression of a more severe pulmonary disease (Novgorodtseva et al., 2013).

Erythrocyte membrane content is considered to be the most reliable, biologically relevant sample of dietary omega-3PUFAs intake. The ratio between omega3 and omega6 PUFA intakes has been studied by using the ratio of ARA to EPA and/or DHA. While Omega-3 Index reflects the amount of EPA and DHA present in RBCs membrane (Cai et al., 2017). In the present work, the fatty acid profiles of total lipid in RBCs from the three studied groups were analysed, to detect potential fatty acids that could be used as biomarkers test for the diagnosis and management of COPD. There are no previous studies examining the use of fatty acid profiles in this context for COPD. Unlike this study, previous studies have investigated the effect of omega-3 PUFA supplementation, either as a fixed-dose therapy or as a dietary supplement, in patients with COPD.

RBC PUFA measurements for the percentage of omega-3 PUFA/total PUFA, ARA: EPA and Omega 3 index showed no significant differences between the three studied groups. No correlation was observed between fatty acid measurements of erythrocyte membranes and patients' health status, and the study findings prove that the fatty acid composition of the erythrocyte membranes in our patients group was unmodified. These results are in agreement with a previous study which analysed the fatty acid profiles of RBCs and found contrasting results showing modification in fatty acids from patients with different types of lung cancers, whereas fatty acid profiles were unmodified in patients with COPD, asthma, and healthy controls (De Castro et al., 2014).

In contrast to the current study, previous study by Novgorodtseva *et al* (2013), revealed an increase of omega-6 PUFAs/omega-3 PUFAs ratio by 3-fold, and a decreased in EPA value by 2-fold in COPD patients when compared to the control group. During the current study, similar work has been published by Kentson *et al.* (2018) in which the omega-3 index was significantly reduced in COPD subjects compared to controls. In that study it was observed that COPD subjects' food intake was not rich in omega-3 PUFA, less healthy, and that they ate smaller food portions than control subjects, which could be the cause of the reduced level of omega-3 PUFAs in patients rather than the disease itself. The mean value for the omega-3 Index in the present study was 5.6 for the COPD group, which is comparable with the level reported in Kentson's study, where the value was 5.16, and 5.79 for controls. However the omega-3 Index value reported in the current study of the controls group was similar to those of patients which may suggest the less healthy diet style of both group subjects. Interestingly, in the current study the omega-3 Index and the percentage of omega-3 PUFA/Total PUFA was lower numerically in the healthy smoker group compared to patients and healthy nonsmokers. However this low level did not reach statistical significance and may be due to smoking effect on healthy smokers' diet style and health status.

Smoking is a major risk factor for COPD especially in developed countries. Smoking can activate oxidative stress causing increase in lipid peroxidation of PUFAs. It also may affect the synthesis and metabolism of fatty acids result in alteration of omega-3 PUFA levels (Scaglia *et al.,* 2016). The literature reports inconsistent findings in relation to the effect of smoking of PUFA composition. In this study, the effects of cigarette smoking on RBCs cell membrane fatty acid levels of COPD patients were examined. It was found that smoking, as quantified by PYH, statistically seemed to have no significant impact on fatty acid composition of COPD subject including; the percentage of omega-3 PUFA/total PUFA, ARA/EPA ratio and omega-3 index.

The current study results are in agreement with Batlle *et al.* (2012) study who observed no effect of smoking status on the levels of PUFA in COPD patients. However, a weak to moderate negative correlation between omega-3 index ratio and smoking habits in current smoker with COPD was observed. These findings indicate that smoking habit may affect the dietary intake of omega-3 PUFA in COPD patients. A similar observation was made by several other research groups, for example; Scaglia *et al.* (2016) reported a lower intake of omega-3 PUFAs by

smokers was associated with lower DHA levels. Additionally, a cross-sectional case-controlled clinical study revealed a decrease in serum omega-3 PUFAs in smokers, especially with COPD compared with nonsmokers (Titz *et al.,* 2016).

In the present study plasma levels of inflammatory biomarkers (CRP and fibrinogen) were found to be significantly higher in the COPD group than in healthy smoking and nonsmoking groups. In addition, several epidemiologic studies indicated a relationship between the low ratio of EPA and DHA intake and high level of CRP. It has been proposed that omega-3 PUFA can modulate the inflammatory process by inhibiting the production of pro-inflammatory factors. Therefore, a possible relationship between the plasma level of inflammatory biomarkers and RBCs cell membrane PUFA composition was investigated. There was no correlation of PUFA with inflammatory biomarkers CRP, as well as fibrinogen, in COPD patients. These findings confirmed that PUFA have no effect on systemic inflammation in patient with COPD. The present results matched a previous 8-week trial, which found that daily supplementation with EPA, DHA, and ALA in 80 patients with COPD did not affect the level of CRP, IL-6, and TNF-α (Broekhuizen *et al.,* 2005). Likewise, a study by Matsuyama *et al* (2005) also reported that diet enriched with omega-3 PUFA did not change serum levels of IL-8 and TNF-α. However it did improve exercise capacity and decrease serum LTB4, sputum LTB4, IL-8, and TNF-α level in patients with COPD. Similarly, the plasma level of CRP was unchanged in COPD patients supplemented with rosuvastatin, omega-3 fatty acids and lycopene (Williams *et al.,* 2016). In contrast, daily supplementation with omega-3 PUFA significantly decreases the serum level of CRP, IL-6, IL-8, and TNFα in the low-intensity exercise group (Sugawara *et al.,* 2010). In line with the previous study result, Batlle *et al.* (2012) observed an inverse association between omega-3 PUFA intake and several serum pro-inflammatory biomarkers level, including CRP, IL-6 and TNFα. However the latter study relied on a food frequency questionnaire to assess the individual dietary data of PUFA intake.

Data from this study showed that pulmonary function including preFEV1% and pre FEV1/FVC ratio were significantly reduced in COPD patients when compared to healthy smoking and nonsmoking groups. A possible protective association between omega-3 PUFA measurements and lung function outcomes was therefore investigated. Unfortunately, there was no relationship between omega-3 PUFA measurements and lung function outcome in COPD

patients. However, the results agree with previous studies which reported that omega-3 PUFA intake does not have a major protective effect on lung function or disease symptoms and severity (Broekhuizen *et al.,* 2005; McKeever *et al.,* 2008).

There are several limitations in previous studies worth to be mentioned. First, the most important limitation is that most of the studies are cross-sectional observational cohort studies. Second, omega-3 PUFA sources are indirectly measured using food frequency questionnaires instead of using laboratory method to analyse RBCs membrane fatty acid. Subsequently, this may lead to inaccurate omega-3 PUFA consumption estimation. Finally, COPD patients and smokers may have changed their lifestyles and dietary habits, reducing fish consumption.

The current study data gave no indication of the possible involvement of RBCs membrane fatty acid composition in the relation with COPD, but the size of the patients sample maybe insufficient for that purpose. A limitation of the present study that might have contributed to the absence of a significant relationship between PUFA measurements and COPD is insufficient statistical power. A post hoc power analysis (Wilcoxon-Mann-Whitney test) could not be performed because the means and standard deviation for the variables were unavailable. The principal finding of this chapter was that patients PUFA profile was not affected by COPD severity. Thus, the relationships between RBCs PUFA and COPD remain controversial in the literature.

6.6 Conclusions

Measurement of PUFAs from RBCs total lipids analysis was considered as a potential useful stable metric for COPD severity. Unfortunately, there was no observed alteration in PUFAs composition in the patients group. Additionally, there was no correlation between patients respiratory function parameters or systemic inflammatory biomarkers and PUFAs measurements. Subsequently our findings indicated that RBC membrane Omega-3 PUFA does not seem to have a major protective effect on lung function or disease symptoms. The results from this study did not support the findings of a number of reports which have revealed that PUFAs composition is modified and omega-3 index is reduced in patients with COPD.

Although consistent with findings in a very recent report by Kentson (2018), an omega-3 index value of 5.6% reported here for COPD patients (and control subjects), is much below the suggested 8-12% that has been associated with achieving health benefits for other conditions. Interventional studies use supplementation of food enriched with omega-3 PUFA to elicit a response. However, there is a failure to evaluate baseline omega-3 and whether supplementation causes any change to achieve higher levels of omega-3 and therefore is unsupportive of a direct benefit from enhancing omega-3 PUFA. This needs to be addressed in future interventional studies. There is no consistent evidence confirming that a diet enriched with omega-3 PUFA reduces chronic inflammation. Only limited evidence shows that the use of omega-3 PUFA supplementation is improving functional capacity in COPD patients. More evidence and further investigation are required to confirm the potential effects of omega-3 PUFA on COPD patient health, before routinely incorporating this therapy within COPD treatment and management (Atlantis Cochrane, 2016). The relationship between omega-3 PUFAs and inflammation in patients with COPD remains unclear. The absence or weakness of a significant relationship may be due to the small patients' number and individual biological variability. Increasing the sample size may have assisted in finding a significant relationship between blood levels of omega-3 PUFA and COPD patients' health status. Collectively the data show that the severity of COPD does not seem to reflect the fatty acid composition of the RBCs membrane, but it is tempting to speculate that enhancing levels of omega-3 from around 5% observed in this study population, to at least 8% may have a different outcome. Further research is needed to study the formation and activity of fatty acid in COPD.

7. Chapter 7: General Discussion and Conclusion/ Future works

COPD is a chronic lung disease characterised by persistent airflow limitation. It is associated with systemic inflammation, extra-pulmonary manifestations, comorbidities and poor outcomes (Barnes 2016). COPD is a major cause of mortality worldwide (Bajpai *et al.,* 2017), and by 2030 COPD is expected to become the 3rd leading cause of death worldwide (Vestbo *et al.,* 2013). It is a complex disease which is influenced by multiple factors such as genetic factors, environmental factors, and genotype–environment interactions (Sandford and Silverman 2002). Severity of COPD is positively associated with systemic inflammation. The presence of increased level of inflammatory markers in circulation, sputum, and BAL fluid suggests that systemic inflammation is one of the potential mechanisms responsible for COPD (Naik *et al.,* 2014). Inflammatory biomarkers are becoming a useful means of characterizing the extent of both airway and systemic inflammation associated with COPD (Barnes 2016). In this regard, disease heterogeneity and a lack of disease specific biomarkers are particularly challenging for the development of effective therapy for COPD. Current therapies are able to attenuate symptoms and prevent exacerbations but they do not prevent the decline in lung function or disease progression. Therefore the development of blood biomarkers would enhance the understanding of COPD pathophysiology and facilitate patient subdivision (Roberts *et al.,* 2015).

In the current programme of studies, a significant decrease was found in the pulmonary functions of COPD patients when compared with healthy controls. These findings are consistent with those of other researchers who have demonstrated that pulmonary function data were significantly lower in COPD patients when compared with controls (El-Shimy *et al.,* 2014). Age, weight and BMI have an impact on subject respiratioty functions when investigating the whole study indviduals. Addionally, pateints parameters including; weight, BMI as well as smoking exposure seemed to have impact on patients' reversibility, but not on disease severity. This may due to low size of patients sample. Furthermore, smoking may impairs the effectiveness of ICS therapy in patients with COPD. However no effect of ICS was found on patients' lung functions.

Blood biomarkers may clinically assist in predicting COPD severity (Augusti *et al.,* 2012; Vestbo *et al.,* 2014; Faner *et al.,* 2014). For example, fibrinogen and CRP, both biomarkers of inflammation were found to be correlated with COPD severity and risk of exacerbations (Detoress *et al.,* 2006; Higashimoto *et al.,* 2009; Vestbo *et al.,* 2011; Augusti *et al.,* 2012, Celli *et al.,* 2012, Duvoix *et al.,* 2013; Fattouh and Alkady, 2014; Manino *et al.,* 2015; Paone *et al.,* 2016). IL-6 was also associated with FEV 1 and FEV1/FVC (Ardestani and Zaerin 2015). Importantly, lung specific proteins such as SP-D and CC16 are also attractive COPD biomarkers. SP-D has been associated with COPD (Vestbo *et al.,* 2014, Lomas et al., 2008), and emphysema and possibly exacerbation frequency (Lomas et al., 2009; Akiki *et al.*, 2016). CC16 may correlate with disease severity and FEV1 decline over time (Lomas *et al.,* 2008; Lomas *et al.,* 2009; Vestbo *et al* 2014). Plasma sRAGE is inversely correlated with decreased lung function, disease severity and emphysema (Change *et al.,* 2013; Gopal *et al.,* 2014; Hoonhorst *et al.,* 2016). Serum levels of CCL18 were correlated with FEV1, exercise capacity, exacerbation frequency and mortality (Pinto-Plata *et al.,* 2007; Sin *et al.,* 2011; Paone *et al.,* 2016).

However, most of the available studies are either observational cohort or small-sized experimental studies, while large clinical trials that evaluating changes in biomarkers in relation to clinical outcome are insufficient. Most of the current biomarker candidates are not lung-specific but may reflect the systemic aspects of COPD and its associated comorbidities. There is a need for lung-specific biomarkers derived from proteins that are either only expressed in the lung tissue (CC16, SPD), or highly over-expressed in the lung (RAGE). Due to the complexity and heterogeneity of COPD, a panel of biomarkers may be required to address personalized treatments of COPD patients, as single biomarker will not be able to identify the different characteristic and phenotypes of COPD (Ongay *et al.,* 2016)

COPD is characterised not only by a local pulmonary inflammation but also by a systemic inflammation. In this study (chapter 4), the associations between circulating inflammatory markers and COPD were investigated. High plasma levels of CRP and Fibrinogen together with a low level of CC16 were observed in COPD patients compared with healthy controls. This is consistent with previous data where elevated plasma inflammation-sensitive protein levels, such as fibrinogen and CRP were associated with the presence of COPD (Agusti *et al.,* 2012; Kleniewska *et al.,* 2016). However, the use of CRP alone as a biomarker for COPD is limited

because of its low specificity as any inflammation or infection not related to the lung, can modify CRP level (Paone *et al.,* 2016). Additionally, the levels of plasma CRP increased as the severity of COPD increased. An increased plasma fibrinogen and CRP level in patients with COPD may suggest a systemic inflammatory effect in these patients. Further clarification is required as to whether systemic inflammation is a primary or secondary related to pulmonary inflammation.

Lung specific proteins can be potential biomarkers that can assist in disease prognosis and severity, predict outcomes, and treatment (Doubková *et al.,* 2016). Findings in this study, from the COPD patient group, indicate the lung-specific protein CC16, could be promising as a local biomarker of an inflammatory process in the airways of COPD patients. However, further studies are required to establish the role of CC16 in COPD patients. An ideal biomarker must be reproducible in stable disease like; SP-D, fibrinogen and CC16, while other biomarkers, including CCL18 and CRP, need further evaluation (Paone *et al.,* 2016). Therefore, novel and possibly more pulmonary specific biomarkers should be tested, perhaps using combinations of (systemic and lung-specific) biomarkers in a 'mini-panel'.

There is no specific accepted systemic biomarker for assessing COPD severity and evaluating the effectiveness of treatment (El Gammal *et al.,* 2015). This study provides evidence that COPD patients have a pro-inflammatory state, with increased circulating levels of inflammatory cytokines and acute-phase reactants. However, circulating TNF-α, IL6, IL8, IP10, SPD and RAGE were not detected in most subjects of the study cohort despite recognized associations with inflammation and COPD. These inconsistencies between studies are often found in the literature and efforts to understand why are necessary. Methodological differences and the heterogeneity of COPD itself may partly explain this.

Assessment of blood markers can be useful for diagnosis, prognosis and treatment of COPD patients. However, due to inconsistent results of published studies of inflammatory biomarkers their evaluation has not been accepted so their routine application requires further studies. The first step of treatment for COPD is to suppress the inflammation process and to prevent its consequences (Heidari 2012). COPD is associated with systemic inflammation but it remains unclear how the systemic inflammation arises. Does it start in parallel to the pulmonary inflammation, or it may due to the spillover of pulmonary inflammation? (Agusti *et al*., 2012).

Of note, unstimulated *ex-vivo* PBMCs in COPD released significantly higher levels of (IL-6, IL-8 and TNF-α) cytokines compared to healthy nonsmoking controls, suggesting that the PBMCs are already activated in the patients' circulation. Altered peripheral lymphocyte functions in COPD patients have previously been reported. Additionally, several immunological functions of circulating T-cells are abnormal and correlated with COPD severity and thus further support that adaptive immune processes contribute to progression of this disease (Zhu *et al.,* 2009). Additionally smoking seemed to have an impact on patients' systemic level of CRP but not on fibrinogen, CC16, CCL18, and CD31. Cigarette smoking, which is a leading risk factor for COPD, can also induce systemic inflammation as the cytokines release from PBMCs of healthy smoker were higher than those of healthy nonsmoking control.

One of the aims of this work was to explore the anti-inflammatory properties of EPA and DHA omega-3 PUFA. Data (chapter 5) indicates that EPA and DHA individually possess potential immunomodulatory effects characterized by significant reductions in PHA-induced IL-6, IL-8 and TNF-α release from *ex-vivo* PBMC. Studies are limited regarding the possibility for omega-3 PUFAs to be used therapeutically in chronic inflammatory diseases such as COPD. Clinical evidence of benefits from omega-3 PUFAs in COPD were reported in observational and interventional studies. Observational studies (de Batlle *et al.,* 2012; Novgorodtseva *et al,* 2013) demonstrate that omega-3 PUFA levels are associated with both systemic inflammation and clinical COPD outcomes. Moreover, a very recent study has reported an increased ratio of (total) omega-6:omega-3 PUFAs in asthma patients (Zhou *et al.* 2018). Data from this study, using RBC-based measurements, found that levels of the EPA and DHA omega-3 PUFAs were generally low across the study population (indicated by an omega-3 index of approximately 5.6% - in itself an interesting finding), but were not correlated with patients respiratory function parameters or systemic inflammatory biomarkers. In a previous report, Novgorodtseva *et al.,* (2013) found a 3-fold increase in the ratio of the omega-6 to omega-3 PUFA (ARA/EPA) in COPD patients compared to controls, whereas ARA/EPA from this study was not different between groups. It is difficult to compare the studies directly but notable that the Novgorodtseva control group comprised both ex-smokers and non-smokers and the COPD patients were all GOLD 1 (mild severity of disease). In this study, controls were separated by smoking status and most (73%) COPD patients were GOLD 2 (moderate severity). However, when both control

groups (healthy smokers and nonsmokers) were combined to re-evaluate the data the outcome remain the same.

Clinical interventional studies tend to use omega-3 PUFAs in nutrient supplement combinations, which makes it difficult to attribute omega-3 PUFAs specific effects. They are also delivered at fixed doses, with compliance recorded by questionnaire, so it is unknown as to whether a therapeutic dose of the omega-3 PUFA is ever achieved *in vivo*. Further, the dose levels of EPA and DHA which was used in previous studies on *ex vivo* lymphocyte proliferation in healthy subjects varied from 0.1 to 7 g/day EPA+ DHA/day. Significant increased lymphocyte proliferation was observed at a dose level of 2.0g EPA+ DHA/day. However, most these studies did not include a formal power calculation (Miles and Calder 2012).

Given the variations observed here between individuals' omega-3 index values, this may help to explain why interventional studies so far are inconclusive. A better approach may be to measure RBC omega-3 pre-and post-intervention, in order to 'personalise' dosing to achieve appropriate *in-vivo* levels. However, there are a few clinical trials ongoing for COPD that are using purified omega-3 PUFAs alone but those results are yet to be confirmed (Wood 2015). More evidence and further investigation are required to confirm the potential effects of omega-3 PUFA on COPD patient health, before routinely incorporating this therapy within COPD treatment and management (Atlantis Cochrane 2016).

As omega-3 PUFAs are natural products they could easily be incorporated as part of a general lifestyle change for the patient and self-monitoring via blood spots is feasible. Studies incorporating the above suggestions are needed on a larger scale and for a longer period of time to more carefully assess the effectiveness of omega-3 PUFAs for treating COPD. This could be in conjunction with biomarkers, antioxidant or other agents (Bajpai *et al.,* 2017). If future studies prove that these therapies are effective and safe it might be more useful to introduce these early in the course of the disease to prevent disease progression and possibly to reduce comorbidities (Barnes 2016).

Limitation

There are some limitations of the present study that deserve to be discussed. First, the definition of COPD was based on post FEV1/FVC <0.70 at a single examination. Although this is the most widely accepted definition for COPD, it represents a simplified case definition for epidemiological purposes and not for clinical diagnosis. Second, this is a clinical-case study looking at a population at a given point in time and not a longitudinal study. Therefore it only provides the frequency and characteristics of the disease in this population taking place when the study was conducted. Third, of the COPD group in this study, all patients used ICS and systemic corticosteroids. This could contribute to the underestimation of the difference in biomarkers between COPD patients and control subjects. Nevertheless, the effect of ICS on inflammatory biomarkers is still controversial.

Fourth, the biology of the inflammatory response is complex and the current study investigates only a limited panel of biomarkers. However, the selected biomarkers were chosen correspond to those investigated by the majority of previous studies, and are often and easily measured in clinical practice. On the other hand, evaluation of markers of oxidative stress and lipid peroxidation; MDA, Hydrogen peroxide and 8-isoprostane, antioxidants; superoxide dismutase 3, glutathione peroxidase, catalase, and ceruloplasmin ferroxidase activity (Bajpai *et al.,* 2017), antioxidant; total bilirubin and uric acid (Lee *et al.,* 2018), extracellular matrix breakdown; MMP-8 and MMP-9 (Duvoix et al., 2012), protease/anti-protease biomarkers, other cytokines include; IL-2, IL-4, and IFN-γ, Adiponectine, Apolipoprotein A1 and lipocallin-1and are suggested to interpret the results accurately (Agusti et al., 2012; Comes et al., 2016). For example, what emerges from literature is that MDA, a lipid peroxidation product, could be used as specific biomarkers of oxidative stress in COPD patients. Furthermore, the use of advanced analytical methods like cytokine arrays, mass spectrometry, or nuclear magnetic resonance, should increase the sensitivity of the assays, and may produce important new information in the future (Liu *et al.,* 2014).

Fifth, this was a single-center study and the number of subjects was not sufficient to be equally categorized to COPD four stages; mild, moderate, sever, very sever; and this may have hampered efforts to clearly define differences between the groups in some cases. Some biomarker data are known to be variable and the PBMCs sample size was sometimes insufficient

to overcome this variability. In addition, direct measurement of inflammatory biomarkers in peripheral blood is known to be difficult due to their short half-lives, low concentrations, and their binding to soluble receptors and the effects of renal clearance (Kawayama et al., 2016). Other factors to consider include; lack of reproducibility in an independent cohort, heterogeneity and subject biological variability of the patients with COPD, pathological characteristics, the use of different methodologies and assays. Therefore, a large-scale and long-term multi-center population-based study with sufficient subjects in each GOLD category may be necessary in the future to investigate the lack of associations between these biomarkers and the presence of COPD (Lee *et al.,* 2018).

Another limitation was the absence of other important measures of COPD, such as diffusion capacity or CT scan. Pervious works (Bailey *et al.,* 2012; Boutou *et al.,* 2013; Šileikienė *et al.,* 2017), in COPD has highlighted that measures other than pulmonary function are important predictors of impaired function and poor outcomes. However, the Lung function remains the primary means of diagnosing and classifying COPD at the present time. For better understanding of the COPD variability between patients, additional parameters sholud be evaluted during patient attendance at routine clinics. The addition of a panel of biomarkers to lung function parameters known to predict mortality in COPD (Cazzola, *et al.,* 2008), alongside lung plethysmography, gas transfer, arterial oxygen, and CT, as well as multiple spirometry will improve diagnosis and prediction of mortality risk in patients with COPD.

Finally, a limitation of the present study that might have contributed to the absence of a significant relationship between variables is the insufficient statistical power. During the planning of the study, no power analysis was performed, and a post hoc power analysis (Wilcoxon-Mann-Whitney test) justifies the sample size. However for some data sample justification could not be performed because the means and standard deviation were unavailable.

Future works

The possibility to continue the current work will be a good opportunity to address some of the limitation, and to make the study data more robust. For example, Apply more pulmonary function and structural test from each patient during clinic presentation such as; CT scan, gas transfer, arterial oxygen, along side spirometry measurments. These tests together could allow clinicians for earlier diagnosis of COPD patients, reduce hospital readmission, and could help governments to plan their healthcare budgets and financial resources. Unlimited resources available will assist to expand the biomarker panel and to add more specific pulmonary markers, and to use more advanced detection methods which will improve the sensitivity and validity of the measurement of several biomarkers in COPD. For example, flow cytometric assay will help to identify various cell types from blood samples of COPD patients, and will allow further investigation of cell characteristics, such as cell count, cytokine production and receptor expression, and apoptosis for different cell types. Furthermore, the use of advanced analytical methods should save time, process more samples, increase the sensitivity of the assays, and may produce new information.

Future investigation should focus on pulmonary marker and other marker related to event and comorbidities usually seen in COPD. For examples, markers for oxidative stress, lipid peroxidation, CVD, angiogenesis, and tissue damage as well as extracellular matrix-degrading enzymes. Apply sample justification analysis prior starting the experiment to estimate the sample size required for the study, to detect a significant relationship. Increase the subject number to be equally categorized to COPD four stages via collecting samples from multi-centres with long-term studies. Additionally, investigate the effect of different concertation range of omega-3 PUFAs on PBMCs biomarker release is worth to be investigated. A concentration range of 100, 200, 300, 400 μM will be a good point to start from. To date, plasma omega-3 PUFA concentrations of participants remained unclear in most the previous studies. It would therefore be useful to investigate the potential relationship between plasma omega-3 PUFAs level and COPD will be interesting, to elucidate the potential influence factors of omega-3 PUFAs. Research into the benefits of omega-3 PUFAs should be continue and include fatty acid blood measurements in COPD patients. This will provide physicians information on individual patient risk classification and anticipated response to treatment that may be of clinical value.

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Appendix A

Study Ethical Approval + Sample Collection Plan

12 April 2010

Dr Dave Singh
Medical Director, Consultant Respiratory Physician
Medicines Evaluation Unit
The Langley Building
Southmoor Road
Wythenshawe Hospital
M23 9QZ

Dear Dr Singh

Full title of study:	**Protocol for Donation of blood and urine samples by Healthy Volunteers and Patient Volunteers for Laboratory Research.**
REC reference number:	**10/H1016/25**
Protocol number:	**1.0**

Thank you for your letter of 12th April 2010. I can confirm the REC has received the documents listed below as evidence of compliance with the approval conditions detailed in our letter dated 26 March 2010. Please note these documents are for information only and have not been reviewed by the committee.

Documents received

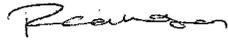The documents received were as follows:

Document	Version	Date
Covering Letter		09 April 2010
Participant Information Sheet: Track changed	2.0	07 April 2010
Participant Information Sheet: Clean	2.0	07 April 2010
Participant Information Sheet: Blood bourne viruses Track changed	2.0	07 April 2010
Participant Information Sheet: Blood bourne viruses Clean	2.0	07 April 2010
Participant Consent Form: Track changed	2.0	07 April 2010
Participant Consent Form: Clean	2.0	07 April 2010

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

This Research Ethics Committee is an advisory committee to the North West Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

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10/H1016/25	Please quote this number on all correspondence

Yours sincerely

Rowen Callaghan
Committee Assistant Co-ordinator

E-mail: rowen.callaghan@northwest.nhs.uk

Copy to: *Miss Sophie Breeze*
                Medicine Evaluation Unit
                The Langley Building
                Southmoor Road, Manchester
                M23 9QZ

Confirmation email from Professor Dave Singh, MEU Medical Director, that they are happy to collaborate on this proposal.

Hi ███

Good to hear from you !

The scientific basis of this work is sound, and I am very happy to collaborate. Could you communicate with Matt Rossall please. I assume that you just want us to collect blood for sending to you ?

For the presentation, its best that you liase with Jonnie and Simon. Our internal presentation program is quiet at the moment, we may be able to do something after summer

Best wishes

Dave

The form can be completed electronically; the sections can be expanded to the size required.

Name of Staff:	Dr X Dr Y
School:	ELS
Name of Research Council or other funding organisation (if applicable):	

1a. Title of proposed research project

Use of PBMC from healthy and patient volunteers to investigate Chronic Obstructive Pulmonary Disease

1b.   Is this Project Purely literature based?

   NO (delete as appropriate)

2. Project focus

To utilise PBMC recovered from whole blood to investigate the effects of potential novel treatments on cell inflammatory responses.

3. Project objectives

1. The main aim of the project will be to obtain whole blood from defined groups of patients with COPD and healthy volunteers. 2. PBMCs will be isolated from fresh whole blood and be either frozen for later use, or immediately used in experiments to test the anti-inflammatory efficacy of potential therapeutic agents.

4. Research strategy

(For example, outline of research methodology, what information/data collection strategies will you use, where will you recruit participants and what approach you intend to take to the analysis of information / data generated)

Subjects will be smokers and ex-smokers with a diagnosis of COPD and a group of healthy individuals who may or may not have a smoking history. All subjects will be recruited by the Medicines Evaluation Unit (MEU), based at University Hospital South Manchester (Wythenshawe) as part of their ongoing studies to investigate airways inflammation in cigarette smokers. Patients with COPD will be classified into 4 subgroups: ex-smokers on inhaled

The form can be completed electronically; the sections can be expanded to the size required.

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Patients with COPD will be classified into 4 subgroups: ex-smokers on inhaled

The Data Protection Act 1998 relates to personal information only. In this regard, no personal data relating to subjects in this proposal will be held or used by UoS staff. As such there are no issues arising.

10. **Are there any other ethical issues that need to be considered? For example - research on animals or research involving people under the age of 18.**

Codes of Practice (CoP's) relevant to the Human Tissue Act (HTA) 2004 apply: (www.hta.gov.uk/guidance/codes_of_practice.cfm). The MRC has issued helpful clarification and guidelines for researchers (www.mrc.ac.uk/pdf-ethics_guide_human_tissue_clarification_april_2005.pdf) although this was produced prior to the CoP's.

Material covered by the act includes 'material…which consists of or includes human cells' and 'includes left-over tissue taken from operations and for diagnostic purposes'; it therefore encompasses this proposal. The main issues are:

1. A license is **not** required to obtain relevant material from the living (CoP 1; para 11).
2. Storage (CoP 5; para. 49-53) and use of relevant material obtained for the purposes covered by the act **does not** require consent where samples are anonymised to the researcher. A licence is **not** required for storage where ethical approval has been granted for a research project.
3. Relevant material can be treated as waste and disposed (CoP 5 para. 63-65) of under current guidelines (by incineration).

Thus, although the HTA 2004 applies to this proposal, there are no additional ethical issues arising at this time.

11. **(a) Does the project involve the use of ionising or other type of "radiation"**

NO

(b) Is the use of radiation in this project over and above what would <u>normally</u> be expected (for example) in diagnostic imaging?

NO

(c) Does the project require the use of hazardous substances?

NO

(d) Does the project carry any risk of injury to the participants?

NO

(e) Does the project require participants to answer questions that may cause disquiet / or upset to them?

NO

If the answer to any of the questions 11(a)-(e) is YES, a risk assessment of the project is required and must be submitted with your application.

12. **How many subjects will be recruited/involved in the study/research? What is the rationale behind this number?**

There is no power calculation for this protocol as there are a number of experiments being performed with the blood obtained. Furthermore, the volume of blood obtained from each subject will vary and in patients there is likely to be variability in their inflammation.

Based on previous published research, as a minimum 10-15 samples for each defined COPD subgroup and the same number of healthy samples will be required to achieve statistically meaningful comparisons. Further subgroups may be defined based on severity to take account of heterogeneity of inflammation between patients. This gives a minimum subject population of 75.

13. **Please state which code of ethics has guided your approach (e.g. from Research Council, Professional Body etc).**

Please note that in submitting this form you are confirming that you will comply with the requirements of this code. If not applicable please explain why.

As a general point, in considering these issues the following MRC guidelines were used:
1. MRC ethics series: Human Tissue and Biological Samples for use in Research, MRC, 2001 (www.mrc.ac.uk/pdf-tissue_guide_fin.pdf)
2. MRC ethics series: Personal information in Medical Research, MRC, 2000 (www.mrc.ac.uk/pdf-pimr.pdf)

Remember that informed consent from research participants is crucial; therefore all documentation must use language that is readily understood by the target audience.

Projects that involve NHS patients, patients' records or NHS staff, will require ethical approval by the appropriate NHS Research Ethics Committee. The University Ethics Panel will require written confirmation that such approval has been granted. Where a project forms part of a larger, already approved, project, the approving REC should be informed about, and approve, the use of an additional co-researcher.

Application Checklist

Name of Applicant: Dr X, Dr Y

Title of Project: Use of PBMC from healthy and patient volunteers to investigate Chronic Obstructive Pulmonary Disease.

The checklist below helps you to ensure that you have all the supporting documentation submitted with your ethics application form. This information is necessary for the Panel to be able to review and approve your application. Please complete the relevant boxes to indicate whether a document is enclosed and where appropriate identifying the date and version number allocated to the specific document (*in the header / footer*), Extra boxes can be added to the list if necessary.

Document	Enclosed? (indicate appropriate response)			Date	Version No
Application Form	Mandatory		If not required please give a reason	24/11/2015	
Risk Assessment Form		Not required for this project			
Participant Invitation Letter		Not required for this project			
Participant Information Sheet	Yes			31/10/2014	6.0
Participant Consent Form	Yes			31/10/2014	6.0
Participant Recruitment Material – e.g. copies of posters, newspaper adverts, website, emails		Not required for this project			
Organisation Management Consent / Agreement Letter	Yes			4/8/2014	
Research Instrument – e.g. questionnaire		Not required for this project			
Draft Interview Guide		Not required for this project			
National Research Ethics Committee consent	Yes			12/4/2010	
Note: If the appropriate documents are not submitted with the application form then the application will be returned directly to the applicant and will need to be resubmitted at a later date thus delaying the approval process					

Study Code: DS-10-01 (DS _____)

Subject Information Sheet

A protocol for donation of blood, urine and stool samples by healthy volunteers and patient volunteers for laboratory research.

This document provides information about the clinical trial in which you have expressed interest, to help you decide whether you wish to participate in the trial. Please read it carefully, and feel free to ask the medical staff about anything that is not clear.

If you agree to take part, you will be asked to sign a form confirming your agreement. You will receive a copy of the form and will be able to keep this information sheet for reference.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Please ask the medical staff about anything that is not clear or if you would like more information.

Thank you for reading this.

1. What is the purpose of the study?

The Medicines Evaluation Unit conducts research into the causes and treatments of diseases such as asthma and chronic bronchitis. To help with this research, blood, urine and stool samples are required from healthy volunteers and patient volunteers on a regular basis. Blood samples may be analysed within The Medicines Evaluation Unit or at Wythenshawe Hospital research laboratories or sent to other establishments such as The University of Manchester/Liverpool or Pharmaceutical companies including outside of the UK. Pharmaceutical companies have a requirement for blood, urine and stool samples to help them in their laboratory research work such as identifying new markers for a variety of diseases, including cancer, diabetes, respiratory, neurological and inflammatory diseases, assessing drug activity and developing new laboratory methods. All the work is all exploratory in nature. It does not involve any diagnostic tests of clinical disease and will not involve any testing for the genes which are associated with inherited diseases. Samples may be stored for up to 15 years and may be used for ongoing research.

2. Why have I been chosen?

You have been asked to participate in this study either because you have a respiratory condition, such as asthma or chronic bronchitis/emphysema or because you are healthy without lung disease.

3. Do I have to take part? What if I change my mind, and what does giving informed consent mean?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and asked to sign the attached consent form prior to participation in the study. This

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does not take away any of your rights, but is to ensure that you have been fully informed regarding the study, written consent is required which is voluntary. However, you are free not to participate, or withdraw from the study at any time without giving your reasons. This would have no effect on your treatment, future medical care or relationship with your doctor.

You can decide which type of sample(s) you are willing to donate e.g. you may be willing to donate blood but prefer not to give urine or stool samples. The study staff will advise you when they contact you what are the specific requirements of the sampling at the proposed visit.

You have the right to ask questions concerning the potential and/or known hazards of this study at any time. The study doctor may withdraw you from the study at any time if it is felt to be in your best interest. You may also be asked to co-operate in having whatever laboratory tests are considered necessary. Providing written informed consent does not mean definite progression into the study.

If you withdraw consent before your samples have been processed then you should inform the study staff. If you withdraw consent thereafter any remaining sample may have to be retained for audit purposes.

4. What is involved? What will happen to me if I take part?
Consent Visit
If you decide to participate in the study you will be asked to sign a consent form. This may occur at the same time as blood donation. On certain occasions you may be asked to undergo Hepatitis/HIV testing prior to donating blood for research. If this is applicable then you will be provided with the blood borne viruses information sheet which gives details about these tests. The study staff will advise you if this is a requirement.

Blood Donation
Before you give a donation of blood you will first be asked about your health and any current medications. Depending on the amount of blood to be given it may be necessary to ensure that you are not anaemic by performing a simple pinprick test to obtain a minute amount of blood from your thumb or finger that will then be analysed. If this result is satisfactory a qualified and experienced member of staff will then obtain the required sample of blood from a vein in your arm or from a capillary via a finger prick. The amount of blood that you will be asked to provide at a single visit to the unit will range from small quantities for example, one or several drops for a fingerprick test, 5 to 20mls (1-4 teaspoons) when taking a sample from a vein or larger quantities of up to approximately 1 pint (470ml). You may have more than one fingerprick during a visit. Each visit will last approximately half an hour.

If you agree to take part in this research program, you may be asked to donate blood on a regular or ad hoc basis. However you will not be asked to donate more than the amount which is stipulated by the blood donation service which is 470mls (just under a pint) up to a maximum of 3 times a year. You will be permitted to take part in other research studies if you have had a suitable gap since your last blood donation and the total amount of blood previously donated is less than a certain amount.

Urine samples
You may be asked to provide either a single urine sample in the unit or given a container to collect urine samples over a period of time at home. Specific instructions will be given to you.

Stool samples
You may be asked to provide either a single stool sample in the unit or given a container to collect stool samples over a period of time at home, following bowel movements. Specific instructions will be given to you.

5. What expenses/compensation will I receive for taking part?
A payment in the region of £20 will be made on completion of each study visit for your time and to compensate for any inconvenience. If travel requirements are incurred you will be reimbursed for these additionally. If you travel by car, you will be able to park in our car park

6. What are the possible disadvantages/risks of taking part?
When a sample of blood is obtained it can sometimes cause temporary localised bruising or swelling which will subside within a few days.

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7. What are the possible benefits of taking part?

This is laboratory research, and although your participation will not provide any direct medical benefit to you, the results will be valuable in furthering medical research or the development of medicine and treatments of potential benefit to others. There will be no financial benefit for yourself from the developments of any new treatments.

Research use of the specimens/samples may result in commercial gain, patents may be filed and research results commercialised. You should not expect to benefit directly from any commercialisation.

8. Are there any restrictions?

Depending on the specific project for which the blood, urine or stool sample is to be used for there may be certain restrictions such as avoiding smoking, alcohol, caffeine or strenuous exercise for up to 24 hours. You may be required to fast overnight e.g. not eat or drink except water. You will be advised of these restrictions by the study staff. You will not be eligible to take part if you use social drugs such as cannabis and a urine test may be required to test for such substances.

9. What if new information becomes available?

If new information becomes available during the course of this research program you will be informed, prior to your continued participation in the study. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue (as stated in the protocol).

10. Would I be compensated for any serious or long lasting injury?

If you are harmed by taking part in this research project, there are arrangements in place to compensate you and the Medicines Evaluation Unit holds appropriate insurance. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been treated, the contact details for complaints are included in section 12 below.

11. Will my taking part in this study be kept confidential?

Confidentiality of your medical records will be maintained. Some or all of your medical information that you have supplied will be kept on computer by the Medicines Evaluation Unit. Access to these is strictly limited, and the requirements of the Data Protection Act will be met. No record of your name or address is provided to any outside institutions and only staff of the MEU, an Independent Ethics Committee, monitors/auditors/Regulatory Authorities (if applicable) may inspect your medical records. This is only permitted under the supervision of the study team. Your GP will be notified of your participation in this research and will be asked to complete a general medical questionnaire. In the event of a publication of the results of the study your personal details will remain confidential. The samples which you give will be anonymised so that you cannot be identified by the scientists carrying out the research.
You should be aware that some countries may not offer the same level of privacy protection as in the UK but the company will take all reasonable steps to protect your privacy.

12. Contact for further information.

If you have any questions regarding this study or in any way concerned about your health, experience a side effect or research related injury while participating in this study please phone the Medicines Evaluation Unit on 0161 946 4050 between the hours of 09:00 am – 5:00 pm Monday to Friday. Outside these hours please contact Wythenshawe Hospital Switchboard on 0161 998 7070 and ask to speak to the Doctor on call for The Medicines Evaluation Unit. Any complaints should be directed to:

Mr D Rogers - Director of Operations – Medicines Evaluation Unit, Langley Building, Southmoor Rd, Manchester M23 9QZ

Thank you for reading this.

Professor. D. Singh - Principal Investigator

Medicines Evaluation Unit
The Langley Building
Wythenshawe Hospital
Southmoor Road
Manchester
M23 9QZ

Tel: 0161 946 4050
Fax: 0161 946 1459

E-mail: enquiries@meu.org.uk
Web: www.meu.org.uk

medicines
evaluation unit

Study Code : DS-10-01 (DS _____)   **Subject Consent Form**

Date of Birth			DB no.				

A protocol for donation of blood, urine and stool samples by healthy volunteers and patient volunteers for laboratory research.

(The patient should complete the whole of this sheet himself/herself)

[Please circle as appropriate]

I have read the information sheet (Version 6.0, 31st Oct 14) for the above study…….   **Yes / No**

I have had the opportunity to ask questions and discuss this study…..   **Yes / No**

I have received satisfactory answers to all of my questions…………….   **Yes / No**

I have received enough information about the study…………………………   **Yes / No**

I have read the blood borne viruses information sheet , discussed   ……………   **Yes / No   NA☐**
this with the study Dr and agree to undergo HIV/Hepatitis testing

I understand that samples are a gift and that I will not benefit from any intellectual………..   **Yes / No**
property that results from the use of these tissues.

I have spoken to the supervising study doctor………………………………….   **Yes / No**

I understand that I am free to withdraw from the study
- at any time
- without having to give a reason for withdrawing
- and without affecting my future care…………………….   **Yes / No**

I consent to permit, under the direction of the study doctor, (if applicable) monitors/auditors/an Independent Ethics Committee and Regulatory Authorities to be granted direct access to parts of my original medical records relevant to the study.. ....   **Yes / No**

I agree to take part in this study………………………………………………….   **Yes / No**

I have received a copy of the information sheet to keep……………………   **Yes / No**

I consent to my samples and/or data being exported outside the UK…………….   **Yes / No**

Signature of **Volunteer**…………………………………………………	Date…………………………….
Name of Volunteer…………………………………………………………	**NB: The patient must date their own signature.**
Signature of **Investigator** …………………………………………….	
Name of investigator……………………………………………………….	**Date**……………………………….
Signature of **clinical staff assisting** or NA☐ ……………………………………	
Name of staff ………………………………………………………………	**Date**……………………………….

Two copies of this form should be signed, one will be retained by the subject, one placed in the Investigator Site File and a photocopy will be placed in the subject's notes.

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DS618: Evaluation of polyunsaturated fatty acids (PUFAs) on inflammatory biomarkers in whole fresh blood of COPD, HS and HNS subjects.

- Friday PM NJ should email Salford group with possible patients for the following week. Up to 2-3 per day maximum.

DS618 Salford e-mail group to include Jeremy Allen [J.T.Allen@salford.ac.uk]; Lucy Smyth [L.Smyth@salford.ac.uk]; Amal Al-Haidose (PG) [A.Al-Haidose@edu.salford.ac.uk]

- For cancellations or additional bookings through the week, MEU should also e-mail Salford Group.
- If too short notice or unable to collect sample on the specified day, Salford to inform MEU ASAP on Academic-MEU@meu.org.uk or 0161 9464065
- Patients who meet the criteria in Appendix 1 on the day of screen (or historical data within 1 year if MEU blood donation visit only) will have 2 additional 10ml EDTA tubes taken.
- MEU to notify Amal of screen failures or non-attendance ASAP (see contact numbers below).
- Blood stored at room temp. in MEU Lab 2 until collection by Salford
- Salford staff to come to MEU reception and state they are here **from Salford Uni to collect samples from the academic team.**
- On collection of samples, the 'Material Transfer Record From' (appendix 2) must be signed by MEU and Salford representative and filed at MEU.
- Suitable Blood transfer packaging to be supplied by Salford.
- Blood will be discarded at the end of day if not collected.

Inclusion/Exclusion Criteria

Inclusion:

1) COPD

   Age 40-80 years

   Post BD FEV1/FVC ratio <0.70

   Pack Years >10

2) Smokers with normal lung function

   Age 40-80 years

   Pre BD FEV1/FVC ratio >0.70

   FEV1>80% predicted

   Current smoker

   Pack years >10

3) Never smokers

   Age 40-80 years

   Pre FEV1/FVC >0.70

   FEV1>80% predicted

   Pack years <1

Exclusion:

1) **All Healthy volunteers:**

   History of a chronic respiratory disorder

2) **All volunteers**

   History of acute respiratory disease within 6 weeks prior to visit

   History of other non-respiratory inflammatory disease

   Blood safety at Dr review:

   Donated 1 pint (500mls) of blood in the last 16 weeks:

Got a chesty cough, sore throat or active cold sore:

   Pregnant or a woman with a baby less than 9 months old:

Taking antibiotics NOW or finished taking them within the last 7 days:

Had hepatitis or jaundice in the last 12 months:

Had acupuncture, ear piercing, body piercing, tattooing or semi-permanent

make-up in the last 4 months:

HIV positive or thinks they may be HIV positive:

Ever injected or been injected with drugs including body building drugs:

Been in contact with an infectious disease or been given

immunisations in the last 8 weeks:

Any known Haemophilia or a related blood clotting disorder who has received clotting factor concentrates: Had a history of anaemia.

Appendix B

Skanlt Software Operating

Introduction to Varioskan LUX

Thermo Scientific™ Varioskan™ LUX is a modular multi-technology microplate reader. Varioskan LUX is controlled by Thermo Scientific™ SkanIt™ Software for Microplate Readers.

End point, kinetic, spectral and multipoint measurements can be carried out in the UV/Vis/NIR range from appropriate microplate formats. In fluorescence intensity, time-resolved fluorescence, luminescence and AlphaScreen™ measurements 6- to 1536-well plates can be used, and correspondingly 6- to 384-well plates in absorbance measurements.

Figure 1. Thermo Scientific™ Varioskan™ LUX

The instrument is always equipped with the following detection technologies:

- Absorbance

- Fluorescence intensity (FI)

Depending on the instrument model, the following optional detection technologies (LAT module) may be included:

- Luminescence

- AlphaScreen

- Time-resolved fluorescence (TRF)

The instrument selects the measurement wavelength either by using filters or monochromators depending on the measurement technology.

Using SkanIt Software

This chapter describes the Session tree which is the main part of the software user interface. You will find information on how to create sessions, view and export measurement results, perform calculations, and create data reports.

The general outline for using the software is:

1. Create a new session or open an existing one.

2. Define the plate layout and protocol.

3. Start the session.

4. View the results and perform calculations.

5. Create result report and export data.

Sessions

The information that is needed to define and run an assay is saved in a *session*. With SkanIt Software you can build sessions for your own assays and run or modify ready-made sessions

Session Structure

The **Session tree** is the main use area in the software. The **Session tree** has five main sections:

1. **Notes** - write notes about a session.

2. **Plate Layout** - define which wells of the microplate you want to measure.

3. **Protocol** - define what you want the instrument to do (e.g., measure, shake).

4. **Results** - view the measurement results and choose your calculation methods.

5. **Report** - create a report of the measurement and calculation results.

Figure 31. The Session tree.
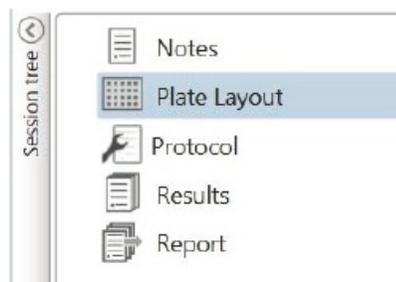
Create and Save a Session

1. Click the application menu tab.

2. Click the **New session** button under **New & Recent**.

3. Click **Save As** or **Save** in the **Home** ribbon.

4. In the **Save as session** window, select the folder where you want to save the session.

   The sessions are saved in the SkanIt Software database.

5. Give the session a name and click **Save**.

Plate Layout

This is where you tell the software which wells to measure (or dispense) and what kind of samples you have in the microplate. The **Pipette content** section is where you define the sample properties. The virtual pipetting section is where you add the samples to the plate.

You can leave the Plate Layout empty. The instrument then measures the whole plate automatically.

Figure 32. The Pipette content section (left.) and virtual pipetting section (right.) in the Plate Layout.
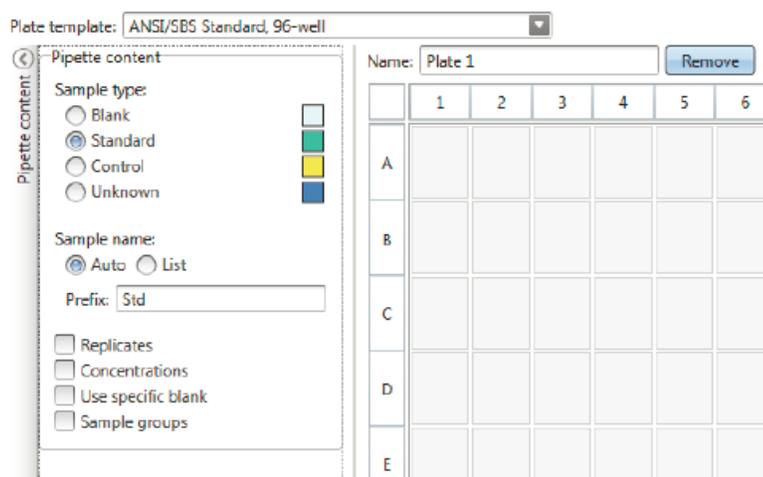
Define the Samples for the Plate

1. Click **Plate Layout** in the **Session tree**.

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2. Select the plate template from the drop-down list.

3. Select the **Sample type** and sample properties.

4. Click the plate wells with the virtual pipette (your cursor) to add the samples.

> **Tip** You can add multiple samples at a time by dragging the pipette across the wells.

To clear or edit a well, right-click on the well.

Figure 33. Example: To add a series of standard samples (concentrations 5,10, 50, 100 and 500 ug/mL), with two replicates side by side, select the pipette content parameters as shown below, and add samples to the plate by painting the wells with the virtual pipette:

Protocol

This is where you define which actions the instrument performs. The instrument runs the actions in the order as listed under the protocol.

Figure 34. In this example the instrument first dispenses liquid to the wells, then shakes the plate, and then measures the fluorescense.
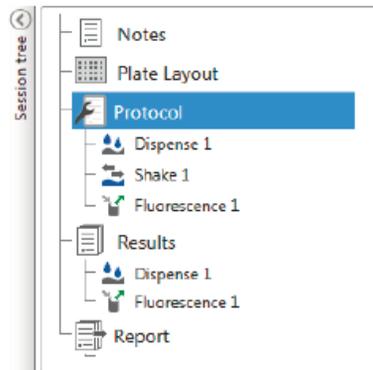

Define a Protocol

1. Click **Protocol** in the **Session tree**.

2. Select the action from the **Protocol** ribbon. The action appears in the **Session tree**.

3. Define the action parameters, e.g., measurement wavelength.

   To change the order of the actions, click the action you want to move, then click the small arrowhead to move it up or down.

Figure 35. To move the action up or down, click the arrowhead icon. To remove the action, click the x-mark.
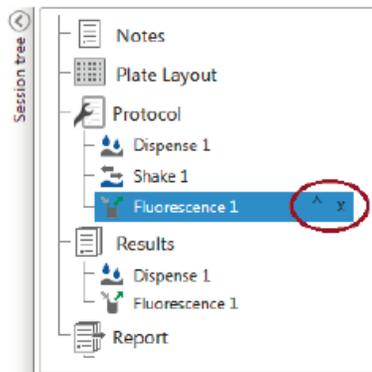
Protocol Actions

Select the protocol actions from the **Protocol** ribbon.

> **Note** The software automatically detects the instrument configuration and shows only those actions that are available.

**Figure 36.** The Protocol ribbon for adding actions.

Results

This is where you can view the measurement results and perform calculations. You can also export measurement and calculation data to use outside SkanIt Software.

View the Results

1. Click the measurement step under **Results** in the **Session tree**.

2. Click the **Plate** or **List** tab to view the results.

Figure 40. The results of a Fluorometric Spectrum measurement with the Plate view open.
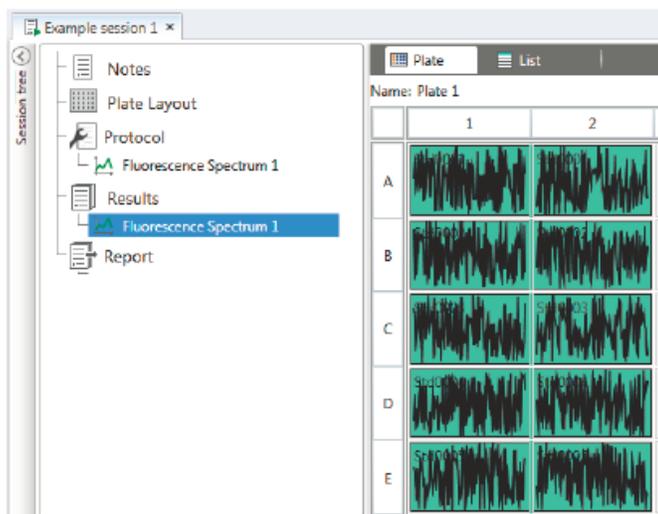
Export Results to Excel

1. In the **Results** view click on the **Export to Excel** tab.

2. Save the data.

> **Tip** You can export the data of several steps into the same file by creating a report. You can create result reports in Excel, PDF, XML and TXT formats.
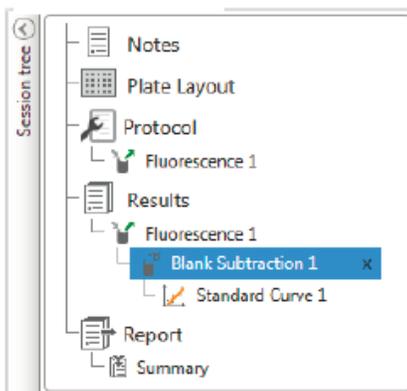
Calculations

The software has built-in calculations that you can use to process data. You can add calculations either before or after a measurement. You can add several calculations to a measurement and also nest calculations.

The calculation uses the result data that is directly above it in the **Session tree**.

Add a Calculation

1. Select the results step in the **Session tree** that you want to use as the source data for the calculation.

2. Click the calculation action on the **Results** ribbon. The action appears in the **Session tree**.

3. Define the calculation parameters (if needed).

4. Click the **Plate** or **List** tab to view the calculation results.

5. Click **Save**.

Figure 41. In this example the Fluorescence Measurement data is the source data for the Blank Subtraction calculation, and Blank Subtraction data the source for Standard Curve.

Calculation Actions

Select the calculation actions from the **Results** ribbon.

Figure 42. The Results ribbon for adding calculations.
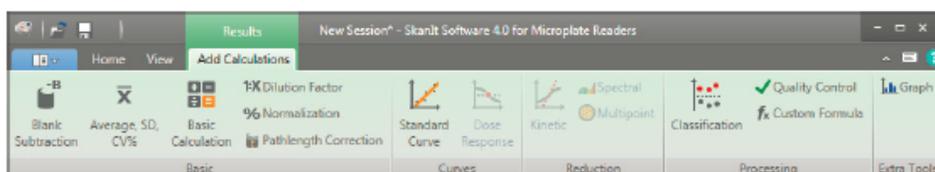
Report

You can create a result report including both measurement and calculation data. You can export the result report to Excel, PDF, XML and TXT formats.

A summary table is automatically created under Report. The summary table shows only the measurement and calculation results of end-point measurements. Kinetic, spectral or multipoint results are not included in the result summary.

You can export any data by selecting the individual result sections to the report.

Create a Data Report

1. Click **Report** in the **Session tree**.

2. Check the sections you want to include in the report from the **Report sections** list.

Figure 43. The Report pane open with Report sections checked.
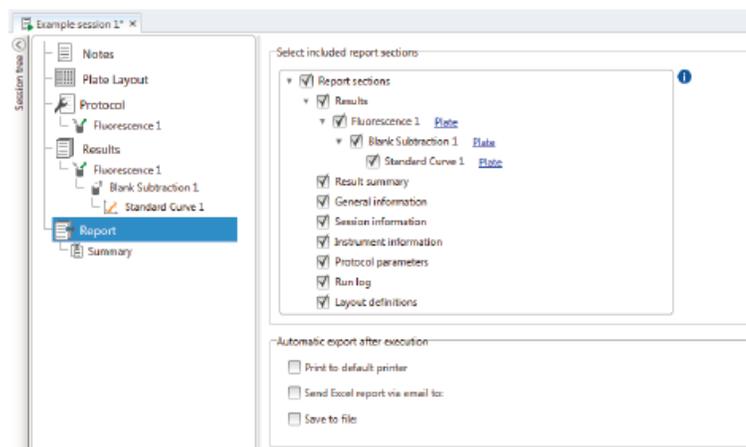
Export a Result Report Manually

1. Click the PDF, Excel, XML, or TXT format on the **Results** ribbon to export the report.

2. Save the report.

The report automatically opens in the format you choose.

Export a Result Report Automatically

You can set the software to export the report automatically after the run to a specific destination.

Before you run a session, you need to select the report content and where to export the report.

1. Click **Report** in the **Session tree**.

2. Check the **Save to file** box in the **Automatic export fter execution** pane.

3. Name the file and click **Browse** to select the destination folder and the file format.

4. Save the session.

The next time you start the session, a report is automatically saved in the destination folder you selected.

Saved Sessions

There are two different kinds of saved sessions:

a. A session that is saved before you have run it.

A session that you have saved but have not run does not have measurement data. You can edit all of the content.

b. A session that has been run.

A session that has been run is automatically saved. You cannot edit the protocol, but you can edit all other content. A green arrowhead icon indicates a saved session with measurement data.

Figure 44. A session with measurement data (green icon) and without measurement data (no icon).

Open an Existing Session

You can open a recent session, or an older session.

Open a Recent Session

1. Click **New & Recent** on the application menu.

2. Select a recent session from the **Open recent session** list.

The session opens in the **Session tree**.