EXPLORING BIOLOGICAL RISK FACTORS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE: OLD AGE AND FEMALE SEX

by

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) has various risk factors including old age and female sex; however, the biological reasoning behind these is not fully understood.

COPD prevalence and mortality increase with age. COPD patients also seem to demonstrate pulmonary and systemic accelerated aging. When people age, repetitive sequences at their chromosomes ends, called telomeres, shorten. In COPD this may occur at an increased rate due to increased cell turnover or DNA damage, caused by inflammation and oxidative stress, and could contribute to lung function decline. Therefore, we measured telomere length in peripheral blood cells of COPD patients using qPCR and examined the relationship with lung function (FEV₁ % predicted, FVC % predicted and FEV₁/FVC) as well as inflammatory marker levels. We found that telomere length was positively related to FEV₁/FVC and negatively related to serum SP-D level, a lung specific marker of inflammation. This supports that COPD is a disease of accelerated aging and suggests that lung inflammation may be involved in the process.

Females seem to be more susceptible to developing COPD than males. A major distinction between males and females is their sex hormone levels. The lung has sex hormone receptors and there are reports of experimental animal studies and observational human studies suggesting that sex hormones have an effect on the lung. Hence, in COPD patients we measured levels of the hormones estradiol, progesterone, testosterone, luteinizing hormone, follicle-stimulating hormone and sex hormone-binding globulin in their serum using ELISAs. The hormone levels of COPD patients fell within normal ranges and had expected relationships with age and BMI. We found a significant negative association between estradiol and FVC % predicted in males; an inverse relationship between progesterone and FVC % predicted in both sexes; and a positive relationship between LH concentration and FEV₁ % predicted in females. These data support that sex hormones affect lung function, though the mechanism by which they do so is unclear due to the scarcity of knowledge in the field.

Telomeres and sex hormones seem to play a role in the risk factors of aging and female sex, respectively, and offer insight into COPD pathogenesis, though more research is needed.
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LIST OF COMMON ABBREVIATIONS
(Listed alphabetically)

α₁-AT: alpha-1 antitrypsin
ABC: Advair, Biomarkers in COPD
BALF: bronchoalveolar lavage fluid
BMI: body mass index
CBG: corticosteroid-binding globulin
COPD: chronic obstructive pulmonary disease
CRP: C-reactive protein
ELISA: enzyme-linked immunosorbent assay
FEV₁: forced expiratory volume in one second
FEV₁/FVC: ratio of forced expiratory volume in one second to forced vital capacity
FSH: follicle-stimulating hormone
FVC: forced vital capacity
GOLD: Global Initiative for Chronic Obstructive Lung Disease
IL: interleukin
LH: luteinizing hormone
MMP: matrix metalloproteinase
NF-κB: nuclear factor-kappa B
qPCR: quantitative polymerase chain reaction
SHBG: sex hormone-binding globulin
SP-D: surfactant protein-D
TGF-β: transforming growth factor beta
TNF-α: tumour necrosis factor alpha
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CHAPTER 1: INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is caused by genetic and environmental interactions and has many risk factors. Some of these risk factors, such as tobacco smoke, are well studied. However, the biological underpinnings of others, including old age and female sex, remain uncertain.

The prevalence, morbidity, and mortality of COPD are known to increase with age\textsuperscript{1}. There is also growing literature supporting a relationship between the pathogenesis of COPD and aging. Nonetheless, the molecular mechanisms behind this remain to be elucidated\textsuperscript{2}. Similarly, there is increasing evidence that women are more susceptible to developing COPD than males\textsuperscript{1,3}, but a void of studies investigating the biological sex differences responsible\textsuperscript{4}.

Therefore, the thesis to follow will explore potential biological explanations for the risk factors of aging and sex in COPD. In COPD patients, aging will be examined by measuring telomere length and sex will be considered by assaying sex hormone levels. Their relationships with disease will be studied by analyzing their associations with lung function measures.
CHAPTER 2: CHRONIC OBSTRUCTIVE PULMONARY DISEASE

2.1 Definition of Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airflow limitation that is not fully reversible, due to increased lung compliance from emphysema and increased airway resistance from chronic obstructive bronchitis. Emphysema is anatomically defined as enlarged airspaces, due to the destruction of alveoli, and presents clinically as dyspnea (shortness of breath) and cough. Chronic bronchitis is defined clinically as the presence of a chronic sputum-producing cough for at least 3 months in 2 consecutive years.

Airflow limitation, which is quantified as forced expiratory volume in the first second of expiration (FEV₁) and forced vital capacity (FVC), is measured by spirometry. In order to assess lung function, FEV₁ and FVC % predicted values, adjusted for height, age, sex and race/ethnicity, and the FEV₁/FVC ratio are calculated. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) spirometric criteria for the diagnosis of COPD is a FEV₁/FVC ratio of less than 0.70. Disease severity is most commonly classified according to GOLD stages, which are defined in Table 2.1 below.

Table 2.1: GOLD Classification of COPD Severity by Spirometric Measures

<table>
<thead>
<tr>
<th>Stage</th>
<th>FEV₁/FVC</th>
<th>FEV₁ % predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>&lt; 0.70</td>
<td>≥ 80</td>
</tr>
<tr>
<td>II</td>
<td>&lt; 0.70</td>
<td>50 to &lt; 80</td>
</tr>
<tr>
<td>III</td>
<td>&lt; 0.70</td>
<td>30 to &lt; 50</td>
</tr>
<tr>
<td>IV</td>
<td>&lt; 0.70</td>
<td>&lt; 30</td>
</tr>
</tbody>
</table>

Abbreviations: FEV₁: forced expiratory volume in one second; FVC: forced vital capacity.
2.2 Epidemiology of Chronic Obstructive Pulmonary Disease

In 2004 nearly 64 million people suffered from COPD and 3 million people died, making COPD the 4th leading cause of death worldwide\(^7\). While mortality rates of other major causes of death have been decreasing, the rate for COPD has been steadily rising. Growing prevalence of tobacco smoking is projected to increase tobacco-related deaths to 8.3 million (10% of deaths) and these deaths encompass not only COPD, but comorbidities of COPD: cardiovascular disease and cancer\(^7\). By 2020, COPD is predicted to become the 3rd leading cause of death worldwide\(^8\).

COPD not only causes mortality, but disability as well. Due to symptoms including dyspnea (shortness of breath), cachexia (muscle wasting) and fatigue, those with COPD have greatly reduced quality of life. In 2004 COPD was the 10th most prevalent cause of moderate and severe disability in the world\(^7\). Burden of disease (quantified as DALYs: disability adjusted life-years) takes into account both the number of years of life lost due to premature death and years of “healthy” life lost due to disability. COPD is forecast to become the 5th leading cause of burden of disease by 2020, from the 13th in 2004\(^7,8\).

Since COPD is such a substantial burden of disease, requiring frequent doctors’ visits, emergency room encounters, drug treatments and hospitalizations, as well as lost work days, it is also an enormous economic burden. There is little data available on the costs caused by morbidity and mortality of COPD, however, from what has been published the global costs easily total in the tens of billions of dollars (USD)\(^9\).
2.3 Causes of Chronic Obstructive Pulmonary Disease

Tobacco smoke, including cigarette, cigar and pipe smoke, is the most common cause of COPD worldwide\(^1\),\(^{10}\). Occupational exposure, in which people are exposed to noxious dust, fumes and/or vapours at work, is also a high risk factor for COPD\(^{11}\).

The effect of outdoor air pollution on COPD is much smaller than that of tobacco smoke and is only responsible for 1 to 2% of COPD\(^1\). On the other hand, indoor air pollution due to the use of biomass fuel for indoor heating and cooking in inadequately ventilated homes is a major risk factor for COPD, particularly in poorer countries\(^1\).

The only confirmed genetic determinant of COPD is a mutation in the alpha-1 antitrypsin (\(\alpha_{1}\)-AT) gene, which codes for a major serine protease inhibitor. However, exposure to noxious particles is usually required along with \(\alpha_{1}\)-AT deficiency for disease to develop and the deficiency is only found in about 1% of COPD sufferers due to the low prevalence of the disease-causing allelic variants\(^{12}\).

It is likely that COPD is a disease of multiple-gene-environment interactions. The prevailing view of COPD is that only about 15% of smokers are susceptible to COPD. However, two studies have shown that if any smokers smoked long enough and accrued enough smoking history, they too would develop COPD\(^{13},^{14}\). As pack-years increase, lung function will decrease, though to varying extents in different people. This variability is probably due to differing genetic susceptibility. The value of 15% is misconstrued from Fletcher & Peto's classic study and the authors themselves wrote that a "spectrum of susceptibility probably exists"\(^{15}\).
2.4 Chronic Obstructive Pulmonary Disease Pathogenesis and Pathophysiology

COPD is an inflammatory disease. Chronic exposure to toxic gases and particles, such as cigarette smoke or others described previously, results in an abnormal chronic immune response and inflammation of the lungs\(^{16}\).

The innate immune system is the body's first line of defense against contaminants and includes the mucociliary escalator and tight junctions between epithelial cells\(^ {17,18}\). Chronic exposure to cigarette smoke breaks down the escalator, resulting in impaired clearance of toxins and, thus, accumulation of inflammatory molecules in the lung. Moreover, the physical barrier provided by the tight junctions is eventually breached, allowing particles to enter the tissue, again causing an influx of inflammatory cells and airway remodeling\(^ {18-20}\). As exposure to noxious particles persists, the cycle of injury and inflammation perpetuates and worsens.

2.4.1 Chronic Bronchitis

Chronic bronchitis is the obstruction of airways larger than 4 mm in diameter due to inflammation and is associated with mucus gland hypertrophy, mucus hypersecretion, and airway remodeling\(^ {3,18}\). Mucus plugging and fibrosis (increased deposition of connective tissue) occludes airflow\(^ {16}\). Immune cells that are characteristic of COPD, neutrophils, macrophages and CD8+ (cytotoxic or killer) T cells, are found infiltrating the bronchial epithelium\(^ {21}\).

2.4.2 Small Airways Disease

While obstruction of the large airways contributes to airflow limitation, the major sites of obstruction in COPD are the peripheral, small airways with diameters less than 2 mm\(^ {22}\). Again, this phenotype of COPD is marked by airway remodeling and by
inflammation, with the influx of high numbers of neutrophils, macrophages and CD8+ T lymphocytes, as well as B cells\textsuperscript{23-25}. The inflammation, fibrosis, and hypertrophy of smooth muscle all contribute to the thickening of the airway walls, which causes considerable narrowing of the small airways, and when enough of the small airways are affected, results in significant airflow limitation\textsuperscript{26}. Hypertrophy of goblet cells, which are normally rarely found in the peripheral airways, also contributes to airflow limitation\textsuperscript{24}.

\textbf{2.4.3 Emphysema}

Emphysema occurs when lung parenchyma, including alveolar walls and alveolar attachments, are destroyed, resulting in enlarged airspaces, loss of elastic recoil and decreased intraluminal pressure and leading to the collapse of small airways\textsuperscript{3}.

Different distributions of emphysematic destruction have been noted. Centrilobular emphysema is localized to the respiratory bronchioles and centres of alveolar sacs\textsuperscript{27}. This type results in more damage to the upper portions of the lungs and is the most common in smokers\textsuperscript{28}. Panlobular emphysema is characterized by uniform involvement of the entire alveolus\textsuperscript{27}. This type usually affects the lower lobes of the lung and is associated with \(\alpha_1\)-AT deficiency\textsuperscript{18, 29}. Once again CD8+ T lymphocytes are a predominant presence, found in the lung parenchyma of alveolar walls\textsuperscript{30}.

\textbf{2.4.4 Inflammation, Oxidative Stress, and Protease-Antiprotease Imbalance}

As described above, COPD is characterized by a constant influx of immune cells, predominantly neutrophils, macrophages and CD8+ T cells, which correlate with disease severity\textsuperscript{31}. These cells are part of the inflammatory response, but also cause inflammation themselves by releasing pro-inflammatory cytokines and chemokines, including TNF (tumour necrosis factor)-\(\alpha\), TGF (transforming growth factor)-\(\beta\), IL
(interleukin)-6, IL-1, leukotriene B₄, MIP (macrophage inflammatory protein)-1α and GM-CSF (granulocyte macrophage-colony stimulating factor), inciting the recruitment of more immune cells¹⁶. Therefore, immune cells that are typically present to help clear toxins and infections from the lung ultimately cause the release of chemicals that promote fibrogenesis, oxidative damage, and proteolytic destruction. Moreover, epithelial cells of the lung release inflammatory mediators, such as IL-1, IL-8 and GM-CSF, further propagating inflammation³³.

Macrophages and neutrophils secrete proteases such as neutrophil elastase, proteinase-3, cathepsins and matrix metalloproteinase (MMPs). Normally, antiproteases such as α₁-AT, secretory leukoprotease inhibitor and tissue inhibitors of MMPs (TIMPs) are able to neutralize these enzymes³⁴, ³⁵. However, an imbalance due to increased inflammation (or α₁-AT deficiency) results in the proteolytic degradation of the alveolar connective tissue as well as stimulation of mucus secretion³⁴.

Another imbalance is important to COPD pathogenesis: the oxidant-antioxidant imbalance. This results in oxidative stress, with sources of reactive oxygen species including cigarette smoke and leukocytes³⁶. Airway epithelium permeability may be affected through damage to glutathione, an antioxidant³⁶. As well, oxidation may damage antiproteases and may activate transcription of inflammatory mediators like TNF-α and IL-8 via NF (nuclear factor)-κB, therefore, causing increased inflammation and proteolytic damage³⁴, ³⁵.

2.4.5 Systemic Inflammation and Oxidative Stress and Comorbidities

In COPD, inflammation and oxidative stress are not confined to the lungs, but occur systemically as well. Comorbidities of COPD include lung cancer, but also systemic
maladies such as cachexia (skeletal muscle wasting), osteoporosis, and cardiovascular diseases (such as endothelial dysfunction and coronary artery disease)\textsuperscript{36-39}. Furthermore, increased numbers of neutrophils and T lymphocytes and increased levels of inflammatory mediators and acute-phase proteins, such as TNF-\(\alpha\), IL-6, fibrinogen and CRP, are found in the peripheral circulation\textsuperscript{40}. However, the cause of these systemic effects is currently unknown\textsuperscript{38}. 
CHAPTER 3: ACCELERATED AGING IN COPD

3.1 INTRODUCTION

3.1.1 Evidence of Accelerated Aging in COPD

COPD is a disease which develops gradually and affects the middle-aged and elderly. Emphysema, one of the components of COPD, is particularly linked to aging. Two studies have found evidence of senescence in emphysematic lungs. Tsuji et. al. found that emphysematous patients (N = 13) had significantly more positive staining for senescence markers (senescence-associated cyclin-dependent kinase inhibitors p16INK4a and p21CIP1/WAF1/Sdi1) in type II epithelial cells and alveolar endothelial cells when compared to asymptomatic smokers (N = 10) and non-smokers (N = 11; p < 0.05 for all, except for p21 staining in endothelial cells between emphysematics and asymptomatic smokers)⁴¹. Similarly, Muller et. al. demonstrated that COPD patients with moderate to severe emphysema (N = 13) have upregulation of senescence-associated β-Galactosidase in their parenchymal lung fibroblasts when compared to asymptomatic smoking controls (N = 15; p = 0.001)⁴². As well, aged lungs share some characteristics with COPD lungs. Janssens et. al. found that aging is associated with enlargement of airspaces and loss of peripheral airway-supporting tissue, resulting in reduced elastic recoil⁴³. As well, the strength of respiratory muscles and vital capacity decrease with aging. However, the morphological changes seen in aged lungs are different from those seen in emphysematous lungs in that there is no destruction of the alveolar walls and the distribution of disease is more homogeneous⁴³.

Mutations in growth and aging related genes have been linked to lung disease. Mice that are homozygous for a mutation in the klotho gene, which codes for a protein...
regulator of growth factor signaling and ion channel activity, develop pulmonary emphysema as they age and have a shortened lifespan\textsuperscript{44,45}. They also present with other phenotypes of aging, such as osteoporosis and arteriosclerosis\textsuperscript{45}. Similarly, when knock-out mice for senescence marker protein (SMP)-30, a protein known to decrease with age, were exposed to cigarette smoke they demonstrated shorter lifespans, airspace enlargement and parenchymal destruction, as well as increased markers of oxidative stress (protein carbonyls and malondialdehyde in lung homogenates and total glutathione in bronchoalveolar lavage fluid [BALF]) and apoptosis. None of these phenotypes were seen in smoke-exposed wild-type mice\textsuperscript{46}. Furthermore, it has been reported that in preliminary studies, levels of Klotho and SMP-30 are decreased in COPD lungs\textsuperscript{2}. Sirtuins (SIRTs), which are histone deacetylases involved in gene expression, cell cycle regulation, inflammation and other roles, are dubbed anti-aging molecules. SIRT\textsubscript{1} has been found to be reduced in the lung tissue of COPD patients (N = 17) when compared to smokers (N = 10; p < 0.001)\textsuperscript{47}. Further histological study of the lungs showed decreased SIRT\textsubscript{1} expression in pulmonary macrophages and in airway and alveolar epithelia\textsuperscript{47}. As well, both Klotho and SIRT\textsubscript{1} are involved in the regulation of MMP-9\textsuperscript{2}.

COPD is also associated with various age-related pathologies such as osteoporosis and cardiovascular diseases\textsuperscript{48,49}. As well, facial wrinkling is associated with both FEV\textsubscript{1} % predicted and degree of emphysema in COPD (adjusted for age, pack-years smoking history, etc.) and the two may share a common susceptibility\textsuperscript{50}.
3.1.2 Telomeres

3.1.2.a Structure

In humans, telomeres are tandem repeats of the sequence (TTAGGG/CCCTAA)$_n$ located at chromosome ends.$^{51}$ They terminate in a 3’ single-stranded G-rich overhang which invades the telomeric repeats to form a large duplex lariat-like structure called a telomeric loop.$^{52,53}$ Telomeres are also associated with a complex of six binding proteins dubbed the shelterin complex.$^{54,55}$ Much is still unknown about the structure and function of the t-loop and telomere-associated proteins and research is ongoing, though they are thought to protect telomere ends.

3.1.2.b Function

Telomeres are believed to have developed during the evolution of linear chromosomes in eukaryotes. Linear chromosomes present problems not found in circular genomes. They suffer the “end-replication problem” whereby semi-conservative replication by DNA polymerases cannot completely duplicate the 5’ end of the lagging strand, resulting in loss of DNA (Figure 3.1)$^{56,57}$. As well, end processing occurs to create the G-rich strand, further shortening DNA.$^{58}$ Furthermore, the exposed ends of linear chromosomes are open to enzymatic attack and may be inappropriately recognized as DNA lesions (specifically, double-stranded breaks) by DNA repair machinery.$^{59}$ Double-stranded breaks are repaired by non-homologous end joining or homologous recombination, which results in chromosomal fusions.$^{50}$

Therefore, telomeres, along with associated binding proteins, act as a sort of buffer zone and cap for chromosomes. Telomeres are lost during replication, instead of essential coding DNA.$^{59}$ and the t-loop and shelterin complex hide chromosome ends.
1) DNA replication begins at the origin or replication where the two parent strands separate, creating a replication bubble with two replication forks at either end. DNA synthesis occurs from 5' to 3'. DNA polymerases require a 3'-OH as a starting point for nucleotide addition, which is provided by an RNA primer. As the replication fork opens (← →), synthesis is occurring in the same direction on one side (leading strand), but in the opposite direction on the other side (lagging strand).

2) DNA replication is completed. Each new strand is composed of one parent strand and one daughter strand (semi-conservative replication). The RNA primers upstream (3') of DNA are removed and replaced with DNA.

3) The RNA primer from the most distal (5') Okazaki fragment is excised, but cannot be replaced with DNA. Therefore each round of replication results in DNA shortening.
from DNA damage surveillance machinery\textsuperscript{54, 55}. When telomeres become too short and lose their end structures, they become dysfunctional and result in fusions, chromosome breaks, break-fusion-bridge cycles, translocations and aneuploidy, causing genomic instability\textsuperscript{61}. Ultimately, telomeres protect from loss of genetic information and maintain chromosome stability.
3.1.3 Telomere Shortening and Aging

As described above, as the number of cellular replications increases, telomere length decreases\textsuperscript{62}. Eventually after a certain number of cell divisions, termed the Hayflick limit, telomeres become dysfunctional and a cell’s “molecular clock” will cease any further divisions\textsuperscript{63, 64}. DNA-damage repair machinery in the p53 and p16/retinoblastoma pathways will recognize the critically short telomeres, stop the cell cycle, and induce senescence, an irreversibly arrested state\textsuperscript{65, 66}. In human cells this generally occurs when telomeres have shortened from 15 to 20 kilobases (kb) in the germ line to 4 to 7 kb\textsuperscript{67}. As previously mentioned, if cell divisions are allowed to occur after this critical point, essential DNA can be lost and/or end-to-end fusions can form dicentric chromosomes, rings and other abnormalities, which cause genomic instability and ultimately apoptosis (programmed cell death). This cellular state is called crisis\textsuperscript{68}.

Of course, the number of cellular replications increases with age and hence telomeres have been proven to shorten with years of aging\textsuperscript{63, 67}. As well, shortening of telomeres has been implicated in many age-related and pre-mature aging diseases\textsuperscript{69}. One example of such a syndrome is dyskeratosis congenita in which symptoms are seen in tissues with high cell turnover and include abnormal skin pigmentation, nail dystrophy and oral leukoplakia. Interestingly, patients often die at a young age from pulmonary fibrosis\textsuperscript{70, 71}. Further, in normal people over 60 years of age, telomere length has been found to be inversely related to age-adjusted mortality (N = 143, p = 0.01)\textsuperscript{72}. Therefore, data show that telomere loss contributes to human aging\textsuperscript{61}. Hence telomere length might be considered a biomarker for biological age.
3.1.4 Possible Causes of Accelerated Aging in COPD

The generalized premature aging in COPD could be due to the increased levels of systemic inflammation and/or oxidative stress associated with the disease. Chronic high levels of oxidative stress could damage telomeres directly, causing telomere shortening and cellular senescence or death. Alternatively, or concurrently, chronic inflammation and oxidative stress could cause increased cell turnover, resulting in shortened telomeres. (See Figure 3.2).

Mild oxidative stress (40% oxygen, 5% CO₂) has been demonstrated to dramatically increase the shortening of telomeres in a human lung fibroblast cell line, with a loss of 500 base pairs (bp) per population doubling (PD) compared to 90 bp/PD under normoxic conditions⁷３. Furthermore, in human lung fibroblast cell lines, reducing oxidative stress (via spin trap α-phenyl-t-butyl-nitron reduction of intracellular peroxide activity) has actually been shown to decrease the rate of telomere shortening, while increasing stress (via addition of hydrogen peroxide) results in higher rates of shortening⁷⁴,⁷⁵. Similarly, in primary skin fibroblasts, rates of telomere shortening correlated with mRNA levels of the antioxidants glutathione peroxidase and copper/zinc superoxide dismutase⁵¹. Oxidative stress induces higher numbers of single-stranded breaks in telomeres than in other parts of the genome which, coupled with the much slower rate of DNA repair in telomeric regions, results in accelerated telomere shortening⁷⁶. Moreover, two studies have found that exposing DNA fragments and human lung fibroblast cell lines to oxidative stress (by incubating with hydrogen peroxide or by UVA irradiation-induced oxidative damage, respectively) resulted in greater formation of DNA damage in GGG
Figure 3.2: A Model of Accelerated Aging in COPD Due to Telomere Shortening

Inflammation and oxidative stress cause increased cell death and, therefore, increased cell turnover, resulting in telomere shortening. Oxidative stress also causes direct damage to telomeres. Eventually telomeres become critically short and dysfunctional, resulting in cellular senescence or apoptosis. In the lungs this results in emphysema. Systemically, this results in comorbidities of COPD. (See text above for a more detailed discussion.)
sequences of telomeric DNA. This is hypothesized to occur due to the obstruction of DNA repair machinery by telomere-binding proteins. Accordingly, a study found higher levels of both plasma oxidative stress markers (protein carbonyl and malondialdehyde) and DNA breaks in peripheral blood leukocytes in COPD patients (smoke- and biomass-related, N = 72) versus controls (N = 36), and a positive correlation between malondialdehyde levels and amount of DNA damage in the (smoke-related) COPD patients (N = 47). Together these studies show that higher levels of oxidative stress in COPD patients can cause increased DNA damage specifically in telomeres, resulting in accelerated rates of telomere shortening.

In emphysema there is increased apoptosis of alveolar cells and, likely to replace those lost cells, increased proliferation. However, it has been shown that in advanced emphysema, rates of proliferation are not high enough. Therefore it has been suggested that as disease progresses there is a high rate of turnover of alveolar cells, which become senescent and no longer able to divide, ultimately resulting in the breakdown of alveoli.

Increased apoptosis is especially high in those with α1-AT deficiency, suggesting a role for protease-antiprotease imbalance in alveolar cell death. As discussed previously, a significant cause of disease progression in COPD is the release of proteases by inflammatory cells, resulting in cellular destruction. A study on emphysematous patients found a positive correlation between lung parenchymal cell death and CD8+ T cells, suggesting that cytotoxic T cells may be involved in alveolar degradation.

Many other chronic inflammatory diseases have also been found to have increased shortening of telomeres, implicating the inflammatory process as a cause of accelerated aging. For instance, the telomere length of liver samples from patients with chronic
hepatitis and liver cirrhosis were significantly shorter than from normal, age-matched samples\textsuperscript{85}. Another example is ulcerative colitis (UC), in which it was shown that telomeres of colonocytes shorten to half the length of those in normal controls within the first 8 years of disease. Moreover, DNA of UC patients showed more signs of damage and leukocytes of UC patients had shorter telomeres\textsuperscript{86}. Yet another disease that illustrates this is rheumatoid arthritis (RA). RA patients had significantly shorter telomeres in peripheral T cells (CD4+ and CD8+) compared to age-matched controls\textsuperscript{87}. 

3.2 RESEARCH AIMS & HYPOTHESIS

COPD patients seem to exhibit accelerated aging, both of the lungs and systemically. Telomeres shorten during aging and at an increased rate in premature aging diseases. Therefore we hypothesized that COPD severity is associated with telomere length. In the following study our primary objective was to determine if the telomere length of COPD patients had an association with their severity of COPD. We measured relative telomere length in their peripheral blood and quantified severity by lung function measures, assessed by spirometry. We expected that telomere length would decrease with worsening lung function.

Our secondary objective was to determine if there was a relationship between telomere length and inflammation. We compared relative telomere length to levels of inflammatory/injury biomarkers: C-reactive protein (CRP), an acute-phase reactant; IL-6, a pro-inflammatory cytokine; and protein surfactant D (SP-D), a lung-specific marker. We anticipated that telomere length would have an inverse relationship with levels of inflammatory markers.
3.3 METHODS

3.3.1 Subject Demographics

3.3.1.a COPD Patient Cohort

Two hundred and eighty-three subjects were recruited for the Advair, Biomarkers in COPD (ABC) study\(^88\). Subject demographics are listed in Table 3.1. The subjects had a clinical diagnosis of COPD following the GOLD guidelines\(^89\). The spirometric criteria were a \(\text{FEV}_1\) less than 80% of predicted and a \(\text{FEV}_1/\text{FVC}\) ratio, post-bronchodilator, less than 0.70. Additional criteria were at least a 10 pack-year smoking history, 4 weeks free of exacerbations and 40 years of age. Participants were excluded if they had known chronic systemic infections, inflammatory conditions, solid organ transplantations, myocardial infarctions or cerebrovascular accidents within the past 3 months prior to study enrolment; had upper respiratory tract infection within the 4 weeks prior to enrolment; were unlikely to survive more than 6 months; were females of child-bearing age; had participated in a drug trial within the 4 weeks prior to study enrolment; or were on chronic oral theophyllines (and unable or unwilling to come off theophyllines for the study period), oral corticosteroids or long-term immunosuppressive agents. All subjects gave informed consent. The study was approved by the University of British Columbia/Providence Research Ethics Board.

Inflammatory markers and lung function were measured by enzyme-linked immunosorbent assays (ELISAs) and spirometry, respectively, as described previously (See reference 88). C-reactive protein (CRP), IL-6 and surfactant protein (SP)-D were measured in serum using commercially available ELISA kits (Alpha Diagnostics, San
Antonio, TX; R&D Systems, Minneapolis, MN; and BioVendor, Czech Republic, respectively).

Table 3.1: Baseline Characteristics of COPD Patient Cohort
Mean ± standard deviation (SD). N = 283.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>68.7 ± 9.0</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>60.4</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>169.4 ± 9.8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>80.1 ± 20.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.7 ± 6.0</td>
</tr>
<tr>
<td>CVD (%)</td>
<td>17</td>
</tr>
<tr>
<td>Current Smoker (%)</td>
<td>32.9</td>
</tr>
<tr>
<td>Pack-Years</td>
<td>62.3 ± 28.6</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>1.37 ± 0.55</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>47.7 ± 16.2</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>2.82 ± 0.87</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>75.4 ± 16.6</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>0.49 ± 0.13</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>3.02 ± 2.44</td>
</tr>
<tr>
<td>CRP (ng/mL)</td>
<td>7,649 ± 17,053</td>
</tr>
<tr>
<td>SP-D (ng/mL)</td>
<td>126.8 ± 190.9</td>
</tr>
</tbody>
</table>

Abbreviations: BMI: body mass index; CVD: cardiovascular disease (stroke or cardiac disease); FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; IL-6: interleukin-6; CRP: C-reactive protein; SP-D: surfactant protein D.

### 3.3.1.b Anorexia Nervosa with Early Emphysema Patient Cohort

Subjects with anorexia nervosa were recruited from the Eating Disorders Program, St. Paul's Hospital (Vancouver, BC) for a study of pulmonary emphysema in chronically malnourished patients (Table 3.2). Spirometry was performed as previously described. All subjects gave informed consent. The study was approved by the University of British Columbia Clinical Ethics Review Board.
Table 3.2: Baseline Characteristics of Anorexia Nervosa Patient Cohort
Mean ± standard deviation (SD). N=40.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36.4 ± 8.9</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>19.3 ± 3.4</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>18.2 ± 4.2</td>
</tr>
<tr>
<td>Duration of Disease (years)</td>
<td>16.6 ± 11.0</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Abbreviations: BMI: body mass index.

3.3.1.c Elderly Subject Cohort

Participants of the T-Cell Responses to Influenza Vaccination in Older Adults study were used as healthy controls (N = 43, 42% male). All of the subjects were 60 years of age and older (mean ± SD: 73.4 ± 5.5 years), most (95.2%) were non-smokers, and none of the patients had COPD, emphysema or chronic bronchitis. All subjects gave informed consent and the study was approved by Institutional Review Board of the University of Connecticut Health Center.
3.3.2 Blood Sample Processing

Blood samples were obtained from subjects via venipuncture. This was performed after a washout period and prior to drug treatment for the ABC study subjects and performed prior to vaccine administration for the Influenza Vaccination study subjects. Blood was collected in Vacutainer Blood Collection Tubes (BD Biosciences, Mississauga, ON) containing ethylenediaminetetraacetic acid (EDTA) as an anti-coagulant. The tubes were centrifuged at $\geq 1,500 \times g$ for 15 minutes at room temperature. Buffy coat fractions were then collected using sterile plastic transfer pipettes, aliquotted, and stored at -80°C.
3.3.3 Telomere Length Measurements

3.3.3.a DNA Extraction

Deoxyribonucleic acid (DNA) was isolated from buffy coat samples using QIAamp DNA Blood Kits (QIAGEN, Mississauga, ON), in accordance with the manufacturer’s Blood and Bodily Fluids Protocol. The concentration of DNA was then measured fluorometrically using a Qubit fluorometer and Quant-iT dsDNA BR (broad range) Assay Kit (Invitrogen, Burlington, ON). Lastly, the DNA was diluted to 1.75 ng/μL in Gibco UltraPure Distilled Water (Invitrogen, Burlington, ON) and stored at -20°C for subsequent use in quantitative PCR.

3.3.3.b Quantitative PCR

A quantitative PCR (qPCR, also known as real-time PCR) protocol was modified from that described by Cawthon\textsuperscript{91}. The primers used were those designed by Cawthon (5’ to 3’):

- tel 1: \texttt{GGTTTTTGAGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGGGTGAGGG GTAATCC

- 36B4u: CAGCAAGTGGGAAGGTGTAATCC
- 36B4d: CCCATTCTATCATCAACGGGTTACAA.

Quantitative PCR was performed in a 384-well Clear Optical Reaction Plate (Applied Biosystems, Foster City, CA). Each well contained a total volume of 20 μL:

- 10 μL QuantiTect SYBR Green PCR Master Mix (QIAGEN, Mississauga, ON)
- 6 μL RNase Free Water (QIAGEN, Mississauga, ON)
- 2 μL primers (Sigma, The Woodlands, TX)
- 2 μL DNA.
The final primer concentrations were tel 1: 270 nM; tel 2: 900 nM; 36B4u: 300 nM; and 36B4d: 500 nM. The final DNA concentration was 0.175 ng/μL. In addition to sample DNA from COPD patients, DNA obtained from the Coriell Institute (Camden, NJ) was run for use as a reference, or calibrator, in relative telomere length calculations (see below). In initial runs the mean Coefficient of Variation (CV) for triplicate T/S ratios (see below for the definition) was 15%; hence, in order to avoid plate effects, samples were subsequently run in singlets so that all samples could be run on one plate. After loading, plates were sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, CA) and centrifuged briefly at 2,500 rpm. The PCR reactions were performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City CA). The telomere cycling profile was 50°C for 2 min then 95°C for 2 min, followed by 30 cycles of 95°C for 15 s and 54°C for 2 min. The 36B4 cycling profile was 50°C for 2 min, 95°C for 2 min, followed by 35 cycles of 95°C for 15 s and 58°C for 1 min. A dissociation stage was added to both cycling profiles.

Telomere length was quantified as a relative T/S (T=telomere, S=single copy gene, 36B4) ratio, calculated according to Cawthon’s formula. Briefly, the relative T/S ratio = $2^{-\Delta\Delta\text{Ct}}$, where $-\Delta\text{Ct} = \Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{calibrator}}$ and $\Delta\text{Ct} = \text{Ct}_{\text{telomere}} - \text{Ct}_{36\text{B4}}$. (See Figure 3.3 for further explanation of Cawthon’s method.)
Figure 3.3: Relative Telomere Length Measurement by Quantitative PCR
Specially designed primers bind to telomere repeats (TTAGGG). Binding of primers and, therefore, fluorescent PCR products are proportional to the number of telomere repeats and, hence, the length of the telomere.

The quantity of telomere repeats is normalized to the quantity of a single copy gene, 36B4, which encodes a phosphoprotein on the large ribosomal subunit.

Lastly, the ratio of telomere repeat copy number to single gene copy number of the samples are standardized to the ratio of a reference/calibrator sample, and this final ratio is the relative telomere length ("relative" to the reference/calibrator).

(See sub-section 3.3.3.b: Quantitative PCR above for methodological details and relative telomere length equation.)
3.3.4 Statistical Analysis

Statistical Package for the Social Sciences (SPSS) 15.0 for Windows (Chicago, IL) was used for statistical analysis. Some continuous variables, including relative telomere length, were natural log-transformed in order to mitigate the biasing effect of extreme outliers.

Analyses performed were two-sample two-tailed t-tests, ANOVA with post-hoc Tukey's test, univariate linear regressions, and multivariate linear regressions. A p-value < 0.05 was considered statistically significant.
3.4 RESULTS

In a non-age matched comparison of women from three cohorts (COPD, anorexia nervosa with early emphysema and normal elderly subjects), COPD subjects had significantly shorter telomeres than either of the other cohorts (Figure 3.4). Women with COPD had telomeres which were 36% shorter than anorexia patients who averaged 33 years younger. Moreover, women with COPD had telomeres which were 41% shorter than elderly subjects 4 years their senior. There was no significant difference in telomere length between the younger anorexic women with early emphysema and the older healthy women. (Note that only women were compared because all of the anorexia patients with early emphysema were female.)
3.4.1 Comparison of Telomere Lengths between Disease Groups

Figure 3.4: The Comparison of Geometric Means of Relative Telomere Length between Women in COPD, Anorexia Nervosa and Elderly Cohorts

Geometric mean ± upper standard error (SE)/lower SE: COPD (N = 112, age = 69): 0.162 ± 0.006; Anorexic (N = 40, age = 36): 0.253 ± 0.019/0.018; Elderly (N = 25, age = 73): 0.277 ± 0.020/0.018. ANOVA: p = 0.02; Tukey’s test: *p < 0.05; relative telomere lengths ln-transformed.
3.4.2 Telomere Length versus Subject Demographics in COPD Patients

An inverse relationship was found between age and relative telomere length in peripheral white blood cells of COPD patients (Figure 3.5). A sex difference was also found: on average females had telomeres 13.9% longer than males (calculated from geometric means of relative telomere length; Figure 3.6).

No association was found between smoking history (pack-years) and telomere length and no difference was found between current and ex-smokers (smoking status).

A positive relationship was found between telomere length and height, when adjusted for age and sex ($R^2 = 0.07$, $\beta \pm SE = 0.007 \pm 0.003$, $p = 0.03$; $N = 283$). No significant relationships were found between telomere length and either weight or BMI.
Figure 3.5: The Relationship between Age (years) and Relative Telomere Length in Circulating Leukocytes of COPD Patients

Univariate linear regression: $R^2 = 0.03$, $\beta \pm SE = -0.006 \pm 0.002$, $p = 0.006$; relative telomere lengths ln-transformed. $N = 283$. 
Figure 3.6: The Comparison of Geometric Means of Relative Telomere Length in Circulating Leukocytes between Men and Women with COPD
Geometric mean ± standard error: Males (N = 171): 0.142 ± 0.004 and Females (N = 112): 0.162 ± 0.006. T-test, *p=0.003; relative telomere lengths ln-transformed.
3.4.3 Telomere Length versus Lung Function in COPD Patients

A strong, but non-significant, trend was seen between relative telomere length and FEV₁ % predicted in COPD patients (Figure 3.7). No association was found between telomere length and FVC % predicted in (Figure 3.8). However, a significant positive relationship was found between telomere length and FEV₁/FVC ratio in COPD subjects ($\beta \pm SE = 0.50 \pm 0.16$; Figure 3.9).

No significant relationships were found between relative telomere length and either IL-6 or CRP. There was a negative relationship between telomere length and SP-D ($\beta \pm SE = -0.10 \pm 0.04$; Figure 3.10).
Figure 3.7: The Relationship between FEV\textsubscript{1} % predicted and Relative Telomere Length in Circulating Leukocytes of COPD Patients
Multivariate linear regression adjusting for age and sex: R\textsuperscript{2} = 0.07, β ± SE = 0.003 ± 0.001, p = 0.053; relative telomere lengths ln-transformed. N=283.
Figure 3.8: The Relationship between FVC % predicted and Relative Telomere Length in Circulating Leukocytes of COPD Patients
Multivariate linear regression adjusting for age and sex: \( R^2 = 0.06, \beta \pm SE = -0.001 \pm 0.001, p = ns; \) relative telomere lengths In-transformed. \( N = 283. \)
Figure 3.9: The Relationship between FEV₁/FVC Ratio and Relative Telomere Length in Circulating Leukocytes of COPD Patients
Multivariate linear regression adjusting for age and sex: $R^2 = 0.09$, $\beta \pm SE = 0.50 \pm 0.16$, $p = 0.002$; relative telomere lengths ln-transformed. $N = 283$. 
3.4.4 Telomere Length versus Inflammatory Marker Levels in COPD Patients

Figure 3.10: The Relationship between Surfactant Protein-D (ng/mL) and Relative Telomere Length in Circulating Leukocytes of COPD Patients
Univariate linear regression: $R^2 = 0.02$, $\beta \pm SE = -0.10 \pm 0.04$, $p = 0.02$; relative telomere lengths ln-transformed. $N = 283$. 
3.5 DISCUSSION & FUTURE DIRECTIONS

Chronic obstructive pulmonary disease patients had significantly shorter telomeres compared to healthy controls of a similar but younger age and compared to substantially younger anorexia nervosa patients with early emphysema (Figure 3.4). This demonstrates that COPD patients undergo accelerated aging and corroborates with two recent studies which reported shorter telomeres (measured by qPCR) in COPD patients compared to healthy age-matched non-smoking and smoking controls\textsuperscript{92,93}. Houben \textit{et. al.} compared telomere length between 102 COPD patients and 20 healthy smoking controls (p < 0.05)\textsuperscript{92} and Savale \textit{et. al.} compared 136 COPD patients to 113 healthy smoking and 46 healthy non-smoking controls (p = 0.0001)\textsuperscript{93}. As well, the telomeres of anorexic subjects were not significantly different from those of the elderly controls, which could suggest that some process associated with their emphysematous phenotype (which seems to be linked to their chronic malnourishment) is causing accelerated aging. Indeed, as discussed previously, there is accumulating evidence that emphysema is a disease of accelerated aging.

Furthermore, in COPD patients there was a significant relationship between disease severity and telomere length of peripheral blood cells. Those with greater airflow obstruction, defined by their FEV\textsubscript{1}/FVC ratio, had shorter telomeres relative to those with less airflow obstruction (Figure 3.9). A similar, but non-significant trend (p = 0.053) was seen with FEV\textsubscript{1} % predicted (Figure 3.7). This is the first study to our knowledge that has found a significant relationship between lung function and telomere length of peripheral leukocytes in COPD patients. Houben \textit{et. al.} stated that they found no association with lung function parameters, which they reported as being FEV\textsubscript{1} %
predicted and diffusion capacity for carbon monoxide (DLCO) % predicted\textsuperscript{92}. Likewise, Savale et al. found no significant correlations between telomere length and FEV\textsubscript{1} % predicted; FEV\textsubscript{1}, L; FVC, L; or FEV\textsubscript{1}/FVC\textsuperscript{93}. This could be attributable to our larger sample size. We also found age (Figure 3.5), sex (Figure 3.6) and height associations with telomere length in COPD patients, which are in agreement with literature.

Interestingly, neither cumulative smoking history (number of pack-years) nor smoking status (current- or ex-smoker) was significantly related to telomere length. Houben et al. and Savale et al. also found no correlation between pack-years and telomere length in COPD patients\textsuperscript{92, 93}. This could be due to different intensities of inflammation and injury from cigarette smoke caused by differing genetic backgrounds. Moreover, study participants could vary in exposures to other risk factors of COPD, such as occupational exposure or second-hand smoke (though data on these variables are not available).

Several studies have found a negative relationship between pack-years and telomere length; however, those seem to exclusively be studies which include healthy smokers along with COPD patients. Morlán et al. found a negative relationship between pack-years and relative telomere length (measured by fluorescence in situ hybridization [FISH]) in a study of 24 healthy smokers and 26 COPD patients (all male; R = \(-0.45\), p < 0.001)\textsuperscript{94}. Similarly, Savale et al. reported a negative association only when patients and healthy smokers were combined in analysis (R = \(-0.37\), p < 0.00001)\textsuperscript{93}. Therefore, the relationship could be due to significant differences in both smoking history and telomere length between COPD patients and healthy smokers. A study by Valdes et al. of only smokers (572 female current- and ex-smokers) found a significant age-adjusted negative
correlation ($R = -0.11$, $p < 0.045$) between pack-years and terminal telomere restriction fragment (TRF) length (a measure of telomere length by Southern blotting) in peripheral leukocytes. This relationship may have been found due to differences in smoking history, as our patients had a much greater history (mean pack-year of 62.3 versus 8.15) and may have already incurred the majority of damage to their telomeres. As well, our patients were older (mean age of 68.7 versus 47.8 years), which could make this relationship more difficult to extricate.

However, we did find a significant inverse association between SP-D, a lung specific marker of inflammation, and telomere length (Figure 3.10). Systemic inflammatory mediator IL-6 and acute phase protein CRP did not associate with telomere length. Again, Houben et al. and Savale et al. also investigated inflammatory markers. In agreement with our results, Houben et al. did not find a significant association for CRP. Savale et al. tested many cytokines involved in COPD pathogenesis: IL-6, IL-8, IL-1β, TGF-β, TNF-α and MCP (monocyte chemotactic protein)-1. Only IL-6 correlated with telomere length ($R = -0.27$, $p = 0.005$). This incongruity for IL-6 could be explained by a two-fold larger range of IL-6 levels in Savale et al.’s patients compared to ours, making the trend easier to detect.

Therefore, there is a growing body of evidence that COPD is a disease of accelerated aging. COPD is generally accepted as being a systemic inflammatory condition, however, with current data it cannot be stated that COPD is anything more than a disease of lung aging and that COPD is a disease of generalized aging. While telomere length was measured in cells found in the systemic circulation, it cannot be discounted that telomere length in leukocytes was affected by passage through the inflammatory and oxidative
milieu of the lungs. This could be supported by our findings that only SP-D, a lung specific marker of inflammation, was related to telomere length, while IL-6 and CRP were not. As well, Tsuji et al. reported telomere shortening in lung epithelial and endothelial cells of emphysema patients, which correlated positively with FEV\(_1\) % predicted (R = 0.39 and R = 0.35, respectively, p < 0.05 for both)\(^{41}\). Measurement of telomere length in non-circulating systemic cells of COPD patients would provide insight into this matter. Whether or not telomere length in lung tissue correlates with telomere length in peripheral blood or non-pulmonary tissue is unknown, although numerous studies have shown that within normal individuals, telomere lengths in various tissues (for example, leukocytes, skin and synovial tissue; cerebral cortex, myocardium, liver, renal cortex and spleen) are significantly correlated\(^{96,97}\).

At present we can only suggest that lung inflammation may play a role in accelerated aging in COPD. Alternatively, the serum SP-D concentrations could be indicating the permeability of the alveolar-capillary interface, which may worsen with aging, rather than marking the level of lung inflammation\(^{98}\).

Therefore, validation studies need to be carried out to determine what, if any, inflammatory mediators, both lung-specific and systemic, relate to telomere length in lung cells, peripheral blood cells and systemic non-circulating cells. Sampling of alveolar and small and large airway cells could tell us which phenotypes of COPD are affected by aging. Furthermore, as previously discussed, oxidative damage could cause telomere shortening, and studies can be done to determine the possible roles of reactive oxygen species and an oxidant-antioxidant imbalance. Moreover, all the studies to date have been cross-sectional and only give us information about associations. Longitudinal
studies monitoring all of these factors, as well as lung function, would give some insight into whether telomere shortening and lung function decline are occurring together and tell us more about the process and potential mechanisms of accelerated aging in COPD patients.
CHAPTER 4: SEX DIFFERENCES IN COPD

4.1 INTRODUCTION

4.1.1 Evidence of Sex Differences in COPD

4.1.1.a Statistics and Studies

COPD has long been thought of as a man’s disease. However, there is increasing evidence that women are more susceptible to developing COPD than men. Historically the total number of deaths due to COPD has been higher in men than in women, and still was worldwide in 2004 (1,620,000 versus 1,405,000). In Canada more men also died from COPD (5,142 versus 4,455). However, mortality rates are continuing to rise in women, whereas they have stabilized and even decreased slightly in men. Furthermore, in Canada in 2005, the prevalence of COPD was higher among women than men (4.8% versus 4.4%). Therefore, if these trends have continued, in 2008 more women should have died from COPD than men. This already occurred in the year 2000 in the United States. This change in mortality rates in westernized countries is thought to be due to a shift in society’s views on smoking. In the past it was improper and taboo for women to smoke; however, since the 1920s, prevalence of smoking among women has been steadily increasing. Along with the slow progression of COPD and delayed onset of symptoms, we are just beginning to see women surpass men in prevalence of disease and mortality rates.

Three US National Health and Nutrition Examination Surveys (NHANES) spanning from 1971 to 1984 found that 5.6% of women with COPD were never-smokers, compared with only 3.7% of men (self-reported, physician-diagnosed). This study took into account potential occupational exposure based on job titles, as well as type of
cooking fuel\textsuperscript{102}. A smaller study also found more female never-smokers with COPD than males (N = 21, 4.2% versus 1.3%, p < 0.001)\textsuperscript{103}. However, none of these studies had data on environmental tobacco smoke exposure (second-hand smoke), which is greater for married women than men, and could have biased the results.

Women seem to be more susceptible to the harmful effects of cigarette smoke, the major cause of COPD. A study on severe, early-onset COPD patients (N = 84), found that the majority of patients were female (71%, p < 0.001). Moreover, of the COPD patients’ first-degree relatives who smoked (ex or current, N = 348), females had significantly lower FEV\textsubscript{1} % predicted and FEV\textsubscript{1}/FVC ratios than males (p < 0.05 for both)\textsuperscript{104}. Another study calculated the rate of lung function decline per pack-year of smoking history in males and females. In one cohort (N = 9,083) they found a loss of FEV\textsubscript{1} of 7.4 mL/pack-year in females compared to 6.3 mL/pack-year in males and in another cohort (N = 4,814) they found a loss of 10.5 mL/pack-year in females versus 8.4 mL/pack-year in males\textsuperscript{105}. In another large study (N = 4,554), they also found for all levels of smoking that compared to males, female smokers had a greater loss of FEV\textsubscript{1} and had a greater rate of decline in FEV\textsubscript{1} compared to never-smokers\textsuperscript{106}. Similarly, a study classified subjects by disease severity and found that for a given severity women had fewer pack-years of smoking history than males (N = 417, p < 0.0001; )\textsuperscript{103}.

There is also a sex bias for diagnosis of COPD. Due to traditional views of COPD, men are more readily referred for spirometric testing and diagnosed with COPD, whereas women are more likely to be diagnosed with asthma\textsuperscript{107, 108}. Hence, it is probable that COPD is more prevalent in women than is currently thought.
4.1.1.b Possible Causes of Sex Differences in COPD

There are generally three reasons considered to be responsible for the sex difference in lung diseases: morphological and physiological differences; immunological differences; and hormonal differences\textsuperscript{109,110}.

Males and females are known to have differences in lung growth and development from birth to early adulthood. Most basically, women have smaller lungs and airways than men; therefore, for a given exposure, the concentration of noxious particles and fumes depositing will be greater\textsuperscript{99}. Furthermore, females suffer a relatively greater degree of airflow limitation for a certain amount of small airway wall thickening\textsuperscript{111}. This is potentially why chronic bronchitis is more common in women\textsuperscript{73} and emphysema is more common in men\textsuperscript{112,113}.

The immune systems of males and females are also different. Females are more likely to mount a $T_{H1}$ (T helper cell) response, compared to a $T_{H2}$ response by males\textsuperscript{114}, the former of which results in the cell-mediated immunity characteristic of COPD. Moreover, women have stronger immune responses than males, with greater immune cell involvement and more antibody production\textsuperscript{114}. As well, autoimmune diseases are frequently reported to be more common in women than men. Some, such as systemic lupus erythematosus, autoimmune thyroid disease and scleroderma have patient populations that are at least 80% female. Still others, such as rheumatoid arthritis, myasthenia gravis and multiple sclerosis, are 60-75% female\textsuperscript{115}. COPD is a chronic inflammatory disease and there have been suggestions that emphysema is an autoimmune disease\textsuperscript{116-118}. 
One of the most basic differences between men and women are differences in their levels of sex hormones. Receptors for all of the sex hormones have been found in the lungs. Sex differences have been seen in animal models of lung disease\textsuperscript{119}. Furthermore, sex hormones have been implicated in other human pulmonary diseases, the most commonly discussed being asthma. There are changes in asthma prevalence, lung function, and respiratory symptoms reported to be associated with life changes that are marked by distinct alterations in sex hormone levels\textsuperscript{110}.

Interestingly, sex hormones have also been reported to modulate the immune response. Changes are seen in severity of autoimmune disease with pregnancy and phases of menstruation, similar to the fluctuations seen in asthma\textsuperscript{114}. More direct evidence is the discovery of estrogen and testosterone receptors on immune cells. They could be involved in modulating antigen presentation, lymphocyte activation, cytokine release, and lymphocyte migration\textsuperscript{114}.
4.1.2 Sex Hormones

4.1.2.a Structure

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) belong to the peptide or protein class of hormones, which are constructed from amino acids. They are glycoproteins made up of two polypeptide units, with distinct carbohydrate chains, connected by two disulfide bridges. LH and FSH are both composed of the same 92 amino acid α subunit, but each have a unique 115 amino acid β subunit which confers them with their specific activities.

Sex hormones belong to the steroid class of hormones. They are derived from cholesterol and share its characteristic structure of a cyclopentanoperhydrophenanthrene nucleus (a carbon skeleton of 4 fused rings arranged in a 6-6-6-5 manner; see Figure 4.1). These include androgens (ex. testosterone), progestogens (ex. progesterone) and estrogens (ex. estradiol).

4.1.2.b Function

LH and FSH are not sex steroids, however, they are involved in the regulation of sex hormones. LH and FSH stimulate the gonads to produce and secrete sex hormones, thereby controlling reproductive function and, particularly, gametogenesis. They do this by increasing transport of cholesterol into steroid synthesis pathways and increasing and decreasing the expression and activity of the different enzymes in those pathways.

Testosterone controls the development and maintenance of male sex characteristics and fertility. It is also involved in the development of some female secondary characteristics, while its absence results in female primary characteristics (the embryonic
default). Estradiol controls the development of female secondary sex characteristics. Progesterone's main function is for promotion and maintenance of pregnancy. 

**4.1.2.c Synthesis, Regulation and Degradation**

LH and FSH are synthesized and secreted in the anterior pituitary gland by gonadotropes (aka gonadotrophs) and, hence, are known as gonadotropins. Their production is triggered by gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus. They are regulated by feedback cycles, whereby sex hormones affect GnRH production, secretion, and receptor number.

All the sex hormones are synthesized by conversion from cholesterol, which occurs primarily in the Leydig cells of the testes and the theca and granulosa cells of the ovaries, but also in the adrenal cortex, adipose tissue and placenta. The process of sex hormone creation is called steroidogenesis (see Figure 4.1 for a more detailed explanation).

Testosterone is synthesized by the gonads, the primary source in males, and also by the adrenal glands. Its production is stimulated by LH. Estrogen secretion occurs in the ovaries and testes under the control of FSH. Estradiol, the predominant estrogen before menopause and most potent estrogen, is created by aromatization of testosterone. The enzyme aromatase is also expressed by adipose tissue, and peripheral conversion is the major source of estradiol in males and postmenopausal female. LH stimulates progesterone synthesis in the adrenal cortex and gonads.

The sex steroids are inactivated when they are processed in the liver, undergoing glucuronidation and sulphation. The products are then secreted into the bile or urine by the gall bladder or kidneys, respectively.
Figure 4.1: Steroidogenesis: The Biological Pathways of Human Steroid Synthesis

Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus in the brain, which stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release from the anterior pituitary. LH and FSH stimulate sex hormone synthesis and secretion in the gonads (testes and ovaries) and the adrenal glands.

The sex hormones are all derived from cholesterol and the first, rate-limiting step in steroidogenesis is conversion of cholesterol to pregnenolone.

The testes are primarily stimulated by LH, which causes Leydig cells to convert cholesterol predominantly to testosterone, but also to progesterone and estradiol. The ovaries are primarily stimulated by FSH to create estrogens. In the ovarian follicle, theca cells convert cholesterol to androgens (mainly androstenedione) and then granulosa cells convert these androgens to estradiol. The ovaries also produce progesterone in response to LH and, to a lesser extent, testosterone.

The adrenal cortex produces testosterone, estradiol and progesterone. There is also peripheral conversion of hormones in the adipose tissue. In males, most estradiol comes from aromatization of testosterone. In females, most testosterone comes from conversion of androstenedione and dehydroepiandrosterone (androgens).

The sex hormones also negatively feedback on GnRH, LH and FSH.

As men age, function of the testes declines slowly, resulting in a gradual decrease of testosterone. On the other hand, when women age they go through menopause, a sudden cessation ovarian function, resulting in an abrupt drop in estradiol and progesterone. Adipose tissue and the adrenal glands continue to produce sex hormones in both sexes.

Abbreviations: GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone.
Figure 4.1: Steroidogenesis: The Biological Pathways of Human Steroid Synthesis
See Figure caption on previous page.
4.1.2.d Transport

Peptide hormones are synthesized as inactive prohormones, which undergo post-translational modification in the Golgi apparatus, and are then stored in secretory granules. As they are composed of amino acids, they are not membrane-permeable, and are released by exocytosis. LH and FSH travel through the systemic circulation. Because steroid hormones are derived from cholesterol, they are highly lipid-soluble and can easily traverse the cell membrane. Therefore, when they are in circulation they are protein-bound, and very little is free and biologically active. Binding proteins help prolong half-life, regulate activity and facilitate delivery of steroid hormones to target tissues. Without them, steroid hormones could not have proper endocrine actions.

Some binding proteins are abundant, but have low affinity and low specificity for hormones; therefore, they only play minor roles in regulation. These include albumin and, to a lesser extent, orosomucoid.

On the other hand, some binding proteins, while in low abundance, have very high affinity and specificity and therefore have large roles in hormone regulation. Sex hormone-binding globulin (SHBG) is a homodimer, with each subunit containing a binding site suitable for androgens and estrogens. It is synthesized primarily by hepatocytes, but also by the testes and brain, and its production is increased by estrogens, but reduced by androgens. Corticosteroid-binding globulin (CBG, also known as transcortin) is a monomer with a single binding site. It binds progesterone as well as glucocorticoids. It is also primarily synthesized by hepatocytes, but by the kidneys, pancreas and lung as well and is upregulated by estrogen. Both SHBG and CBG levels are inversely related to body mass index (BMI).
4.1.2.e Receptors and Signaling

Gonadotropin hormones signal by binding to G protein-coupled receptors (GPCRs) and activating adenylate cyclase (AC) and cyclic AMP (cAMP), resulting in a kinase cascade\textsuperscript{120}.

Since steroid hormones can easily pass through the plasma membrane, they act on intracellular receptors, which can be cytosolic and translocate to the nucleus or can be nuclear. Before being bound by their ligands, the receptors are bound to heat shock proteins (HSPs) and are inactive. Receptor-hormone binding causes dissociation from HSPs, formation of dimers, and exposure of nuclear localization signals. These sex hormone-receptor complexes then function as transcription factors. The receptors have three domains: a variable amino-terminal that affects transcription; a conserved, receptor-specific DNA-binding domain (DBD) that binds hormone response elements (HREs) of DNA and influences dimerization; and a conserved carboxy-terminal involved in ligand binding, dimerization, and transcription\textsuperscript{122}.

4.1.2.f Hormone Level Changes with Age

LH and FSH levels are low before puberty and rise slightly during and after puberty. In females, the peak levels increase more, especially with regard to LH, though levels rise and drop with the menstrual cycle, being highest at ovulation. After menopause, levels rise dramatically and remain steady\textsuperscript{122}.

All the sex hormones increase during puberty, when sex characteristics are developing and sexual maturation is occurring. In females estradiol increases the most, and during adulthood estradiol and progesterone levels change with the menstrual cycle. After menopause, when the ovaries dysfunction and atrophy, estradiol and progesterone
levels fall to amounts found in girls and men, or even lower. Testosterone, as it is not predominantly produced in the ovaries, is not affected. In males, testosterone is the major hormone. When males age, the testes do not undergo the same abrupt termination of function as the ovaries do in females, but instead experience a gradual decline in function; therefore, testosterone levels falls slightly with advanced aging\textsuperscript{122}.
4.1.3 Sex, Sex Hormones, and Lung Function

4.1.3.a Animal Studies

Receptors for all of the sex hormones have been shown to be expressed in the lung\(^{119}\). Various animal models suggest that estradiol and progesterone increase relaxation and reduce contractility of bronchial smooth muscle\(^{125}\).

There are also animal models for various lung diseases, including allergic airway disease and, more relevant to COPD, pulmonary fibrosis and emphysema.

Allergic airway disease models show increased susceptibility in females in mice and rat models. Females had more severe bronchial inflammation\(^{126}\) and, accordingly, greater levels of serum immunoglobulin E\(^{127,128}\). Ovariectomy of females reduced inflammation to the level of controls, which was reestablished by estrogen replacement\(^{129}\), and administration of progesterone also increased disease\(^{130}\). Castration of males rendered them similar to females\(^{126}\).

In a rat model of pulmonary fibrosis, induced by bleomycin treatment, female rats demonstrated a greater severity of fibrosis and higher mortality\(^{131}\). In fact, female rats had an 80% mortality rate while no male rats died within 3 weeks of treatment. Fibrosis, as determined by histological staining (Masson’s trichrome) for collagen deposition, assay for hydroxyproline content and Northern blotting for expression of fibrogenic cytokine mRNAs, was diminished by ovariectomy and then subsequently restored by estradiol replacement. Furthermore, in plasma, estradiol levels positively correlated with hydroxyproline content in the lung (R = 0.41, p = 0.029)\(^{131}\). Conversely, in a mouse model of bleomycin-induced fibrosis, male mice demonstrated a decline in static compliance compared to saline-treated controls (p < 0.05), while female mice did not\(^{132}\).
Castration resulted in a female-like response in males, while exogenous androgen induced a male-like response in females. Ovariectomized mice and male and female estrogen receptor knock-out mice showed no differences from the normal female response and from wild-type males and females, respectively. However, there were no sex differences in the histological assessment of severity, collagen content of the lung (measured by colourimetric assay) or number of inflammatory cells in BALF\textsuperscript{132}.

In a cigarette smoke-induced pulmonary emphysema model, female mice developed signs of emphysema more quickly than male mice (10 versus 16 weeks)\textsuperscript{133}. Alveolar airspace enlargement was assessed morphometrically by staining inflated, fixed, sectioned lungs with hematoxylin and eosin (H&E) and then determining $L_m$ (mean linear intercept). Both sexes developed emphysema at the same time only when males were given 2.5 times the dose of cigarette smoke administered to the females. The mice developed other changes that were also characteristic of COPD, such as mucus cell hypertrophy and hyperplasia in the large airways and increased levels of inflammatory cells and MMPs\textsuperscript{133}.

Data on the effect of sex hormones on lung disease is limited and conflicting. Though there seems to be evidence of sex hormone-mediated sex differences, even in the same bleomycin-induced pulmonary fibrosis model, results are opposite for mice and rat models. While female rats appear to be more susceptible and estrogen is implicated in disease development, male mice seem to be more susceptible and their androgen levels, but not estrogen, mediate lung function decline.
4.1.3.b Human Studies

Progesterone is known to be a strong stimulator of ventilation. However, little else is known about the effect of sex hormones on the lung. Information about the role of sex hormones in human lung function comes primarily from research on asthma, as well as from hormone replacement therapy (HRT) and oral contraceptive (OC) use. Therefore, the information gleaned from this data is specific to women and is predominantly about so-called “female” sex hormones (estrogens and progestogens). (Research on hormones in COPD specifically will be discussed in the subsequent section: 4.1.4: Sex Hormones and COPD.)

Asthma is known to be more common in males during childhood, but after puberty, as sex hormone levels rise, becomes more common in females.

In women with asthma, their respiratory symptoms and lung function seem to change throughout the menstrual cycle, during which estradiol and progesterone levels rise and fall. During menstruation estradiol and progesterone levels are low. Then during the follicular phase the level of estradiol increases rapidly, before dropping slightly at ovulation, during which time progesterone level begins to rise. Next, in the luteal phase, the estradiol level is maintained and the progesterone level continues to rise. Finally, levels of both hormones drop and the menstrual cycle begins again. Some research has found worsening of asthma symptoms perimenstrually, during the luteal phase when estradiol and progesterone levels are high. This indicates that estradiol and progesterone are detrimental to the lungs. However, other research suggests the opposite. Many asthmatic women report that their symptoms worsen premenstrually, when estradiol and progesterone levels are falling, and during menstruation, when levels are lowest. As
well, women describe deterioration of their symptoms from the end of the follicular phase to the end of the luteal phase, a period in which estradiol levels fall from their peak concentration\textsuperscript{125}. These studies suggest that estradiol and progesterone are beneficial for asthma.

Pregnancy also comes with hormonal fluctuations, as estrogens (estradiol and estrone) and, more so, progesterone levels rise dramatically. Many women with asthma report improvement in symptoms, but many also report experiencing no changes or worsening of symptoms\textsuperscript{125}.

For women, the final major change in hormone levels occurs with menopause. As menopause begins, estradiol and progesterone levels fall (while levels of estrone, a much weaker estrogen, rise) and LH and FSH levels rise. After the menopausal transition, these changes stabilize. A large study was conducted on women (N = 1,274) who were perimenopausal, menopausal, and postmenopausal (some subjects had asthma and COPD). Women who were amenorrheic (no menstruation for at least 6 months; lower estradiol and higher LH and FSH) had significantly lower FEV\textsubscript{1} and FVC values and more respiratory symptoms than those who had regular periods\textsuperscript{136}. On the contrary, there is also evidence that prevalence of asthma decreases at menopause\textsuperscript{125}.

Multiple studies on HRT have found that in postmenopausal women, HRT (estrogen or estrogen + progesterone/progestin) significantly improves FEV\textsubscript{1} and FVC (both L and % predicted) and airway obstruction, with the best, and sometimes only, results coming from combined therapy\textsuperscript{137-139}. This was found for both comparisons of HRT users versus non-HRT users (N = 2,149)\textsuperscript{89} and pre- versus post-HRT use for 3 months (N = 25 and N = 82, respectively)\textsuperscript{138, 139}. Similar results have been found for OC use\textsuperscript{140,}
which are thought to smooth out hormonal fluctuations that usually occur during the menstrual cycle.\textsuperscript{25}

One study has looked at hormone levels and lung function in men (N = 2,197, mean age = 66.0 years), some of whom were smokers (25.4%) and some of whom had emphysema or chronic bronchitis (4.7%)\textsuperscript{141}. FEV\textsubscript{1} and FVC % predicted correlated positively with testosterone (R = 0.16 and R = 0.10, respectively) and negatively with estradiol (R = -0.05 and R = -0.02, respectively), LH (R = -0.06 for both) and FSH (R = -0.07 and R = -0.05, respectively; all p < 0.05). However, after adjusting for age, waist circumference, smoking and physical activity score in multivariate regressions, only testosterone was significantly associated with FEV\textsubscript{1} and FVC % predicted (β = 0.08, p = 0.001 and β = 0.05, p = 0.033, respectively)\textsuperscript{141}.

Although these data are conflicting and may not all be applicable to the study of COPD, they do show that sex hormones may have an effect on the lungs.
4.1.4 Sex Hormones and COPD

There is a void of research on sex hormones in COPD patients. Only a few studies have evaluated hormone level differences between COPD and controls and only a few have investigated the relationships between sex hormone concentrations and inflammatory marker levels. To compound this problem, all of the research reported has been conducted only on men. Moreover, exceptionally few studies have examined sex hormone levels in COPD patients in relation to disease severity and lung function.

A study by Makarevich measured levels of estradiol, testosterone, LH and FSH in 159 male patients (mean age = 49.0 years, range = 38-60 years) with COPD (based on the British Thoracic Society’s guidelines: FEV₁ % predicted < 75%), as well as in 32 age- and smoking history-matched healthy males. Hormone levels were compared between groups. Estradiol levels were higher and testosterone levels were lower in men from all stages of COPD compared to controls (p < 0.05). Moreover, testosterone levels decreased as COPD severity increased. LH and FSH levels were higher in COPD patients, but only significantly in those with mild and moderate disease.

Van Vliet et al. carried out a study to evaluate the effect of hypogonadism on muscle weakness and exercise intolerance in COPD. In support of Makarevich’s results, they found that testosterone levels were lower and LH and FSH levels were higher in COPD (N = 78, mean age = 66 years) compared to controls (N = 21, mean age = 63 years).

In a study on erectile dysfunction in COPD patients, Karadag et al. found that testosterone levels were lower in the COPD group (based on GOLD guidelines; N = 95, mean age = 63.5 year) than in age-matched controls with normal lung function (N = 30,
mean age = 61.1; p = 0.001). They also measured estradiol and found no difference in its levels (p = 0.420). In another study Karadag et. al. again evaluated sex hormone levels in men with COPD (N = 103; mean age = 65.5 years) as compared to age-matched controls (N = 30). Testosterone levels were lower and LH levels were higher in COPD patients, which is in agreement with Makarevich’s findings. Dissimilarly, FSH levels were not significantly different. Karadag et. al.’s findings changed, however, for LH and FSH when the analysis divided stable patients from those suffering exacerbations. Comparisons of LH levels had less significance and levels were only borderline-significantly higher in exacerbated patients (p = 0.050). For FSH, patients in the exacerbation group had significantly higher levels while patients in the stable group remained not significantly different.

Furthermore, they compared hormone levels of GOLD 1 and 2 patients (FEV$_1$ ≥ 50% predicted) with GOLD 3 and 4 patients (FEV$_1$ < 50% predicted). Again, testosterone levels were lower in more severe COPD. Moreover, there was a positive correlation between testosterone and FEV$_1$ % predicted (R = 0.23, p = 0.040) No significant differences were found for LH or FSH levels. However, there was a negative correlation between LH and FVC % predicted (R = 0.26, p = 0.020). In contrast, Van Vliet et. al. found no differences in sex hormone levels between GOLD stages.

Lastly, Karadag et. al. found that sex hormones had no correlations with pack-years, TNF-α levels or IL-6 levels. Van Vliet et. al., however, found some significant relationships. In their COPD patients, free testosterone (but not total) correlated inversely with pack-years (R = -0.22, p = 0.052). Moreover, CRP correlated negatively
with free testosterone ($R = -0.28$, $p = 0.014$); IL-8 correlated positively with FSH and LH ($R = 0.27$, $p = 0.021$ and $R = 0.29$, $p = 0.012$, respectively); and soluble TNF receptor correlated positively with FSH ($R = 0.23$, $p = 0.045$).\textsuperscript{143}

Due to the differences in age the actual hormone concentrations cannot be compared between studies.

Also, there have been reports on the effect of smoking on hormone levels in healthy men and pre and postmenopausal women. In men, the effect of smoking is unclear as it has been associated with both higher and lower testosterone levels and others have found no difference.\textsuperscript{141,146} As discussed above, male COPD patients have been reported to have lower testosterone.\textsuperscript{142-145} The reports in female smokers are also mixed, though generally, in premenopausal women smoking slightly elevates estradiol and significantly elevates progesterone (with a corresponding fall in LH level).\textsuperscript{148} In postmenopausal smokers, progesterone and testosterone levels are significantly higher than in nonsmokers.\textsuperscript{147,149}
4.2 RESEARCH AIMS & HYPOTHESIS

There is growing evidence of a disparity between the sexes in their susceptibility to COPD. A fundamental difference between men and women is their levels of sex hormones. Sex hormone receptors have been found in the lungs and there is evidence that sex hormones can affect lung function. Hence, we hypothesized that sex hormones could be a driving force behind the sex difference in COPD susceptibility.

Our main objective was to determine if the hormone levels of male and female COPD patients had any associations with their lung function. Serum levels of estradiol, progesterone, testosterone, LH, FSH and SHBG were measured and lung function was assessed by spirometry. Another objective was to investigate whether there were any relationships between hormone levels and levels of inflammatory markers, as COPD is an inflammatory disease and there is evidence of hormonal modulation of the immune response. We also examined the hormone levels in each sex to see if they fell within normal ranges and if they had the same relationships with age and BMI as in healthy people.
4.3 METHODS

4.3.1 Subject Demographics

The ABC cohort described previously (section 3.3.1.a COPD Patient Cohort) was divided by sex (Table 4.1). Subjects younger than 55 years of age were excluded in order to remove any female subjects who were in menopause.

Table 4.1: Baseline Characteristics of COPD Patient Cohort Separated by Sex
Mean ± standard deviation (SD), t-test, *p < 0.05. N = 223, all patients 55 years of age and older.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Male</th>
<th>Female</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=140</td>
<td>N=83</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>71 ± 7</td>
<td>69 ± 8</td>
<td>0.25</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>175 ± 7</td>
<td>160 ± 7</td>
<td>*&lt;0.001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>85 ± 18</td>
<td>70 ± 17</td>
<td>*&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28 ± 5</td>
<td>27 ± 6</td>
<td>*0.28</td>
</tr>
<tr>
<td>Current Smoker (%)</td>
<td>36</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>Pack-Years</td>
<td>68 ± 30</td>
<td>57 ± 29</td>
<td>*0.008</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>1.5 ± 0.6</td>
<td>1.1 ± 0.4</td>
<td>*&lt;0.001</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>46 ± 16</td>
<td>51 ± 16</td>
<td>*0.02</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.3 ± 0.7</td>
<td>2.2 ± 0.5</td>
<td>*&lt;0.001</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>75 ± 16</td>
<td>77 ± 16</td>
<td>0.36</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>0.47 ± 0.14</td>
<td>0.50 ± 0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>3.3 ± 2.7</td>
<td>2.8 ± 2.1</td>
<td>0.19</td>
</tr>
<tr>
<td>CRP (ng/mL)</td>
<td>7,825 ± 16,741</td>
<td>8,618 ± 19,653</td>
<td>0.69</td>
</tr>
<tr>
<td>SP-D (ng/mL)</td>
<td>143 ± 266</td>
<td>116 ± 55</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Abbreviations: BMI: body mass index; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; IL-6: interleukin-6; CRP: C-reactive protein; SP-D: surfactant protein D.
4.3.2 Blood Sample Processing

Blood samples were obtained from subjects via venipuncture. This was performed after a washout period and prior to drug treatment. Blood was collected in Vacutainer Blood Collection Tubes (BD Biosciences, Mississauga, ON) without anti-coagulant. To obtain serum, the tubes were allowed to clot for a minimum of 30 minutes and then centrifuged at $\geq 1,500 \times g$ for 15 minutes at room temperature. Serum fractions were collected after centrifugation using sterile plastic transfer pipettes, aliquotted, and stored at -80°C.
4.3.3 Hormone Level Measurements

4.3.3.a Enzyme-linked Immunosorbent Assays

The sex hormones estradiol, progesterone, testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), as well as sex hormone-binding globulin (SHBG), were measured in serum samples by enzyme-linked immunosorbent assays (ELISAs; DRG International, Inc., Mountainside, NJ), according to the manufacturer’s instructions. ELISAs for estradiol, progesterone and testosterone were competitive and ELISAs for FSH, LH and SHBG were sandwich.

ELISAs were in a 96-well plate format. Standards, samples (neat) and controls were run in duplicates. The mean CVs for ELISAs were: estradiol: 12.8%; progesterone: 10.3%; testosterone: 5.2%; FSH: 5.6%; LH: 7.6%; and SHBG: 5.4%. For each hormone ELISA all of the samples were run concurrently and internal controls were included on each plate. Any samples with a value greater than the second lowest standard and a CV greater than 15% were rerun. Samples above the upper limit of detection were diluted and rerun. Samples below the lower limit of detection were entered as the value of the lower limit for analysis.
Figure 4.2: The Principle of a Competitive ELISA
1) Microwell plate is coated with antibody for hormone.
2) Sample containing hormone & enzyme-conjugated hormone are added and incubated, allowing for binding of hormones.
3) Plate is washed, removing unbound hormone.
4) Substrate is added and cleaved by enzyme to produce a colour change.
4.3.3.b Free Hormone Concentration Calculations

Free estradiol and testosterone levels were calculated using the total estradiol, total testosterone and SHBG levels measured by ELISA in Vermeulen, Stoica and Verdonck’s formula\(^{150}\), based on the law of mass action:

\[
FH = \frac{H - N \times FH}{K_s \times (S - H + N \times FH)}
\]

where \(FH\) = free hormone concentration; \(H\) = total hormone concentration; \(N = K_a\) (association constant of albumin) \(\times\) albumin concentration; \(S = \) SHBG concentration; and \(K_a\) = association constant of sex hormone-binding.

The free hormone level can be solved for using the quadratic equation:

\[
FH = x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}
\]

where \(a = N \times K_s\); \(b = K_s \times S - K_s \times H + N\); and \(c = -H\).

The albumin concentration was assumed to be 43 g/L (molecular weight: 69,000 g/mol), as done by Vermeulen, Verdonck and Kaufman\(^{151}\). See Table 4.2 for the association constant values used.

**Table 4.2: Association Constants of Albumin and Sex Hormone-Binding Globulin for Estradiol and Testosterone**

<table>
<thead>
<tr>
<th></th>
<th>Estradiol</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_a) (L/mol)</td>
<td>(4.21 \times 10^4) (*)</td>
<td>(3.6 \times 10^4) (\dagger)</td>
</tr>
<tr>
<td>(K_s) (L/mol)</td>
<td>(3.14 \times 10^8) (*)</td>
<td>(1.0 \times 10^9) (\dagger)</td>
</tr>
</tbody>
</table>

\(K_a\): association constant of albumin; \(K_s\): association constant of sex hormone-binding globulin. \(*\) Södergård *et. al.*\(^{152}\), \(\dagger\) Moll *et. al.*\(^{153}\)
4.3.4 Statistical Analysis

Statistical Package for the Social Sciences (SPSS) 15.0 for Windows (Chicago, IL) was used for statistical analysis. Some continuous variables, including hormone levels, were natural log-transformed in order to mitigate the biasing effect of extreme outliers.

Analyses performed were two-sample two-tailed t-tests, Pearson correlation, univariate linear regressions, and multivariate linear regressions. A p-value < 0.05 was considered statistically significant.
4.4 RESULTS

4.4.1 Hormone Levels of COPD Patients

The median measured hormone and binding globulin levels of both sexes were within the expected ranges for their age groups (Table 4.3). Men and women significantly differed from each other in all hormone levels. Men had higher estradiol, progesterone, and testosterone levels and women had higher FSH, LH, and SHBG levels.

Table 4.3: Median Sex Hormone Levels and Expected Ranges in Males and Females

Expected ranges in adult men and postmenopausal women\textsuperscript{122}.  
All levels statistically different between males and females: t-test, $p < 0.05$.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Median (Expected Range)</th>
<th>Male N=140</th>
<th>Female N=83</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol pg/mL</td>
<td>29.4 (10-50)</td>
<td>16.6 (10-30)</td>
<td></td>
</tr>
<tr>
<td>Progesterone pg/mL</td>
<td>0.18 (0.2-1.4)</td>
<td>0.06 (0.1-0.8)</td>
<td></td>
</tr>
<tr>
<td>Testosterone pg/mL</td>
<td>3.75 (0.02-10)</td>
<td>0.38 (0.05-0.5)</td>
<td></td>
</tr>
<tr>
<td>Follicle-Stimulating Hormone mIU/mL</td>
<td>13.1 (1-15)</td>
<td>73.4 (15-125)</td>
<td></td>
</tr>
<tr>
<td>Luteinizing Hormone mIU/mL</td>
<td>9.2 (1-6)</td>
<td>37.2 (9-52)</td>
<td></td>
</tr>
<tr>
<td>Sex Hormone Binding Globulin nmol/mL</td>
<td>29.5 (6-44)</td>
<td>42.9 (8-85)</td>
<td></td>
</tr>
</tbody>
</table>
4.4.2 Hormone Levels versus Age and BMI in COPD Patients

The relationships of the hormones to both age and BMI were examined in order to elucidate whether they were the same in COPD patients as in healthy individuals. Log-estradiol had a negative relationship with age in females ($\beta \pm SE = -0.021 \pm 0.009$), but had no significant relationship in males (Figure 4.3a-b). Neither log-progesterone nor testosterone was related to age in either sex (Figure 4.3c-f). Log-FSH, LH and SHBG were not related to age in females, but were all positively related to age in males ($\beta \pm SE = 0.024 \pm 0.009, \beta \pm SE = 0.030 \pm 0.008, \beta \pm SE = 0.014 \pm 0.006$, respectively; Figure 4.3g-l).

Log-estradiol was not related to BMI in either sex (Figure 4.4a-b). Log-progesterone and testosterone were not associated with BMI in women, but were inversely related in men ($\beta \pm SE = -0.039 \pm 0.012, \beta \pm SE = -0.034 \pm 0.011$, respectively; Figure 4.4c-f). Log-FSH, LH and SHBG were all negatively related to BMI in both sexes (women: $\beta \pm SE = -0.037 \pm 0.009, \beta \pm SE = -0.033 \pm 0.009, \beta \pm SE = -0.041 \pm 0.012$; men: $\beta \pm SE = -0.037 \pm 0.011, \beta \pm SE = -0.031 \pm 0.010, \beta \pm SE = -0.037 \pm 0.007$, respectively; Figure 4.4g-l).
Figure 4.3: The Relationship between Age (years) and Sex Hormone Levels in COPD Patients.

Age versus Estradiol levels (pg/mL) in (a) Females ($R^2 = 0.06, p = 0.03$) and (b) Males ($p = ns$); Age versus Progesterone levels (pg/mL) in (c) Females ($p = ns$) and (d) Males ($p = ns$); Age versus Testosterone levels (ng/mL) in (e) Females ($p = ns$) and (f) Males ($p = ns$).

Univariate linear regression; hormones ln-transformed. Females: $N = 83$, Mean age = 69; Males: $N = 140$, Mean age = 71.
Figure 4.3 continued: The Relationship between Age (years) and Sex Hormone Levels in COPD Patients.

Age versus Follicle-Stimulating Hormone (FSH) levels (mIU/mL) in (g) Females (p = ns) and (h) Males ($R^2 = 0.06, p = 0.005$); Age versus Luteinizing Hormone (LH) levels (mIU/mL) in (i) Females (p = ns) and (j) Males ($R^2 = 0.10, p < 0.001$); and Age versus Sex Hormone Binding Globulin (SHBG) levels (nmol/mL) in (k) Females (p = ns) and (l) Males ($R^2 = 0.04, p = 0.02$).

Univariate linear regression; hormones ln-transformed. Females: N = 83, Mean age = 69 years; Males: N = 140, Mean age = 71 years.
Figure 4.4: The Relationship between Body Mass Index (kg/m\(^2\)) and Sex Hormone Levels in COPD Patients.
BMI versus Estradiol levels (pg/mL) in (a) Females (p = ns) and (b) Males (p = ns); BMI versus Progesterone levels (pg/mL) in (c) Females (p = ns) and (d) Males (R\(^2\) = 0.07; p = 0.002); BMI versus Testosterone levels (ng/mL) in (e) Females (p = ns) and (f) Males (R\(^2\) = 0.19; p <0.001).
Univariate linear regression; hormones ln-transformed. Females: N = 83, Mean BMI = 27 kg/m\(^2\); Males: N = 140, Mean BMI = 29 kg/m\(^2\).
Figure 4.4 continued: The Relationship between Body Mass Index (kg/m²) and Sex Hormone Levels in COPD Patients.

BMI versus Follicle-Stimulating Hormone (FSH) levels (mIU/mL) in (g) Females ($R^2 = 0.16, p < 0.001$) and (h) Males ($R^2 = 0.07, p = 0.001$); BMI versus Luteinizing Hormone (LH) levels (mIU/mL) in (i) Females ($R^2 = 0.14, p < 0.001$) and (j) Males ($R^2 = 0.06, p = 0.004$); and BMI versus Sex Hormone Binding Globulin (SHBG) levels (nmol/mL) in (k) Females ($R^2 = 0.14, p = 0.001$) and (l) Males ($R^2 = 0.17, p < 0.001$).

Univariate linear regression; hormones ln-transformed. Females: $N = 83$, Mean BMI = 27 kg/m²; Males: $N = 140$, Mean BMI = 29 kg/m².
4.4.3 Hormone Levels versus Lung Function in COPD Patients

Log-estradiol was negatively related to FVC % predicted ($\beta \pm SE = -7.01 \pm 1.94$) and positively related to FEV$_1$/FVC in males ($\beta \pm SE = 0.037 \pm 0.016$). No other significant relationships were found between estradiol and lung function measures in either sex (Figure 4.5).

Log-progesterone was negatively associated with FVC % predicted in males ($\beta \pm SE = -4.90 \pm 1.74$) and females ($\beta \pm SE = -5.13 \pm 2.36$). Progesterone had no significant associations with either FEV$_1$ % predicted or FEV$_1$/FVC (Figure 6.4).

Log-testosterone and log-FSH had no significant relationships with any of the spirometric measures (Figures 4.7 and 4.8).

Log-LH was positively associated with FEV$_1$ % predicted in females ($\beta \pm SE = 7.06 \pm 3.34$). There were no other significant relationships (Figure 4.9).
**Figure 4.5:** The Relationship between Estradiol (pg/mL) and Lung Function (FEV₁ % Predicted, FVC % Predicted, FEV₁/FVC) in COPD Patients.

Estradiol levels (pg/mL) versus in FEV₁ % predicted in (a) Females (p = ns) and (b) Males (p = ns); versus FVC % predicted in (c) Females (p = ns) and (d) Males (R² = 0.09, p < 0.001); and versus FEV₁/FVC ratio in (e) Females (p = ns) and (f) Males (R² = 0.13, p = 0.02).

Multivariate linear regression adjusting for age and BMI; hormones ln-transformed. Females: N = 83; Males: N = 140.
Figure 4.6: The Relationship between Progesterone (pg/mL) and Lung Function (FEV₁ % Predicted, FVC % Predicted, FEV₁/FVC) in COPD Patients. 
Progesterone levels (pg/mL) versus in FEV₁ % predicted in (a) Females (p = ns) and (b) Males (p = ns); versus FVC % predicted in (c) Females (R² = 0.06, p < 0.03) and (d) Males (R² = 0.06, p = 0.006); and versus FEV₁/FVC ratio in (e) Females (p = ns) and (f) Males (p = ns).
Multivariate linear regression adjusting for age and BMI; hormones ln-transformed. Females: N = 83; Males: N = 140.
Figure 4.7: The Relationship between Testosterone (ng/mL) and Lung Function (FEV1 % Predicted, FVC % Predicted, FEV1/FVC) in COPD Patients.
Testosterone levels (ng/mL) versus in FEV1 % predicted in (a) Females (p = ns) and (b) Males (p = ns); versus FVC % predicted in (c) Females (p = ns) and (d) Males (p = ns); and versus FEV1/FVC ratio in (e) Females (p = ns) and (f) Males (p = ns).
Multivariate linear regression adjusting for age and BMI; hormones ln-transformed.
Females: N = 83; Males: N = 140.
Figure 4.8: The Relationship between Follicle-Stimulating Hormone (mIU/mL) and Lung Function (FEV$_1$ % Predicted, FVC % Predicted, FEV$_1$/FVC) in COPD Patients.

FSH levels (mIU/mL) versus in FEV$_1$ % predicted in (a) Females (p = ns) and (b) Males (p = ns); versus FVC % predicted in (c) Females (p = ns) and (d) Males (p = ns); and versus FEV$_1$/FVC ratio in (e) Females (p = ns) and (f) Males (p = ns).

Multivariate linear regression adjusting for age and BMI; hormones ln-transformed. Females: N = 83; Males: N = 140.
Figure 4.9: The Relationship between Luteinizing Hormone (mIU/mL) and Lung Function (FEV\textsubscript{1} % Predicted, FVC % Predicted, FEV\textsubscript{1}/FVC) in COPD Patients.

LH levels (mIU/mL) versus FEV\textsubscript{1} % predicted in (a) Females (R\textsuperscript{2} = 0.14, p = 0.04) and (b) Males (p = ns); versus FVC % predicted in (c) Females (p = ns) and (d) Males (p = ns); and versus FEV\textsubscript{1}/FVC ratio in (e) Females (p = ns) and (f) Males (p = ns). Multivariate linear regression adjusting for age and BMI; hormones ln-transformed. Females: N = 83; Males: N = 140.
4.4.4 Hormone Levels versus Inflammatory Marker Levels

Levels of inflammatory markers, IL-6, CRP and SP-D, were not different between sexes (Table 4.1). After adjusting for age and BMI in linear regressions, IL-6 and SP-D had no significant relationships with hormone levels (total and free estradiol, progesterone, total and free testosterone, FSH, and LH) in either sex. The only significant relationships found were for CRP. CRP had a positive relationship with estradiol in females ($R^2 = 0.34$, $\beta \pm SE = 0.37 \pm 0.18$, $p = 0.04$). CRP also had a negative relationship with SHBG in males ($R^2 = 0.07$, $\beta \pm SE = -0.47 \pm 0.21$, $p = 0.02$).
4.4.5 Measured Total Hormone Levels versus Calculated Free Hormone Levels

The total testosterone and estradiol levels measured by ELISA correlated strongly with the free levels calculated from formulas based on the law of mass action (R = 0.94, p < 0.001 and R = 0.98, p < 0.001, respectively; Figure 4.10).

Nearly all statistically significant relationships found for total hormone levels remained significant and some additional relationships were found for free levels. The only change was for the relationship between CRP and estradiol in females, which became borderline significant with a p-value of 0.50 (R² = 0.39, β ± SE = 0.41 ± 0.21). In females there were positive associations between BMI and both ln-free estradiol (R² = 0.10, β ± SE = 0.034 ± 0.011, p = 0.004) and ln-free testosterone (R² = 0.07, β ± SE = 0.033 ± 0.014, p = 0.02).
Figure 4.10: The Relationship between Measured Total Hormone Levels and Calculated Free Hormone Levels
(a) Estradiol (pg/mL): R = 0.98, p < 0.001. (b) Testosterone (ng/mL): R = 0.94, p < 0.001. Pearson correlation; hormones ln-transformed. N=223.
4.5 DISCUSSION & FUTURE DIRECTIONS

The serum concentrations of sex hormones in our cohort of COPD patients all fell within the ranges expected for normal healthy people of the same age and sex. The hormone levels also all differed significantly between sexes (Table 4.3).

The relationships between hormone levels with age and BMI were also as expected (Figures 4.3 and 4.4, respectively). In females, the shutting down of the ovaries results in a decrease in estradiol levels with age. In males, there is also a decline in gonadal function, though it is much more gradual. A drop in estradiol levels results in increased FSH and LH levels, due to the decreased negative feedback, and they reach a maximum after menopause. In males, the decline in testosterone levels also affects regulation of FSH and LH, resulting in increases in their concentrations. Lastly, SHBG levels are known to increase slightly with age in males.

For BMI, we see a positive relationship with estradiol in females, as expected due to peripheral conversion in adipose tissue. The negative association between progesterone and BMI in males is somewhat unexpected as progesterone is primarily made by the adrenal glands. The decrease in testosterone could be due to increased peripheral conversion or due to increased SHBG sequestration, as SHBG levels are inversely associated with BMI. The inverse relationships between LH and FSH levels and BMI may be due to the positive association of other hormones, which regulate LH and FSH, with BMI. As mentioned above, SHBG concentration is known to be inversely related to BMI, though the mechanistic link between the two is uncertain.

Determining the relationships between hormone levels and lung function was our primary objective. In analyses correcting for age and BMI, no significant relationships
were found between either testosterone or FSH and any of the lung function measures: FEV₁ % predicted, FVC % predicted, and FEV₁/FVC (Figures 4.7 and 4.8, respectively). We found a significant negative association between estradiol and FVC % predicted in males only (Figure 4.5d). Males also showed a positive association between estradiol and FEV₁/FVC (Figure 4.5f), though this is likely driven by the relationship with FVC % predicted. Intriguingly, the same inverse relationship was found in both sexes between progesterone and FVC % predicted (Figure 4.6c & d). Finally, there was a positive relationship between LH concentration and FEV₁ % predicted in females (Figure 4.9a).

The negative relationship between progesterone and FVC % predicted shows that COPD patients with high progesterone levels generally have poor lung function while those with low progesterone levels have better lung function. The beta coefficient for this relationship was larger in females than in males, perhaps indicating a greater negative effect of progesterone on female lung function. Unfortunately little is known and literature is contradictory about the effect of sex hormones outside of the reproductive system. Therefore, it is difficult to hypothesize potential mechanisms by which this relationship could have transpired.

The relationship observed for progesterone could be a by-product of another process and progesterone could merely be a marker of another mechanism. Progesterone circulates in the blood bound to albumin and CBG. CBG, as its name of corticosteroid-binding globulin implies, also transports glucocorticoids (cortisol and corticosterone)154. Interestingly, CBG has been found to be a member of the serine protease inhibitor (serpin) superfamily, to which α₁-AT belongs to155. As described previously, serine proteases such as elastase, proteinase-3 and cathepsins play a role in COPD pathogenesis.
CBG, however, has no protease-inhibiting action. Instead, it exists in a highly stressed conformation and releases its ligand when proteolytic cleavage between two specific amino acids results in a conformational change. It has been shown that neutrophil elastase cleaves CBG on the surface of activated leukocytes at sites of inflammation. Therefore, it seems that CBG provides targeted delivery of glucocorticoids (anti-inflammatories) to sites of inflammation and remodeling where proteinases have amassed. It follows then that cleavage of CBG could also result in increased release of progesterone in COPD lungs, which are marked by inflammation and remodeling. Since COPD is also a disease of systemic inflammation progesterone could also be released in the systemic circulation, or could leak into the circulation from the lungs. Thus, people with more extensive fibrosis could have greater serum progesterone levels and worse FVC % predicted.

We also investigated whether hormone levels were related to any markers of inflammation. After adjusting for age and BMI, we found a strong positive relationship between CRP and estradiol in females (R² = 0.34) and a negative relationship between CRP and SHBG in males. Correspondingly, a study of postmenopausal females (not using hormone therapy; N = 221) found that estradiol, free estrogen index (molar ratio of total estradiol to SHBG, estimating the amount of free estradiol) and free androgen index (molar ratio of testosterone to SHBG, estimating the amount of free testosterone) were positively related to CRP, while SHBG was negatively related to CRP (Spearman correlation adjusted for age, BMI and development of cardiovascular disease, p ≤ 0.0001 for all). CRP is an acute-phase protein typically secreted after injury or during infection and is involved in the clearing of pathogens. Conversely, CRP can promote
inflammation by activating the complement cascade; inducing protease and cytokine release via NF-κB activation in endothelial and mononuclear cells; and repressing repairing ability in endothelial cells\textsuperscript{161}. Considering this with our findings on lung function, estradiol could stimulate release of CRP which, in turn, could damage the lungs and impair lung function.

The low levels of sex hormones found in older people, like our patient population, may render difficulties in detecting significant relationships, as with low levels it is inherent that the range of concentrations will be narrow. Validation studies in other cohorts are needed to confirm or refute the present findings.

However, since all of the COPD patients in our study had hormone levels within the normal, expected ranges for healthy individuals there is a question of whether sex hormones affect the lungs differently in COPD patients. Of course there is also the possibility that sex hormones, whether directly or indirectly, do not affect lung function in COPD. Measurement of hormone levels in healthy age- and weight-matched smokers and non-smokers using the same assay would be worthwhile, as it would provide a more informative basis of comparison for COPD patients. Moreover, especially due to the fact that COPD patients’ hormone levels were in the normal range, it would be important to determine the relationships between hormone levels and lung function in those healthy controls. If sex steroids do play a role in the pathogenesis and sex difference in COPD, then we would expect to see some kind of distinction between the groups, whether it be a different type of relationship (positive, negative or none) or a difference in the relationship (slope).
Furthermore, our study has limitations since it is cross-sectional. This inhibits our ability to speculate on causation, and, while we hypothesize that sex hormones affect lung function, changes in lung function could affect hormone levels instead. As well it results in data with a lot of scatter due to inter-individual differences. A longitudinal study would be ideal, as one could follow changes in baseline hormones levels and lung function over time in the same individuals. Additionally, an ideal study would be one that followed subjects from a younger age, perhaps even before they were diagnosed with COPD, while the disease is developing, and that followed women before menopause as well as after.

If these findings are validated, then there is also much work to be done to elucidate the pathway by which sex hormones are related to lung function (or vice versa). Further, if there is a cause-and-effect between the two, future studies will need to be carried out to determine the mechanisms responsible, most likely with in vivo studies using animal models or in vitro studies using cell culture. Much more research will need to be done on the non-reproductive effects and pathways of action of sex hormones.
CHAPTER 5: CONCLUSION

We found that COPD patients have shorter telomeres in their peripheral blood cells than healthy people and COPD patients with worse lung function have shorter telomeres compared to those with better lung function. Telomere length in COPD patients is related to SP-D, suggesting that inflammation in the lungs plays a role in their aging process. These data support the concept that COPD is a disease of accelerated aging and offer insight into why aging is a risk factor for COPD. COPD is caused by long, extended exposures to noxious particles and gases and, therefore, develops slowly over time. Telomere shortening and aging are inherently intertwined and the two also occur over time. These processes appear to be hastened in COPD patients and eventually contribute to the symptoms, phenotypes, and comorbidities of COPD.

We also determined that COPD patients over 55 years of age have hormone levels within the range of healthy individuals and experience the same changes in hormone levels with age and BMI as normal people. Lung function in COPD patients is related to progesterone, estradiol and LH levels, and their estradiol and SHBG levels are related to CRP concentration. This provides evidence for a role of sex hormones in the lungs (or vice versa), possibly by interactions with inflammatory molecules. However, due to the extremely limited knowledge of the effect of sex hormones outside of the reproductive system, much more work is needed to determine if and how sex hormones are involved in the sex difference in susceptibility to COPD. This could potentially be a direct effect or an indirect effect, perhaps through modulation of the immune system or other, potentially very complicated, pathways.
Therefore we have demonstrated that telomeres play a role in the accelerated aging of COPD patients and that sex hormones may affect lung function and could be a cause of the sex difference seen in COPD. Our data suggest many more avenues of research to be done on the two COPD risk factors of elderly age and female sex.
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160. Joffe HV, Ridker PM, Manson JE, Cook NR, Buring JE, Rexrode KM. Sex hormone-binding globulin and serum testosterone are inversely associated with C-reactive protein levels in postmenopausal women at high risk for cardiovascular disease. Ann Epidemiol 2006;16:105-12.