Accelerated Aging in COPD:
The Relationship of Telomere Length
and Mortality in COPD

by

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Abstract

The evidence for the role of accelerated aging in COPD progression is growing and the senescence hypothesis is one possible molecular pathway by which COPD develops. Telomeres are used as a biomarker of cellular aging, and cellular aging is accelerated by the presence of oxidative stress and inflammation. Previous studies have shown that telomeres of peripheral blood cells are significantly shorter in COPD patients, but no studies to date explored the relationship of telomere length to important health outcomes such as mortality. Using samples from Lung Health Study (LHS), we examined the role of telomere length and polymorphisms in genes involved in aging process in health outcomes in COPD patients.

There were no significant differences in age, sex, BMI, race of cumulative smoking exposure (pack-years) among 4 groups, divided on basis of telomere length. However, the risk of all cause mortality was similar across the first 3 quartiles (short telomere) but dropped significantly in the 4th quartile (longest telomere, hazard ratio (HR), 1.30). Compared to individuals in the 4th quartile of relative telomere length, the remaining participants had significantly higher risk of cancer mortality (HR, 1.48). Smoking status did not make a significant difference in leukocyte telomere length but when compared to non-COPD, age matched control group, all smoker groups in LHS had shorter telomeres.

We also investigated the role of SNPs that were previously associated with leukocyte telomere length in disease outcome. Although no SNPs were associated with leukocyte telomere length, several SNPs of telomere biology genes were associated with cardiovascular and lung cancer mortality.

The rate of telomere attrition is influenced by both extrinsic factors, such as inflammation and oxidative stress and intrinsic factors such as genetic predisposition. Here, we have shown that accelerated aging of peripheral blood cells, indicated by short telomeres, seems to play a role in disease outcome of COPD. Although we failed to show significant associations between leukocyte telomere length and genetic polymorphisms of telomere regulatory genes, the SNPs may contribute to risk of mortality. Further research is needed to elucidate the pathways underlying these observations.
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LIST OF COMMON ABBREVIATIONS (in alphabetical order)
A1-AT: alpha-1 antitrypsin
BMI: body mass index
COPD: chronic obstructive pulmonary disease
CD: cluster of differentiation
FEV₁: forced expiratory volume in one second
FEV₁/FVC: ratio of forced expiratory volume in one second to forced vital capacity
FISH: fluorescence in situ hybridization
GWAS: genome-wide association study
HSC: hematopoietic stem cells
LHS: lung health study
MAF: minor allele frequency
MEN1: multiple endocrine neoplasia 1
MRE11A: meiotic recombination 11
qPCR: quantitative polymerase chain reaction
RECQL5: RecQ protein like 5
ROS: reactive oxygen species
SNP: single nucleotide polymorphism
TNKS: tankyrase
UCP2: uncoupling protein 2
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CHAPTER 1. INTRODUCTION

1.1 Introduction

The pathogenesis of chronic obstructive pulmonary disease (COPD) is obscure. What is well known is that COPD is rare before 40 years of age even among heavy smokers and its incidence increases rapidly with aging. Furthermore, COPD frequently co-aggregates with other age-related co-morbidities such as osteoporosis, cardiovascular disease and dementia (1,2). Together, these data suggest that COPD is related to the aging process (3). Telomeres are biomarkers of aging. Telomeres are DNA caps located at the end of chromosomes (4), protecting DNA against degradation and remodeling, and preventing gene-to-gene fusion or other gene rearrangements that may lead to cancerous changes (5-7). Owing to the end-replication problem in mature somatic cells, telomere repeats are lost with each replicative cycle, until a critical length is reached at which point cells undergo apoptosis or other disruptive events (8). The rate of telomere attrition is under influence of both genetic and environment determinants. Studies of telomere length in newborns (9) and twin and family studies (10-13) suggest that telomere length is in large part attributable to genetic determinants. Also, telomere shortening is accelerated by the presence of reactive oxygen species (ROS) or inflammation (14-16). Telomere length may thus be a robust indicator of human “biological age”. Previous studies in COPD suggest that telomeres of peripheral leukocytes are significantly shorter in COPD patients than in healthy control subjects (17-19). However, the relationship of telomere length in peripheral leukocytes to important health outcomes such as mortality and cancer has not been explored in COPD. Using samples from the Lung Health Study (LHS), we examined the role of telomere length and health outcomes in the COPD patients. We hypothesized that COPD patients with longer telomeres would have a lower mortality rate than COPD patients with short telomeres and that telomere
length serves as a predictor of mortality among COPD patients. Because telomere length is under the influence of gene-environment interactions, we hypothesized that telomere length in COPD patients is associated with candidate SNPs that were previously reported to be associated with leukocyte telomere length and that there would be significant association between disease outcome and the presence of SNPs.

1.2 COPD as a disease of early senescence

COPD is a chronic disease that is characterized by airflow limitation and associated with significant systemic manifestations (20). The airflow limitation (21,22) is caused by a combination of increased resistance in small conducting airways (23,24), and increased lung compliance due to emphysematous destruction of the lung parenchyma (25). Although COPD is predominantly a lung disease, there is increasing evidence that it is associated with significant extra-pulmonary manifestations including atherosclerosis, coronary artery disease (26), anxiety and depression (27), weight loss, skeletal muscle dysfunction (28), osteoporosis (29) and cancer (20). The presence of these extra-pulmonary manifestations of COPD contributes to the overall morbidity and mortality of COPD patients (30,31). The major risk factor for COPD is cigarette smoking and the resulting protease/anti-protease imbalance is suggested to drive the emphysematous changes in the lung. Yet, the exact pathogenesis of COPD is unknown. However, it is clear that the risk of COPD increases with aging and in animals, genetic alterations leading to an accelerated aging state are associated with premature emphysema. Furthermore, many of the extra-pulmonary manifestations related to COPD (such as atherosclerosis and osteoporosis) are also related to the aging process. This has led some investigators to suggest that COPD is a disease of accelerated aging. In this chapter, we will review the possible mechanisms of aging
that might be germane to COPD and in particular the possible role of telomere attrition in the pathogenesis of COPD.

1.3 Aging – a multifaceted process

Aging is defined as progressive decline in homeostasis after reaching maturity, resulting in increased risk of disease or death (32). Biological aging is normally linked with chronological aging, but can occur earlier in life as a result of a failure to protect DNA against oxidative injury leading to perturbed cell maintenance or repair and organ failure (33). To distinguish the cellular process from aging of whole organs or organisms, cellular senescence is designated more specifically as replicative senescence (34). The concept of replicative senescence was first described by Leonard Hayflick, when he showed that cultured mammalian cells undergo a limited number of cell divisions before they become arrested in the cell cycle, permanently losing their potential for replication (35). Cellular aging not only occurs after exhaustion of a pre-determined proliferative capacity (intrinsic senescence), but also can be induced by extrinsic factors, such as oxidative stress or radiation (36). The concept of ‘free radical theory of aging’ initially proposed by Harman and has been extensively supported by a number of in vivo and in vitro studies (16,37-40). It provides a plausible explanation for the pathogenesis of diverse degenerative conditions involving different organs, including Alzheimer’s disease, cataracts, rheumatoid arthritis, osteoporosis, cardiovascular disease and COPD (41). According to the free radical theory of aging, the normal aging process is accelerated by reactive oxygen species (ROS), when their damaging effects are not fully counterbalanced by anti-oxidative defenses. This leads to deterioration of organ function and eventually to organ failure. Although the precise mechanism has not been fully elucidated, it has been shown that ROS accelerate cellular aging either by causing telomere attrition and thus, depleting the replicative capacity of the cell
or by directly inducing DNA damage, a process that is independent of telomere attrition (Fig 1) (14). ROS may also activate redox sensitive transcription factors, such as activator protein-1, and nuclear factor κB (NF-kB) leading to the generation of pro-inflammatory molecules and propagation of a pro-inflammatory state. Consistent with this theory, nearly all of the age related chronic diseases demonstrate persistent chronic inflammation both locally and systemically (36).
Figure 1 Models of cellular aging

Cellular aging occurs when the replicative capacity of the cell is depleted and this is set by the inherent telomere length. Also any agent that damages DNA causes cell cycle arrest and cellular senescence. Although the precise mechanism has not been fully elucidated, it has been shown that ROS accelerate cellular aging either by causing telomere attrition and/or by directly inducing DNA damage, a process that is independent of telomere attrition.
1.4 COPD and aging

The precise pathogenesis of pulmonary emphysema is not known. However, the experience with alpha-1-antitrypsin deficiency suggests that an imbalance in the protease-antiprotease pathways may be important in this process. It is believed that cigarette smoke recruits and activates neutrophils and macrophages which in turn unleash a cocktail of proteases such as elastase that degrade the extracellular matrix of the airways, leading to emphysematous changes (42,43). However, this hypothesis does not fully explain why alveolar cells are lost in emphysema, suggesting other process(es) must be at play. It is important to note that the prevalence of COPD increases almost exponentially with aging. Before 40 years of age, COPD is extremely rare but by 70 years of age, the prevalence is as high as 30%, even among ex smokers or lifetime non-smokers (44), indicating a strong link between aging and COPD.

The basic premise of the senescence hypothesis (Fig 2) is that oxidative stress related to cigarette smoking increases cell turnover and induces direct DNA damage, causing the cells to age. Over time, senescence induces premature death or aberrant behaviour in the cells. For instance, senescence can turn on the inflammatory (or down-regulate anti-inflammatory) genes in the cells, causing them to become pro-inflammatory. Consistent with this notion, Aoshiba and Nagai showed that older mice (24 months old) had significant up-regulation of pro-inflammatory cytokine genes in their lungs compared with those of younger mice (12 weeks old), suggesting that senescent cells induce a pro-inflammatory state in the lungs (45). A pro-inflammatory milieu in turn causes cells to proliferate and undergo rapid cell turnover. However, senescent cells have impaired replicative capacity and thus over time, this promotes premature death and apoptosis. At the organ and tissue level, this can lead to accelerated loss in function and structure leading to premature emphysema.
Oxidative damage delivered to the lung and chronic inflammation related to environmental irritants such as cigarette smoking or air pollution can not only induce senescence in the lungs but may also impact on the circulating blood cells such as peripheral blood leukocytes. Circulating cells may “pick-up” the oxidative signal in the pulmonary circulation during their journey, resulting in several important downstream effects. First, oxidative stress on blood immune cells such as lymphocytes can induce senescence in these cells and down-regulate both humoral and cellular immunity, making individuals more susceptible to infections (46). Second, senescent immune cells may lose their ability to “self-recognize”, making them more prone to producing “auto-antibodies” and causing autoimmune inflammation (47,48). Consistent with this notion, patients with COPD have been shown to harbour increased numbers of circulating senescent T cells, which express an autoimmune phenotype (49). Autoimmunity has been postulated to explain the persistence of lung inflammation and progression of emphysema despite many years of smoking cessation (50).

Several clinical observations support the hypothesis that accelerated aging may play a role in the pathogenesis of COPD. First, lung function declines in healthy individuals over 30 years of age. This is associated with several structural changes such as progressive distal air space enlargement, with loss in the gas exchange surface area and the supporting alveolar attachments in the peripheral airways (51). Although these structural changes are thought to be non-destructive, in contrast with smoking-induced emphysema (52), the physiological consequences of the structural changes are similar to cigarette-induced COPD in that there is a loss of elastic recoil pressure, an increase in residual volume and functional residual capacity and over-inflation of the lungs (51). Second, skin wrinkling which is associated with aging is related to the extent of emphysema in smokers and independent of sun exposure (53). It is postulated
that smoking induces elastin fibre degradation and matrix remodeling in the lungs similar to that which occurs with aging in the skin, resulting in skin wrinkling. Consistent with this postulate, the degree to which skin wrinkling occurs in smokers correlates with the quantitative measurements of emphysema as assessed by computed tomographic (CT) scanning (53). Third, in vitro exposure of human epithelial cells to cigarette smoke results in an increased expression of senescence-associated β-galactosidase (SA-β-gal), a marker of cellular senescence (54). Interestingly, cultured lung fibroblasts from patients with emphysema also show increased expression of SA-β-gal compared with those from healthy smokers (55,56) suggesting that the accelerated aging process affects more than one type of cell. Furthermore, the proliferative capacity of lung fibroblasts is impaired in patients with emphysema compared with control smokers, suggesting cellular senescence.

Fourth, there are compelling animal data suggesting the importance of cellular senescence in the pathogenesis of COPD. Senescence marker protein-30 (SMP30) is a multifunctional protein which provides protection against deleterious changes related to aging. Transgenic mice, with deficiency in SMP30 (i.e. SMP30 knockout mice) contain lungs that demonstrate significantly larger mean linear intercept than wild type mice when exposed to cigarette smoke, suggesting more emphysematous changes in the lungs (57). The Klotho gene encodes a membrane protein that regulates oxidative stress and cellular senescence. Homozygous mutant Klotho mice have a shorter life span, and age related disorders. Indeed, mice with deficiencies in the Klotho gene develop emphysematous changes in the lungs following normal development (58). The Klotho deficient mice also demonstrate increased expression of matrix metalloproteinase-9 (MMP-9) and decreased expression of tissue inhibitor of matrix metalloproteinase (TIMP) compared with age-matched wild type mice, implicating a disturbance
in the protease-antiprotease balance in the development of emphysema (59). Sirtuin 1 (SIRT1) is an anti-inflammatory and anti-aging protein. Environmental stress such as cigarette smoke decreases SIRT1 levels in rat lungs, leading to increased expression of inflammatory cytokines (60). COPD lungs have reduced expression of SIRT1 possibly owing to post-translational oxidative modification induced by cigarette smoke (61). Taken together, previous studies support the hypothesis that accelerated aging has an important role in the pathogenesis of COPD.
Oxidative stress from various sources (cigarette smoking) leads to increased cell turnover and direct DNA damage, resulting in senescence of cells exposed to the stress. Senescent cells exhibit pro-inflammatory characteristics and undergo premature cell death. For example, senescence of alveolar and airway cells leads to arrested tissue repair and chronic inflammation. Senescent leukocytes often demonstrate autoimmunity and are less efficient in removing pathogens. Reduced tissue repair leads to emphysematous changes in the lung and chronic inflammation results in tissue damage and remodeling, which together lead to disease progression. Owing to an increased number of senescent immune cells, COPD patients are more prone to pulmonary infections, which may lead to an increased risk of exacerbations. Also genetic instability due to telomere dysfunction leads to increased risk of developing cancer.
1.4.1 Telomeres, the marker of biological aging

Telomeres are regions at the end of chromosomes containing repeats of TTAGGG (4), which protect DNA against degradation and remodeling, and thereby confer stability to chromosomes (5-7). Telomeres are subjected to shortening during mitotic cell division because the ends of the chromosome do not replicate in their entirety (7,62-64). Telomere shortening may also occur because of oxidative damage that can break unrepaired single stranded DNA (16,65,66). Standard DNA polymerases are unable to fully correct this defect, causing a gap in the ends of the chromosomes (15). To overcome incomplete replication of telomeres, most immortal eukaryotic cells express telomerase (Fig 3), which is a reverse transcriptase enzyme that synthesizes and adds de novo nucleotides to the 3’ ends of telomeres (66-68). In humans, telomerase activity is abundant in germ cells, adult stem cells and activated immune cells; however, it is generally absent or poorly expressed in terminally differentiated (structural) cells and resting immune cells. In the absence of telomerase activity, telomere attrition occurs with each replicative cycle until a critical length is reached, which results in apoptosis or other disruptive cellular event (8).

Telomere length reflects several important factors such as heredity, telomerase activity (69), the efficiency of telomere binding proteins (70), the rate of cellular proliferation, and oxidative stress in the milieu (16). Telomere length is under influence of both genetic and environment determinants. Both twin and family studies have estimated the heritability of mean leukocyte telomere length to range from 44 to 87% (10-13). Although telomere length is partly heritable, there are major differences in telomeric length even among monozygotic twins (71), which suggests that environmental factors play a major role in telomere attrition and aging.
The study of telomere length is of interest in human health because telomere loss and cellular senescence are likely to have functional implications for health and disease such as infection (72), atherosclerosis (73), cardiovascular diseases (74), osteoporosis and osteoarthritis (75). Although there are variations in the telomere length across different tissues (73), within a given subject, telomere lengths from various organs and tissues are significantly correlated (76). In one study, investigators showed that the telomere lengths of cells from different organs (cerebral cortex, myocardium, liver, renal cortex and spleen) from the same human subjects ranging from neonates to centenarians were significantly correlated (76).

Several studies have shown significant relationship of reduced telomere length in peripheral blood leukocytes to increased risk of bladder, head and neck, lung, renal cell cancers and cardiovascular mortalities (74,77-80). Other studies have shown a relationship of reduced telomere length in peripheral leukocytes to diabetes mellitus, coronary artery disease and ulcerative colitis (81). In these studies, leukocyte telomere length (LTL) was used as a biomarker of aging (75) based on the assumption that telomere length in circulating leukocytes reflects the physiologic age of individuals (72). Importantly, the relationship of telomere length to various disease processes is independent of chronological age suggesting the incremental value of telomere measurement as a biomarker of biological or cellular aging beyond chronological age (72). Together, these data suggest that telomere length serves as a useful indicator of chromosomal stability, telomerase activity, proliferative capacity, and the aging process of the cells. Since COPD is an age-dependent process, the assessment of telomere length may be useful in better understanding the pathogenesis of the disease.
Figure 3 Structure and function of Telomerase.

Telomerase comprises two subunits, telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC). Telomerase adds telomere repeats to the 3’ end of chromosomes by reversely transcribing the sequence in the template region of TERC (sequence shown in yellow box)
1.4.2 Short telomeres in COPD patients

A number of studies have examined telomere length to determine the role of accelerated aging in COPD patients (Table 1). Alveolar cells of emphysematous patients exhibit increased rate of cell death, proliferation and turnover when compared to that of asymptomatic smokers’ and nonsmokers’(82). For instance, in one study, COPD patients had shorter telomeres in alveolar type II epithelial and endothelial cells compared with nonsmokers. Interestingly, in this study, the telomere length of alveolar cells correlated significantly with FEV\(_1\) (r=0.39, P<0.05) (83). In another study, the telomere length of circulating leukocytes of COPD patients was significantly shorter than that of control subjects and within COPD patients, telomere length was significantly related to serum markers of inflammation (r=-0.27, P=0.005) and to the partial pressure of arterial oxygen (r=0.27, P<0.002) (19). Mui et al. showed that the telomere length of circulating leukocytes in COPD patients positively correlated with the extent of airflow obstruction (\(\beta\pm\) standard error of the mean =0.50\pm 0.16, P=0.002) and inversely related to a lung specific inflammatory marker, surfactant protein D (SP-D) (\(\beta\pm\) standard error of the mean =-0.10\pm 0.04, P=0.02) (84). Some studies failed to find a significant relationship between telomere length in circulating leukocytes and presence of COPD, probably owing to small sample size and suboptimal statistical power (Table 1). Together, these data suggest that telomere length in the lungs and in the peripheral circulation are biomarkers for COPD and disease progression. Additional work will be needed in the future to determine the clinical relevance of this observation.
<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>Controls</th>
<th>Sources of subjects</th>
<th>Cells</th>
<th>Telomere measurement</th>
<th>Telomere length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muller</td>
<td>13 emphysema patients</td>
<td>15 controls</td>
<td>Tumor resection and lung volume reduction surgery</td>
<td>Lung fibroblasts</td>
<td>Terminal restriction fragment (southern blotting)</td>
<td>Not different between patients and controls</td>
<td>(55)</td>
</tr>
<tr>
<td>Morla</td>
<td>26 COPD patients</td>
<td>26 never smokers, 24 smokers with normal lung functions</td>
<td>Not given</td>
<td>Peripheral blood lymphocytes</td>
<td>FISH (telomere PNA kit / FITC)</td>
<td>Decreased with age and pack years in smokers Not different between COPD and non-COPD smokers</td>
<td>(85)</td>
</tr>
<tr>
<td>Tsuji</td>
<td>13 emphysema patients</td>
<td>10 asymptomatic smokers, 11 asymptomatic non-smokers</td>
<td>LVRS for patients, tumor resection for controls</td>
<td>Paraffin embedded lung tissue</td>
<td>FISH (telomere PNA kit/FITC)</td>
<td>In alveolar type II cells and endothelial cells decreased in smokers vs. non-smokers Not different between COPD and non-COPD smokers</td>
<td>(83)</td>
</tr>
<tr>
<td>Houben</td>
<td>102 COPD patients</td>
<td>19 age matched controls</td>
<td>Pulmonary rehab centre (patients), respondents to a newspaper ad (controls)</td>
<td>Peripheral blood lymphocytes</td>
<td>qPCR</td>
<td>Decreased in COPD patients Decreased with age in patients and controls Not related to smoking history or lung function Relationship with SOD level</td>
<td>(18)</td>
</tr>
<tr>
<td>Savale</td>
<td>136 COPD patients</td>
<td>113 age and sex matched smokers, 42 nonsmokers with normal lung function</td>
<td>Two French centres (patients), respondents to a newspaper ad (controls)</td>
<td>Peripheral blood lymphocyte</td>
<td>qPCR</td>
<td>Decreased in COPD patients vs. control smokers or non smokers Decreased with age in patients and controls Decreased with IL-6 level but not lung function</td>
<td>(19)</td>
</tr>
<tr>
<td>Mui</td>
<td>283 COPD patients</td>
<td>40 anorexia nervosa patients, 43 healthy, elderly, non smokers</td>
<td>Recruited for study (COPD), eating disorder program (anorexia nervosa)</td>
<td>Peripheral blood lymphocyte</td>
<td>qPCR</td>
<td>Decreased with decreased lung function Decreased with increased SP-D level</td>
<td>(17)</td>
</tr>
<tr>
<td>Tomita</td>
<td>8 COPD patients (lung biopsy)</td>
<td>9 healthy non smokers, 10 age matched current smokers (BAL) 9 healthy smokers, 8 healthy non-smokers (lung biopsy)</td>
<td>Recruited for study (controls)</td>
<td>Alveolar macrophage from BAL</td>
<td>QFISH</td>
<td>Reduced in smokers than non smokers No difference between healthy smokers and smokers with COPD Shorter telomeres in smokers (with and without COPD) than non smokers</td>
<td>(86)</td>
</tr>
</tbody>
</table>

Table 1 Literature review of telomere length and COPD
1.4.3 Telomere biology of peripheral blood leukocytes

Peripheral blood leukocytes are comprised of different types of cells with immune function that are derived from the hematopoietic stem cells (HSC) in the bone marrow. Leukocytes can be mainly grouped into myeloid and lymphoid lineages (Fig 4). The myeloid progenitor cells give rise to granulocytes (neutrophils, eosinophils and basophils), monocyte/macrophage and mast cells. Lymphoid progenitor cells give rise to T and B lymphocytes and natural killer cells. Once fully differentiated, leukocytes of myeloid lineage do not undergo further cell divisions; however leukocytes of lymphoid lineage retain the ability to proliferate and differentiate upon antigenic stimulation. The lifespan of leukocytes may be regulated by various factors and telomeres and telomerase have been implicated for their roles in regulating replicative lifespan.

It is widely known that germ cells and some stem cells express telomerase while most terminally differentiated cells do not express this enzyme for they do not require substantial cell divisions. In most cases, telomerase expression is down-regulated as the cell matures and differentiates to a specific cell type (Fig 4) (87,88). Lymphoid derived cells are long lived and undergo proliferation when activated. Both CD4+ and CD8+ T cells express high levels of telomerase activity upon antigenic stimulation and this telomerase activation is not affected by aging (89). Like T lymphocytes, B cells lose telomeres with age, but the rate of telomere shortening is slower than T cells (89) and this is due to high telomerase expression in activated B cells. Myeloid derived cells are generally short lived and do not undergo subsequent cell division upon activation (90-93). Therefore, the telomere lengths of granulocytes and monocytes/macrophages reflect the telomere length of progenitor cells. Telomere length of various sub-populations of leukocytes varies and the rate of attrition changes with aging (Fig 5). The telomere length of germ cells stays constant through age due to constitutive telomerase
expression (94). HSC have longer telomeres than cells from the lymphoid and myeloid lineages (95). The difference in telomere length among these four types of cells reflects the loss of telomeres during their differentiation which is then affected by the rate of proliferation and expression of telomerase. The pattern of telomere loss in HSC and in lymphocytes with age is similar, while myeloid cells display a continuous loss of telomere length, which may reflect the loss of telomeres in myeloid progenitor cells. Telomeres are generally shorter in granulocytes than in lymphocytes, but with age, telomeres become longer in granulocytes than in lymphocytes. This could be due to a significant increase of memory T cells that have shorter telomeres and a reduction in the number of naïve T lymphocyte in the elderly (95).

Recent studies showed that telomerase is expressed in HSC in a regulated fashion. Metcalf and Moore showed that murine embryonic stem cells can undergo 20-80 more doublings than equivalent cells from adult bone marrow (96). Vaziri et al. (97) showed that telomeres of human HSCs purified from adult bone marrow are shorter than that of foetal liver or umbilical cord blood. They also demonstrated age dependent decline in telomere length in HSCs. Other numerous studies reported that embryonic and foetal HSC have greater proliferative potential than adult HSCs both in vivo (98,99) and in vitro (100-102). Serial transplantation experiments of stem cells showed replicative limitations of HSC, which could be interpreted as providing support for telomere-related restriction in the proliferative potential of HSCs (96,103). Given that all HSCs show age and proliferation dependent shortening of their telomeres, any event that could lead to shortening of telomeres could also lead to depletion of the replicative potential of HSC.

HSCs that reside in a low-oxygen compartment of the bone marrow exhibit a higher G0 (non-dividing phase of the cell cycle) activity which suggests that quiescent HSCs are physically
located in the low oxygenic niche to allow long term protection from ROS related oxidative stress (104). HSCs migrate to more oxygenic perivascular areas when they are mitotically active (105), which might increase the intracellular ROS level. A number of studies showed faster telomere shortening under mild oxidative stress when compared to equivalent cell types in normal culturing conditions (106,107). More importantly, the accelerated telomere shortening induced by oxidative stress can be inhibited by treating cells with antioxidants (108). This strongly suggests that the main mechanisms of senescence under low and mild stress conditions is telomere shortening and that telomere shortening itself is stress dependent. Taking this concept to HSC, free radicals from cigarette smoke exert oxidative stress on proliferating HSCs in the perivascular niche of the bone marrow, shortening telomere length. This in turn results in shortening of telomeres of subsequent cell types that are derived from HSCs. Furthermore, chronic systemic inflammation in COPD patients increases the output of leukocytes from the bone marrow, which then shortens telomeres of HSCs even further.

In our study, peripheral blood leukocytes were assessed for telomere length not only because they were readily available, but also because it reflects the amount of stress on immune cells due to cigarette smoke and/or other environmental stresses.
Telomerase expression is highly regulated during development of leukocytes. Telomerase expression is shown by the color of the background, with the level of activity indicated by the intensity of the color (blue), darkest being the highest, white being the lowest. Telomerase is expressed during the development in both lymphoid and myeloid lineages but down regulated in mature, resting lymphoid and myeloid cells. Upon antigenic activation, lymphocytes are capable of expressing telomerase, whereas myeloid cells are not.
Figure 5 Changes in telomere length of various cell populations with aging.

The telomere length of germ cells stays constant through age due to constitutive telomerase expression. Hematopoietic stem cells have longer telomeres than cells from the lymphoid and myeloid lineages. The difference in telomere length among these four types of cells reflects the loss of telomeres during their differentiation which is then affected by the rate of proliferation and expression of telomerase. The pattern of telomere loss in hematopoietic stem cells and in lymphocytes with age is similar, while myeloid cells display a continuous loss of telomere length, which may reflect the loss of telomeres in myeloid progenitor cells.
1.4.4 Aging of the immune system
The senescence hypothesis suggests that the chronic complex interaction between the airways/lung tissues, and noxious inhalants (the most common one being cigarette smoke), leads to COPD in genetically susceptible individuals. The immune system is a complex and highly regulated network of innate and adaptive immunity. Evidence suggests that immunity deteriorates with age (109). Both branches of the immune system are affected by aging, but adaptive more so than innate. The key inflammatory cells that are implicated in COPD are macrophages, CD8+ T cells, and neutrophils.

1.4.4.a Neutrophils
Neutrophils are the front line defensive cells of the immune system and a major source of ROS, inflammatory cytokines and tissue damaging enzymes (110). They are strongly implicated in both the generation of mucus hyperplasia in chronic bronchitis and the destruction of lung parenchyma in emphysema. Analyses of induced sputum (111,112) and BAL fluid (113,114) demonstrated increased neutrophil counts and neutrophil derived enzyme levels in COPD patients.

Although the total number of neutrophils and macrophages in the peripheral blood remains unchanged with age (43, 44), their function appears to be affected. Neutrophils of older adults have impaired ability to kill infected cells but produce more ROS (46) and sustain exaggerated expression/production of pro-inflammatory cytokines, which prevents apoptosis of neutrophils. In summary, the ability of senescent neutrophils to proliferate and kill infectious agents is reduced but increased ROS production and impaired apoptosis may result in accumulation of senescent neutrophils in either the systemic circulation or local tissues where they may induce substantial local damage.
1.4.4.b Macrophages

Macrophages account for the majority of inflammatory cells found in bronchoalveolar lavage (BAL), regardless of the subject’s smoking status (and disease status) (115). Compounds released by macrophages include ROS, chemotactic factors to recruit other immune cells, inflammatory cytokines, and an array of matrix metalloproteinase enzymes (MMPs). Many studies have demonstrated possible role of macrophages in emphysematous destruction of lung tissue, via MMP production. In animal models, administration of aerosolized alveolar macrophages induced emphysema (116) as does overexpression of MMP-1 ((117). Deficiency of MMP-12 in mice appears to have a protective effect against cigarette smoke induced lung destruction (118). Use of an MMP inhibitor attenuated the inflammatory response as well as the severity of emphysema in guinea pigs upon exposure to cigarette smoke (118). Gene expression analysis of alveolar macrophages in COPD patients showed increased expression of MMP-12 by 3.5 fold (119). Studies of emphysematous lung tissue from human subjects have shown a direct relationship between alveolar macrophage density in the parenchyma and the severity of lung destruction (120).

Macrophages from aged mice showed impaired antigen presentation and reduced MHC class II molecule expression. However, upon activation by mitogen, macrophages from older persons produce more pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α in vitro than those from younger subjects (48, 49).

1.4.4.c T Lymphocytes

T cells develop in the thymus and play a pivotal role in cell-mediated immunity. In COPD, increased CD8+ cell numbers are found in airway walls (121). The primary function of CD8+ cells is to fight viruses either by cytolysis of infected cells or induction of apoptosis (122). Activated CD8+ cells have the potential to damage the surrounding tissue via release of lytic
substances such as perforin and granzyme (123). Indeed, CD8+ cells from COPD patients display higher levels of perforin expression and increased cytotoxic activity than the ones from control subjects (124).

With aging, thymic involution occurs, resulting in changes in T cell profile in the peripheral blood of older adults. Also the diversity of the T cell receptor repertoire is reduced significantly with thymic involution. With aging, lymphocyte production in bone marrow is reduced, resulting in decreased output of naïve T cells. A significantly greater number of CD8+ cells are found in older individuals (125).

1.4.4.d How does this tie in with COPD?
An initiating event of COPD pathogenesis is likely the exposure to noxious inhalants such as cigarette smoke, which induces pro-inflammatory responses and in turn recruits inflammatory cells. As a result, various inflammatory cells accumulate in the lungs. Alveolar macrophages produce elevated level of MMPs, which causes lung parenchymal destruction. In addition, cigarette smoke also impairs ingestion of neutrophils by macrophages and decreases the ability of macrophages to phagocytose the apoptotic cells. The resulting decreased clearance of recruited inflammatory cells along with increased production and release of ROS, proteases and pro-inflammatory cytokines lead to persistent inflammation. Antigen presenting cells (APC) induce a Th1 response from CD4+ T helper cells, which leads to cell mediated immunity. Once recruited, CD8+ cytotoxic T cells and innate immune cells activate a biological cascade of airway remodeling (126) and pulmonary parenchyma destruction tilted toward protease rather than antiprotease (127,128) activity and induce early, self-perpetuating cellular death (126,129,130). Each of these processes has been shown independently to induce changes similar to emphysema in murine lungs. Later, as inflammatory mediators increase and local pulmonary
damage occurs; a cycle of ongoing self-perpetuating inflammation and pulmonary damage sets in and the vicious circle worsens due to aging of the immune system.
1.5 Method of telomere length measurement

There are a number of methods to measure telomere length. Of many methods that are available, the most commonly used are Southern blotting, quantitative fluorescence in situ hybridization (Q-FISH), flow–fluorescence in situ hybridization (flow-FISH) and quantitative polymerase chain reaction (qPCR).

1.5.1 Southern blot

Southern blotting was first described by Southern in 1975 (131), and can be used to analyze the structure of DNA and telomeres. Measurement of telomere length by Southern hybridization requires minimally fragmented DNA. After extraction, the DNA is digested into fragments by a restriction enzyme. The fragments are then separated by electrophoresis and hybridized with radiolabeled probes specific for telomere sequences (TTAGGG). The length of the telomere/terminal restriction fragments (TRF) is measured quantitatively by a densitometer (131,132).

Although Southern blotting is often used, there are several disadvantages to this approach. First, it is technically challenging to isolate minimally fragmented DNA for analysis because DNA is very viscous and the strands are very long and thus susceptible to mechanical cleavage (132). Another drawback is that the resulting TRF represents the mean length of telomere of all chromosomes, and the results can be heterogeneous depending on the specificity of the restriction enzyme that is employed (133). This limitation can be surmounted, however, by simultaneously deploying several different restriction enzymes, which will produce very small subtelomeric regions and enhance the accuracy of the measurement (132). Third, Southern blotting is time consuming and is low throughput. Notwithstanding these limitations, Southern blotting is still considered the gold standard for measuring telomere length from biological samples (132).
1.5.2 Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization allows fluoro-labeling of telomeres by adding specific probes labelled with a fluorescent tags directly to telomere sequences (134). Using a flow cytometer (flow FISH (135)) or other detection systems (e.g. quantitative FISH (Q-FISH) (136)), the telomere length of individual cells can be quantified with some precision.

1.5.3 Quantitative-FISH

Lansdorp et al. developed a Q-FISH method by using peptide nucleic acid (PNA) probes, which conferred several advantages over the conventional method that employed oligonucleotides. PNA probes form more stable complexes with DNA than those formed by oligonucleotide probes, which in turn generates stronger and more specific hybridization signals and thus enhances sensitivity (134,137). Using this method, the fluorescence value from signals produced by individual telomere repeats is calculated and expressed as telomere fluorescence units (TFU). 1 TFU corresponds to approximately 1kb of TTAGGG repeats (134,138). Telomere lengths obtained by Q-FISH have been shown to correlate well with the TRF values obtained by conventional Southern blotting analysis (134,137). Q-FISH and modified Q-FISH methods are performed on metaphase spreads which can be problematic when studying cellular senescence because senescent cells (or cells approaching senescence) are likely to enter mitosis (138-140). This can lead to variable results in telomere lengths of cells arising from a mixed population that contain both senescent and proliferating phenotypes.

1.5.4 Flow-FISH

Flow-FISH is a modified form of Q-FISH, which combines FISH with a flow cytometry method. Flow cytometry allows segregation of cells into subtypes from a mixed population of cells and at different phases of the growth cycle (141). When flow cytometry is used in combination with Q-FISH, the telomere length of each individual cell type may be separately
determined from a heterogeneous mixture of cells. Another advantage of this method is the ability to measure telomere length in all cells, including senescent cells because it uses interphase spreads (140). With these advantages, flow-FISH can eliminate most problems associated with Q-FISH alone.

In the flow-FISH procedure, fluorescently labeled PNA probes are used in the hybridization process. Cells are then treated with specific antibodies tagged with fluorescent dyes to allow segregation of cells into different subtypes. Using the fluorescence-activated cell sorter (FACS), the cells are sorted based on the intensity of the fluorescence signal emitted. The signal generated by the respective probes can be detected by different channels (140). Telomere length values are calculated as the ratio of telomere fluorescence signal of the sample to that of an internal control. A significant correlation has been found in the telomere length between that obtained by flow-FISH and that by Southern blotting (140).

1.5.5 Quantitative polymerase chain reaction (qPCR)

Cawthon described the measurement of telomere length by a quantitative polymerase chain reaction (qPCR). The PCR primers are modified such that tandem repeats of TTAGGG and CCCTAA are followed by two mismatched bases, which prevent hybridization between primers. In qPCR, the ratio of telomere repeat copy number (T) to a single copy gene copy number (S) is determined. This ratio (T/S) is proportional to the average telomere length (142). qPCR facilitates accurate measurement of telomere length because it does not include subtelomeric regions which are highly variable among individuals.

Monochrome multiplex quantitative PCR method is a modified form of qPCR that uses specifically designed primers to yield PCR products of fixed length. By knowing the length and the base composition of products, it is possible to accurately estimate the melting temperature of
each product. By changing thermal cycling and using a single intercalating dye, this method enables the determination of the relative copy numbers of two different DNA sequences (e.g. a single copy gene and telomeres) in a multiplex qPCR. This modified method has advantages in that because both the single copy gene and the telomeres are measured in the same reaction well, the results are highly reproducible. Moreover, the method is cost-effective and high throughput. (143).

The major disadvantage of qPCR is that the absolute length of telomere cannot be determined. This problem is solved by using an oligonucleotide with a known number of repeats as described by O’Callaghan et al. (144). In this method, an 84mer oligonucleotide that contains only repeat fragments of TTAGGG in known quantities is diluted to establish a standard curve. Then, by using the conversion factor calculated from the standard curve, the absolute telomere length can be estimated (144). Since Cawthon first described the use of qPCR to measure telomere length in 2002, there have been numerous studies that have used this method. With further refinements to this technique, more accurate and reproducible results will be obtained in future experiments (144).

1.5.6 Telomere biology, epidemiology and its pitfalls

A number of aging related diseases and genetic and environmental factors that predispose to them have been linked to shorter leukocyte telomeres (19,70,74,75,83,85,145-150). Although longitudinal evaluation gives us useful information such as changes in telomere length over a period of time, cross sectional studies are frequently used due to the challenges associated with the longitudinal design. First, there are very few repositories that have longitudinally collected leukocyte DNA samples. Second, there are methodological limitations. Current techniques for measuring telomere lengths are inherently limited in their ability to detect small differences in
telomere length. Coefficient of variations (CVs) of flow-FISH (151) and Q-FISH are > 5% or unreported. The more frequently used Southern blot analysis and the qPCR methods (142) have lower CVs but they also are not able to pick up small differences. For this reason, in order to perform a longitudinal study, sequential samples of leukocyte DNA that have been collected for a prolonged period of time to detect substantial changes (or detectable changes) are required. For the above reasons, studies of association between telomere length and chronic diseases are often based on cross sectional studies. Therefore, large cohorts are needed in cross sectional analyses in order to capture the impact of a given biological factor on telomere dynamics, which could be variable among individuals.

1.5.7 Telomere length measurement in a large epidemiological study

Results from studies using traditional Southern blotting methods are generally consistent. As individuals get older, their telomere length shortens. In the presence of chronic disease, the rate of attrition is generally accelerated. Twin studies have also indicated that telomere length predicts the likelihood of mortality (152,153). The use of Southern blotting in large clinical or epidemiological studies is often discouraged by the cost, labor intensiveness and degree of expertise needed to undertake the analyses. Consequently, qPCR has provided an effective high throughput alternative with which to measure telomere lengths in peripheral blood cells in larger clinical studies. (19,146,154-156). Comparison of relative T/S ratio obtained by qPCR and mean TRF length measured by traditional Southern Blot method showed strong correlation between these two approaches ($r^2 = 0.677$, $P=1.4915 \times 10^{-24}$) (142). For practical reasons, qPCR has become the most commonly used technique in epidemiological telomere research over the past years.
1.6 Lung health study

The Lung Health Study (LHS) a randomized and controlled trial designed to study the effect of a smoking intervention program and the use of an inhaled bronchodilator (ipratropium bromide) on the rate of decline in lung function among smokers with mild to moderate COPD.

The study design, spirometric techniques, smoking intervention programs, and methods used for collection of vital status and hospitalisation data in the original LHS have been reported previously (157). Smokers of both sexes, aged 35-60 years with mild to moderate airflow obstruction (defined as FEV₁/FVC <0.7 and 55<FEV₁ % predicted<90) were recruited in 10 centers across North America. Amongst the participants recruited, individuals who had a history of cancer (except carcinoma in situ or basal cell carcinoma of the skin), myocardial infarction (in the past 2 years), angina, heart failure, stroke (in the past 2 years), renal failure, cirrhosis or other serious liver diseases, pulmonary embolism, disorders of the central nervous system, narrow angle glaucoma, or any other major diseases which could have compromised follow up were excluded from the cohort (158).

After enrolment and baseline measurements, the study participants were asked to visit the study centre annually for follow up for five years. At these visits spirometric parameters were measured before and after two puffs of isoproterenol. The largest single FEV₁ and FVC were recorded and converted to percentages of the predicted normal value using the formula of Crapo and coworkers (159). In addition, the health status, symptoms, and health service utilization over the preceding 12 months were captured using a detailed questionnaire. At these visits salivary cotinine levels were measured to verify biochemically the smoking status of the participants. Participants were categorized as sustained quitters if they were validated non-smokers at each annual visit. Participants who were smokers at each annual visit were continuing smokers. Those
whose behaviour varied were classified as intermittent quitters. At year five, 5413 participants were alive and were eligible for venipuncture. Of those, 4803 provided blood samples (89% of eligible participants). After collection the venous blood samples were separated into various components and then shipped to the LHS data coordinating centre on dry ice and kept in -70 °C freezers until use.

At year 5 the participants were also asked to consent for additional follow up. During the additional follow up, the vital status and hospitalisation records of participants were captured biannually. If a participant had been admitted to hospital, copies of essential documents were obtained from hospital record rooms. The mortality and morbidity review board was responsible for classifying the causes of death for all participants who died during the study by reviewing death certificates, necropsy reports, relevant hospital records and summaries of interviews with attending physicians or eye witnesses. These data were supplemented by a National Death Index which provided the date and cause of death for all US study participants (160). Mortality end points were classified into: coronary heart disease, cardiovascular disease (which also included coronary heart disease), lung cancer, other cancer, respiratory disease excluding lung cancer, other, and unknown.
1.7 Research aim and hypothesis

Although exact pathogenesis of COPD has not been elucidated, evidence shows that accelerated senescence due to cigarette smoke contributes to COPD pathogenesis. Previous studies have the short telomeres in COPD patients compared to healthy controls, but no studies have shown the effect of cigarette smoke in COPD patients. We hypothesized that the telomere of peripheral blood leukocytes shortens at a faster rate in response to cigarette smoke and that telomere length of peripheral blood cells of COPD patients will be significantly associated with smoking status.

Our secondary objective was to determine if there was a relationship between telomere length and mortality. We stratified the cohort into 4 identical groups based on telomere length and compared the proportion of all-cause, cancer, lung cancer, cardiovascular, and non-neoplastic respiratory mortality in each group. We hypothesized that the first quartile (the shortest telomere group) would have a higher mortality rate than the fourth quartile (the longest telomere group).
Chapter 2. METHODS AND MATERIALS

2.1. Subjects

Subjects with mild to moderate COPD who took part in this study were from the LHS. The detailed study design is described in Section 1.8. A mid-life comparison group was used as a non-COPD control, and the participants of this group were recruited randomly without regard to health or disease status and were 40-50 years old (n=56). The biological samples from the recruited participants were processed by Dr. Angela Brooks-Wilson’s lab.

2.2 DNA extraction

Deoxyribonucleic acid (DNA) was extracted from buffy coat of blood collected at year 5. Extraction of DNA was performed following standard procedures in different centres that conducted the study.

The concentration of DNA (LHS and mid-life control) was then measured spectrophotometrically using Nanodrop (Thermo Scientific, Wilmington, USA). Lastly the DNA was diluted to 1ng/µL in 1x Tris-EDTA buffer and stored at -20°C for subsequent use in quantitative PCR.

2.3 Measurement of telomere length

Peripheral blood leukocyte telomere length was measured using the modified qPCR protocol described by Cawthon (142). The reference single copy gene used was 36B4 and the primers used were those designed by Cawthon (5’-3’):

tel 1: GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT

tel 2: TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTATCCCTA

36B4u: CAGCAAGTGGGAAGGTGTAATCC

36B4d: CCCATTCTATCATCAACGGGTACAA.
Telomere length measurement was performed in triplicate for each LHS sample in a 384 well Clear Optical Reaction Plate (Applied Biosystems, Foster City, CA). Reference DNA obtained from the Coriell Institute (Camden, NJ) was run on each quantitative PCR plate in triplicate to account for interplate measurement variation (Fig. 6). Each well contained a total volume of 20 μL:

10 μL QuantiTect SYBR Green PCR Master Mix (QIAGEN, Mississauga, ON)
8 μL RNase Free Water (QIAGEN, Mississauga, ON)
2 μL primers (Sigma, The Woodlands, TX)
5 ng DNA (dried down prior to adding mastermix)

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 0.25 ng/μL. After loading, plates were sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, CA) and centrifuged briefly at 2,500 rpm. The reactions were performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City CA). The thermal cycling profile for both telomere and single copy gene amplification began with 50°C for 2 min then 95°C for 2 min. For telomere PCR, there followed 30 cycles of 95°C for 15 s and 54°C for 2 min. The 36B4 cycling profile was 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) ratio, calculated according to Cawthon’s formula (142). Cycle threshold (Ct) is the cycle at which the fluorescence exceeds the threshold and serves as a tool for calculating the amount of starting
template amount in the sample. Briefly, the relative T/S ratio = $2^{-\Delta \Delta Ct}$, where $-\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$ DNA and $\Delta Ct = C_{telomere} - C_{36B4}$. 
Figure 6 Telomere length measurement by qPCR protocol

Primers that are specific to telomere and 36B4 gene bind to the target sequence. Fluorescent double stranded DNA dye binds the PCR product and the fluorescent signal is measured as cycle threshold (Ct). The quantity of telomere repeats is normalized to the quantity of 36B4, a single copy gene which encodes a phosphoprotein on the large ribosomal subunit.

In each plate, LHS samples were run in triplicate and reference DNA is run to control for inter-plate variation. The ratio of telomere repeat copy number to single gene copy number of the sample is standardized to the ratio of a reference sample, and this final ratio is denoted as the relative telomere length.
2.4 Statistical analysis

The telomere lengths (T) of peripheral blood leukocytes were standardized to the reference single copy gene (S) to yield a T/S ratio. For analytic purposes, the participants were divided into quartiles based on their T/S ratio. The clinical characteristics were then compared using a chi-square test for dichotomous variables (using appropriate degrees of freedom) and one-way analysis of variance (ANOVA) for continuous variables. The primary endpoint for this study was all-cause mortality. We compared the risk of all-cause mortality across the quartiles over the follow-up period using the Kaplan-Meier (K-M) method for the univariate analysis and a Cox proportional hazards model for the multivariate analysis. The K-M survival curves across the T/S quartiles were compared using a log-rank test. In the multivariate model, we included the following covariates: age, gender, body mass index (BMI), smoking status through the first 5 years of follow-up, pack-years of smoking, blood pressure at year 5, and FEV₁ at year 5. However, FEV₁, BMI, blood pressure measurements and pack-years of smoking did not significantly affect the results of the model. Thus, they were dropped in the final analysis. A similar approach was used for cause-specific mortality endpoints. T/S ratios across the smoking groups were compared using the Kruskal-Wallis test, as the data were non-normally distributed. Correlations between the T/S ratio and variables such as FEV₁ at year 5 and age were tested using the Spearman test for non-parametric variables. P-values less than 0.05 (using a two-tailed test) were considered significant. All analyses were conducted using SAS (version 9.1, Carey, N.C.).
Chapter 3. RESULTS

3.1 Validation of qPCR method

To test the validity of qPCR method to telomere length measurement, we compared the relative telomere length (average T/S ratio) in whole blood DNA samples from 14 non-COPD individuals measured in triplicate by qPCR to the telomere lengths of these same DNA samples as measured by the Flow-FISH approach. Figure 7 shows the strong correlation in relative telomere lengths measured by flow FISH technique in lymphocyte population (r=0.813, p < 0.001), naïve T cells (r=0.797, p < 0.001), and memory T cells (r=0.702, p=0.003).
Figure 7 The relationship between methods of telomere length measurement (qPCR vs. Flow-FISH) in non COPD control
3.2 Clinical characteristics of COPD patients

We were able to purify 4,578 genomic samples. Samples (n=165) with concentrations lower than 1 ng/µL were excluded from the analysis. Samples with a high coefficient of variation (＞5%) and those that did not amplify were also excluded, leaving 4,271 samples (93.3%) for the final analysis (Fig 8). Table 2 shows the main clinical characteristics of the LHS participants according to the relative length of peripheral blood leukocyte telomeres. Participants were divided into four equal groups based on the T/S ratio and clinical characteristics were compared among the four groups. There were no significant differences (p＞0.05) in age, sex, BMI, race or cumulative smoking exposure (pack-years) among the groups. The percentage of various smoking groups (continuous smoker, intermittent smoker, sustained quitter) showed a significant difference (p=0.029) between the quartiles.
Figure 8 Number of participants excluded from analyses and reasons for exclusion

At year five, 5413 participants were alive and were eligible for venipuncture. Of those, 4803 provided blood samples (89% of eligible participants). DNA was extracted and purified from the buffy coat compartment from a total of 4578 samples. Samples with concentration below 1 ng/μL were excluded and 4413 samples were used for telomere length measurement. Samples with greater than 5% coefficient of variation (CV) and with no amplification were excluded. Data analyses were carried out using telomere length measurement of 4271 subjects.
<table>
<thead>
<tr>
<th>Demographics</th>
<th>Peripheral blood leukocyte telomere length</th>
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<tbody>
<tr>
<td></td>
<td>Quartile 1 (1068)</td>
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<tr>
<td><strong>Number of subjects</strong></td>
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</tr>
<tr>
<td><strong>Age (years)</strong></td>
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<td><strong>Gender (% male)</strong></td>
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<tr>
<td><strong>BMI</strong></td>
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<tr>
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<td><strong>Sustained quitters (%)</strong></td>
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<td><strong>Pack years</strong></td>
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<td><strong>CHD mortality (%)</strong></td>
<td>1.22</td>
</tr>
<tr>
<td><strong>Respiratory mortality (%)</strong></td>
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</table>

Table 2 Clinical and demographic features of the LHS participants according to telomere length in peripheral blood cells

Definition of abbreviations: FEV$_1$: forced expiratory volume in 1 second; BMI: body mass index. Study participants were divided into 4 identical groups based on the telomere length, 1$^{st}$ quartile being the shortest and 4$^{th}$ quartile being the longest. Continuous data are presented as mean ± SD and tested with one factor analysis of variance (ANOVA).* significantly different among different groups based on the ANOVA (p<0.05)
3.3 Telomere length association with baseline characteristics

The median (relative) telomere length (T/S ratio) was 0.3256 (interquartile range: 0.2124 to 0.5000). Telomere length was non-normally distributed with no significant differences between males (n=2685; median=0.3246; interquartile range, 0.2133-0.4987) and females (n=1586; median=0.3268; interquartile range, 0.2102 – 0.5016). There was no significant relationship between T/S ratio and age (r=-0.020; p=0.195), FEV₁% predicted (r=0.001; p=0.946) or BMI (r=0.011; p=0.474).
3.4 Telomere length and smoking status

Because cigarette smoke is suggested to accelerate telomere attrition (85), we evaluated the effect of smoking status on leukocyte telomere length. There was no significant difference in telomere length between the quitter group and the smoker groups (sustained quitters versus intermittent quitters: \( p=0.074 \); sustained quitters versus continuous smokers: \( p=0.605 \)).

We also compared telomere length of various smoker groups in the LHS to mid-life comparison group. Telomere length of control was significantly longer than all three smoker groups in the LHS (sustained quitter: \( p<0.001 \); intermittent quitter: \( p<0.001 \); continuous smoker: \( p<0.001 \)) (Fig 9). The significant difference existed when controlled for age.

Because mid-life control participants’ samples were collected recently compared to LHS genomic samples, Advair, Biomarkers in COPD (ABC) study samples were also compared to rule out the storage issue. ABC study (161) subjects had a clinical diagnosis of COPD, following the GOLD guidelines (162). There was no significant difference in leukocyte telomere length between LHS and ABC subjects (\( p=0.8941 \)), suggesting that storage condition is not the main driver of difference between mid life control and LHS shown in figure 9.

Comparison of lung function measurement, mortality and other clinical variables among smoker groups are published (160,163).
Figure 9 Telomere length in smoker groups in LHS and mid life controls.

Mid-life control (n=53, age=45.34); sustained quitter (n=753, age=54.07), intermittent quitter (n=1,192, age=53.61), and continuous smoker (n=2,319, age=53.26). Results are expressed as mean ± standard error. Significantly different when compared to mid-life control group based on multivariate linear model (* p<0.001).
3.5 Mortality risk prediction by telomere length

During a median follow-up of 7.51 years (interquartile range, 7.41 to 7.58 years) from blood collection (1994) to 2001, 399 of 4271 participants (9.34%) died; 202 from cancer (4.73%), 127 from lung cancer (2.87%), 52 from cardiovascular disease (1.22%) and 37 from non-malignant respiratory causes (0.87%). The T/S ratio was significantly related to all-cause mortality but the risk was non-linear (Table 3). The risk of all-cause mortality was similar across the first 3 quartiles (p>0.05) of T/S ratio but dropped significantly in the 4th quartile (hazard ratio (HR), 1.30; p=0.0331 comparing quartiles 1-3 to quartile 4). Adjustments of covariates made no significant differences to the overall results (adjusted HR, 1.29; p=0.0425). These data were largely driven by cancer mortality (Table 3). Compared to individuals in the 4th quartile of T/S ratio, the remaining participants had significantly higher risk of cancer mortality (adjusted HR, 1.48 p=0.0324). There was no statistically significant relationship between increased lung cancer mortality and telomere length, owing to the relatively small number of lung cancer mortality cases during follow-up (adjusted HR, 1.48; p=0.0886). The Kaplan Meier survival analyses of all-cause mortality and cancer mortality are shown in Figure 10 and 11, respectively.
<table>
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<th>1&lt;sup&gt;st&lt;/sup&gt; vs 4&lt;sup&gt;th&lt;/sup&gt; quartile</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; vs 4&lt;sup&gt;th&lt;/sup&gt; quartile</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; vs 4&lt;sup&gt;th&lt;/sup&gt; quartile</th>
<th>1-3 versus 4&lt;sup&gt;th&lt;/sup&gt; quartile</th>
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</thead>
<tbody>
<tr>
<td>Total Mortality (unadjusted)</td>
<td>1.22 (0.91, 1.63)</td>
<td>1.19 (0.88, 1.59)</td>
<td>1.51 (1.14, 2.01)</td>
<td>1.30 (1.02, 1.66)</td>
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<tr>
<td>Total Mortality (adjusted)*</td>
<td>1.20 (0.90, 1.61)</td>
<td>1.18 (0.88, 1.59)</td>
<td>1.48 (1.12, 1.96)</td>
<td>1.29 (1.01, 1.64)</td>
</tr>
<tr>
<td>Cancer Mortality (unadjusted)</td>
<td>1.45 (0.95, 2.20)</td>
<td>1.32 (0.86, 2.03)</td>
<td>1.67 (1.11, 2.51)</td>
<td>1.48 (1.04, 2.11)</td>
</tr>
<tr>
<td>Cancer Mortality (adjusted)*</td>
<td>1.43 (0.94, 2.16)</td>
<td>1.35 (0.88, 2.07)</td>
<td>1.65 (1.10, 2.49)</td>
<td>1.48 (1.04, 2.11)</td>
</tr>
<tr>
<td>Lung Cancer Mortality (unadjusted)</td>
<td>1.43 (0.84, 2.43)</td>
<td>1.51 (0.89, 2.56)</td>
<td>1.54 (0.91, 2.61)</td>
<td>1.50 (0.95, 2.35)</td>
</tr>
<tr>
<td>Lung Cancer Mortality (adjusted)*</td>
<td>1.40 (0.83, 2.38)</td>
<td>1.53 (0.91, 2.60)</td>
<td>1.51 (0.89, 2.56)</td>
<td>1.48 (0.94, 2.33)</td>
</tr>
<tr>
<td>CVD Mortality (unadjusted)</td>
<td>2.10 (0.85, 5.14)</td>
<td>2.01 (0.81, 4.98)</td>
<td>2.32 (0.95, 5.64)</td>
<td>2.14 (0.97, 4.75)</td>
</tr>
<tr>
<td>CVD Mortality (adjusted)*</td>
<td>2.09 (0.85, 5.12)</td>
<td>1.95 (0.79, 4.84)</td>
<td>2.19 (0.90, 5.33)</td>
<td>2.08 (0.94, 4.61)</td>
</tr>
<tr>
<td>Respiratory Mortality (unadjusted)</td>
<td>1.73 (0.58, 5.18)</td>
<td>1.57 (0.51, 4.81)</td>
<td>3.05 (1.11, 8.40)</td>
<td>2.11 (0.82, 5.41)</td>
</tr>
<tr>
<td>Respiratory Mortality (adjusted)*</td>
<td>1.69 (0.57, 5.06)</td>
<td>1.59 (0.52, 4.86)</td>
<td>3.02 (1.10, 8.32)</td>
<td>2.09 (0.81, 5.36)</td>
</tr>
</tbody>
</table>

Table 3 Hazard ratio of total and disease specific causes of mortality according to telomere length

*adjustments were made for age, sex, smoking status (i.e. sustained smokers, intermittent quitters, quitters). Inclusion of other variables such as FEV1% predicted, body mass index and pack years of smoking made no differences to the results.
Figure 10 Cumulative all cause mortality in subjects with short telomere (Q1-3) and longest telomeres (Q4)

Subjects are divided into quartile 1, 2, and 3 (short telomere) versus quartile 4 (longest telomere). P value was derived from multivariable Cox models. The hazard ratio between Q1-3 and Q4 is 1.48 (95% CI, 1.02-1.66)
Figure 11 Cumulative cancer mortality in subjects with short telomeres (Q1-3) and longest telomeres (Q4)

Subjects are divided into quartile 1, 2, and 3 (short telomere) versus quartile 4 (longest telomere). P value was derived from multivariable Cox models. The hazard ratio between Q1-3 and Q4 is 1.48 (95% CI, 1.04-2.11)
3.6 Discussion and future directions

The most important and novel finding from our study was that the telomere length of peripheral blood leukocytes of patients with COPD was significantly related to the risk of all-cause and cancer mortality over a median follow up of 7.5 years, independent of possible confounders such as chronological age, smoking status and lung function. Interestingly, the relationship was non-linear with the lowest risk observed in the group with the longest T/S ratio, suggesting a threshold effect. We also found that although smoking status was not associated with differential telomere lengths, the telomere length of peripheral leukocytes from LHS participants, who all had significant smoking history (on average more than 40 pack-years) at the time of blood collection, was significantly shorter (by more than 50%) than that of healthy subjects of similar age. Together, these data suggest that smoking accelerates telomere attrition, leading to short telomeres and in its extreme, short telomeres are associated with increased risk of cancer and total mortality in COPD patients.

One of the strengths of this study was its longitudinal design. The cohort was made up of mild to moderate COPD patients, who were passively followed for a significant amount of time, providing a unique opportunity to examine the relationship of telomere lengths to clinical outcomes in COPD patients. Another strength of this study was the large sample size. Previous clinical studies examining the relationship between telomere length and incidence of COPD have demonstrated mixed results owing largely to small sample sizes.

The mechanism that underlies the association between short telomeres in leukocytes and mortality in COPD patients is unknown, but at least two explanations are possible. The first is that oxidative stress from environmental oxidants (cigarette smoke) and chronic inflammation lead to accelerated shortening of telomeres, and predispose COPD patients to adverse outcomes. Several clinical studies have shown a significant relationship of reduced leukocyte telomere
length to increased risk of developing coronary artery disease (81), bladder, head and neck, lung, renal cell cancers and cardiovascular mortalities (74,77-80)(164)(162). Taken together these studies suggest that cellular senescence is likely to contribute to poor clinical outcomes of COPD patients. In the LHS, cancer mortality contributed to more than 50% of all deaths. Short telomeres may contribute to carcinogenesis in several ways. Under normal circumstances, cells with short telomeres undergo cell cycle arrest; however, in the presence of mutations in the tumor suppressor pathways such as those related to p53, cells with telomere dysfunction persist and drive chromosomal changes (e.g. translocation and end to end fusion) that result in cancer formation (165,166). Indeed, several studies have shown a significant association between the onset of telomere dysfunction and chromosomal instability during early carcinogenesis (133,167), which was present in both neoplastic tissues, and also in peripheral blood leukocytes (79,80,168).

However it is also possible that COPD patients who had an adverse health outcome were genetically predisposed to accelerated telomere shortening. Mammalian telomeres are associated with a wide range of proteins whose function is to maintain chromosomal integrity such as shelterin proteins, telomerase, and other non-shelterin telomere maintenance proteins (169-171). In addition to telomere length attrition from repeated cell divisions, telomeres may also malfunction due to genetic variation in telomere maintenance genes. Therefore polymorphisms of the genes encoding those proteins/enzymes may be important factors in telomere length variations. Telomere length is under influence of both genetic and environmental determinants. Both twin and family studies have estimated the heritability of mean leukocyte telomere length to range from 44 to 87% (10-13) and wide inter-individual variation in telomere length was found at birth (9), suggesting that inter-individual variation in telomere length is partially attributable to genetic determinants. The above explanations are not mutually exclusive, as
shorter telomeres in leukocytes of COPD patients that develop cancer might reflect both constitutionally short telomeres and further shortening by oxidative damage.

There were some limitations to the present study. First, although the qPCR method of quantifying telomere length is well accepted and widely used (for high throughput analysis), its signal to noise ratio is still relatively low, which may have led to an underestimation of the relationship between T/S ratio and adverse outcomes in our study. Nevertheless, it was reassuring that the qPCR methods produced similar results to those produced by flow FISH. Second, most of the patients in this study had mild to moderate disease; thus, the relationship of T/S ratio to health outcomes across the full range of COPD severity is unknown. This may also explain the lack of significant association between T/S ratio and lung function in our study. Furthermore, all of the LHS participants had heavy smoking exposure history before blood samples were collected (on average more than 40 pack-years of smoking), which may have obfuscated the relationship between smoking cessation (status) and T/S ratio in our study. Third, the LHS did not have biological materials from lungs. Thus, the relationship of T/S ratio in peripheral blood leukocytes to that in lung tissues is uncertain. Lastly, telomere length was measured using genomic samples taken from buffy coat which also contains terminally differentiated cells.

In summary, we have shown that COPD patients who have reduced telomere lengths in their peripheral blood leukocytes are at higher risk of total and cancer-related mortality. Our results are consistent with previous studies demonstrating a link between reduced telomeres and increased risk of cancer mortality in non-COPD elderly populations (172,173). Variables that were previously shown to be associated with telomere length such as age, gender and BMI, did not make a significant difference in leukocyte telomere length in COPD patients. This may be
due to the limitations of the study that are listed above. COPD is a worldwide epidemic and is projected to increase as smoking frequencies rise and the population ages (174,175). Our results indicate that some COPD patients have short telomeres. This may be a distinct phenotype that is associated with increased risk of cancer and total mortality. Since telomeres can be readily measured in the peripheral circulation, telomeres are a promising biomarker in identifying this phenotype. Future studies will need to address the function of genetic variants in telomere function and the impact on clinical outcomes of COPD.
CHAPTER 4. SINGLE NUCLEOTIDE POLYMORPHISM, TELOMERE AND MORTALITY

4.1 Single nucleotide polymorphisms, telomeres and COPD

A single nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide (A, T, C or G) in the genome differs between members of a species. SNPs are one of the most important types of genetic alterations and account for 90% of all human genetic variation. More than 99% of human DNA sequences are the same, and any variations in DNA sequence can have a major impact on how humans respond to pathogens and various environmental factors.

SNPs can be found in coding or non-coding regions of genome, with or without any effects on the gene product. For instance, SNPs in a coding exon could alter the structure of the protein, affecting the function of the protein whereas SNPs found in a promoter region could influence the gene expression. Therefore, individuals with different SNPs could have slightly different metabolic pathways, which in turn affect disease susceptibility and other phenotypes. This makes SNPs valuable for understanding human disease pathogenesis.

Several groups identified SNPs that are associated with susceptibility to complex diseases, such as Alzheimer disease (176), sickle cell anemia (177), hyper-lipidemia (178) and different types of cancer (179). COPD is a heterogeneous disease with several different clinical phenotypes and little is known about the genetic contribution to COPD pathogenesis. Although smoking is the principal cause of COPD, less than 15-20% of smokers develop COPD, suggesting the role of gene-environment interactions. A number of genome-wide association studies (GWAS) and genotyping studies identified various loci that are associated with risk of developing COPD and reduced lung function. Genotyping of Japanese elderly people who died
with emphysema revealed a significant association of a polymorphism of the SERPINE2 gene and the severity of the disease which was assessed macroscopically. The SERPINE2 gene encodes an antiprotease and of 12 examined serine protease inhibitor SNPs, only rs975278 in the SERPINE2 gene was significantly associated with emphysema (odds ratio (OR) = 1.54; 95 CI = 1.29-3.15; p= 0.002) (180). Genotyping using COPD cases from the National Emphysema Treatment Trial (NETT), and control smokers from Normative Aging Study (NAS) showed several SNPs that were significant at p<0.01. A replication study using the Boston Early Onset COPD cohort (BEOCOPD) cohort replicated this finding and the most significant SNP (rs11046966, p=2.3 x 10^{-4}; after Bonferroni correction) is located 3’ to the sex determining region Y-box 5 (SOX5) gene. SOX5 knockout (KO) mice demonstrated impaired embryonic lung development and reduced expression of fibronectin and various extracellular matrix components which are critical for lung morphogenesis during development (181). GWAS of the Bergen cohort (COPD case control cohort) identified several significant associations that reached the level of genome wide significance. The top 100 SNPs were then validated in the International COPD Genetics Network (ICGN) population and 8 were further replicated in NETT, NAS and BEOCOPD. Two SNPs at α nicotinic acetylcholine receptor (CHRNA3/5) locus were identified and these SNPs were also associated with lung cancer (182).

It is important to note that SNPs are not absolute indicators of disease development. Someone who has inherited certain SNPs that are linked to a given chronic disease may not or may develop the disease. These associations are difficult to establish because a single altered gene may make only a small contribution to the disease.
4.2 Is telomere length under genetic regulation?

Telomeres are specialized nucleoprotein complexes which protect the end of linear chromosomes. The very end 3’ terminus of a telomere is not a double-stranded blunt end, but a single-stranded G-rich overhang (Fig 12). Electron microscopy revealed that human and mice genomes have this G-overhang which is evolutionarily conserved and essential to protect the end of chromosome from DNA repair machinery by forming the t-loop. The single-stranded overhang invades the double-stranded portion of telomeres, forming a loop which protects the single stranded end (t-loop) with displacement loop (D-loop) at the invasion site (183).

Mammalian telomeres are associated with a wide range of proteins whose function is to maintain chromosomal integrity. The shelterin complex consists of 6 interdependent telomeric core proteins which are telomeric-repeat-binding factor (TRF1), TRF2, TRF1-interacting protein2 (TIN2), the transcriptional repressor/activator protein (RAP1), protection of telomeres 1(POT1) and the POT1 and TIN2-organizing protein TPP1 (169,170). This complex helps the formation of the t-loop and protects the telomere from degradation and inappropriate DNA repair, thereby avoiding end-to-end fusion, atypical recombination and premature senescence (184) (shown in Fig 12). In addition to the shelterin complex, there are a large number of telomere maintenance proteins, including proteins involved DNA repair (MRE11, ERCC1, Rad 50), helicases (WRN and BLM helicase) and regulators of the cell cycle (tankyrase 1 and 2) (171). Furthermore, telomerase reverse transcriptase (TERT) and its telomere template containing RNA component (TERC) are collectively called a telomerase and this enzyme adds telomeric repeats to elongate telomeres. Besides telomere length attrition from repeated cell divisions, telomeres may also malfunction due to genetic variation in telomere maintenance genes. Therefore
polymorphisms of the genes encoding these proteins/enzymes are important factors in telomere length variation.

One of the aims of this thesis is to determine whether telomere length attrition in COPD patients is associated with SNPs that are known to be associated with leukocyte telomere length. Studying SNPs that are associated with leukocyte telomere length will provide important information in the role of accelerated aging in COPD pathogenesis.
Mammalian telomeres consist of TTAGGG repeats with a single-stranded 3’ overhang of the G-rich strand. The single-stranded overhang can invade the double stranded portion of the telomere, forming protective loop (t-loop) with displacement loop (D-loop) at the invasion site. Components of the shelterin complex bind to the double and single stranded telomeric DNA, stabilizing the structure.
4.3 Candidate SNPs regulating leukocyte telomere length

Several GWAS and candidate gene studies identified associations of SNPs with the telomere length of peripheral blood leukocytes. Most of SNPs with significant associations were found in telomere maintenance genes, and genes that regulate the cell cycle.

A GWAS performed in a twin cohort identified 2 SNPs (rs2162440 and rs7235755) that were associated with shorter leukocyte telomere length (5 years of age related attrition based on estimates of loss with age) (185). Both SNPs were found 48Kb linkage disequilibrium (LD) block within a gene desert, between the Bruno-like 4 (BRUNOL4) and VPS34 (also known as PIK3C3) genes. A gene desert is a region with no known genes in a 500kb region. Members of the BRUNOL gene family encode RNA binding proteins with highly a conserved RNA recognition motif whose role is to control normal cell functions, regulating events such as RNA processing, mRNA transport, stability and translation. VPS34 is a component of the phosphoinositide (PI) 3-kinase family which includes proteins that regulate several aspects of cell physiology. The homologous gene to VPS34 in yeast is known to be involved in the telomere length regulatory pathway (185). A GWAS of emphysema cohorts (ECLIPSE, NETT and COPD cohort from Norway) showed presence or absence of emphysema was associated with a SNP in Bicaudal D homolog 1 (BICD1) (P=5.2 x 10\(^{-7}\) with mild emphysema vs. control; P=4.8x 10\(^{-8}\) with moderate and more severe emphysema vs. control subjects) (186). Fine mapping of chromosome 12 revealed a SNP in a BICD1 intron and expression analysis showed that this SNP influenced BICD1 gene expression. The C allele of the SNP (rs2630678) causes cellular aging via disruption of nuclear factor-Y (NF-Y) binding. The SNP is associated with telomeres that were shorter by 604 base pairs, equivalent to ~15-20 years of age related telomere attrition (187), which suggests that a mechanism linked to accelerated aging may be involved in
the pathogenesis of emphysema. Codd et al. identified associations of rs12696304 and rs16847894 with telomere length at a locus that includes TERC, which encodes the RNA template component of telomerase (188).

A study of telomere length and variants of genes encoding telomere associated proteins identified 13 SNPs from 4 genes that were associated with the telomere length of leukocytes. Multiple endocrine neoplasia 1 (MEN1) SNPs (rs669976, rs524386, rs2957154, rs670358) showed inverse association with telomere length. MEN1 is a strong tumor suppressor gene that encodes menin and known to be a negative regulator of TERT (189). Through TERT, MEN1 may affect telomere length maintenance, because TERT expression has been shown to stimulate telomerase activity (189). A genetic variant in the RecQ protein like 5 (RECQL5) gene showed inverse association (P=0.025) with telomere length of peripheral blood measured by qPCR. RECQL5 is a helicase which serves as a guardian of the genome and its deficiency leads to genomic instability, premature aging and an increased susceptibility to cancer (190). Tankyrase (TNKS) is a poly ADP-ribose polymerase and it is a positive regulator of telomere length through modifying TRF1, allowing telomerase to bind and elongate telomeres (191). Six SNPs found in introns of the TNKS gene showed inverse association with telomere length (192). Two SNPs (rs12270338, rs13447720) in the meiotic recombination 11 (MRE11A) gene were positively associated with telomere length. MRE11A senses DNA damage and is involved in modulating t-loop formation (193).

For the purpose of our study, we selected 18 SNPs that were shown to be associated with leukocyte telomere length from previous GWAS and candidate gene studies (PubMed search terms: telomere length polymorphism). We studied these SNPs (genetic factors) as well as
smoking history of the participant (environmental factors) to give insight into the role that telomere length plays in COPD pathogenesis and the mechanisms of accelerated aging in COPD.

4.4 Candidate SNPs analyses

A clean data set obtained from Johns Hopkins University (Boston, MA) was used for the analyses. A total of 4397 samples (2164 continuous smokers, 1163 intermittent smokers and 756 non-smokers) were genotyped recently in a GWAS. Because relative telomere length was not normally distributed, log telomere length was used instead. All analyses were adjusted for the top 5 principal components (PCs). The PCs were calculated by using EIGENSTRAT (191) and this analysis detects and corrects for most of the genetic variance due to ancestry differences. A total of 23 SNPs were chosen from selected studies and 18 SNPs that were in the LHS data base were used in analyses (Table 4). LHS samples were genotyped on the Human 1M-Duo DNA Analysis BeadChip from Illumina.

Linear regression models were used to estimate the association with log telomere length for each SNP independently. Models were adjusted for smoking status (categorized into continuous smokers, intermittent smokers and sustained quitters), age (continuous variable), gender and the 5 top PCs. Logistic analyses were performed to estimate the association between SNPs and the mortality status, and the association between SNPs and telomere length and the mortality status. For each test, the analyses were run twice since the telomere length variable was treated both as a continuous and a binary variable. Statistical analyses were performed using PLINK software 1.07(http://pngu.mgh.harvard.edu/purcell/plink).
<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>Chr</th>
<th>Location; Known gene function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10511887</td>
<td></td>
<td>9</td>
<td>Located in gene desert (chromosome 9p21.1)</td>
<td>(188)</td>
</tr>
<tr>
<td>rs10903314</td>
<td>TNKS</td>
<td>8</td>
<td>Intron; Telomere length regulation through modification of telomerase activity</td>
<td>(192)</td>
</tr>
<tr>
<td>rs11249943</td>
<td>TNKS</td>
<td>8</td>
<td>Intron</td>
<td>(192)</td>
</tr>
<tr>
<td>rs11991621</td>
<td>TNKS</td>
<td>8</td>
<td>Upstream</td>
<td>(192)</td>
</tr>
<tr>
<td>rs17150478</td>
<td>TNKS</td>
<td>8</td>
<td>Downstream</td>
<td>(192)</td>
</tr>
<tr>
<td>rs12549064</td>
<td>TNKS</td>
<td>8</td>
<td>Intron</td>
<td>(192)</td>
</tr>
<tr>
<td>rs6990300</td>
<td>TNKS</td>
<td>8</td>
<td>Intron</td>
<td>(192)</td>
</tr>
<tr>
<td>rs12270338</td>
<td>MRE11A</td>
<td>11</td>
<td>Intron; a part of complex which senses DNA damage and modulates t-loop formation</td>
<td>(192)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Also regulates telomerase dependent telomere elongation</td>
<td>(185)</td>
</tr>
<tr>
<td>rs13447720</td>
<td>MRE11A</td>
<td>11</td>
<td>Upstream</td>
<td>(185)</td>
</tr>
<tr>
<td>rs2162440</td>
<td>BRUNOL4,</td>
<td>18</td>
<td>48 kb LD block within a gene desert, between the Bruno-like 4 (BRUNOL4) and VPS34 (also known</td>
<td>(192)</td>
</tr>
<tr>
<td></td>
<td>VPS 34</td>
<td></td>
<td>as PIK3C3) genes</td>
<td>(192)</td>
</tr>
<tr>
<td>rs7235755</td>
<td>BRUNOL4,</td>
<td>18</td>
<td></td>
<td>(192)</td>
</tr>
<tr>
<td></td>
<td>VPS34</td>
<td></td>
<td></td>
<td>(192)</td>
</tr>
<tr>
<td>rs2957154</td>
<td>MEN1</td>
<td>11</td>
<td>Downstream; strong tumor suppressor gene that encodes menin, which is a negative controller of</td>
<td>(194)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TERT</td>
<td>(195)</td>
</tr>
<tr>
<td>rs524386</td>
<td>MEN1</td>
<td>11</td>
<td>Downstream</td>
<td>(192)</td>
</tr>
<tr>
<td>rs670358</td>
<td>MEN1</td>
<td>11</td>
<td>Downstream</td>
<td>(188)</td>
</tr>
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<td>rs669976</td>
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<td>11</td>
<td>Intron</td>
<td>(192)</td>
</tr>
<tr>
<td>rs6822</td>
<td>NDUFA8</td>
<td>9</td>
<td>Intron; encodes mitochondrial protein which is a part of the oxidative phosphorylation enzyme</td>
<td>(192)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>complex</td>
<td></td>
</tr>
<tr>
<td>rs660339</td>
<td>UCP2</td>
<td>11</td>
<td>Negative regulator of ROS production in mitochondria</td>
<td>(192)</td>
</tr>
<tr>
<td>rs820152</td>
<td>RECQL5</td>
<td>17</td>
<td>Upstream; a helicase with a role in protecting and stabilizing the genome; deficiency leads to genomic instability, premature aging</td>
<td>(192)</td>
</tr>
</tbody>
</table>

Table 4: List of candidate SNPs selected from the previous literature which investigated genetic polymorphisms in telomere length regulation.

Abbreviations: Chr: chromosome; UCP2: uncoupling protein 2
4.5 Research aim and hypothesis

Cigarette smoke is the major risk factor for COPD. However, only 10-15% of heavy smokers develop COPD, suggesting a role of genetic predisposition in disease pathogenesis. In the previous chapter, we were able to show that telomere length of peripheral blood cells of COPD patients was associated with cancer mortality and all cause mortality. We hypothesized that if indeed peripheral blood cell telomere length is a predictor of mortality, genetic polymorphisms in genes involved in telomere maintenance will also be associated with the risk of mortality. Because we have SNPs data available for LHS cohort, we tested the association between candidate SNPs and mortality outcome as well as telomere length of peripheral blood cells.

We aimed to determine the relationship between candidate SNPs that were shown to be associated with leukocyte telomere length in the literature and the telomere length in the LHS cohort. We hypothesized that SNPs that were associated with telomere length in the general population would have the same effect in the LHS, which is a cohort of COPD patients.

We also sought to determine whether there was a relationship between the SNPs that are known to be associated with leukocyte telomere length and disease outcome in COPD patients. We expected that the candidate SNPs will have a significant association with mortality.
4.6 Results

4.6.1 Candidate SNPs and leukocyte telomere length

Table 5 shows the association between the candidate SNPs and leukocyte telomere length measured by qPCR. The log relative telomere length was the dependent variable and the 18 candidate SNPs were used as the independent variables. There were no significant SNPs in the linear regression analysis adjusting for PCs.
<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>Chr</th>
<th>BP</th>
<th>Minor allele</th>
<th>Major allele</th>
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<th>Beta</th>
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</tr>
</thead>
<tbody>
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<td></td>
<td>9</td>
<td>31827334</td>
<td>G</td>
<td>A</td>
<td>0.247</td>
<td>0.001</td>
<td>0.957</td>
</tr>
<tr>
<td>rs10903314</td>
<td>TNKS</td>
<td>8</td>
<td>9504516</td>
<td>T</td>
<td>C</td>
<td>0.242</td>
<td>0.036</td>
<td>0.075</td>
</tr>
<tr>
<td>rs11249943</td>
<td>TNKS</td>
<td>8</td>
<td>9645273</td>
<td>C</td>
<td>A</td>
<td>0.187</td>
<td>0.024</td>
<td>0.273</td>
</tr>
<tr>
<td>rs11991621</td>
<td>TNKS</td>
<td>8</td>
<td>9443992</td>
<td>T</td>
<td>C</td>
<td>0.172</td>
<td>0.03</td>
<td>0.193</td>
</tr>
<tr>
<td>rs12270338</td>
<td>MRE11A</td>
<td>11</td>
<td>93787112</td>
<td>A</td>
<td>C</td>
<td>0.218</td>
<td>-5.00E-04</td>
<td>0.983</td>
</tr>
<tr>
<td>rs12549064</td>
<td>TNKS</td>
<td>8</td>
<td>9479437</td>
<td>C</td>
<td>A</td>
<td>0.171</td>
<td>0.018</td>
<td>0.429</td>
</tr>
<tr>
<td>rs13447720</td>
<td>MRE11A</td>
<td>11</td>
<td>93804974</td>
<td>G</td>
<td>A</td>
<td>0.229</td>
<td>-0.001</td>
<td>0.958</td>
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<td>rs17150478</td>
<td>TNKS</td>
<td>8</td>
<td>9678444</td>
<td>G</td>
<td>A</td>
<td>0.182</td>
<td>0.021</td>
<td>0.34</td>
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<tr>
<td>rs2162440</td>
<td>BRUNOL4</td>
<td>18</td>
<td>33468004</td>
<td>T</td>
<td>C</td>
<td>0.193</td>
<td>0.005</td>
<td>0.812</td>
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<td>rs2957154</td>
<td>MEN1</td>
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<td>64341563</td>
<td>G</td>
<td>A</td>
<td>0.25</td>
<td>-0.033</td>
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<td>rs524386</td>
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<td>64341535</td>
<td>C</td>
<td>T</td>
<td>0.088</td>
<td>0.033</td>
<td>0.262</td>
</tr>
<tr>
<td>rs660339</td>
<td>UCP2</td>
<td>11</td>
<td>73366752</td>
<td>T</td>
<td>C</td>
<td>0.402</td>
<td>0.03</td>
<td>0.081</td>
</tr>
<tr>
<td>rs669976</td>
<td>MEN1</td>
<td>11</td>
<td>64330165</td>
<td>C</td>
<td>T</td>
<td>0.099</td>
<td>0.03</td>
<td>0.291</td>
</tr>
<tr>
<td>rs670358</td>
<td>CDC42BPG</td>
<td>11</td>
<td>64348255</td>
<td>A</td>
<td>G</td>
<td>0.104</td>
<td>-0.02</td>
<td>0.462</td>
</tr>
<tr>
<td>rs6822</td>
<td>NDUFA8</td>
<td>9</td>
<td>1.24E+08</td>
<td>A</td>
<td>G</td>
<td>0.154</td>
<td>-2.00E-04</td>
<td>0.994</td>
</tr>
<tr>
<td>rs6990300</td>
<td>TNKS</td>
<td>8</td>
<td>9585271</td>
<td>G</td>
<td>A</td>
<td>0.312</td>
<td>0.012</td>
<td>0.514</td>
</tr>
<tr>
<td>rs7235755</td>
<td>BRUNOL4</td>
<td>18</td>
<td>33470259</td>
<td>A</td>
<td>G</td>
<td>0.193</td>
<td>0.004</td>
<td>0.85</td>
</tr>
<tr>
<td>rs820152</td>
<td>RECQL5</td>
<td>17</td>
<td>71127683</td>
<td>C</td>
<td>T</td>
<td>0.371</td>
<td>-0.02</td>
<td>0.254</td>
</tr>
</tbody>
</table>

**Table 5 Association of candidate SNPs and leukocyte telomere length**

The linear regression was performed by using PLINK. The log relative telomere length was the dependent variable. The 18 candidate SNPs were used as the independent variables. In total, 3797 individuals were included. There were no significant SNPs in the default linear regression analysis.

Abbreviations: BP: base pair; MAF: minor allele frequency; P: P-value for linear regression
4.6.2 Candidate SNPs and mortality

Logistic regressions were performed to determine the association between mortality, and the presence of candidate SNPs. The mortality status was the dependent variable and the 18 candidate SNPs were used as the independent variables. All analyses were adjusted for principal components.

Table 6 summarizes the odds of mortality for each candidate SNPs, determined by logistic regression. One SNP of MEN1, rs670358, was associated with increased risk of lung cancer (OR=1.524, p=0.0322). Five SNPs in TNKS (rs10903314, rs11249943, rs11991621, rs12549064, rs17150478) were associated with increased risk of cardiovascular mortality. Two MEN1 SNPs were inversely associated with cardiovascular mortality (rs524386, OR: 0.2357, p=0.0437; rs669976, OR: 0.2112, p=0.03036). Two SNPs in chromosome 18 gene desert, between the BRUNOL4 and VPS34 (also known as phosphoinositide 3-kinase) genes showed significant association with non-neoplastic respiratory mortality. Also, the same SNPs were border-line non-significant in lung cancer mortality.
Table 6 Association between candidate SNPs and mortality. ORs and P values presented apply to logistic regression. OR >1 means that the odds of mortality is greater among minor allele carrier of given SNP and vice versa.

ORs and P values presented apply to logistic regression. Statistically significant (P<0.05) ORs are bolded and marked with asterisk.

<table>
<thead>
<tr>
<th>SNP</th>
<th>All cause</th>
<th>Cancer</th>
<th>Lung cancer</th>
<th>CVD</th>
<th>Respiratory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>P</td>
<td>OR</td>
<td>P</td>
<td>OR</td>
</tr>
<tr>
<td>rs10511887</td>
<td>0.8882</td>
<td>0.2104</td>
<td>0.9402</td>
<td>0.633</td>
<td>0.8524</td>
</tr>
<tr>
<td>rs10903314</td>
<td>1.054</td>
<td>0.58</td>
<td>0.8929</td>
<td>0.4031</td>
<td>0.8434</td>
</tr>
<tr>
<td>rs11249943</td>
<td>1.188</td>
<td>0.08789</td>
<td>0.9583</td>
<td>0.7714</td>
<td>0.9922</td>
</tr>
<tr>
<td>rs11991621</td>
<td>1.095</td>
<td>0.3914</td>
<td>0.9289</td>
<td>0.6275</td>
<td>0.8975</td>
</tr>
<tr>
<td>rs12270338</td>
<td>1.117</td>
<td>0.2418</td>
<td>1.122</td>
<td>0.3767</td>
<td>1.204</td>
</tr>
<tr>
<td>rs12549064</td>
<td>0.9998</td>
<td>0.9986</td>
<td>0.8587</td>
<td>0.3327</td>
<td>0.7796</td>
</tr>
<tr>
<td>rs13447720</td>
<td>1.086</td>
<td>0.3807</td>
<td>1.088</td>
<td>0.5161</td>
<td>1.19</td>
</tr>
<tr>
<td>rs17150478</td>
<td>1.155</td>
<td>0.1623</td>
<td>1.054</td>
<td>0.7154</td>
<td>1.065</td>
</tr>
<tr>
<td>rs2162440</td>
<td>1.086</td>
<td>0.4018</td>
<td>1.156</td>
<td>0.2769</td>
<td>1.364</td>
</tr>
<tr>
<td>rs2957154</td>
<td>0.9413</td>
<td>0.5184</td>
<td>0.9686</td>
<td>0.8037</td>
<td>0.9508</td>
</tr>
<tr>
<td>rs524386</td>
<td>0.9533</td>
<td>0.7365</td>
<td>1.12</td>
<td>0.5402</td>
<td>1.3</td>
</tr>
<tr>
<td>rs660339</td>
<td>0.9912</td>
<td>0.9137</td>
<td>0.8614</td>
<td>0.1902</td>
<td>1.002</td>
</tr>
<tr>
<td>rs669976</td>
<td>0.9738</td>
<td>0.8438</td>
<td>1.051</td>
<td>0.7813</td>
<td>1.088</td>
</tr>
<tr>
<td>rs670358</td>
<td>1.211</td>
<td>0.1223</td>
<td>1.354</td>
<td>0.06499</td>
<td><strong>1.524</strong>*</td>
</tr>
<tr>
<td>rs6822</td>
<td>1.043</td>
<td>0.6981</td>
<td>0.9288</td>
<td>0.6367</td>
<td>0.9131</td>
</tr>
<tr>
<td>rs6990300</td>
<td>1.127</td>
<td>0.1692</td>
<td>1.007</td>
<td>0.9529</td>
<td>0.9056</td>
</tr>
<tr>
<td>rs7235755</td>
<td>1.089</td>
<td>0.3874</td>
<td>1.158</td>
<td>0.2693</td>
<td>1.367</td>
</tr>
<tr>
<td>rs820152</td>
<td>0.9822</td>
<td>0.8295</td>
<td>1.069</td>
<td>0.5561</td>
<td>1.077</td>
</tr>
</tbody>
</table>
4.6.3 Candidate SNPs, leukocyte telomere (continuous variable) and mortality

Logistic regressions were performed to determine the effect of leukocyte telomere length and the presence of candidate SNPs on mortality risk. Neither SNPs nor telomere length showed significant association with any mortality.

Sensitivity analysis was carried out by performing the analyses after excluding the top 5 outliers. The results were all unaffected for all the mortality variables.
4.6.4 Candidate SNPs, leukocyte telomere (binary variable) and mortality

Multivariate logistic regressions were performed to determine the relationship between leukocyte telomere length and the presence of candidate SNPs as predictor variables, and the risk of mortality as an outcome. The participants were divided into 2 groups for the analyses: 1st-3rd quartile (short telomere group) and 4th quartile (longest telomere group).

Short telomere (1st -3rd quartile) group had increased risk of all cause mortality when MRE11A SNP was added in the model (rs13447720: OR=1.319, p=0.04677). For cancer mortality, odds of mortality were significantly higher in shorter telomere length group for all 18 SNPs. Leukocyte telomere length was a significant predictor of all cancer mortality and the odds ratio of mortality in relation to telomere length and SNPs were similar to odds ratios of mortality in relation to telomere length only, suggesting that the association is mainly driven by telomere length, not SNPs. ORs of telomere length for both CVD mortality (1.668-1.788) and respiratory mortality (1.731- 1.786) were higher than cancer mortality (1.55-1.572), but failed to reach statistical significance.

Table 7 shows the risk of mortality for each SNP with leukocyte telomere length. Three SNPs (rs11991621, rs10903314, rs11249943) in TNKS gene, one SNP (rs17150478) in MRE11A were significantly associated with increased risk of cardiovascular mortality. Two SNPs (rs2162440: OR=1.36, p=0.04845; rs7235755: OR=1.372, p=0.04691) in chromosome 18 gene desert were associated with both respiratory mortality and lung cancer mortality.

Sensitivity analysis was carried out by performing the analyses after excluding the top 5 outliers. The results were all unaffected for all the mortality variables.
<table>
<thead>
<tr>
<th>SNP</th>
<th>All cause OR (P-value)</th>
<th>Cancer OR (P-value)</th>
<th>Lung cancer OR (P-value)</th>
<th>CVD OR (P-value)</th>
<th>Respiratory OR (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10511887</td>
<td>0.8905 (0.2212)</td>
<td>0.9447 (0.6602)</td>
<td>0.8551 (0.3468)</td>
<td>0.6703 (0.1489)</td>
<td>1.008 (0.9799)</td>
</tr>
<tr>
<td>rs10903314</td>
<td>1.06 (0.5408)</td>
<td>0.9001 (0.4393)</td>
<td>0.8499 (0.3478)</td>
<td><em><em>1.687</em> (0.0269)</em>*</td>
<td>1.054 (0.8609)</td>
</tr>
<tr>
<td>rs11249943</td>
<td>1.192 (0.08342)</td>
<td>0.9615 (0.7897)</td>
<td>0.9957 (0.9813)</td>
<td><em><em>1.874</em> (0.0105)</em>*</td>
<td>0.6938 (0.3228)</td>
</tr>
<tr>
<td>rs11991621</td>
<td>1.099 (0.3691)</td>
<td>0.9346 (0.657)</td>
<td>0.9031 (0.598)</td>
<td><em><em>1.989</em> (0.0053)</em>*</td>
<td>0.768 (0.4752)</td>
</tr>
<tr>
<td>rs12270338</td>
<td>1.121 (0.2269)</td>
<td>1.129 (0.3517)</td>
<td>1.21 (0.2346)</td>
<td>1.268 (0.3338)</td>
<td>1.037 (0.9064)</td>
</tr>
<tr>
<td>rs12549064</td>
<td>1.002 (0.9884)</td>
<td>0.8607 (0.3413)</td>
<td>0.7814 (0.227)</td>
<td><em><em>1.763</em> (0.02594)</em>*</td>
<td>0.7627 (0.465)</td>
</tr>
<tr>
<td>rs13447720</td>
<td>1.09 (0.3597)</td>
<td>1.095 (0.4862)</td>
<td>1.197 (0.2593)</td>
<td>1.422 (0.1389)</td>
<td>0.9707 (0.9228)</td>
</tr>
<tr>
<td>rs17150478</td>
<td>1.16 (0.1515)</td>
<td>1.061 (0.6841)</td>
<td>1.071 (0.7073)</td>
<td><em><em>1.736</em> (0.02891)</em>*</td>
<td>0.8023 (0.5378)</td>
</tr>
<tr>
<td>rs2162440</td>
<td>1.09 (0.3822)</td>
<td>1.162 (0.2584)</td>
<td><em><em>1.37</em> (0.04845)</em>*</td>
<td>1.351 (0.2274)</td>
<td><em><em>1.768</em> (0.04037)</em>*</td>
</tr>
<tr>
<td>rs2957154</td>
<td>0.9358 (0.4795)</td>
<td>0.9596 (0.7488)</td>
<td>0.9432 (0.7179)</td>
<td>1.097 (0.7026)</td>
<td>0.5619 (0.09713)</td>
</tr>
<tr>
<td>rs524386</td>
<td>0.9539 (0.7395)</td>
<td>1.12 (0.5395)</td>
<td>1.301 (0.23)</td>
<td><em><em>0.238</em> (0.04497)</em>*</td>
<td>1.085 (0.8497)</td>
</tr>
<tr>
<td>rs660339</td>
<td>0.9933 (0.9338)</td>
<td>0.8644 (0.2008)</td>
<td>1.004 (0.9762)</td>
<td>1.22 (0.3546)</td>
<td>1.223 (0.4324)</td>
</tr>
<tr>
<td>rs669976</td>
<td>0.9743 (0.8469)</td>
<td>1.051 (0.7808)</td>
<td>1.089 (0.7035)</td>
<td><em><em>0.2128</em> (0.03102)</em>*</td>
<td>0.7926 (0.6214)</td>
</tr>
<tr>
<td>rs670358</td>
<td>1.205 (0.1316)</td>
<td>1.343 (0.07251)</td>
<td><em><em>1.514</em> (0.03516)</em>*</td>
<td>1.284 (0.4269)</td>
<td>0.5722 (0.2828)</td>
</tr>
<tr>
<td>rs6822</td>
<td>1.047 (0.6782)</td>
<td>0.933 (0.6578)</td>
<td>0.9174 (0.6615)</td>
<td>1.045 (0.882)</td>
<td>1.262 (0.4858)</td>
</tr>
<tr>
<td>rs6990300</td>
<td>1.131 (0.159)</td>
<td>1.012 (0.9227)</td>
<td>0.9096 (0.5434)</td>
<td>1.462 (0.09268)</td>
<td>1.111 (0.7059)</td>
</tr>
<tr>
<td>rs7235755</td>
<td>1.093 (0.3684)</td>
<td>1.164 (0.2512)</td>
<td><em><em>1.372</em> (0.04691)</em>*</td>
<td>1.354 (0.2234)</td>
<td><em><em>1.771</em> (0.03967)</em>*</td>
</tr>
<tr>
<td>rs820152</td>
<td>0.9788 (0.7972)</td>
<td>1.063 (0.5911)</td>
<td>1.072 (0.6268)</td>
<td>0.7768 (0.284)</td>
<td>1.239 (0.4112)</td>
</tr>
</tbody>
</table>

**Table 7:** Association between candidate SNPs, leukocyte telomere length, and mortality.

ORs and P values presented apply to multivariate logistic regression. OR >1 means that the odds of mortality is greater among minor allele carrier of given SNP and vice versa. Statistically significant (P<0.05) ORs are bolded and marked with asterisk.
4.7 Discussion and future directions

Studies have shown the link between telomere length of peripheral blood cells and cancer mortality (172), cardiovascular mortality (72) and all cause mortality (153). In LHS, we have shown that leukocyte telomere length is significantly associated with increased risk of all cause mortality and cancer mortality. Based on what has been shown, we hypothesized that if indeed leukocyte telomere length is a strong predictor of mortality, polymorphisms of genes that are involved in telomere maintenance would also be associated with mortality.

When we tested the association between the telomere length and the SNP genotype of the same subject, there were no SNPs that were associated with leukocyte telomere length. This could be due to the narrow range of telomere length of this cohort. Telomere length is influenced by age, gender, smoking status and disease status. The LHS cohort had an age restriction (35-60 years), and all subjects were affected (COPD), which means the distribution of telomere length could be too narrow to see a significant association. It is also possible that the association between leukocyte telomere length and candidate SNPs that was shown in general population is simply not present in the COPD population. Another possibility for the lack of association is the accurate measurement of telomere length. Leukocyte telomere length was measured using qPCR, which only measures the amount of amplicon. The crudeness of the method may have made it difficult to detect small differences in telomere length. A single SNP may only have a small effect on telomere length and the method may not have been sensitive enough to detect small changes.

We also tested for the association between candidate SNPs and the risk of mortality. The study revealed that several polymorphisms in genes (TNKS and MEN1) that are involved in telomere maintenance and regulation were significantly associated with lung cancer and
cardiovascular mortality. While the reason for the association between the SNPs and mortality is unclear, considering the known functions of the genes, these variants may work through regulating telomere length. TNKS is a known telomere length regulator which modifies telomerase through TRF1. Tankyrase releases TRF1 from telomeres, allowing access of telomerase to telomeres. Tankyrase 1 inhibition in human cancer cells enhances telomere shortening and promotes premature cell death (196). Inhibition of tankyrase may directly induce cell senescence by abrogating telomerase activity and tankyrase has been studied as a target for cancer therapy. MEN1 is a potent tumor suppressor gene and affects telomerase activity (197). MEN1 has been implicated in various endocrine cancers and it may be related to telomere function. The variants of TNKS and MEN1 gene may regulate telomere length through modifying the level of expressions and this may lead to protection from cardiovascular and lung cancer mortality.

We also looked at effect of telomere length and SNPs on risk of mortality. Short telomeres were significantly associated with increased risk of cancer mortality when combined with SNPs effect. This association was primarily driven by telomere length, which is consistent with current knowledge in the role of telomeres in carcinogenesis. Furthermore, the range of ORs of telomere length for both CVD mortality (1.668-1.788) and respiratory mortality (1.731- 1.786) were higher than cancer mortality (1.55-1.572), but failed to reach statistical significance. This may be due to a small number of deaths (% CVD mortality: 1.159%; % respiratory mortality: 0.816%). Also the fact that significant associations were found only when telomere length was treated as binary variable, not as continuous variable, suggests that there may be a threshold effect, which is consistent with what we found in telomere and mortality analysis.
CHAPTER 5. CONCLUSION

We found that mild to moderate COPD patients with shorter telomeres in their peripheral blood cells had higher risk of mortality than those with longest telomeres in LHS cohort. Association between leukocyte telomere length and mortality has been shown in general population. Danish twin study (152) showed that persons with shorter telomeres were more likely to die during follow-up than those with longer telomeres (HR=1.61, p=0.001). Also among individuals over the age of 60 years, those with short leukocyte telomere length had higher mortality rate from heart disease (HR=3.18, p=0.0079) and infectious disease (HR=8.54, p=0.015) (72).

Telomere length was not associated with smoking status, determined by the first 5 year follow-up. This could be due to the nature of the cohort; everyone in LHS was smokers and had long history of smoking (over 15 pack years). qPCR measures the amount of PCR product and due to the crude nature of the method, the resolution is not high. Therefore the minute changes in telomere length due to 5 years of smoking cessation may not have been detected. It is also possible that ongoing chronic inflammation in COPD patients even after smoking cessation drove the telomere shortening. We showed that mid-life control group, which is a non COPD cohort, had significantly longer telomeres than all smoker groups in LHS. Shorter telomere in peripheral circulation was associated with elevated pro-inflammatory cytokines in COPD patients (19), suggesting a major role for inflammatory severity in telomere shortening.

We failed to show significant association between leukocyte telomere length and the candidate SNPs in LHS. However, some of the SNPs were associated with risk of mortality in LHS. Future studies such as replication study in different cohorts or functional analysis of the
candidate SNPs are needed to elucidate the mechanism on how SNPs are associated with mortality.

In summary, we have demonstrated that telomeres are predictive of mortality in COPD patients, especially cancer mortality and genetic variants of telomere biology genes are associated with mortality.
References


(123) Garcia-Sanz JA, Velotti F, MacDonald HR, Masson D, Tschopp J, Nabholz M. Appearance of granule-associated molecules during activation of cytolytic T-lymphocyte precursors by defined stimuli. Immunology 1988 May;64(1):129-134.


