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ROLE OF ULTRA-SHORT TELOMERES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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STATEMENT (DECLARATION)

Hereby I declare that this thesis study is my own study, I had no unethical behaviour in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behaviour of breeching patent rights and copyright infringement during the study and writing of this thesis.

Hüseyin Çağsın

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Abbreviations List

TERT	Telomerase reverse transcriptase
TERC	Telomerase RNA component
TL	Telomere Length
NHP2	Non-histone protein 2
NOP10	Nucleolar protein 10
GAR1	H/ACA ribonucleoprotein complex subunit 1
DKC	Dyskerin
TCAB1	Telomerase Cajal body protein 1
TRF	Telomeric repeat binding factor
POT1	Protection of telomeres 1
RAP1	Repressor/activator protein 1

TIN	TRF1-interacting nuclear factor
TPP1	Adrenocortical dysplasia protein homologue, ACD
TSA	Trichostatin A
Pol II	RNA polymerase II
TFIIB	Transcription factor II B
UVA	Ultraviolet A
GTS	Gradual telomere shortening
ATS	Abrupt telomere shortening
SSS	Sudden Senescence Syndrome
FISH	Fluorescence in situ hybridization
PNA-FISH	Fluorescence in situ hybridization using peptide nucleic acid probes
hMSC	Human Mesenchymal Stem Cell

PBMC	Peripheral blood mononuclear cell
ROS	Reactive oxygen species
COPD	Chronic Obstructive Pulmonary Disease
NF-KB	Nuclear Factor Kappa B
BAL	Bronchoalveolar Lavage
SASP	Senescent associated secretory phenotype
FEV1%	Forced expiratory volume in second
BW	Bronchial washing
WBC	White blood cell

Turkish Summary

Telomer biyolojisi, ozellikle de doku spesifik ultra-kisa telomerler, KOAH gelisimi ve ilerleyisi hakkinda bilgilerimize katkida bulunabilir ve prognoz icin prediktif bir marker olarak kullanilabilir. Bu hipotezi sinamak adina, KOAH hastalarindan alinan akciger dokusunda ve lokositlerde telomer boylari arastirilmistir.

Calismadaki tum hastalarda post-bronkiodilator orani %70'ten azdi (FEV1%). Ultra-kisa telomerleri bulmak adina Universal tek telomer uzunlugu analizi (U-STELA) kullanilmistir. Bulgulara gore, bronkoalveolar lavaj (BAL) sonrasi elde elilen hucrelerde, ultra-kisa telomer olgusu lokositlere gore stastiki acidan anlamli sekilde, $t(62)=5.771$, $p<0.00001$, fazla oldugu goruldu. FEV1% BAL orneklerinde ultra-kisa telomer gorulen hastalarda daha dusuk (50.6% vs 81.6%: $p<0.001$) bulundu. Ayni zamanda bu hastalarin daha yasli olduklari gozlemlendi ($p=0.014$). Yasa ($p<0.0001$), sigara kullanilmasina ($p<0.0001$) ve cinsiyete ($p=0.71$) gore normalize edilmis regresyon modeline gore BAL da gorulen ultra-kisa telomerler ($p=0.05$) FEV1% ile iliskilendirildi fakat lokositlerdekilerde bir iliski saptanamadi ($p=0.33$), Ultra-kisa telomerler ayni zamanda paket-yili ile de iliskilendirildi ($p=0.045$) .GOLD klasifikasyonuna gore, ultra-kisa telomerleri olan hastalarda hastaligin derecesi daha yuksek bulundu ($p=0.006$). Bu tezde vurgulanmak istenen ana konulardan biri, KOAH calismalarinda dogru doku kullanildiginda hastalik derecesinin daha iyi irdelenebilecegidir. Bu calisma, KOAH derecesi ile ultra-kisa telomerler arasinda iliski oldugunu gosteren ilk calismadir. Ayni zamanda, Koahli hastalarda telomer boyu ile paket yili ve dusuk akciger fonksiyonu arasindaki doz bagimli iliski gosterildi.

Anahtar kelimeler:KOAH, ultra-kisa telomerler, BAL, doku spesifik, paket yili

English Summary

In COPD research, telomeres and telomere biology can be a usefull tool. A small subset of telomere biology, that is tissue-specific ultra short telomeres (UST), could be a meaningful contributor for the understanding of the COPD development. In addition to this, USTs might also be used as a predictive biomarker for prognosis of COPD. Telomere lengths (TL) in leukocytes and bronchoalveolar lavage cells were investigated in COPD patients, in order to test this hypothesis,.

All patients showed a post-bronchodilator ratio of less than 70% post-bronchodilator predicted value of forced expiratory volume in second (FEV1%). Universal Single Telomere Length Analysis (U-STELA) was used for UST detection.

In the results, UST were found to be present at a significantly higher rate in BAL samples than the leukocytes after quantitative comparison $t(62)=5.771$, $p<0.00001$. The patients with USTs in BAL had lower FEV1% than the patients who did not had USTs (50.6% vs 81.6%: $p<0.001$). Similarly, the rate of presence of USTs in BAL increased as the patients get older ($p=0.014$). Only USTs in BAL ($p=0.05$) were found to be associated with FEV1% when variables such as ever smoking ($p<0.0001$), sex ($p=0.71$) and age ($p<0.0001$), were normalized according to the regression model. Furthermore, USTs were also associated with pack-years ($p=0.045$). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification ($p=0.006$) was found to be higher in the patients with USTs.

Therefore, here it is postulated that the investigation of the COPD at a molecular level should be carried out in the representative tissue (lung tissue) and not in leukocytes. The COPD severity and presence of USTs in the lung tissue is found to be positively correlated, in a novel manner. The association between USTs in COPD

patients, low lung function and pack-years of smoking is shown to be at a dose-response manner.

Keywords: tissue-specific; BAL; COPD; ultra-short telomeres; pack-years

1 Introduction and Aim

Telomeres are involved in a number of complex diseases as telomeres are important regulators of replicative capacity, biomarkers of cellular aging and protectors of DNA integrity. In this thesis, the role of tissue specific USTs in Chronic Obstructive Pulmonary Disease (COPD) is investigated.

Although the associations between TL and the COPD risk is currently contradictory, COPD molecular pathogenesis might be influenced from deficient telomere shelterin and constitutive short telomeres. A small subset of telomere biology, that is tissue-specific ultra short telomeres (UST), could be a meaningful contributor for the understanding of the COPD development. In addition to this, USTs might also be used as a predictive biomarker for prognosis of COPD

The primary aim of this project is to evaluate the importance of the ultra short telomeres instead of the mean TL in a tissue specific manner in the molecular basis of COPD. Therefore, the USTs were evaluated in both the lung tissue and the blood cell samples for differentiation in tissue specificity. It is thought that the comparison of the lung tissue from bronchoalveolar lavage (BAL) sampling and peripheral blood, provided a distinguishable choices of primarily affected tissue and indirectly affected tissue.

The secondary aim of this project to evaluate whether the USTs could be used in the evaluation of the severity and causation of the COPD. Therefore, patients with and without the USTs were compared in terms of disease severity, smoking status and age.

2 General Information

2.1 Telomere Biology

Telomeres are specialised structures that are localised at the ends of each chromosome. They are composed of tandem repetitions of TTAGGG together with telomere specific proteins. There is a 3' overhang, which is a result of 3' end of the DNA is approximately 35-60bps longer than the 5' end of the DNA, at the ends of the chromosomes which interacts with the telomeric proteins. The chromosome ends are protected by this complex very effectively by avoiding the DNA repair mechanisms to recognise the ends of chromosomes as double strand breaks. Thus, the cellular integrity is preserved and cellular arrest is prevented. The evidence shows that the chromosomal end protection is achieved by formation of telomeric loop where the 3'-overhang is annealed to the telomere-repeat-block by a triple helix formation at the the upstream part. The formation of the telomeric loop is called the 'cap' which also includes the shelterin proteins that also mediate the telomerase activity. Furthermore, the capping of the telomeres prevent end-to-end fusions between the chromosomes, preventing genomic instability and providing DNA integrity. (Turner *et al*, 2019).

In human cells, telomerase is selectively active depending on the tissue type. Active telomerase is seen in actively dividing cells and consist of several subunits. These subunits are the catalytic subunit TERT, RNA component TERC, non-histone protein 2 (NHP2), nucleolar protein 10(NOP10), GAR1 (encoding H/ACA ribonucleoprotein complex subunit 1), dyskerin (DKC) and telomerase Cajal body protein 1 (TCAB1).The shelterin complex is composed of six proteins. Telomeric repeat binding factor 1 (TRF1) and TRF2 bind to the double helix region of the

telomere and prevent addition of more telomeric repeats. Both TRF1 and TRF2 recruit the protection of telomeres 1 (POT1)which binds to the single stranded telomeric DNA. The repressor/activator protein 1 (RAP1) is then recruited by TRF2 and bind to it however in humans, it does not directly bind to the telomeric DNA. TRF1-interacting nuclear factor 2 (TIN2) is centrally localised in shelterin complex where it bind to TRF1, TRf2 and TPP1 (adrenocortical dysplasia protein homologue, ACD) which forms a bridge between POT1 and TIN2. The shelterin complex caps the telomeres when it is completed with the discussed proteins (Maciejowski and de Lange, 2017; Palm and de Lange, 2008).

In the case of any deficiency in one of these subunits can result in dysfunctional telomeres which may result in cellular senescence. For example POT1 is involved in ATR-dependent DNA damage response as POT1 deficiency is associated with increased apoptosis and cell cycle arrest (Wang *et al*, 2013). On the other hand, TRF2 is involved in DNA damage response which is ATM dependent where TPP1/POT1 complex inhibits the occurance of separate telomere 5' end-resection pathways. (Kibe *et al*, 2016). One pathway is ATM-activated and is a limited resection, which is inhibited by the presence of functional TRF. The other pathway is that the ATR-stimulated extensive resection which is inhibited by the presence of functional TPP1/POT1(Kibe *et al*, 2016) (Figure 1).

Figure 1: Shelterin proteins and their interactions with telomere

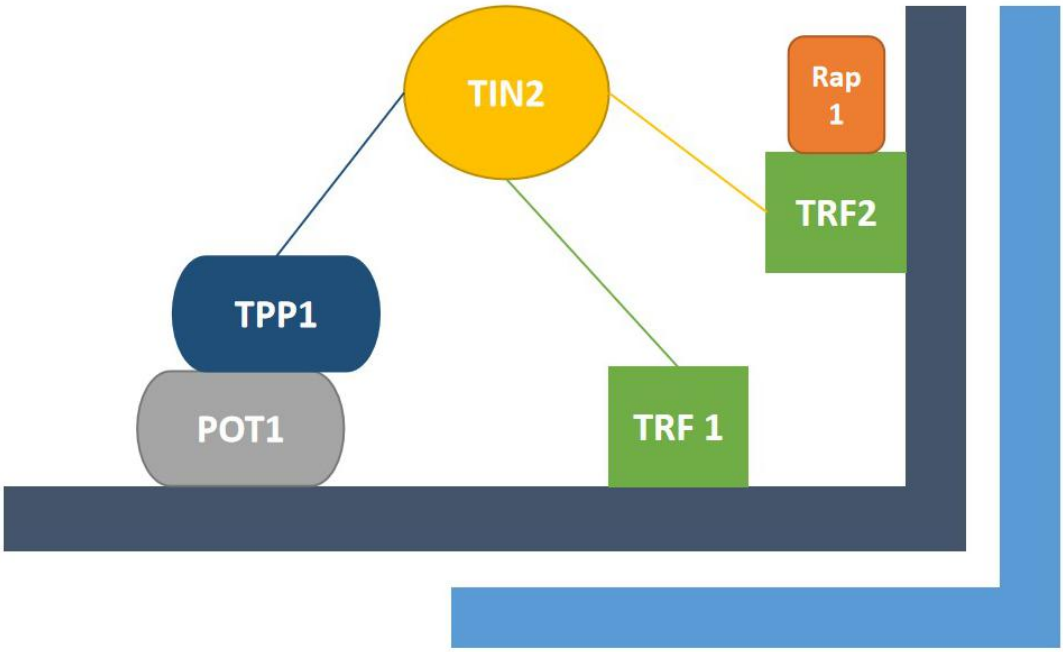

Figure 1: Both TRF1 and TRF2 are bound to the telomere at double stranded region. RAP1 binds to TRF2, POT1 binds to the 3' overhang, TPP1 acts as a connector between POT1 and TIN2. TIN2 also have interactions with TRF1 and TRF2. Blue strand is the 5' end strand, grey strand is the 3' end strand with the 3' end overhang.

2.2 Telomere Length and Replication Potential

Leonard Hayflick showed the somatic cells have a replicative capacity in the 1960s which is now known as the Hayflick limit (McCormick *et al*, 2015). Despite this revelation, it was not associated with the end replication problem up until after a decade and another decade was required to show the association of telomere shortening and passage number and replicative capacity (Longo *et al;* Chen *et al*, 2017; He *et al*, 2018). Furthermore the evidence shows that the TL is negatively

associated with the organismal and tissue age, in nearly all cell types, as the TL decreases as the donor age increases (Turner *et al,* 2019).

Moreover, the cellular aging process can be influenced by the telomere shortening. Telomeres can be shortened to a certain degree that they can be regarded as critically short telomeres. At this point, the associated proteins can no longer sustained and the formation of shelterin complex is halted. Thus, the shelterin inhibition of the DNA damage responses are relieved such cells are driven to cellular arrest (Longhese 2008). The cellular arrest is mediated through ATM or ATR pathways. Each pathway increases the p53 phosphorylation, p21 expression and inhibition of the cyclin dependent kinases to interrupt the normal progression through cell cycle. The activated p53 and reduced amount of cyclin dependent kinases naturally stops the cell cycle and initiate cell cycle arrest (Shay 2003; Munoz-Espin and Serrano, 2014). When cell cycle is interrupted, there are two options for the cell; senescence where the cell loses its division potential and apoptosis where the cell is destined to programmed cell death. Although the molecular mechanism behind the choice of cells between senescence and apoptosis is not clear, the evidence suggests that the apoptosis is triggered if the cell has detrimental damage and can not function properly under the dealt circumstances. In any case, the cell had lost its division potential even though the molecular characteristics of senescence and apoptosis are quite different (Vicencio *et al* 2008).

The aging itself can be attributed to senescence as the accumulation of the senescent cells would lead to a increased number of undividing cells is a particular tissue, thus reducing the growth and repair potential of that tissue. In addition to this, senescent cells secrete proteases, chemokines, growth factors and inflammatory cytokines to

the extracellular matrix which in turn affect the dividing cells which is called the senescence associated secretory phenotype (Hansel et al, 2020). Generally this triggers the immune removal of the senescent cells, although this function can be reduced as the organism ages. Therefore, the telomere shortening and associated accumulation of senescent cells both contribute to the cellular and organismal aging.

2.3 Telomerase in maintenance of telomere length

The TL can be naturally increased in a group of dividing cell by increasing the effects of telomerase which can be done through either up-regulation of the activity or increased expression. The TL maintenance in the human cells where telomerase is utilised, the telomerase regulation depends on several ways. The telomerase activation is limited to the hTERT mRNA expression level. Thus, hTERT expression regulation at the transcription level is a crucial mechanism in the control of the hTERT levels in a given cell. Several genes such as MYC, p53 and BRCA1, had been identified to be either positive or negative regulators of hTERT transcription by the hTERT promoter region cloning. In addition to this, these genes are also implicated in chromatin remodelling which suggests telomerase activation may also be involved (Blasco, 2004). On the contrary, several reports suggest that chromatin remodelling process also effects telomerase expression, although this effect was found to be indirect (Benetti *et al*, 2007; Blasco, 2007)

On the other hand, it is shown that the hTERT gene can be silenced by histone modifications (Cenci *et al*, 2003). The hTERT expression can repressed by the hTERT promoter specific histone hypoaceltylation which is a reversible effect as the repression of the hTERT expression can be inhibited by Trichostatin A (TSA)

treatment. TSA treatment resulted in an increase in Pol II and TFIIB tracking and increase in promoter specific histone acetylation, further supporting that the telomerase expression can be regulated through histone modifications.

In addition to the hTERT expression, telomerase recruitment to the telomeres is also a significant mechanism for TL maintenance. Naturally, both positive and negative feedback mechanisms in TL contributes to the homoeostatic nature of the TL maintenance. The shelterin proteins also contribute to this complex interaction. Shelterin complex does not only protect telomeres from the DNA damage responses but also regulates the lengths of the telomeres as shelterin complex proteins strongly controls the amount of telomerase to be recruited to the telomeres (Dubrana *et al*, 2001).

Moreover, the telomere structure also have a significant role in the telomere maintenance and elongation. It has been suggested that telomere structure is regulated by both shelterin proteins (Dubrana *et al*, 2001; Yanada *et al*, 2006) and chromatin modifications (Peters *et al,* 2001; Gonzalo *et al*, 2006). The telomerase mediated telomere elongation can be inhibited by the formation of G-quadriplex at the site of the guanine rich 3' overhang. The G-quadriplex formations interact with the RNA component of the telomerase thus disable the telomerase in telomere elongation (Moye *et al*, 2015). Telomeric chromatin structure suggests that the histone modifications found in these structures are consistent with the constitutive heterochromatin domains (Garcia-Gao *et al,* 2004). The region of constitutive heterochromatin is tightly packed, repetitive genomic DNA which is transcriptionally inactive. In this region heterochromatin protein 1 (HP1) is bound to the high levels of trimethylated H3 and H4 histones. These histones are also trimethylated in telomeres

(Gonzalo *et al,* 2006; Peters *et al,* 2001). The evidence from mice, drosophila melonagaster and yeast have shown that the alterations in the telomeric heterochromatin due to histone modifications might alter the telomere elongation process. This is found to be correlated with impaired recruitment of the telomerase and impaired telomere function (Cano *et al*, 1999; Fanti *et al*; 1998; Greider and Blackburn, 1985). The hypo-methylated subtelomeric regions are shown to mediate the telomere elongation in humans, suggesting that this mechanism is tightly conserved among species (Schreurs *et al,* 2005). In addition to this, both telomeric and subtelomeric DNA can be methylated for repression of the transcription which results in the telomere shortening. The main reason for this shortening is again the inability to recruit telomerase for the addition of telomeric repeats (Gonzalo *et al,* 2005; Oullette *et al,* 2000). Taking account the evidence, it is indicated that the chromatin modifications may influence the TL regulation through regulation of telomerase recruitment.

2.4 Telomere length measurements in different tissues

It has been previously shown in human fibroblasts that telomere shortening is accelerated through oxidative stress and when oxidative stress is suppressed by antioxidants, the rate of telomere shortening decreases and the replicative lifespan gets enhanced (von Zglinicki, 2002). As a response to the oxidative stress-induced telomere shortening or loss, replicative senescence is induced. It was also shown that in human WI-38 fibroblasts which were exposed to UVA irradiation, 8-oxodG at the GGG triplet in telomere sequence were generated due to the oxidative stress and it was suggested that oxidative stress have role in enhancement in the reduction in the

TL where TL was measured as TRF length in WI-38 fibroblasts (Kawanishi and Oikawa, 2004).

Both the end replication problem and the degradation of the 5' end of the telomeres by the action of exonucleases lead to gradual telomere shortening (GTS) (Rubelj, 2010). GTS, in turn, reduces the proliferation potential of the cultured cells gradually as TL is a rate limiting factor in proliferation. This explains why non-transformed somatic cells after experiencing several divisions, become senescent, begin to age and undergo apoptosis. In addition to the GTS, abrupt telomere shortening (ATS) can occur by the invasion of the 3'- telomeric overhang at the telomere –sub-telomere border part by the telomeres themselves and following this, recombination occurs that causes deletion of distal repeats by the formation of t- loop (Rubelj, 2010). ATS is recognised as DNA damage which can result in cell cycle arrest and Sudden Senescence Syndrome (SSS).

For the telomere dynamics in lymphocyte progenitor cells, research groups have studied TL, both mean length and individual TL on each chromosome, in primary lymphocyte cultures from individuals at different ages. Given that native lymphocytes are constantly produced in lymphatic tissues from progenitor cells, the characteristics of the telomeres in the native lymphocytes most likely represent the telomere status in the corresponding stem cells (Lin *et al,* 2016).

Several groups, by using mean TL have shown that telomeres are lost at a rate of about 50bp a year, except the initial 1-12 months of life, where the telomere loss is is at an increased rate. The separate chromosome's telomeres can be investigated by a FISH-based assay that has clearly showed humans have a similar telomere profiles although there are specific characteristics to each individual. This telomere profile

was demonstrated to be conserved – in spite of the gradual loss of total TL - throughout life, and only showed slight signs of degeneration in elderly individuals. Furthermore, it was shown that the length of the individual telomeres was determined by telomere-near sequences (Lee *et al*, 1998).

The maintenance of a specific profile, combined with this lifelong similarity of relative length of the same telomere in chromosomes identical by descent over 80 years of life (Graakjaer *et al,* 2007) suggest that there is a conservation of the relative TL in lymphocyte progenitor cells, either due to a very low degree of telomere erosion/elongation in the stem cells or due to a very strict regulation of the balance between telomere erosion and elongation in the stem cells.

Previously, by utilizing the PNA-FISH method and computer-assisted telomere quantifier software assay, the telomere profile in mesenchymal stem cells has been investigated by PNA FISH and the TLs were quantified by computer software. It was observed that the telomere profile of human mesenchymal stem cells is very much like the profile of lymphocytes ((Graakjaer *et al,* 2007). In that study, hMSCs were cultured until they became senescent, samples were collected from both early and late passages and TL were analysed for each sample to show the effects of cell division in hMSC telomeres. When the TL profile of hMSC cell samples was compared with the mean TL analysis of telomeres, a significant correlation was detected. This profile suggests that in mesenchymal stem cells the degree of random fluctuation in the telomere dynamics is low, as it is for lymphocyte progenitor cells.

Chromosomal instability can be caused by short telomeres, thus, contribute to tumorigenesis (Zhao *et al,* 2014). In human cancers, USTs have also been detected and it is suggested that mean TL shortening does not contribute to the chromosomal

instability but the USTs do (Capper *et al,* 2007; Heman *et al*, 2001). Furthermore, the evidence suggests that USTs are produced as a result of the abrupt shortening of telomeres (Friis-Ottossen *et al*, 2014). Overall, in the light of these, the cell division potential may highly depend on TL (Cherif *et al,* 2003).

It has been shown that telomeric sequence is a highly conserved sequence between eukaryotes however the length of telomeres in different species is not the same (Cherif *et al,* 2003). As well as differences among species, different TLs are reported in different tissues in adults (Gadalla *et al,* 2010; Lin *et al*, 2010; Friedrich *et al*, 2000) whereas in fetuses and newborns the TL is identical (Gadalla *et al,* 2010; Friedrich *et al*, 2000; Youngren *et al,* 1998). The variety in the TLs of different adult tissues might be because of different replicative turnovers, cellular micro-environment and cell specific regulatory pathways (Lin *et al*, 2016). It has been identified that there is an association between TLs of peripheral blood mononuclear cells (PBMCs), T cells, B cells and monocytes in an individual, however, the alterations in the TL may not be associated with human diseases (Lin *et al,* 2015). As the circulating immune cells, which are particularly composed of T and B cells, are exposed to the same micro-environment in circulation, the difference in the rate of TL alterations among different cells is reported and this is due to the cell type-specific intracellular responses (Lin *et al*, 2016). The link between telomere shortening and several different diseases and the changes in the TL that occur as a consequence of exposures to social and environmental factors have emphasized the requirement for different approaches to evaluate TL accurately and consistently (Montpetit *et al,* 2014). Therefore, telomere studies in senescence-related tissue-specific diseases are crucial to determine if the telomere attrition is accelerated

or the risk of developing senescence-related diseases is higher in people with short constitutional telomeres.

To quantify the TL in different cell types, various methods have been established, in spite that measuring tissue specific TL yields more information about the specific cell type and pathologies related to that cell type. This is evidenced at evaluation of the TL in cardiac samples to suggest a link with cardiac pathologies (Sharifi-Sanjani *et al*, 2017a). Recently it is reported that telomere shortening takes place in a cell type-specific way as it is shown for the first time that cardiomyocytes form patients who suffer from heart failure have considerably shorter telomeres compared to healthy subjects (Sharifi-Sanjani *et al*, 2017b). Therefore, it can be hypothesized that complex, senescence-related diseases can be better understood with tissue-specific telomere studies.

2.5 Mechanisms of Telomere shortening

In humans, telomeres get shorter at each cell division because of the incomplete replication of DNA at the very ends of the chromosomes. As a result of the normal DNA replication, the 5' strand of the DNA where RNA primer is attached is shorter than the 3' strand of the DNA where a short 3' overhang is generated. This is called an end replication problem (Serakinci *et al,* 2008). This is attributed to be the main reason for gradual telomere shortening due to replication. It can be varied between cell types as the cellular turnover of the tissues differ drastically depending on the cellular requirements in growth and repair.

In most of the cell types, telomere shortening is prevented by the activity of the telomerase enzyme as it provides elongation of the telomeres. Despite that, the

telomerase is not present in all cell types. After the early stages of embryogenesis, the activity of the telomerase is down-regulated/silenced in most human cells. This is one of the main reasons shortening of the telomeres in proliferating cells (Graakjaer *et al*, 2004).

Inflammatory diseases that can be manifested due to USTs. The molecular mechanisms of the telomere associated disease phenotypes are similar in spite of different pathologies. Inflammation is one of the most striking molecular event underlying these phenotypes (Hasset *et al*, 2014), oxidative stress (Domej and Oettl, 2014), senescence (Aoshiba and Nagai, 2009) and apoptosis (Aoshiba *et al,* 2012). Telomere shortening can occur by several different mechanisms. One of the major mechanism is the end replication problem faced during the cell division. This mechanism leads to telomere shortening after the cell division at a rate of 30 ± 50 bp, because the ends of the telomeres can not be fully replicated (Martens *et al*, 2000). In DNA replication, an RNA primer is required by the DNA polymerase for replication initiation. DNA polymerase can initiate DNA replication when RNA primer is bound to the 3' end of the DNA. When replication is complete, RNA primer dissociates from the template DNA. DNA can not be fully replicated at the site of RNA primer. Therefore the DNA molecule in the daughter cells becomes shorter than the previous DNA molecule (Zhao *et al*, 2014). Other factors contribute to the telomere shortening such as telomere repair problem, ROS and inactive telomerase (Jiang *et al*, 2007; Grach, 2013).

The second mechanism is the shelterin-mediated telomere repair problem (Grach, 2013. In the end repair problem, extreme ends of the chromosome are not repaired completely and direct damage-mediated telomere shortening is triggered. In direct

damage-mediated telomere shortening, even the damage repair at the chromosome ends cannot be initiated and this also leads into telomere shortening. In the shelterin-mediated telomere repair problem, damage generated at telomeres cannot be detected and repair by the proteins that take part in DNA damage response as t-loop prevents the access of these proteins and also prevents the DNA repair pathways. This, in turn, leads to the accumulation of damage and shortening of telomeres.

The link between telomere shortening and several different diseases and the changes in the TL that occur as a consequence of exposures to social and environmental factors have emphasized the requirement for different approaches to evaluate TL accurately and consistently (Montpetit *et al*, 2014).

2.6 Role of telomere shortening in aging

The cellular and organismal aging differs as it is also the case for the unicellular and multicellular organisms. The multicellular organisms age differently as the cell to cell communications and the interactions might have a significant effect on the process of aging. It is shown that increasing the TL in mice might also increase the lifespan of the organism (Thomas-Loba *et al,* 2008). Although the mice used in this study were cancer resistant, there have been successful attempts to not only increase the TL but also not increase the neoplastic potential (Bernardes *et al*, 2012). Furthermore, it is also shown that the increasing the TL, also increases the replicative potential of the human cells (Rubio *et al,* 2002). the evidence on longer mean telomeres is contradictory. There are reports that show that longer mean TLs are associated with less aging related disease pathologies in humans (Codd *et al,* 2013).

However, there also reports that show the risk of having lung adenocarcinoma and hypertension are associated with longer mean telomeres, both of which can be considered as aging related disease pathologies (Izziki *et al,* 2015; Zhang *et al*, 2015).

In all essence, telomeres whether short or long, are associated with aging related diseases. The telomeres that can send the cell to senescence are termed as UST (Bendix *et al*, 2010). These telomeres are shorter than 1500bps and may induce senescence in a given cell even if the mean TL is considered in a normal range. On the other hand, although longer telomeres are implicated in high proliferative capacity of the cells, the tumorigenesis potential may also increase, tipping the balance of benefit-loss in the wrong direction. The TL homoeostasis is crucial for the healthy growth and repair of the tissue and it has been implicated in aging related diseases.

2.7 Chronic Obstructive Pulmonary Disease (COPD) as an Aging Disease

COPD is characterised by progressive airflow limitation in the lungs where age of onset is over 60 years of age although patients with alpha-1 anti-trypsin deficiency might present symptoms at an earlier age than the general population since the alpha-1 anti-trypsin deficiency predisposes to the COPD development (Strange, 2010). The disease is a progressively chronic disease which presents cough and sputum (Miravitlles, 2011). COPD patients show small airway obstruction (Hogg *et al*, 2004) followed by emphysema (McDonough, *et al*, 2011). Generally, all of these symptoms are coupled with frequent exacerbations. As COPD is a progressive disease, the rate of emphysema increases as the COPD progresses (Hurst *et al*, 2011).

Although the symptoms may differ, the molecular basis of them are similar. The most established molecular mechanisms behind the COPD associated phenotypes are inflammation (Hasset *et al*, 2014), oxidative stress (Domej and Oettl, 2014), senescence (Aoshiba and Nagai, 2009) and apoptosis (Aoshiba *et al,* 2012).

Smoking induced oxidative stress in the pulmonary epithelium (Faux *et al,* 2009). In addition to this, the decrease in antioxidants and their activity, present increased oxidative stress in COPD patients (Tager *et al*, 2000). Oxidative stress produces external reactive oxygen species (ROS) in the lungs. These can be combined with inflammatory ROS within the lung tissue and contribute to development of COPD (Rahman and Adcock, 2006). Oxidative stress induces inflammation in pulmonary cells by activating stress kinases and transcription factors such as NF-KB (Puig-Vilanova *et al*, 2015). Inflammation in the lung occurs by the recruitment of proinflammatory cells such as macrophages to the lungs as a response to infection or injury. In COPD cases, the exacerbations due to oxidative stress provide the necessary signals for the recruitment. Supportively, macrophage counts are higher in BAL samples of COPD patients when compared with healthy controls. There also is an increase in macrophage number as the severity of COPD increases (Vlahos and Bozinovski, 2014).

Human cells are known to undergo apoptosis in response to certain stimuli such as oxidative stress, inflammation and presence of chemokines in senescent associated secretory phenotype SASP (Demedts *et al*, 2006). In COPD patients, the rate of apoptosis is increased and remain at the same level well after elimination of exposure (Hodge *et al*, 2005). One of the factors in COPD development, emphysema, occurs due to high levels of apoptosis in the ling tissue. Therefore it can be stated that

apoptosis can trigger the development of COPD and can be triggered due to COPD(Padowski *et al*, 2009). Apoptosis may be a result of COPD at first, but then it effectively helps COPD to progress by producing emphysematous lesions. Altogether, both inflammation and oxidative stress, act upon triggering apoptosis and therefore increase the cellular turnover in the lung tissue causing cellular aging. Despite the exact role of aging in COPD development remains to be revealed, cellular aging in the lung tissue has a contributing role in molecular basis of COPD. (Mercado *et al*, 2015).

2.8 Role of Telomeres in COPD

Telomeres are shown to become an important factor in all of the molecular mechanisms discussed above (Artandi and Attardi, 2005). TL was shown to be an important determinant in aging through senescence and apoptosis of the lung tissue and eventually leading to COPD (Mui *et al* 2009). COPD patients are also shown to have shorter leukocyte TLs than healthy people linking TL to COPD directly (Lee *et al*, 2012). Although there are reports on TLs in lung tissue (Amsellem *et al*, 2011) most of the current evidence is based on the information from TLs in leukocytes. The evidence in the literature about TL association with COPD risk is conflicting. The shorter mean TL can be found to be moderately associated with COPD risk (Rode *et al*, 2013) despite the previous evidence supports a very strong association between short telomeres and COPD (Savale *et al*, 2009). Furthermore, telomere shortening takes place in a cell type-specific manner in compex diseases (Sharifi-Sanjani *et al*, 2017b). The fact that the contradictory mean TL associations with COPD and tissue

specific nature of telomere involvement in complex disease, raises the questions on the possibility of the involvement of USTs in the molecular basis of COPD.

3 Materials & Methods

3.1 Subject Selection

The subjects in this study were patients admitted to Erciyes University Hospital to undergo bronchoscopy aged from 31 to 73 years (median 63). In total 32 subjects were included. Out of 32 patients 17 of them had lung cancer (53%) and 1 patient had lung metastasis (3%). All patients had persistent airway obstruction, defined as a post-bronchodilator ratio of less than 70%. FEV1% was used to express lung function where patients' FEV1% varied from 19% to 86% (mean 56%). Therefore, all patients were diagnosed with severe to mild COPD (Karloh *et al*, 2016). 20 cigarettes per day per year was used as a definition of pack years.

3.2 Tissue selection and DNA extraction

USTs were evaluated in leukocytes and BAL (lung tissue including neutrophils, alveolar cells, macrophages) collected from the involved patients. The bronchial washing was carried out with a pre-wash and 20ml of isotonic NaCl in the opposite bronchial tree from the routine procedures. The peripheral blood samples were obtained after the BAL sampling from the same patients. A mesenchymal stem cell line was, because it was previously studied and telomere length is known, was used as an internal control for U-STELA, which were recultured from and existing immortal stock (Serakinci *et al*, 2007). In these cells, TL and existence of USTs were previously shown by our group. From all the patients involved in the study, both the peripheral blood (leukocytes) samples and the BAL samples were collected. The extraction of DNAs were carried out using commercial DNA extraction kits from RTA technologies. The ethical approval for the study was obtained from Near East

University to pursue the study within the university facilities (Ethical Application Ref: YDU-2015/30-202) where all patients were informed about the study and consents were obtained.

3.3 Selection of telomere measurement method

To date, there are several methods available for TL investigation. As above mentioned due to the nature of the telomere biology and complexity of variation among tissues, presents specific problems in TL measurement in research. While all of these methods have advantages and disadvantages over other methods, it is crucial to account that the method of choice in a given study should consider type of tissue, what aspect of telomere shortening is being assessed, what is the medical condition, what molecular aspect is being assessed and which or which combination of telomere measurement methods are optimal. To be able to differentiate between available methods, a literature search was conducted to identify the most suitable method to achieve the aims of this study (Table 1). Here, U-STELA was used to evaluate the presence of USTs. The other methods of TL measurements did not specifically fill the need in this research. One if the main reasons of that USTs were evaluated, is because senescence can be induced in alveolar cells when USTs are present. As COPD is associated with alveolar senescence, it was decided to utilise U-STELA.

Table 1: Comparison of telomere length measuring methods

Method	Required Material/ DNA Amount	Target Telomere Size	Advantages	Disadvantages
Q-FISH	15 to 30 metaphases	Total and/or single Telomere length in a single cell	• Enables characterization of single telomeres in metaphase • Both mean and individual telomere lengths can be assessed	• Viable cells are required for the telomere length assessment • Requires specialised equipment in terms of florescence microscopy • Florescent signal must be translated into length unit
qPCR	10ng	Telomere length to single gene copy ratio	• Faster than other methods thus better suited for population studies. • Can use little amount of DNA	• Variation between research groups and labs • No standardized references • Error prone due to technical difficulties

TRF	1 µg	Mean telomere length in a cell population	• Comparative to previous evidence and other methods • Presents a gold standard in telomere length measurement	• Detection of only mean length on telomeres and does not detect ultra short telomeres • Requires more than 1µg of DNA • Subtelomeric regions may influence telomere length data in different populations
U-STELA	40-100 pg	Ultra short telomere length	• Enables ultra short telomere detection • Efficient in senescence associated disease studies • Well characterized subtelomeric region enables production of reliable data	• Does not detect total telomere loss • Does not detect telomeres longer than 8kb

Table 1: the most frequently used telomere length measurement methods are shown. qPCR and TRF methods provide mean telomere length. Q-FISH provides single cell, single telomere detection. U-STELA provides the detection of USTs

3.4 Universal STELA (U-STELA)

The application of U-STELA differed slightly from the previously described (Bendix *et al,* 2010). Subsequent to the DNA isolation, 1 µg DNA from each sample was digested using restriction endonucleases MseI and NdeI.For the required digestion, 1 µl of MseI and 0.5 µl of NdeI was used with 5 µl cut-smart buffer and water was added to make up to 50 µl reaction. The reactions were incubated at 37^{o}C for 1 hour. To inactivate the reactions, they were incubated at 65^{o}C for 20 minutes. After inactivation, 0.05 µg of digested DNA was added to 3 µl of 12 mer and 42 mer pan-handles to make up 7 µl reactions. Following the addition of panhande sequences, the reaction temperatures were decreased from 65^{o}C to 16^{o}C for 49 minutes. T4 DNA ligase working solutions were prepared to have 20 units in 0.5 µl. 1.5 µl of T4 DNA ligase buffer with the T4 DNA ligase were rapidly added to the reactions followed by addition of 6 µl dH2O at 16oC to make 15 µl reactions. Subsequently the reactions were incubated overnight. After the incubation, 0.5 µl of T4 DNA ligase, 1 µl of T4 DNA ligase buffer, 2.5 µl telorette working solution 6 µl dH2O were added to make 25 reactions. These reactions were then Samples were incubated at 35oC overnight followed 20 minutes incubation at 65oC for inactivation. This was then followed by a PCR reaction containing, 40 pg ligated DNA, 0.1 µM adapter and teltail primers, 6 µl of failsafe master mix and 0.5 µl of the failsafe enzyme with a total volume of 12 µl. PCR conditions were not changed (Bendix *et al,* 2010). The amplicons were run on a 0.8% agarose gel at 70 V for 3 hours for separation. The gel was then subjected to serial washing to prepare for transfer. The separated DNA was transferred to a positively charged nylon membrane (Amersham). DNA fragments in the blot were hybridized to the DIG-labelled telomeric probe

overnight at room temperature and incubated with a DIG-specific antibody with AP fragments. Chemiluminescence was detected with CDP-Star (Roche). All experiments were triplicated. The threshold for the ultra-short telomeres was defined as 1.5 kb as established previously. (Figure 2)

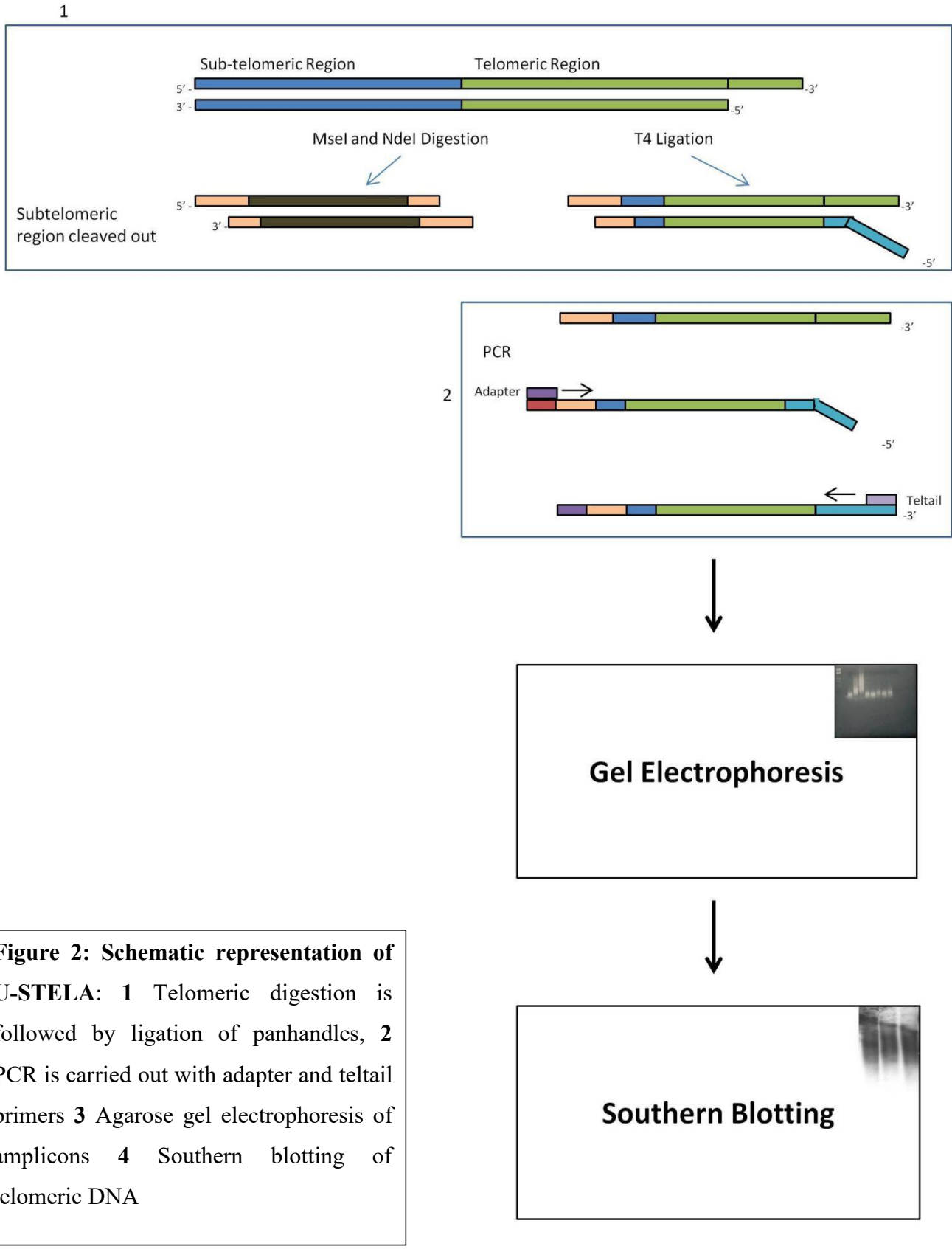

Figure 2: Schematic representation of U-STELA: **1** Telomeric digestion is followed by ligation of panhandles, **2** PCR is carried out with adapter and teltail primers **3** Agarose gel electrophoresis of amplicons **4** Southern blotting of telomeric DNA

3.5 Statistical analysis

All the quantitative variables were represented as arithmetic mean standard deviation for the descriptive statistics purposes. The frequency and percentages were used for categorical data representation. The Fisher Exact Chi-Square test was used for categorical variables in evaluation of the associations of USTs with variables such as age, sex, smoking status, and COPD grades. Furthermore Mann-Whitney U test was used for continuous variables for such associations. p-values ≤ 0.05 were regarded as statistically significant. Multiple linear regression model (entry method) was used to test the whether USTs (both in BAL and leukocytes) are related with lower lung function and are associated with FEV1% adjusted for smoking status, sex and age. . SPSS (IBM SPSS version 23.0) statistical program was used for all statistical analyses.

4 Results

4.1 Ultra-short telomere presence

Overall, USTs were found in 26 BAL samples (81.1%) and only in 3 leukocyte samples(9.3%). 22 participants in this study either a current smoker or formerly smoked (69%) whereas 10 participants never smoked (31%). There were USTs in 18 subjects' samples who are ever smokers (81.8%). Figure 3 represents the UST determination. Gender was not significant factor in UST presence in both BAL and leukocytes. The 13 (76.5%) subjects who had lung cancer had USTs whereas 4 subjects who had lung cancer did not. Similar to these results, in 13 out of 15 subjects who did not had lung cancer, USTs were present (86.7%). when presence of USTs were compared with relation to lung cancer, it was found that the lung cancer incidence is not associated with UST presence (p=0.659).

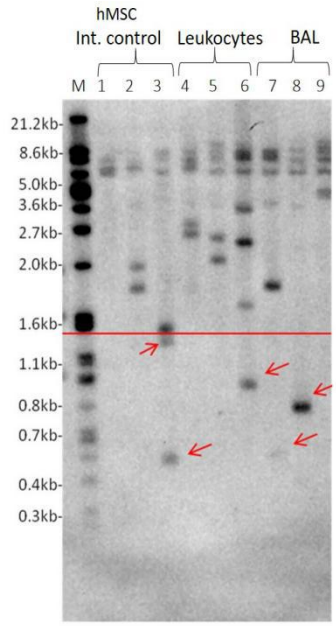

Figure 3: USTELA representation. 1. Marker, 2-4: hMSC internal control at different passages (p X, X, X), 5-7: Leukocyte samples, 8-10: BAL samples. The DNA fragments below the red horizontal line are USTs. Red arrows point to the USTs which were smaller than 1.5kb threshold.

4.2 Lung function correlation with respect to ultra-short telomere presence

FEV1% was lower in ever smoker (current or former) than in never smokers (51.3% versus 67.8%; p=0.04). In addition to this, the FEV1% was higher in subjects without USTs in BAL (81.6% vs 50.6%: p<0.001) when compared with subjects with USTs. Furthermore, when leukocyte samples were compared with regards to USTs presence, subjects with USTs had lower FEV1% (37.3% vs 58.5%: p=0.051) although it was narrowly above statistical significance. Moreover, USTs in BAL samples were positively correlated with increased age (p=0.014) as subjects with USTs in BAL were significantly older. Unlike the leukocyte samples (p=0.33), USTs in BAL samples (p=0.05) were significantly associated with was associated with FEV1% when adjusted for ever smoking, age and sex [(p<0.0001),(p<0.0001), (p=0.71)] in the regressions model generated.

4.3 COPD groups and tissue specificity of ultra short telomeres

Table 2 shows the categorical analysis that was carried out in the participating patients with COPD. USTs presence in the BAL samples were significantly at a higher rate when compared to the leukocyte samples of the same patients $t(62)=5.771$, $p<0.00001$, providing an indication that TL investigation should be carried out in the correct tissue sample. The patients who had UST and who did not were also compared to investigate whether telomere shortening in either in BAL samples or leukocytes were associated with COPD progression. The patients who had USTs were also had higher GOLD classification if the BAL samples are investigated (p=0.006) (Table 3). Conversely, when leukocytes were investigated, the UST presence is not correlated with smoking status, age (71.7), gender and COPD grade (p>0.05).

Table 2 COPD Patients- Categorical analysis

	COPD Patients (n=32)
	Mean ± SD
Age (years)	60.0 ± 10.5
	n (%)
Smoking History	
Smoker	13 (40.1)
Former Smoker or non Smoker	19 (59.9)
Gender	
Male	26 (81.3)
Female	6 (18.7)
Presence of USTs in leukocytes	3 (9.3)
Presence of USTs in BAL	26 (81.3)
COPD Grade	
Grade 1-2	15 (46.9)
Grade 3-4	17 (53,1)

Table 2: USTs were found in 26 BAL and 3 leukocyte samples

Table 3 :The relationship between the presence of ultra-short telomeres and age, smoking history, and COPD grading

	BAL + (n=26)	BAL – (n=6)	p	Leu + (n=3)	Leu – (n=29)	p
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Age (years)	62.6 ± 7.8	48.8 ± 14.2	**0.014***	71.7 ± 1.2	58.8 ± 10.3	NaN
	n (%)	n (%)		n (%)	n (%)	
Smoking History						
Ever Smoker	18 (81.2)	4(11.8)		3 (13.6)	19 (86.4)	
			1.00**			0.534**
Never Smoker	8 (80)	2 (20)		0 (0.0)	10 (100.0)	
COPD Grade						
Grade 1-2	9 (60.0)	6 (40.0)	**0.006 ****	1 (6.7)	14 (93.3)	1.00**
Grade 3-4	17 (100.0)	0 (0.0)		2 (11.8)	15 (88.2)	

Table 3: BAL+= Presence of USTs in BAL samples. BAL-= Absence of USTs in Bal samples. Leu+= Presence of USTs in leukocyte samples. Leu- Absence of USTs in leukocyte samples. *Results were obtained with the Mann-Whitney U test. ** Results were obtained with Fisher Exact test. NaN: No statistics were calculated due to inadequate sample size in one Leu (+) group (n=3).

4.4 Telomere length measurement is not associated with the region of bronchial washing

The difference in BW sites in the lung were investigated to assess if the region of BW. To investigate whether the BW influence the TL measurement in regards to USTs presence. There was not a correlation between BW region and presence of UST ($p>0.05$) (Figure 4). 32 patients had a median age of 63 ranging from 31 to 73 years, total pack-years 25 (20cig/day/year) ranging from 0 to 106 and FEV1% was 47%, ranging from 19% to 86%. 3 quarters of the patients were male. The 3 patients who had UST in the leukocytes also had UST in the BAL samples. 23 patients had USTs only in BAL samples but not in leukocytes (72%). Only 6 patients did not had USTs in either BAL samples or leukocyte samples (19%) because all the telomeres in the samples from these patients were longer than 1.5kbs.

Figure 4: Bronchial tree showing the different locations of the bronchial washing sample (BW) location

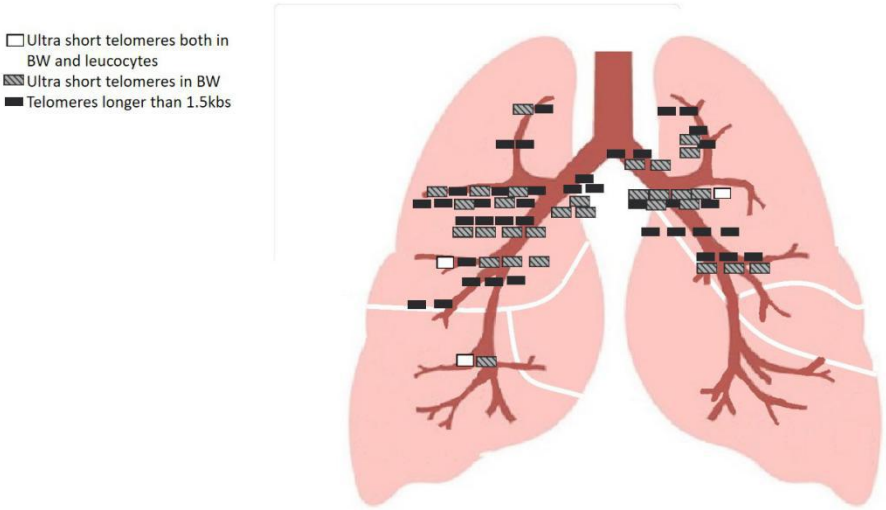

Figure 4: Presence of USTs in both BW and leukocytes (white bars), USTs only in BW (shade bars), and only telomeres longer than 1.5kbs (black bars).

4.5 Evaluation of the lung tissue showed association of ultra-short telomeres with higher pack years and lower lung function

All of the patients in the study were COPD patients with different grades. There were 8 grade 1 patients (25%), 7 grade 2 patients (22%), 14 grade 3 patients (44%) and 3 grade 4 patients (9%). Similar to the UST presence, COPD severity was also significantly associated with old age ($p=0.005$). However, the direct relationship between smoking and COPD grade was not observed as COPD grade and higher pack-years were not significantly associated ($p=0.88$).

When the relationship between total pack-years and UST presence was investigated in BAL samples, it was found that patients with USTs also had higher pack years when compared with patients without USTs (32 to 16; $p=0.045$). On the other hand, the lung function in terms of FEV1% was lower (51% to 82%; $p<0.001$) (figure 5a).

Together with these, despite the association between UST presence in BAL and age is not at a statistical significance ($p=0.06$), USTs were present in older patients (63 to 49 years).

When leukocyte samples were considered, only age was at a statistical significance with relation to UST presence (72 to 59 years; $p<0.001$). Furthermore total pack-years was found to be higher in patients with leukocyte USTs (77 to 24 years; $p=0.058$), and lower FEV1% (37% to 58%; $p=0.12$) but these values were not at a statistical significance (figure 5b).

Figure 5: Distribution of total pack-years, age and lung function with respect to ultra-short telomeres

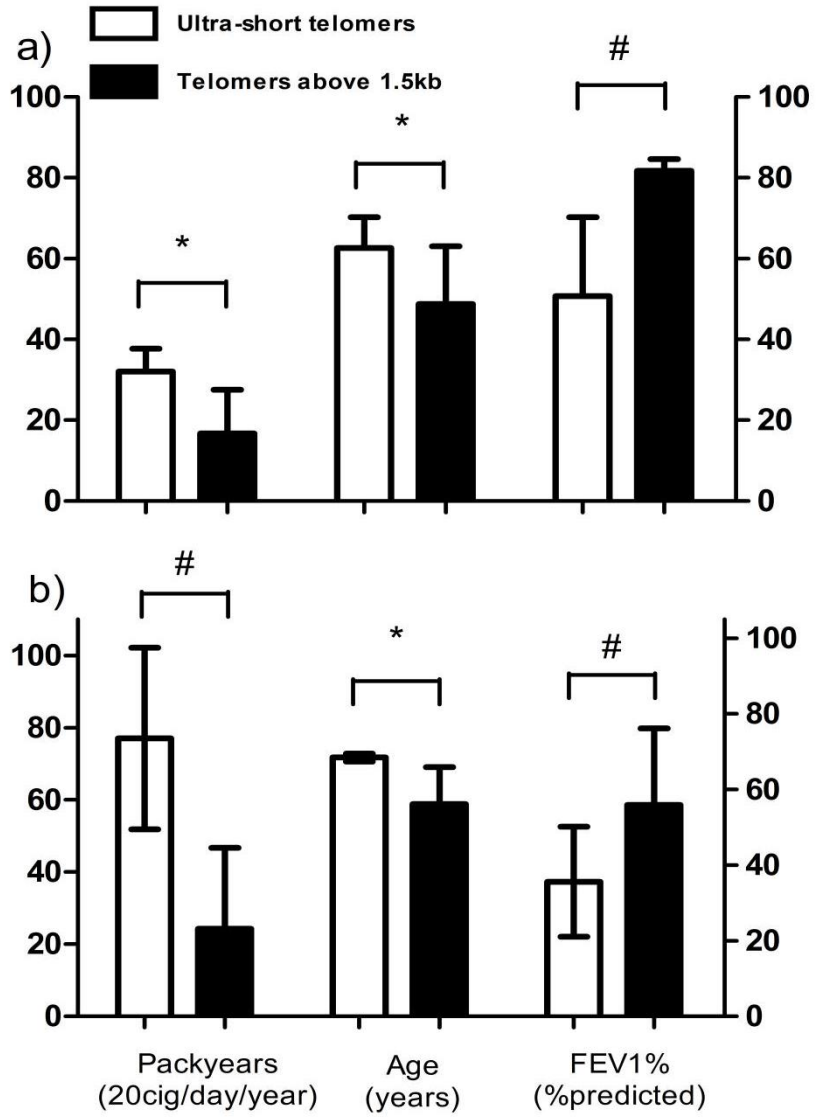

Figure 5: USTs (white bars) and telomeres longer than 1.5kbs (black bars) in bronchial washing samples (a) and leukocyte samples (b). *=p<0.05, #=p>0.05, FEV1%= predicted value of Forced Expiratory Volume in 1 second.

4.6 Lung function, age and total pack years incidence

The patients were grouped into three to compare the lung function, age and total pack-years incidences. These groups were consisting of, patients with USTs; only in BAL samples, both in BAL and leukocyte samples and patients without USTs. age and total pack-years incidences were increased from patients without USTs to patients who had USTs only in BAL samples to patients who had USTs in both BAL and leukocyte samples. Conversely lung function incidence was decreasing from patients without USTs to patients who had USTs only in BAL samples to patients who had USTs in both BAL and leukocyte samples (all $p<0.01$) (Figure 6).

Figure 6: Incidence comparison of total pack-years, age, and lung function

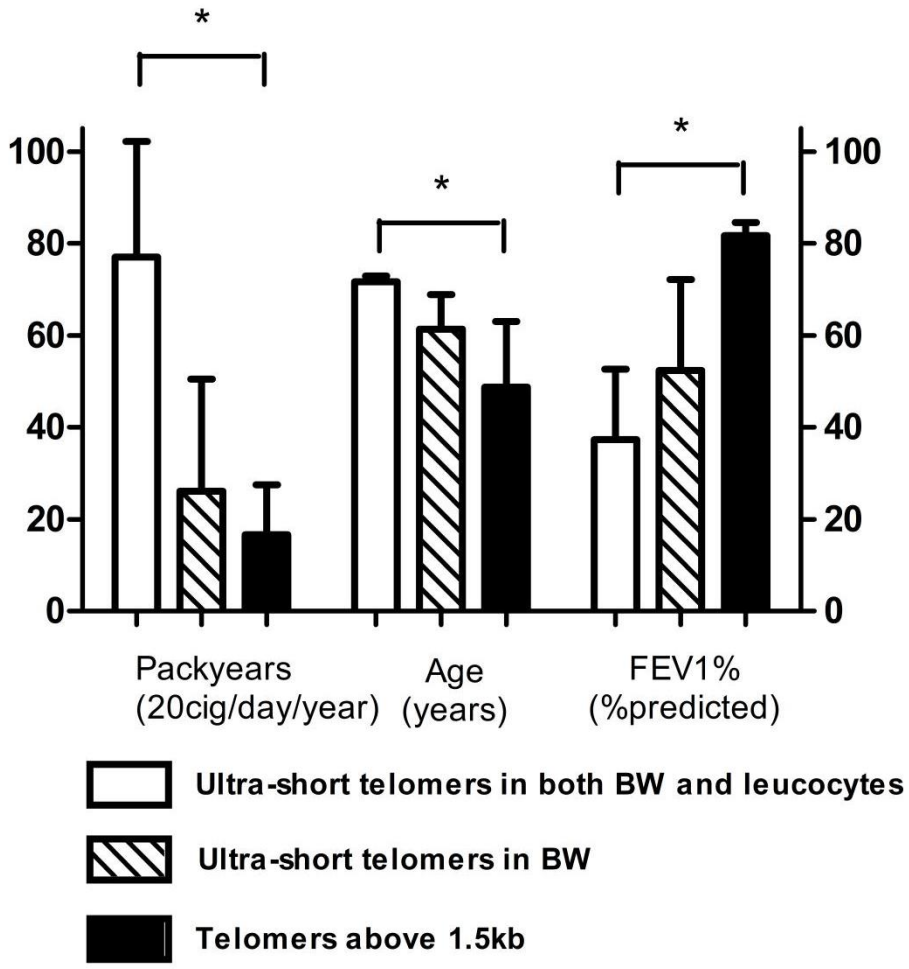

Figure 6: Subjects with USTs compared to subjects with telomeres longer than1.5kbs in both bronchial washing samples and leukocyte samples(white bars); subjects with USTs only in bronchial washing samples (shaded bars) and subjects with telomeres longer than1.5kbs (black bars). *=$p<0.05$, FEV1%= predicted value of Forced Expiratory Volume in 1 second.

5 Discussion

Here it is shown that the involvement of USTs in complex diseases such as COPD should be studied in the affected tissue (BAL) rather than systemic cells in leukocytes, provide insight on the value of tissue specific sampling. Our best knowledge presents this work to be the first one to show a association between UST presence in the lung tissue and COPD further supporting the notion that identification of ideal tissue type instead of utilising leukocytes in evaluation of molecular basis of complex disease is pivotal in order to advance our knowledge. USTs have a great potential to be used as a biomarker for COPD development and prognosis in a easy and cost-effective manner although this area should be researched extensively to fully comprehend the involvement of USTs in the COPD molecular genetics.

TL measurements were often done only in leukocytes (Karloh *et al,* 2016; Lee *et al* 2012; Mui *et al,* 2009). If the main aim of the such studies is to provide ageing related telomere shortening, the use of leukocytes may be satisfactory and adequate. However, here is is evidenced that, in COPD and other diseases that result in higher cellular turnover, tissue specificity might prove to be a more efficient way in determining the importance of USTs in molecular basis of chronic diseases. In COPD the major damage is to the lung tissue, therefore, research which involves alveolar cells from patients diagnosed with COPD should be new frontiers in the field. Here, it is shown that the difference between the affected tissue and the systemic leukocytes might be substantial in relation to telomere shortening. Although telomere shortening is directly proportional with age and and individual's way of life, the cellular stresses that the tissue of interest endure should also be accounted. In

order to fully account and thus, fully comprehend the extend of the COPD biology, the associations between ultra short telomeres and COPD risk factors should also be considered.

Patients diagnosed with COPD are likely to be older as it is a progressively chronic disease. In addition to this, as the age increases, it is expected to have more severe COPD manifestations. Therefore, we support the previous evidence that shows the association between aging and abrupt telomere shortening. Since even a single UST can trigger the cellular arrest, the evaluation of USTs in senescence associated diseases for increasing our knowledge on disease mechanisms, emerges as a better suited approach than investigation of average telomere shortening (Harbo *et al*, 2013). The mean TL decrease might not be at a significant level although the alveolar cell could accumulate ultra short telomeres to trigger senescence. Once the senescence is triggered the repair of the injured lung tissue is impaired (Li e*t al*, 2018). Therefore, here, it is strongly suggested that the evaluation of the USTs might provide the missing link between the telomere biology and COPD manifestations in order to prevent COPD development and if COPD is manifested, reduce the rate of progression.

Moreover, it is suggested here that not only disease development but also disease progression may be influenced by telomere dynamics. The accelerated telomere shortening might be caused by the increased age as it increases cellular aging (Vidacek *et al*, 2010), therefore provide a basis for BAL samples having USTs. On the other hand, lung tissue has slower renewal because of slower response from stem cells to damage than the leukocytes, supporting the absence of USTs in the leukocytes (Yamada *et al*, 2016). The active telomerase in bone marrow progenitor

cells might be another reason for less number of USTs in leukocytes (Roake and Artandi, 2020). In the light of these results, here, it is hypothesized that USTs might also be used as a prognostic marker for patients diagnosed with COPD provided that these patients have frequent bronchoscopy procedures as routine checks and BAL samples are accessed. Furthermore, this is also the first report showing the association between USTs and smoking in lung tissue in patients diagnosed with COPD with respect to total pack-years. BW does not affect the TL measurement method that had been utilised here, eliminating the location bias. In addition to this, the samples from the BAL were taken on the opposite branch of the routine procedure eliminating the involvement of the TL biased, such as tumour, cells.

Here, it is also shown that the higher incidence of pack years and lower lung function and response is USTs which is dose dependent. Therefore, it provides a connection between USTs and persistent airway limitation that depends on total tobacco smoke exposure, together with aging related disease.

Previously, the evidence on smoking and TL relationship were based on leukocytes (Bendix et al, 2010), lung tissue from mice (Savale *et al, 2009).* They were also based on other disease models (Birch *et al*, 2015). In contrast, we do not report any association between TL and smoking and FEV1% in leukocyte samples from the patients diagnosed with COPD in this study. Although there were USTs in leukocyte samples, these patients had USTs in BAL samples as well therefore patients who only had USTs in leukocyte samples were absent in the study group. Conversely, 72% of the patients only had USTs in BAL samples. These results suggest that the cells of lung tissue may be subjected to the smoking induced DNA damage prior to it is inflicted in leukocyte cells generating USTs in lung tissue before USTs are present

in leukocytes. Similar to previous reports, the results presented here indicate that smoking generate DNA damage in lung tissue and abrupt telomere shortening is an evidence of DNA damage in COPD patients. Therefore, it is highly recommended that lung tissue should be the choice of tissue when TL is investigated.

Here we report that patients diagnosed with COPD had shorter TL as the age increased which corroborate the earlier evidence (Walters *et al*, 2014Savale *et al*, 2009; Houben *et al*, 2009). it is expected that telomeres become shorter due to natural or accelerated aging. The accelerated aging may be considered as a risk factor for COPD. In addition, cellular aging is enhanced by other COPD risk factors which leads to shorter TL. The results presented here provide a exemplary representation since the subjects involved in the study has a The wide range of age. Moreover, the inclusion of middle aged patients diagnosed with COPD showed that the USTs are not only found in the elderly.

FEV1% was found to be moderately associated with leukocyte TL previously in a large cohort (Rode *et al,* 2013). these results have been replicated in other pulmonary diseases(Albrecht e*t al*, 2014) despite TL was more strongly associated with FEV1% in patients diagnosed with COPD. Here we present that USTs are strongly associated with FEV1% in the lung tissue despite the sample size smaller when compared to the previously discussed evidence. The reason behind our results can be credited to COPD patients have lung tissue as primary injury sites therefore increasing the cell turnover, then in turn increase the USTs presence.

One of the methods used to measure the TL is the qFISH. PNA-FISH is a modified version of qFISH and used as a method to measure average TL using metaphase cells and fluorescently labelled PNA probes $(CCCTAA)_3$. PNA probes specifically

hybridize to denatured telomeric repeats. The TL is measured by the detection of relative fluorescence to a previously known TL. PNA-FISH can be used to measure TL in a single or all chromosomes in a human diploid cell. Most commonly it is used in scarcely available cells as 15 to 30 metaphase cells are sufficient to obtain reliable TL data. PNA-FISH is also a hybridization-based method and can detect telomeres shorter than 0.5 kb and chromosome fusions (Krejci *et al*, 1998). However, PNA-FISH can only be used to measure TL in mitotically active cells but cannot be used to measure TL in cells that are not actively dividing. This technique is also labour and technically intensive. Interphase, PNA-FISH is a modified version of this method that overcomes the problem with non-dividing cells.

On the other hand, Interphase, PNA-FISH can not differentiate between specific chromosomes and thus belonging telomeres and does not detect telomeres shorter than 0.5 kb (Montpetit *et al,* 2014). This technique is well established and used in primary hematopoietic cells (Wand e*t al,* 2016) bone marrow-derived cells (Hwang *et al*, 2016), leukocytes (Squassina *et al*, 2016) and cancer cells (Kammori *et al*, 2015). showing that PNA-FISH can be a viable method for different tissues and a broad range diseases from breast cancer to physiological disorders.

qPCR is a version of PCR where amplification of DNA is monitored real time. qPCR can be used to measure TL (Cawthon, 2002). Telomeres cannot be amplified using standard PCR primers because of the repeating sequence. The complementary primers would form primer dimers. This problem was overcome by a combination of the use of complementary primers to the G and C rich telomeric regions that are not complementary to other telomeric regions and lower annealing temperatures in the

first two cycles of qPCR. The amplifications are done in higher temperatures after the initial annealing of primers to the telomeric DNA.

However, the technicality of qPCR in telomere measurement and the irrelativeness in the TL measurement brings several limitations. The comparison of amplified telomere to the reference gene in two different test tubes lowers the qPCR reliability/sensitivity in TL measurements (Montpetit *et al,* 2014). Although there have been several modifications to the original method, TL measurement with qPCR still has reproducibility and reliability problems (Gadalla *et al*, 2016; Aviv *et al*, 2011; Cawthon, 2009). The main advantage of the qPCR methods in TL measurements is that the amount of DNA requirement is extremely low when compared with TRF. However, in measuring cultured hMSC TL, because of the availability of large quantities of DNA, qPCR methods would not be optimal. qPCR has also been used in various cells such as leukocytes, (Cordoba-Lanus *et al*, 2017; Gadalla e*t al*, 2016), bone marrow (Panero e*t al*, 2015), tumour tissue (Ma *et al,* 2017) and adipose tissue (Yamada *et al,* 2015) although leukocyte TL is used to derive disease risk or relationship affecting other tissues may not be informative in some cases (Gadalla e*t al*, 2016).

TRF assay is used to measure the mean TL. TRF analysis consists of a slightly modified Southern blot for telomere-containing genomic DNA to establish the TLs in a cell mass (Oullette *et al*, 2000; Harley *et al*, 1990). In a TRF assay, isolated genomic DNA is digested with a combination of frequent cutter restriction endonucleases that digest the DNA to the last available restriction site before the beginning of the telomere. This DNA is run on a gel and hybridized to a telomere specific probe. The resulting Southern blot will display a signal distribution that

represents the distribution of terminal restriction fragments that contain telomeric sequences as well as any undigested adjacent subtelomeric DNA (Mender and Shay, 2015).

Although TRF assay is the gold standard for telomere total length measurements it is not exempt from limitations. The subtelomeric DNA may result in the overestimation of TLs which can skew the results as opposed to other methods. However, the main pitfall of TRF assay is that it only measures the average TL which does not fully explain several molecular mechanisms including senescence. The average TL has a nearly linear decrease after subsequent cell divisions (Counter e*t al*, 1992). In contrast, the senescent cells are mostly seen in very late passages in cell culture (Smith and Whitney, 1980). It has been implicated that a single or few numbers of USTs in particular cells can trigger DNA damage response and subsequently induce senescence while the average TL does not indicate senescence induction (Abdallah e*t al*. 2009). Therefore TRF is not always the best choice in telomere-associated senescence measures. On the other hand, the average TL measurement by TRF is strongly correlated with age (Behrens *et al*, 2017) implicating that TRF is a suitable method for the detection of aging-related telomere shortening in bone marrow (Ma *et al*, 2015) and leukocyte samples (Ren *et al*, 2009).

STELA is a method that is designed to eclipse TRF and qPCR in abrupt telomere shortening detection and the presence of USTs. This method uses specific primers for single subtelomeric sequences and aims to amplify these telomeric regions (Baird e*t al*, 2003). However, only XpYp, 2p, 11q, 12q, and 17p subtelomeric sequences are amplifiable with this method (Britt-Compton *et al*, 2006. Therefore, the modified STELA, which is U-STELA was later developed.

Universal STELA (U-STELA) was developed to overcome several pitfalls of the STELA[31]. U-STELA provides a method to detect all USTs independent of chromosomal location. The novel approach in U-STELA is to anneal a linker or telorette to the G rich 3' overhang of the telomeric sequence that is a product of restriction digestion after DNA isolation The U-STELA uses two ligation steps where firstly, a double-stranded specifically designed oligo is annealed and ligated to the subtelomeric region. Then, the telorette is annealed and ligated to the complementary C rich 5' end, generating telorette tail which is non-complementary to the 3' overhang. Telorette tail generates a annealing site for the specific primer used in U-STELA called teltail. The subtelomeric region that the primers are annealed can be accounted in the TL measurement hence providing an advantage over TRF assay. U-STELA uses very little amount of DNA, thus it is suitable to be used for TL measurement in cells with very low DNA yield. Also, it is the choice of method in measuring the length of USTs (Bendix e*t al,* 2010).

On the other hand, U-STELA cannot measure TLs of more than 8 kb. It is also very sensitive to template DNA amount used since more than 100pg of DNA would result in smears forming due to PCR artefacts. Similar to other methods, U-STELA has also been used in studies with a broad range of cells including placental cells (Garcia-Martin *et al*, 2017) epithelial cells (Friis-Ottosen e*t al*, 2014), cartilage (Harbo *et al*, 2013), human mesenchymal stem cells (Harbo e*t al*, 2012) and fibroblasts (Bendix e*t al*, 2010) making U-STELA a very powerful tool in measuring TL in specific tissues that are affected with a particular inherited disease. Overall our results further support that U-STELA may be the most suitable choice of available methods to measure USTs in lung tissues due to discussed characteristics.

Generally, TRF is used for the telomere shortening measurement caused by replication. Other methods that have been discussed here are also detecting mean TLs. Exceptionally, a Universal single TL assay (STELA) can measure telomere shortening that can be caused by cellular stress, which contributes to senescence. Telomere shortening caused by oxidative damage, generates only a few critically short telomeres. Universal STELA, allows visualizing these USTs which are an advantage for increasing our understanding of the molecular pathogenesis of many age-related diseases.

The presence of a few USTs, can lead telomeric uncapping because of distal erosion. Telomere shortening has been associated with generation of anaphase bridges as a result of sister chromatid fusions (SCF). Additionally, chromosomal instability can be initiated through SCF when USTs are present (Soler *et al,* 2005). Furthermore, genetic instability can be triggered only by a single telomere loss loss of a single telomere may result in genetic instability, involving chromosomes with or without telomeres by fusion break fusion (F-B-F) cycles (Sabatier *et al*, 2005). Thus, our group's previous findings are further supporting the occurrence of F-B-F cycles in the irradiated hMSC-telo1 cell line in which we have shown that right after IR, the telomere profile is lost. Moreover, investigation of USTs in senescence-associated diseases is extremely important in increasing our knowledge on disease mechanisms as only a single UST can trigger entry into senescence instead of lower mean telomere length (Harbo *et al*, 2013).

Alveolar senescence, a marker of the severity in chronic diseases, is shown to be increased in patients with emphysema (Tsuji e*t al*, 2006). Senescent cells contribute to inflammation in the lung tissue similar to oxidative stress. This in turn contributes

to inflammation and senescence (Kanaji *et al*, 2014) in the lung tissue and finally, inflammation further induces oxidative stress and senescence because of inflammatory cell activities. Altogether, telomere shortening in the lung tissue is expedited through oxidative stress, apoptosis and inflammation because of high cell turnover lung function is reduced because of USTs causing senescence. Previously USTs were shown to be caused by stress-induced telomere shortening that lead to chondrocyte senescence in human osteoarthritic knees (Harbo e*t al*, 2013), thus measuring short telomeres in defective organs instead of mean TL is more informative. Furthermore, in light of the above-mentioned discussion, we suggest that U-STELA is the most informative approach to date to develop further understanding of disease pathophysiology or other telomere shortening related chronic diseases such as COPD.

There are also limitations in this thesis. While 80% of the patients had USTs in BAL samples and involved patients represent a wide range of FEV1% (19-86%), there is a lack of control group who are not smokers and do not have airway obstruction. Inclusion of such a control group would have been exemplary, however during the sampling period, bronchoscopy eligible patients who fit in with our inclusion criteria were not identified. As bronchoscopy is a highly invasive procedure and resorted according to the patients' need for such a procedure, only COPD patients who were eligible for bronchoscopy were included.

Previously it is shown that COPD patients encounter increased macrophage counts (Vlahos and Bozinovksi, 2014). Moreover, it is reported that the COPD grade is in a direct relation with macrophage counts. Therefore, it would have been strengthen our

results if the BAL cells were sorted and USTs were quantified. Despite these discussed limitations, there are significant associations shown in this study.

Although the COPD development is highly influenced by smoking, we failed to show any direct tissue relationship in any of the COPD grades. On the other hand, there was a direct tissue relationship between COPD grades and lung function. There could be several reasons behind these results including the size of the sample and absence of exacerbation registration. Therefore, we suggest that the involvement of USTs in the development of COPD and as a potential biomarker for progression should be studied with a larger group.

Secondary risk factors including air pollution, lifestyle, and occupation should also be considered. These risk factors should not be overlooked as there was no data collected in this study and may influence the results. Due to the cross-sectional approach taken in this thesis, longitudinal effects between TL, total pack-years, and lung function cannot be studied.

6 Conclusions

The presented results suggest that USTs are involved in the molecular pathogenesis of COPD. USTs might be used as a tissue-specific predictive biomarker for COPD. Furthermore, this study emphasizes the need to investigate the correct specific tissue to get a representative evaluation of the stage or advancedness of a given disease, such as COPD. The USTs are associated with increased pack-years, age, and decreased lung function in patients diagnosed with COPD irrespective of BW location. Pack-years is an important variable in telomere shortening and thus lower lung function in COPD. The effects of smoking can be determined at an earlier stage if the telomere evaluation is carried out in the lung tissue.

Often TL measurements were done in leukocytes. This approach is mostly acceptable and sufficient to provide base aging-related telomere attrition. However, in the case of diseases with high cell turn over such as COPD, tissue and cell-specific methods and evaluation of USTs may be more effective in the characterization of the molecular basis of such diseases. Thus, here, it is suggest that the U-STELA approach is to be preferentially considered as the method of choice when measuring the length of telomeres in particular senescence-associated diseases.

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8. Curriculum Vitae

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High School	Turkish Maarif College	2007

Job Experience	**Institution**	**Time (Year-Year)**
Research Asistant	Near East University	2013-2020

Foreign Languages	**Reading**	**Speaking**	**Writing**
English	Very Good	Very Good	Very Good
French	Intermediate	Beginner	Beginner

Computer Programs	Level
Microsoft Word	Very Good
Microsoft Powerpoint	Very Good
Microsoft Excell	Very Good
SBSS	Good
Adobe Acrobat	Good

Scientific Publications

1. Serakinci N, Cagsin H, Mavis M. Use of U-STELA for accurate measurement of extremely short telomeres. Stem Cells and Aging, 2018. Humana Press.
2. Pirzada RH, Orun O, Erzik C, Cagsin H, Serakinci N. Role of TRF2 and TPP1 regulation in idiopathic recurrent pregnancy loss. Int J Biol Macromol, 2019. 15;127:306-310
3. Serakinci N & Cagsin H. Turning stem cells homing potential into cancer specific drug delivery machines. Ann Transl Med, 2019. Suppl 3:S148
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