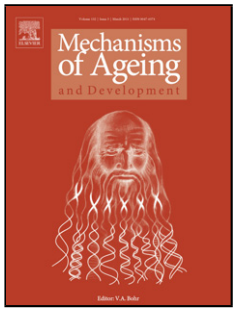

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Long-term tobacco exposure and immunosenescence: paradoxical effects on T-cells telomere length and telomerase activity

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Highlights

- It is known that telomere length shortening is associated with replicative senescence and can be counteracted by telomerase activity; however, up regulation of the latter can also favour cell immortalization.
- Current smokers without COPD, with a lifespan tobacco exposure had telomerase up regulation and attenuation of telomere shortening.
- If causal, the positive effects of tobacco exposure might be mitigated by the chronic pulmonary inflammatory process.

Abstract

Immunosenescence are alterations on immune system that occurs throughout an individual life. The main characteristic of this process is replicative senescence, evaluated by telomere shortening. Several factors implicate on telomere shortening, such as smoking. In this study, we evaluated the influence of smoking and Chronic Obstructive Pulmonary Disease (COPD) on cytokines, telomere length and telomerase activity.

Blood samples were collected from subjects aged over 60 years old: Healthy (never smokers), Smokers (smoking for over 30 years) and COPDs (ex-smokers for ≥15 years). A young group was included as control. PBMCs were cultured for assessment of telomerase activity using RT-PCR, and cytokines secretion flow cytometry. CD4+ and CD8+ purified lymphocytes were used to assess telomere length using FlowFISH.

We observed that COPD patients have accelerated telomere shortening. Paradoxically, smokers without lung damage showed preserved telomere length, suggesting that tobacco smoking may affect regulatory mechanisms, such as telomerase. Telomerase activity showed diminished activity in COPDs, while Smokers showed increased activity compared to COPDs and Healthy groups.

Extracellular environment reflected this unbalance, indicated by an anti-inflammatory profile in Smokers, while COPDs showed an inflammatory prone profile. Further studies focusing on telomeric maintenance may unveil mechanisms that are associated with cancer under long-term smoking.

Introduction

Cellular senescence is a natural aging process marked by the cessation of cell cycle, in which proliferating cells become resistant to growth and present deficient signalling in response to DNA damages. Senescence occurs throughout the cell lifespan and is a key point from organism development to wound healing (1). An important aspect of senescence is cell maintenance since a narrow limit of proliferation is known after a defined number of divisions. This event is called replicative senescence, with the telomeres as its major components. Telomeres are DNA-protein complexes which protect the end of chromosomes during the cell division process. They are non-coding repeats of "TTAGGG" that preserve chromosomes from degradation, fusion or atypical recombination (2). During the cell division, telomeres are not fully replicated due to the

DNA polymerase inefficiency in completing the replication of the extremities from linear molecules, which leads to the shortening of these structures (3). To mitigate the telomere shortening, telomerase adds terminal repeats to the remaining telomeres. Telomerase is a ribonucleoprotein enzyme associated with the immortalization of cells through the maintenance or elongation of telomeres. This enzyme is usually inactive in most somatic cells but is highly detected in tumor cells (4, 5). Therefore, effective estimation of telomerase activity would provide valuable data for better clinical evaluation and deeper understanding of the biological importance of this enzyme.

In normal cells, the activity of the telomerase enzyme is regulated during the development of life, but it ceases during embryological differentiation in somatic cells. However, in some tissues such as germ cells, activated lymphocytes, and some populations of stem cells, this activity is sustained (6). The fundamental understanding of the role of telomeres and telomerase in aging and disease indicates that relatively small and subtle metabolic, environmental, or systemic changes that affect the abundance or function of telomerase can influence the telomere length and, consequently, increases the risk of disease (7). The deregulation of telomerase functioning results in accelerated shortening of telomeres and proliferative deficiencies. In the presence of low levels of telomerase cells enter the non-proliferative state called replicative senescence, establishing a system in which not all telomeres are stretched during a given cell cycle, even when telomerase is normally expressed (8, 9). This anti-tumorigenic mechanism to prevent uncontrolled cell division fails if a rare cell escapes senescence and aberrantly resumes telomerase expression to restore telomere length maintenance, leading to "replicative immortality," a hallmark of cancer (10, 11).

Several factors are implicated to the acceleration of telomere shortening, such as the exposure to tobacco. However, there is still no consensus in the literature on the effect of smoking on telomere length, with both association(12-21) and no association (22-28) being reported in previous studies. Although a small proportion of smokers do not develop respiratory diseases (29, 30), it has been hypothesized that shorter telomeres may contribute to susceptibility to the harmful effects of cigarettes (31) . Nonetheless, it has been shown the involvement of the increased expression of the telomerase enzyme, responsible for telomeric maintenance, in lung cancer. As the disease is primarily caused by tobacco damage, it is suggested that substances in cigarettes can reactivate the telomerase. The mechanisms involved in telomerase reactivation are still not well

understood and may be due to epigenetic processes, such as methylation (32). Moreover, long-term smoking damages cells of the innate immune system and inhibits the production of immune molecules, favoring the growth and colonization of pathogens. Tobacco suppresses the phagocytosis of bacteria and apoptotic cells and also inhibits the maturation of dendritic cells with decreased expression of IFN-α (33). In addition, it causes a reduction in the cytotoxicity of NK cells with a decrease in IFN-γ and TNF-α (34). In general, cigarettes can suppress immune activation by decreasing the expression and secretion of effector cytokines in peripheral blood (35).

COPD is classically characterized as an inflammatory disease mainly induced by exposure to respiratory agents, as cigarette smoke. On healthy people, immune response for an inflammatory process aims to prevent lung injuries, but on COPD patients there is an abnormal immune response which causes a lung remodeling and an exacerbated inflammatory response, named inflammaging. Innate and adaptative immunity are involved in the chronic inflammation of COPD, but the exact mechanisms of it are still being discussed. Literature information agrees of immunosenescence and inflammaging are driving mechanisms of disease development (36, 37). Besides that, negative correlation has been described between inflammation and telomere length (38). Moreover, in COPD patients the telomere shortening description is commonly found in literature (15, 39).

In the present study, we evaluated the influence of smoking and Chronic Obstructive Pulmonary Disease (COPD) on cytokine secretion, telomere length and telomerase activity, which can assist in the investigation and establishment of mechanisms involved in the process of cell senescence.

2 Material and Methods

2.1 Study population and ethics

A total of 96 individuals (both man and woman), distributed in four groups were evaluated: COPD patients (COPD group, n = 24), smokers without evidence of lung disease (Smokers group, n = 26), healthy aged subjects (Healthy group, n=25), and young subjects (Youngs, n = 21). This sample size was estimated from our previous works on TL of aged individuals (40-42). COPD individuals were recruited from the Ambulatório de Doenças Obstrutivas do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo. All COPD subjects were, ex-smokers, who had smoked for at least 15 years (34.2 ± 13.5 years of smoking) and have quitted smoking for at least 12

months prior admission to the study, were over 60 years old, with BMI <35kg/m², and classified by the Global Obstructive Lung Disease consensus (GOLD) as level 2 to 4 (43). Sampling was done after at least 30 days with stable disease, i.e., with neither secondary respiratory infections nor new symptoms or treatment change. Smokers were recruited from candidates to smoking cessation groups. They were all current smokers, with the following inclusion criteria: age over 60 years old, BMI <35kg/m², and absence of pulmonary disease confirmed through clinical history, symptoms and spirometry examination. Healthy subjects were aged over 60 years old, with BMI <35kg/m², who have never smoked (defined as a patient who had smoked less than 80 cigarettes in life). They were recruited from an association of almost 300 older adults of both genders and different origins and professions, some of them still actively working, who volunteered to spend 6 h/week at our institution to help in the assistance of patients. Both the healthy aged, Smokers groups and Young described themselves as healthy and eventually having non-severe comorbidities under medical control, such as hypertension, non-insulin dependent diabetes, arrhythmia, depression/anxiety, etc., common for their age (Supplementary Table 1). The young control group was recruited from the laboratory staff, including men and women aged between 18-30 years old who have never smoked, with BMI <35kg/m². This group was designed to verify the net effect of aging in all three aged groups, irrespective of they being healthy, smokers or COPD.

The major exclusion criteria for all four groups were absence of cancer/chemotherapy, chronic diseases out of control, auto-immune diseases, insulin-dependent diabetes, or use of antibiotics, sustained non-hormonal anti-inflammatory therapy, corticosteroids or other immunomodulatory drugs. Baseline information regarding anthropometric and demographic data, tobacco exposure, and medical history was collected from the hospital's medical records and questionnaires (Supplementary Table 1). All individuals agreed to the research by signing the written consent form. This investigation was approved by the Ethics Committee from the Hospital das Clínicas da Faculdade de Medicina da USP (#4.207.522).

2.2 Blood collection and sample procedures

Peripheral blood mononuclear cells (PBMCs) were obtained from blood samples (50 mL) and isolated by Ficoll-Hypaque gradient separation, then cryopreserved. Cells were defrosted and incubated overnight in RPMI/10% normal human AB serum (SAB)

to recover homeostasis. Trypan blue staining was used to assess cell viability and only cell suspensions with over 90% viability were used.

2.3 Telomere length determination using FlowFish

CD4+ and CD8+ T cell subsets were negatively selected from PBMCs using magnetic microbeads from MACS Cell Separation Reagents (MiltenyiBiotec, Germany) according to the manufacturer's instructions. Telomere length was analyzed in these subpopulations using the Telomere peptide nucleic acid (PNA) kit/FITC (Dako, CO, UK) according to the manufacturer's instructions and as previously described (44). Cell line 1301 was used as telomere length control lineage. Briefly, the DNA of one million cells (of both subject and control cells) was denatured for 10 min at 82 °C in hybridization solution with or without a FITC-conjugated PNA telomere probe and then hybridized overnight in the dark at room temperature. Hybridization was followed by two 10-min washes with a Wash Solution at 40 °C. Then, the samples were suspended in DNA staining solution to identify cells in G0/1 phase. Data were acquired using a FACSFortessa flow cytometer (BD Biosciences, CA, USA) by the acquisition of $1x10^5$ cells. Cells without and with PNA probes were acquired to calculate mean fluorescence intensity (MFI). Data were analyzed using FlowJo software, version 10.0 (BD Biosciences, CA, USA). Relative telomere length (RTL) was calculated as the ratio of the telomere signals (FITC fluorescence) of the sample and the control 1301 cell line, with correction for the DNA index of G0/1 cells and converted to kilobase pairs using the telomere length of the 1301 cell line (23,480 bp) as reference (44).

2.4 Relative telomerase activity of PBMCs

The protocol of telomerase activity was performed on total PBMC cells as described by Pinto et al., 2020 (45). Briefly, PBMCs were defrosted and maintained in RPMI with SAB for 24 hours to recover homeostasis. Then, aliquots of 2 million cells were incubated for 72 hours in two different conditions: unstimulated and PHA-stimulated. After this period cells were harvested, centrifuged, and lysed with a CHAPS buffer at -80°C.

We used Luna SYBRGreen 1X (New England Biolabs, Massachusetts), 0.25µM of each primer: TS (5'-AATCCGTCGAGCAGAGTT-3') and ACX (5'-GCGCGG(CTTACC)3CTAACC-3') (5, 46) in a 25µL final reaction. Standard total

protein concentration (0.15 mg/mL of protein) was used as input to test the cell lysates (n=32), while the number of cells was used as input (starting at 10^6 cells) to produce the standard curve (HEK cells). The cycle conditions were one cycle at 95°C for 60 s followed by 40 cycles of 15 s at 95°C and 30 s at 60°C and additional melting curve cycle. The reaction was performed in an Applied Biosystems 7500 Real-Time PCR (Applied Biosystems, California). Each plate contained tested samples, negative and positive controls, and standard curve.

In this assay, the numbers are not absolute, but relative values: the PCR signal of patients' samples are compared with a HEK curve (10^6cells to 10^2cells). Our assay is normalized by subtracting unstimulated values from stimulated values, being these two conditions carried out simultaneously in the same plate, thus under identical conditions. Some experiments carried out to evaluate the reproducibility of the test showed less than 10% variation of the results (data not shown).

2.5 Cytokine quantification

Secretion of tumor necrosis factor (TNF), interferon-gamma (IFN-γ), IL-10, IL-6, IL-2 and, IL-4 was quantified on PHA-stimulated supernatants from PBMCs cultures using the Cytometric Bead Array Kit (BD Biosciences, CA, USA). Samples were analyzed by flow cytometry (LSRFortessa, BD Biosciences, CA, USA). The detection limits in the supernatant assays were (pg/mL) as follows: TNF = 3.8; IFN-γ ≥ 3.7; IL-10 ≥ 4.5; IL-6 ≥2.4; IL-4 ≥ 4.9; and IL-2 ≥ 2.6.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). D'Agostino & Pearson omnibus normality test, and Shapiro-Wilk normality test were used to determine parametric and nonparametric data. Kruskal-Wallis analysis with Dunn's post-test was used to compare non-parametric and the ANOVA with Dunnett's post-test was used to compare parametric data. Information of the test used for each variable was included in the Figure subtitles. Chi-square test was used to compare the percentage of comorbidities. The Spearman test was used for correlation studies. Statistical significance was set at $p < 0.05$.

3 Results and discussion

3.1 Telomere length

As expected, CD4+ and CD8+ T cells from aged individuals, either healthy donors or COPD patients presented shortened telomeres compared with the young group (Figure 1). However, surprisingly, (a) the TL from both COPDs and aged healthy donors showed no statistical difference, and (b) aged smokers showed, in fact, longer telomere lengths than both COPD and healthy aged groups, particularly of the CD8+ cell subset ($p < 0.01$; for CD4+ cells there was only a not statistically significant trend).

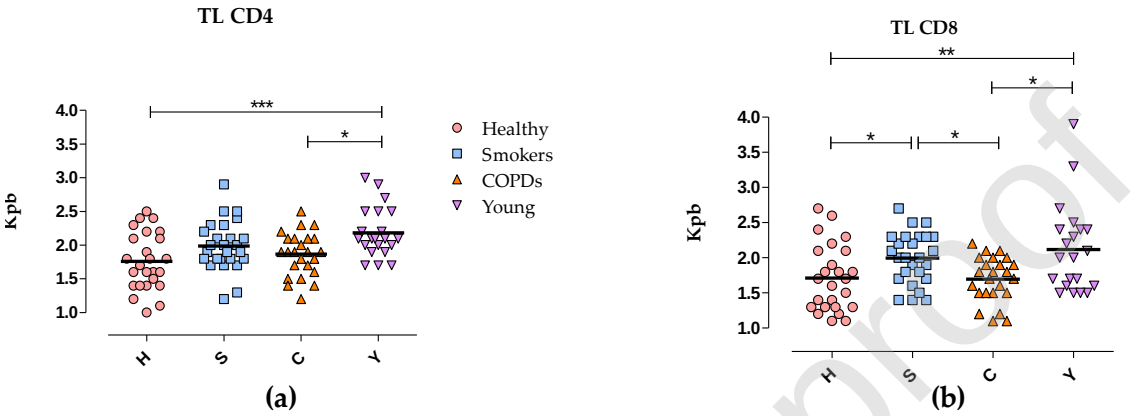

Figure 1: Comparison of telomere length (TL) in CD8+ and CD4+ T lymphocytes from the Healthy (n=25), Smokers (n=26), Chronic Obstructive Pulmonary Disease (n=24) and Young (n=21) groups. **(a)** CD4+ T lymphocyte population in the Youngs showed greater TL than the Healthy and COPD, but it was observed similar TL between Youngs and Smokers group (ANOVA with Dunnett's post test). **(b)** Differences in TL were more pronounced in CD8+ T lymphocytes: Young group also showed greater telomere length than COPD and Healthy individuals, but not when compared to Smokers. CD8+ T lymphocytes from Smokers also showed greater telomere length compared with COPD patients and Healthy individuals (Kruskal-Wallis test with Dunn's post test). Each symbol point represents an individual and the horizontal line the group medians. * < 0.05; ** $p <$ 0.01; *** $p < 0.001$.

Patients with COPD are known to have a persistent inflammatory background due to the pulmonary airway architectural destruction, continuous cell activation and repeated infections. In these patients, T lymphocytes are attracted to the inflammatory sites in the lungs and subject to multiple rounds of activation, thereby releasing proinflammatory mediators (47) and perpetuating the gradual process of lung tissue destruction (48). These activated T lymphocytes transit between the inflammatory foci (lungs) and raining lymph nodes, through the lymphatics but also reach the peripheral blood. There is evidence that CD8+ predominates over the other T cell types in the COPD lung infiltrates and have enhanced functional capabilities, playing a major role in the pathogenesis of the lung disease (49). This inflammatory background and repeated T cell activation leading to

several rounds of proliferation would result in accelerated telomere length (TL) shortening, especially of the CD8+ cells. In fact, Wan et al. (50) analyzed COPD cohorts and found a correlation between both the number and severity of the exacerbation episodes and the telomere shortening. Furthermore, Rutten et al. (51) reported a direct correlation between telomere shortening and the impairment in pulmonary function tests. However, there are scarce studies comparing the TL in blood cells of COPD patients with control groups. Cordoba et al. (52) showed that PBMCs from COPD patients had shorter telomeres than smokers although we observed such behaviour only in the CD8+ lymphocytes subpopulation. Rode et al. (53) evaluated a large cohort and found that shorter telomere length was associated with decreased lung function and with increased risk of COPD, although these associations were markedly attenuated after adjustment for age and other confounders. Their hypothesis to explain the shorter telomere length in COPD as compared with healthy smokers is that the subjects more susceptible to the effects of smoking would have a genetic predisposition to decreased TA and shorter TL: for the same cumulative exposure to smoking, subjects who have developed COPD would have genetically-driven lower telomerase activity and, consequently, shorter telomeres than healthy smokers. On the other hand, a possible mechanism for the lack of difference on TL between COPD and Healthy is the use of statin. According to Boccardi et al. (54), the use of statins can reduce the rate of telomere shortening. Even though there was no significant difference in the percentage of dyslipidemia between the groups ($p = 0.052$), COPD patients had a higher frequency of the disease and higher period of medication use (Supplementary Figure 1 and data not shown).

We also expected to find shortened TL in smokers. A meta-analysis study from 200 retrieved 84 reports on TL and smoking, most of them using leukocytes as the study object. The majority of reviewed studies reported either no correlation or TL shortening in tobacco exposed individuals compared with non-exposed individuals (55). A single study showed a trend to higher peripheral blood leukocytes telomere length in smokers compared to non-smokers in a population with a wide age range window (20 to 80 years old), although there was also a trend for increased decline in TL in the smokers (56). However, more recently, a study of twins discordant for smoking habit showed significantly higher RTA and TL values in the smokers, as well as lower DNA methylation at CpG sites of their catalytic subunit (*hTERT*), suggesting a possible functional implication of the smoke- related epigenetic modification of TA (57). Another factor possibly further

contributing to the longer TL in our cohort of smokers is the more frequent use of statin in this group due to the higher percentage of dyslipidemia (42%), as also observed in COPD patients (Supplementary Figure 1 and data not shown).

On the other hand, the large variability in the results observed in the literature may be ascribed, at least in part, to differences in the cell populations analyzed (whole blood, total leukocytes, PBMC, etc.) and methods employed. Analyses of pools of different types of cells have the inconvenient that each cell type may exhibit a different proliferative flow and peculiar telomere shortening dynamics, which may remain uncovered in cell pool analyses. E.g., B lymphocytes have longer TL and higher telomerase activity than T lymphocytes (58, 59). Our study analyzed highly purified (>90% purity) subpopulations and telomere length was obtained by Flow-FISH. Gutierrez-Rodrigues et al. (60) compared several methods for TL assessment, such as Flow Fish, qPCR and telomere restriction fragment (TRF) by Southern blot. TRF and Flow-FISH showed a good agreement but the Flow-FISH was more precise and reproducible, with higher specificity and at least equal sensitivity, compared with the qPCR. Flow-FISH was described as the most extensive quantitative reference data available and the first telomere length measurement validated for clinical diagnosis (61). When compared to PCR, FlowFISH had a superior reproducibility with an intra and interassay coefficient of variation of 2.2% and 2.5% for lymphocytes (62).

To evaluate whether the shortening of the telomere occurs at the same level for both CD4+ and CD8+ subpopulations, we assessed the correlation of the TL in these cells subpopulations within each evaluated group (Figure2).

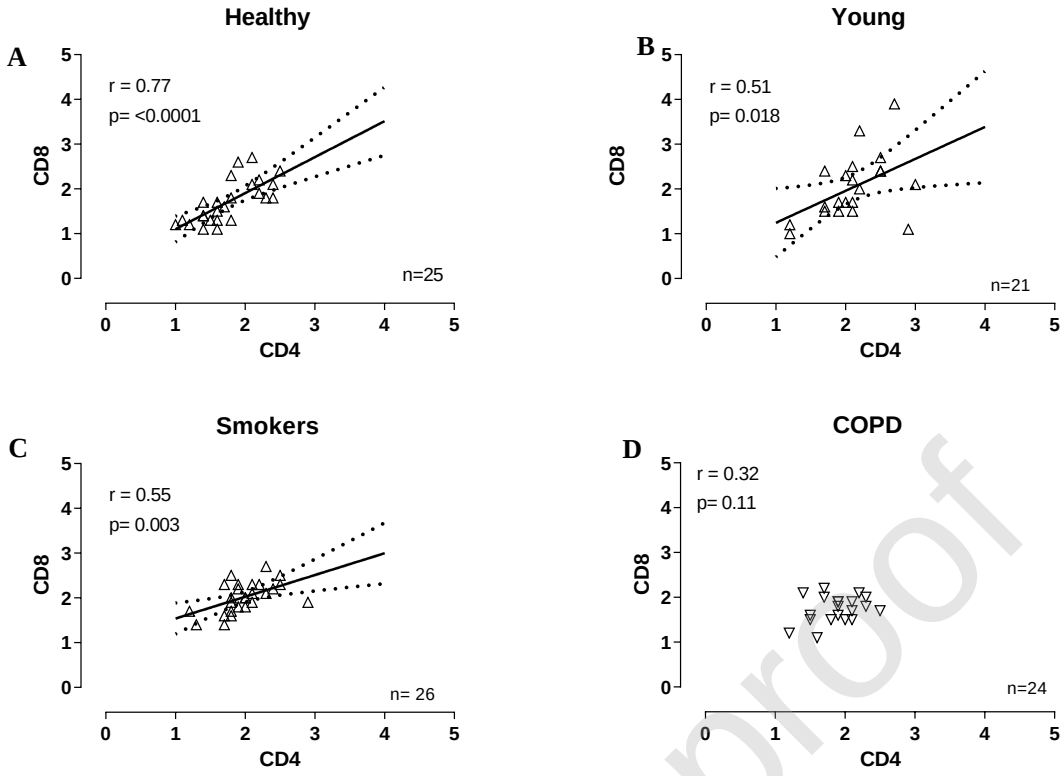

Figure 2: Telomere length correlation between CD4+ and CD8+ lymphocytes with the Spearman test. The trend lines show the rate of telomere difference within lymphocytes populations. A positive correlation was observed in Healthy, Young and Smokers groups. In COPD, no correlation was observed suggesting that in this group telomere shortening in CD4+ and CD8+ occurs at a different level.

CD4+ and CD8+ lymphocytes seem to suffer similar magnitude of telomeric shortening in Healthy, Smokers and Youngs. However, only in the COPD group, there is a greater telomere shortening in the CD8 lymphocytes than in the CD4 lymphocytes (CD4: 1.86±0.32 and CD8: 1.72±0.36, p=0.03 Paired T test), which can be explained by the greater activity of these cells especially in the lung, with their subsequent re-circulation. This data is corroborated by the absence of correlation between the TL of the CD4 and CD8 T lymphocytes in the COPD group (Figure 2). Pulmonary lymphocytes are overly activated in COPD and actively secrete inflammatory mediators involved in pathogenesis (48, 63). These cells pass through the inflammatory focus, organs and lymph nodes; however, part of these cells enter the lymphatic and blood circulation (64). Evidence from previous studies indicates that both the number and functional activity of CD8+ are increased in COPD, highlighting the involvement of these cells in the pulmonary pathogenesis (49). In general, there is a combination of two mechanisms, the

CD8+ lymphocytes migrate from the blood to the lungs in an attempt to fight the disease, and in the same way, the highly activated CD8+ lymphocytes leave the lung through the peripheral blood (65).

3.2 Telomerase activity

Commonly, in healthy individuals' telomerase activity is only present in cells with extended proliferation potential, such as germline, embryonic tissues, and self-renewing stem cell populations. Other tissues show inactivated telomerase and restricted proliferation programs. Deregulation of telomerase has direct consequences (66). For instance, reactivation of telomerase is associated with approximately 90% of human cancers, while insufficient telomerase activity is linked to stem cell telomere inherited disorders (67). Thus, we hypothesized that the unexpected findings in the COPD and smokers could be related to differences in relative telomerase activity (RTA).

Due to the limited cell yields after CD4+ and CD8+ purification, telomerase activity could only be assessed in PBMCs. In resting, unstimulated PBMCs there were no differences in the RTA among all four groups (healthy, 28.6 ± 7.8%; smokers 28.9 ± 6.6%, COPD 32.9 ± 6% e youngs 28.4 ± 6.4%). PHA stimulation significantly increased RTA in all four groups.

As it was observed in the telomerase assays, the young group presented significantly higher RTA than healthy aged and COPD patients, but not smokers, who had slightly higher RTA (not statistically different) than the two other aged groups (Figure 3). These results agree with the TL data and raise the hypothesis that cigarette smoking would be associated with upregulation of the RTA, slowing the progressive TL shortening promoted by aging. This also could contribute to the results of the COPD group, which due to the extensive inflammatory and infectious background that results in repeated rounds of T cell proliferation, exhibit shortened telomeres compared with healthy smoker individuals; however, the previous tobacco exposure would be associated with an enhancement of RTA and, consequently, protection from accelerated TL shortening, resulting in the lack of difference when compared with the healthy (non-smoker) group. Alternatively, an unidentified selection bias could have resulted in the selection of healthy aged individuals with risk factors for increased telomere shortening. This group was randomly selected from a group of almost 300 old adults and we were not able to detect

selection biases for individuals with risk factors for increased telomere shortening, although yet unknown confounding factors cannot be excluded.

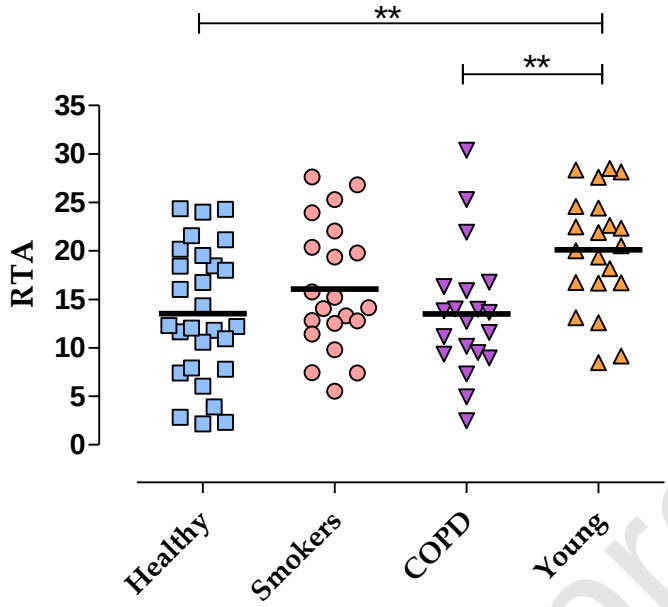

Figure 3: Comparison of relative telomerase activity (RTA) among evaluated groups. RTA is the difference between the telomerase activity in unstimulated and PHA-stimulated PBMCs. The Young group (n=21) showed significantly higher RTA compared to the healthy (n=28) and COPDs (n=20) group, but not to the Smokers group (n=21). Smokers showed a discrete higher RTA compared with COPD and healthy subjects, but no statistical difference was observed. Each symbol point represents an individual and the horizontal line the group medians. ** $p < 0.01$ (ANOVA with Dunnett's post test).

Also, factors present in the extracellular environment, such as cytokines, may interfere in the regulatory mechanisms of RTA, and therefore, modify the molecular structure of the telomeres (68, 69). COPD is characterized by its systemic inflammation with an important role of inflammatory cytokines (70). Cigarette compounds are far-reaching and complex, bringing both pro-inflammatory and immunosuppressive effects together, changing the balance of the extracellular environment (28). The observation that cigarette smoking can have a positive effect on human telomerase activity has already been shown in the literature not only in PBMC (57), but also in normal bronchial epithelium (71), and vascular smooth muscle cells (72). We used two different techniques (telomere length measured by Flow Cytometry and telomerase activity by a PCR) and both pointed to the same direction, giving unexpected but consistent results.

3.3 Cytokines secretion

Therefore, we evaluated a set of pro and anti-inflammatory cytokines (IL-6, IL-2, IL-10, TNF, IFN-γ, IL-17 and IL-4) released in the supernatants of PBMCs cultures (Table 1). In non-stimulated cultures, cytokines release was generally undetectable, except for IL-6, which was spontaneously released in large amounts by all four groups examined, but no differences among them could be detected (Kruskal-Wallis, $p = 0.54$). In PHA stimulated cultures the IL-6 levels were increased, being in most instances above the detection limit of the assay in all four groups (data not shown). IL-17 was undetectable in all four groups in either non-stimulated or PHA-stimulated conditions. PHA stimulation induced strong release of IFN-γ, TNF, IL-10 and IL-2 and a mild increase in IL-4 levels in all four groups. Of note, IFN-γ level in the smokers was statistically lower than in healthy and COPD groups. Slightly higher IL-4 levels were found in the Healthy group, showing statistical difference when compared to the Youngs (Table 1). The PHA-induced release of IL-6 (range: 588 - 11880 pg/mL, p = 0.54) and IL-2 (58 - 1622 pg/mL, p = 0.24) was similar among all groups.

Table 1: Quantification of cytokines from PHA-stimulated PBMCs culture supernatant

	Healthy	**Smokers**	**COPD**	**Young**	***P***
IFNγ (pg/mL)	3397* (615.5 - 3746)	1338*# (3.7 - 2976)	2923# (1343 - 3546)	3160 (1156 - 3460)	**0.02**
TNF (pg/mL)	1953 (1068 - 5929)	1742 (734.8 - 3654)	2121 (1014 - 3619)	1742 (376.7 - 5155)	0.9
IL-4 (pg/mL)	14.6& (5.4 - 35.3)	8.6 (4.9 - 40.5)	5.7 (4.9 - 15.2)	4.9& (4.9 - 11.1)	**0.01**
IL-10 (pg/mL)	139.8 (49.6 - 215.3)	121.7 (16.1 - 276.7)	100.2 (45.7 - 192.8)	104.3 (26.6 - 130.3)	0.5
IL-2 (pg/mL)	223.9 (40.8 – 2212)	598.7 (111.4 – 1785)	452.3 (110.3 – 1589)	158.6 (38 – 1076)	0.7
IFNγ / IL-10 ratio	18.6 (3.8 - 55.1)	7.9$# (0.2 - 16.9)	14.8# (8.9 - 35.6)	26.3$ (17.2 - 50.6)	**0.01**
IFNγ / IL-4 ratio	108.1 (20.6 - 418)	54.9$# (0.7 - 333.3)	343.3# (62.9 - 674.6)	261.4$ (117 - 689.3)	**0.01**

Values presented as median (interquartile range). Symbols show the statistical difference ($p < 0.05$) indicated by Kruskal-Wallis with Dunn's post-test. (*) Difference between healthy versus

smoker. (#) Difference between smokers and COPD. (&) Difference between healthy and Youngs. ($) Difference between smokers and Youngs.

Assessing the IFN-γ/IL-10 and IFN-γ/IL-4 ratios could be a better representation of the cytokine balance and help to delineate the Th-1 or Th-2-prone profiles among the study groups. In fact, lower IFN-γ/IL-10 and IFN-γ/IL-4 ratios were found in the Smokers, which showed a statistically significant difference compared to Youngs and COPDs. In summary, our findings showed a different profile of cytokines secretion under influence of cigarette smoke. Healthy smokers seem to be switched to an anti-inflammatory cytokines balance. Other studies also described enhancement of Th-2 responses in association with smoking, although the cytokines characterizing this enhancement differed. Byron et al. (73) found higher levels of IL-4 in smokers compared with non-smokers in a cohort of 20 to 65 years old subjects. Cozen et al. (74) found greater IL-5 and IL-13, but not IL-4 responses associated with smoking in a cohort of twins, where one or both siblings were smokers. In this study participants were aged 39 to 69 years old and had no sign of respiratory diseases, indicating that lifestyle, psychological and environmental factors can affect the extracellular milieu and the inflammatory process (42, 69, 75). However, our findings differ from Zeidel et al. (76), who described higher plasma levels of IFN-γ in smokers aged 45 to 65 years old compared with non-smokers at the same age. COPDs patients, who quitted tobacco but developed pulmonary damage, showed a higher release of IFN-γ, and higher IFN-γ/IL-4 and IFN-γ/IL-10 ratios than smokers, probably reinforcing the pro-inflammatory background (inflammaging) that characterizes aged individuals. Some other authors (77-79) described a decreased IL-10 level in COPD patients compared to current smokers, which corroborate with our data from IFN/IL-10 ratio. Moreover, smoking and alcohol consumption are related to alterations in cellular dynamics that implicate in telomere shortening promoted by changes in the extracellular milieu and the cytokines balance as well as stimulate replication and activation of immune cells (27).

Table 2: Linear correlation between relative telomerase activity and cytokines levels among the four groups.

	Healthy		**Smokers**		**COPDs**		**Youngs**	
Cytokine	***IFN-γ***	***IL-10***	***IFN-γ***	***IL-10***	***IFN-γ***	***IL-10***	***IFN-γ***	***IL-10***

Spearman r	0.41	0.47	0.11	0.38	0.47	0.11	0.44	0.45
P value	**0.04**	**0.02**	0.6	0.11	**0.03**	0.6	**0.04**	**0.03**

Values presented are Spearman correlation (r) analysis. Statistical significance $p < 0.05$

Hence, we performed correlation analyses to investigate the possible impact of cytokines on RTA (Table 2). Upon PHA activation RTA was increased and both IFN-γ and IL-10 release were upregulated, showing a weak positive correlation in both the Healthy and Youngs groups. In the smoker's group, RTA and cytokine release showed no correlation, possibly due to contrasting association with tobacco exposure, which upregulates RTA but negatively affects Th-1 responses. In the COPD group, the IFN-γ showed a weak correlation with RTA, yet no correlation was observed between RTA and IL-10, probably due to the pro-inflammatory microenvironment in these individuals. Thus, our data suggested a lower Th-1 response in smokers, as indicated by the low IFN-γ/IL-4 and IFN-γ/IL-10 ratios. Hyperreactivity and bronchial inflammation are increased in ex-smokers and active smokers (80, 81), possibly triggering a counteracting mechanism aiming to regulate Th-1 responses through the induction of Th-2 cytokines. In fact, smoke appears to directly immunomodulatory PBMCs, driving the differentiation of Th-2 cells and the production of IL-4, IL-5 and IL-13 (73). According to Cozen et al. (74), there is a positive correlation between smoking burden and Th-2 cytokines. Other studies showed that cigarettes induce a higher production of TGF-β in smokers not affected by COPD than in smokers who developed COPD (82). Moreover, cigarette components, by increasing oxidative stress levels, indirectly modulate p21. This molecule plays an important role in regulating the progression from G1 to S in the cell cycle, in addition to being a major inhibitor of apoptosis (83). However, smoking also inhibits the expression and secretion of effector cytokines such as IFN-γ and TNF, which decreases the levels of immune responses (84). High levels of TGF-β may be a possible mechanism for the decreased IFN-γ in smokers. Finally, the higher RTA and lower IFN-γ in the smoker group as compared to the healthy group could be explained by a short-term effect of current smoking on RTA (with no effect after smoking cessation, e.g. in past-smoker COPD patients).

Conclusion

Our data suggest that lymphocyte stimulation increases cytokines such as IFN-γ and IL-10 in individuals who have a regulated immune system such as the healthy and young groups. It is well established that COPD patients have an inflammatory microenvironment (48, 49, 63-65). Accordingly, compared with healthy smokers, our COPD patients had a stronger Th-1 response (high levels of IFN-γ and higher IFN-γ/IL-4 ratio), and no compensatory anti-inflammatory response (e.g., no increased IL-10 levels), resulting in a higher IFN-γ/IL-10 ratio. Meanwhile, in smokers, there was only a discrete inflammatory reaction caused by tobacco smoking, with low IFN-γ and IL-10 activation. However, telomerase activity is still augmented by some specific mechanism that helps to maintain telomere length. In this way, we can hypothesize that the enhanced telomerase activity associated with cigarette smoking would favor abnormal cell proliferation in active smokers. Consequently, these cells would be able to continue to proliferate without losing telomeric sequences. The COPD patients included in this study have been ex-smokers for at least 15 years, so they would also be susceptible to the modulation caused by cigarettes during the period of life they were smokers. However, after the establishment of the lung injury, and the inflammatory pool generated by the disease, patient's cells were compromised, and upregulation of telomerase seems to be switched off. Besides, one of the COPD's characteristics is the chronic activation of immune cells, causing them to constantly enter replicative cycles, which promotes telomere shortening.

The lymphocytes compartment in the elderly is altered and presents a senescent profile, in particular cells with a CD28- phenotype, and a lower number of B lymphocytes, besides the lower telomerase activity. Thus, the greater number of cells with short telomeres and high differentiation profiles will naturally induce less telomerase activity in the PBMCs in the studied cohort. Furthermore, estimations using PBMCs fail to explain the mechanisms implicated in our findings. The proportion of each cell type varies between individuals, preventing a better understanding of the role of telomere maintenance mediated by the environment, lifestyle, and immunosenescence. *Hematopoietic disorders can also alter telomere dynamics and telomere shortening, so medullary epigenetic activation mediated by tobacco exposure could be an important issue, warranting further studies (85).*

A critical limitation of this study was the use of PBMCs to investigate telomerase activity instead of purified lymphocyte subpopulations. Although estimation of RTA in

CD4+ and CD8+ lymphocytes, (as it was performed for inferring TL) would be ideal and bring more accurate information to elucidate the mechanisms involved in RTA, due to the low yield of purified cells we could only assess RTA in PBMCs. Additionally, it was not possible to evaluate TGF-β and other interleukins (e.g., IL-13) in the culture supernatants due to the long period of incubation used (72 hours). These extracellular factors are potentially involved in the senescence process, but their peak concentration occurs at 12-24 hours in culture. Finally, despite the number of aged subjects studied in each group (n = 24-26) may have precluded powerful statistical analyses, our conclusions are supported by concordant results obtained in using both telomere length and telomerase activity methodologies.

Telomeres shortening at each cell division acts as a barrier to replicative immortality, mediating the interruption of the cell cycle and the occurrence of apoptosis. However, some cell clones manage to escape this modulation via reactivation of telomeric preservation mechanisms. The most common mode of escape is the upregulation of the telomerase reverse transcriptase (TERT), the catalytic unit of the telomerase enzyme, responsible for DNA binding and nucleotide recognition. Its expression upregulates telomerase activity, which in turn can lead to the process of cellular immortalization that has been widely described in cancer. Immunosenescence is an inevitable process and can occur in a healthy or pathological condition. COPD seems to enhance senescence, with many alterations, especially on immune cells. Otherwise, tobacco smoke may be associated with a retard of senescence but may enhance cell immortalization and dysfunction. Thus, future work must be carried out focusing on the genetic mechanisms of telomerase and telomeric maintenance in these study groups to clarify the mechanisms and effects of smoking.

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