Telomerase activity, telomere length and hTERT DNA methylation in peripheral blood mononuclear cells from monozygotic twins with discordant smoking habits

Francesca Marcon, Ester Siniscalchi, Cristina Andreoli, Alessandra Allione, Giovanni Fiorito, Emanuela Medda, Simonetta Guarrera, Giuseppe Matullo, Riccardo Crebelli

Paper: Telomerase activity, telomere length and hTERT DNA methylation in peripheral blood mononuclear cells from monozygotic twins with discordant smoking habits

ENVIRONMENTAL AND MOLECULAR MUTAGENESIS, 58 (8), 2017, pp: 551-559

DOI: 10.1002/em.22127

Environmental and Molecular Mutagenesis

TELOMERASE ACTIVITY , TELOMERE LENGTH AND hTERT DNA METHYLATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM MONOZYGOTIC TWINS WITH DISCORDANT SMOKING HABITS.

Journal:	*Environmental and Molecular Mutagenesis*
Manuscript ID	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Marcon, Francesca; Istituto Superiore di Sanità, Siniscalchi, Ester; Istituto Superiore di Sanità, Environment and Primary Prevention Andreoli, Cristina; Istituto Superiore di Sanità Allione, Alessandra; Human Genetic Foundation and University of Turin, Medical Sciences Fiorito, Giovanni; Human Genetic Foundation and University of Turin, Medical Sciences Medda, Emanuela; Istituto Superiore di Sanità Guarrera, Simonetta; Human Genetic Foundation and University of Turin, Medical Sciences Matullo, Giuseppe; University of Turin, Medical Sciences Crebelli, Riccardo; Istituto Superiore di Sanità, Environment and Primary Prevention
Key Words:	telomerase activity, smoke, telomere length, twins, htert dna methylation

Telomerase activity, telomere length and *hTERT* DNA methylation in peripheral blood mononuclear cells from monozygotic twins with discordant smoking habits.

Marcon Francesca§°, Siniscalchi Ester§, Andreoli Cristina§, Allione Alessandra*, Fiorito Giovanni*, Guarrera Simonetta *, Medda Emanuela^, Matullo Giuseppe *, and Crebelli Riccardo§

§Department of Environment and Primary Prevention and ^National Centre for Epidemiology, Surveillance and Health Promotion, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy; *Human Genetics Foundation (HuGeF), Via Nizza 52, 10126 Torino and Dept. Medical Sciences, University of Turin, Via Santena 19, 10126 Turin, Italy

°To whom correspondence should be addressed: Department of Environment and Primary Prevention, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy. Tel. +39 06 49902680; Fax: +39 06 49903650; Email: francesca.marcon@iss.it

Running title: telomerase activity and tobacco smoke

Keywords: telomerase activity, smoke, telomere length, twins, *hTERT,* DNA methylation

ABSTRACT

The primary cause of lung cancer is smoking; since dysfunctions of telomerase activity are involved in the pathogenesis of lung cancer, an association between telomerase reactivation and tobacco smoke has been proposed. In this work an investigation has been performed to assess the influence of tobacco smoke exposure on telomerase activity (TA) in peripheral blood mononuclear cells (PBMCs) of healthy smokers. The methylation status of the catalytic subunit of telomerase *hTERT* was concurrently investigated to assess the possible association between epigenetic modifications of *hTERT* and telomerase activity. Besides, the influence of smoke on telomere length (TL) has been evaluated. Healthy monozygotic twins with discordant smoking habit were selected as study population to minimize inter-individual differences due to demographic characteristics and genetic heterogeneity. Statistically significant higher values of TA and TL were observed in smokers compared to non-smoker co-twins. The multivariate analysis of data showed, besides smoking habits ($p=0.02$), an influence of gender ($p=0.005$) and BMI ($p=0.001$) on TA and a borderline effect of gender ($p=0.05$) on TL. The DNA methylation analysis, focused on 100 CpG sites mapping in *hTERT*, highlighted nine CpG sites differentially methylated in smokers. When co-twins were contrasted, selecting as variables the intra twin difference in TA and *hTERT* DNA methylation, a statistically significant inverse correlation ($p=0.003$) was observed between TA and DNA methylation at the cg05521538 site. In conclusion, these results indicate an effect of tobacco smoke on TA and TL and suggest a possible association between smoke-induced epigenetic effects and TA in healthy smokers.

INTRODUCTION

Eukaryotic chromosomes are capped with telomeres, stretches of TTAGGG repeats which play a pivotal role in the maintenance of genome stability, preventing chromosome ends from being recognized as double strand breaks and processed by DNA damage repair [Blackburn, 1991, Palm, de Lange, 2008, O'Sullivan, Karlseder, 2010]. Due to incomplete DNA replication at the ends of chromosomes, telomeres shorten progressively with cell divisions [Makarov et al., 1997, Cech, 2004]. Telomerase, a ribonucleoprotein reverse transcriptase, counteracts the progressive telomere erosion, adding TTAGGG repeats at the end of chromosomes and allowing cell proliferation [Greider, Blackburn, 1996].

Telomerase activity (TA) is normally only present in proliferating cells of renewable tissues, and barely detectable in differentiated somatic cells [Chiu et al., 1996, Norrback et al., 2001]. However, modifications of the enzyme may occur triggering telomerase reactivation, providing the cells with an unlimited capacity of replication [Hooijberg et al., 2000, Kim et al., 1994]. This event is thought to be critical in cell immortalization, and a rate limiting step in carcinogenesis [Hiyama, Hiyama, 2003, Artandi, DePinho, 2000, Smith et al., 2009].

A dysfunction of telomerase activity has been shown to be involved in the pathogenesis of lung cancer [Jeon et al., 2012, Shibuya et al., 2001], and since the primary cause of lung cancer is smoking, an association of telomerase reactivation with tobacco smoke exposure has been proposed [Capkova et al., 2007]. Indeed different levels of telomerase activity has been reported in short-term cultures of human bronchial epithelial cells obtained from smokers, ever-smokers and non-smoker, with a strong correlation between telomerase activity and number of packs years [Yim et al., 2007]. Similar findings have been also reported in tumor cells of lung cancer patients, in which telomerase activity correlated positively with the history of tobacco smoke addiction addiction [Targowski et al., 2005].

In this work a pilot investigation has been performed to assess the influence of tobacco smoke exposure on telomerase activity in somatic cells of healthy smokers. Peripheral blood lymphocytes were selected as target cells, because of their ease availability and their acknowledged role as reporter cells for systemically induced effects.

In addition, as tobacco smoke is known to affect DNA methylation profile of blood cells [Allione et al., 2015, Shenker et al., 2013, Belinsky et al., 2005, Breitling et al., 2011, Harlid et al., 2014, Lee et al., 2016] and telomerase activity may be modulated by epigenetic changes affecting its catalytic subunit (*hTERT*) [Lewis, Tollefsbol, 2016], the methylation status of

hTERT gene was investigated to assess the possible association between smoke-induced epigenetic modifications of *hTERT* and telomerase activity.

Together with telomerase activity, also telomere length (TL) was concurrently evaluated as a functionally related end-point and possible sensitive biomarker of smoke related oxidative stress. Indeed, due to the high content of guanine, telomere DNA is highly sensitive to oxidative damage, and oxidative stress and inflammation have been identified as major determinants of telomere erosion [von Zglinicki, Martin-Ruiz, 2005, Houben et al., 2008].

Healthy monozygotic twins (MZ) with discordant smoking habit were selected as study population to minimize inter-individual differences due to demographic characteristics (age, gender) as well as genetic heterogeneity. All subjects fell within a relatively small age range, which excluded elderly individuals in order to minimize the well-known effect of aging on telomere length. Thus, any difference in the end-points investigated could directly be attributed to the non-shared environment experienced by co-twins, which included as main factor the exposure to cigarette smoke.

MATERIALS AND METHODS

Study population

Twenty-two pairs of monozygotic twins discordant for smoking habits were enrolled for a cross-sectional study conducted in accordance with the principles of Good Clinical Practice and approved by the Independent Ethics Committee of the University of Rome ‘‘Tor Vergata’’. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Information on demographic data, medical history, lifestyle, dietary habits, occupational and environmental exposures was collected by questionnaire. Saliva samples were taken from all subjects to verify the zygosity status. Testing for zygosity was performed using the AmpFiSTRs Identifier kit (Applied Biosystems). All the analyses were carried out on coded samples.

Detailed information on the smoking habits of all study subjects was retrieved as previously described [Andreoli et al., 2011].

Determination of telomerase activity

In brief, peripheral blood mononuclear cells (PBMC) were separated from whole blood by Histopaque-1077 (Sigma-Aldrich, St.Louis, USA) and stimulated with 1% phyto-haemagglutinin (PHA HA15, Remel Inc., Santa Fe, USA) for 48 hours; at the end of the incubation period cells were harvested by centrifugation and cell extracts obtained by incubation with NP-40 lysis buffer containing 1% protease inhibitor mix (GE Healthcare, UK). Protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad, Munchen, Germany). Immortalized 293 human embryonic kidney cells were chosen as telomerase-positive reference sample to construct a standard curve for normalization of unknown samples. Two negative controls samples were also included in each run of analysis, i.e. a cell lysate from a heat-inactivated sample, and lysis buffer alone to check for the presence of contamination in the lysis buffer. Telomerase activity was determined by the real-time, quantitative TRAP (Q-TRAP) protocol using a fluorescent-based assay [Herbert et al., 2006]. In the first step of the protocol, the telomerase substrate and dNTPs are used for the addition of telomeric repeats by telomerase, while in the second steps, specific primers for these products are used for amplification. For the assay, 20 µl of TRAP master mix per sample containing 1X Sybr Green Master Mix (including ROX as passive reference dye, Bioline), 100 ng TS primer (5’-AATCCGTCGAGCAGAGTT-3’), 100 ng ACX primer (5’-GCGCGGCTTACCCTTACCCTTACCCTAACC-3’), 1mM EGTA, water. The real-time

PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 60 sec. Each sample was prepared in triplicate; only experiments with standard curves with R^2>98% were considered for analysis. Values were expressed as relative telomerase activity (%RTA) in comparison to that of the positive control.

Telomere length determination

Genomic DNA was isolated from whole blood samples using Puregene Core Kit (Qiagen, Hilden,

Germany), according to the manufacturer's instructions and stored at −80°C.

A non-radioactive chemiluminescent assay was applied to determine telomere length using the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostics, Indianapolis, USA). Briefly, 1.5 µg of DNA were digested with 20 units of RsaI and HinfI for 2 h at 37°C. The sequence specificity of enzymes is such that telomeric DNA and sub-telomeric DNA is not cut, due to the sequence characteristics of the repeats, while non-telomeric-DNA is digested to low molecular weight fragments. After digestion, samples were loaded on a 0.5% agarose gel and run for 21 h at 35V. Gels were treated with HCl, denaturalized and neutralized, and then transferred to a nylon membrane by Southern blotting for 12–18 h. DNA fragments were indirectly visualized by hybridization with a digoxigenin (DIG)-labeled probe complementary to the telomeric repeat sequence (3h, 42°C). Finally, images were digitalized using a densitometer and the mean telomeric length (MTL) determined using the formula: MTL = ∑(MWi x ODi)/∑(ODi) where ODi is the densitometer output and MWi is the length of the DNA at position I [Kruk et al., 1995]. Sums were calculated over the range 1.6-12.2 kb.

DNA methylation array

Genomic DNA was isolated from whole blood samples as described above. Bisulphite conversion of 500 ng of each DNA sample was performed using the EZ-96 DNA Methylation GoldTM kit (Zymo Research, Orange, CA, USA). Bisulphite-converted DNA was hybridized on the Illumina HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA), following the Illumina Infinium HD methylation protocol.

The GenomeStudio Methylation Module v1.0 software (Illumina Inc., San Diego, CA) was used to convert on-chip fluorescent methylation signals into numerical values (β-values) between 0 and 1, which represent the methylation percentage of each analyzed CpG site. Data pre-processing was performed using in-house script written for the R statistical computing

environment and described elsewhere [Campanella et al., 2015]. DNA methylation values whose detection p-value was below 0.01 were set to missing. Both probes and samples were excluded from the analysis if the global call rate was less than 95%.

Statistical analyses

Arithmetic means, standard deviation and percentage distribution of categorical variables by smoke habits were used to describe the data. Analysis of variance (ANOVA) was undertaken to estimate between-pairs and within-pairs means of squares and to evaluate the homogeneity of variances (F ratio). In order to quantify the similarity within pairs for the two end-points analyzed, intra-class correlation (ICC) coefficient was computed as well. Pearson correlation coefficient was estimated to test the degree of relationship between telomerase activity and telomere length.

Differences in TA and TL between smokers and non smokers were assessed by Wilcoxon signed-rank test. Moreover, the effect of smoking habits on TA and TL was evaluated by robust multivariate linear regression model (to account for twin clustering), adjusting for age, gender and BMI as possible confounders.

To identify differentially methylated CpGs sites between smokers and non-smokers plied a linear mixed model including DNA methylation as dependent variable, smoking status, age, gender and white blood cell percentages as fixed effect and twin pair as random effect was applied [Carlin at al., 2005, Davies et al., 2014]. Association between DNA methylation values and telomerase activity/telomere length was also investigated using Pearson correlation coefficients.

Statistical analyses were conducted using Intercooled STATA for Windows (version 11.2; StataCorp, College Station, TX, USA) and R-software (https://www.R-project.org/).

RESULTS

Telomerase activity and telomere length in co-twins

Demographic characteristics and information on smoking habits of the study population are summarized in Table I, while individual values of TA and TL are presented in Table II.

To compare the similarities between and within twin pairs, an analysis of variance has been performed. The results obtained confirmed the higher degree of similarity of scores of co-twins compared to those of unrelated subjects (variance between pairs/variance within pairs for TA and TL, (F)=2.65, p=0.03 and F=19.4, p<0.001, respectively). Intra-class correlation coefficients estimated to evaluate the level of similarities between the two members of each twin pair (Figure 1) highlighted statistically significant correlations between co-twins for TL (ICC=0.90, p<0.001) and TA (ICC=0.46, p=0.03) values, suggesting a significant influence of familiarity (genetic and shared environmental factors) on these end-points.

Influence of smoking habits on telomerase activity and telomere length

The paired analysis of data from co-twins showed statistically significant higher values in smokers compared to non-smokers both for TA (Wilcoxon signed-rank test, p=0.03) and for TL (p=0.04) (Table II). The effect of smoking was most consistent for TA: a large prevalence of smoker twins (13/16, 81.2%) displayed higher TA values compared to the non- smoker co-twin and only three smokers had lower TA compared to the non-smoker co-twin (12.5%). (Figure 2). Overall, a significant correlation (r=0.46; p=0.008) between telomerase activity and telomere length values was observed within the whole study population (Figure 3.)

The influence of smoking habits on TA and TL was confirmed in a multivariate analysis of data, in which age, gender and body mass index (BMI) were considered as covariates (Table III). This analysis highlighted, besides smoking habits (p=0.02), an influence of gender (p=0.005) and BMI (p=0.001) on TA, and a borderline effect of gender (p=0.05), besides smoking habits (p=0.02), on TL. This model also suggested a positive association between age and TL, even though this association did not attain statistical significance (p=0.09), possibly in consequence of the relatively young sample (mean 31.5, range 23-46 years, Table I).

Influence of smoking on hTERT methylation

In order to probe the possible involvement of epigenetic modifications in the modulation of TA elicited by tobacco smoke, the influence of smoking habits on methylation of *hTERT* was

assessed. This analysis was based on DNA methylation data on 100 CpG sites mapping in *hTERT* (supplementary data), obtained in a previous epigenome-wide DNA methylation study on the same subjects [Allione et al., 2015]. Since this analysis was only focused on 100 CpG sites, no correction for multiple testing was deemed necessary and alpha = 0.05 was used as threshold of significance to define differentially methylated CpG sites.

In this model the addiction to tobacco smoke was associated with significantly lower DNA methylation in seven *hTERT* CpG sites, while significantly higher DNA methylation was detected in two CpG sites (Table IV). No further association was disclosed considering the intensity and/or duration of tobacco smoke as variables (data not shown).

Overall, the nine CpG sites differentially methylated in smokers were all consistently methylated (>70%), except cg05521538 that showed medium methylation level (from 20 to 70%). The effect of smoking habits on methylation levels was – albeit statistically significant - relatively small in absolute terms (1 to 3.5 %), but confirmed and validated in DNA blood samples obtained from the Turin Bladder Cancer Study (TBCS) [Matullo et al., 2005]. The analysis of *hTERT* DNA methylation in 18 current smokers and 10 never smokers enrolled as controls in the TBCS showed for all CpG sites the expected direction of difference, which attained statistical significance for cg26221342 (Wilcoxon test, p=0.01) (data not shown).

Association between telomerase activity and hTERT methylation

The correlation between methylation at the nine CpG sites differentially methylated in smokers and non-smokers and telomerase activity was investigated by Spearman correlation analysis. No statistically significant correlation between TA and DNA methylation was observed in an unbiased analysis of the whole study population (data not shown). However, when genetically homogeneous individuals (co-twins) were contrasted, selecting as variables the intra-twin pair difference (delta=smoker-non smoker) in TA and *hTERT* DNA methylation, a statistically significant inverse correlation (p=0.003) was observed between TA and DNA methylation of cg05521538 locus. Figure 4 shows that most of the twin couples is distributed in the right bottom of the graphic (delta TA>0; delta cg05521538<0), where the smokers have higher TA and lower DNA methylation of cg05521538 than the non smoker co-twins, indicating an inverse relationship between TA and methylation of cg05521538 in individuals with the same genetic background. This inverse correlation holds true also for the twin couple in the upper left side of the figure.

DISCUSSION

Tobacco smoke exposure has been associated to the reactivation of telomerase in bronchial epithelial cells of healthy smokers and in tumor cells of lung cancer patients [Greider, Blackburn, 1996, Chiu et al., 1996], suggesting a role for telomerase reactivation in the etiology of smoke-related tumors [Capkova et al., 2007]. Smoke has also been proposed as possible modulator of telomere dynamics through the increase of oxidative stress [Houben et al., 2008].

In this study, the effect of the exposure to tobacco smoke on telomerase activity and telomere length has been investigated in a group of healthy monozygotic (MZ) twins with discordant smoking habits. This study population, previously characterized for smoking habits and clinical parameters [Andreoli et al. 2011, 2015], and consisting of young adults to avoid the confounding effect of aging on telomerase activity and telomere length, provided a convenient model to assess the influence of smoking habits on biological parameters ruling out the effect due to genetic heterogeneity.

The results obtained highlighted a significantly higher average TA in blood cells of smoking subjects compared to non smoking subjects. The trend to a higher TA in smokers was highly reproducible among all twin couples: in nearly 81% of cases (i.e. 13 out of total 16 twin couples) the smoker twin displayed a relatively higher TA compared to the non-smoker co-twin. This result is in agreement with recent published data showing a smoke-related increase of TA in normal bronchial epithelium [Yim et al., 2007], but discrepant from previous results on PBMCs from healthy individuals reporting no differences in TA according to smoking status [Jeon et al., 2012, Getliffe et al., 2005, Narducci et al., 2007, Rentoukas et al., 2012]. However, these investigations were not specifically designed to evaluate the effects of smoking habits on TA. Consistently with the results obtained with TA, also TL proved to be significantly higher in smokers compared to non-smokers. The strong correlation observed between TA and TL among the study subjects strengthen the biological plausibility of the results obtained, even though conflicting findings on the effects of smoking on TL have been reported in other studies on PBMCs [Muezzinler et al., 2013, McGrath et al., 2007, Valdes et al., 2005, Morlà et al., 2006, Weischer et al., 2014, Huzen et al., 2013, Song et al., 2010], which suggest a complex relationship between tobacco smoke exposure and telomere maintenance. These apparently conflicting findings could be interpreted in view of a recent study characterizing the repair of oxidative base damage in mammalian telomeres [Wang et al., 2010]. In particular, Wang and coworkers propose that the oxidative stress generated by

the burden of reactive oxygen species may result in either telomere shortening or lengthening, depending on the type and level of DNA damage induced. Mild oxidative base damage of telomeric DNA sequences may moderately decrease the binding of telomeric proteins in favor of telomerase, promoting the telomerase-dependent telomere repeat addition and telomere lengthening; on the other hand, extensive oxidative base damage may severely deplete telomere protein complex in telomeres and attenuate telomere recombination, replication as well as the resolution of DNA strand breaks, finally leading to telomere shortening.

This picture is consistent with the increase of TL observed in smokers in the present study, which mainly involved light smokers who reasonably experienced a mild level of oxidative stress.

The mechanism by which the exposure to tobacco smoke may influence telomerase activity is not elucidated. The higher level of telomerase expression observed in epithelial bronchial cells of smokers has been linked to the higher proliferative potential of these cells compared to cells from never-smokers [Yim et al., 2007]; however, this explanation is hardly applicable to resting blood cells which express telomerase activity only upon mitogen stimulation. Also inflammation cytokines, increased in smokers [Andreoli et al., 2015], have been associated with telomerase activation [Shen et al., 2013], but this mechanism is unlikely to play a role under the *ex vivo* culture conditions applied to stimulate PBMCs to express telomerase activity. Rather, the picture is reminiscent of the induction of a stable epigenetic modification induced by tobacco smoke exposure, retained in resting cells *ex vivo*.

This possibility was explored assessing the influence of smoking on the methylation of *hTERT*, the gene coding for the telomerase reverse transcriptase subunit. No smoke-related difference in methylation of *hTERT* promoter was observed in a previous study on the same population [Ottini et al., 2015]. Here we extended the analysis to CpG sites located in *hTERT* gene, characterized in a previous epigenome-wide DNA methylation study using Methylation450 Illumina BeadChip [Allione et al., 2015], and identified nine loci differentially methylated in smokers and non-smokers. The effect was relatively small, but statistically significant and validated in another population enrolled in the Turin Bladder Cancer Study (TBCS) [Matullo et al., 2005]. However at variance to the present work, in the TBCS only for one CpG site the difference between smokers and non-smokers attained statistical significance, highlighting the greater statistical power of the discordant MZ twin model applied herein [Tsai, Bell, 2015].

The functional implications of DNA methylation of *hTERT* was tentatively tested by a quantitative comparison of TA and DNA methylation levels at the nine CpG sites differentially methylated in smokers and non-smokers. This analysis highlighted an inverse correlation between DNA methylation of cg05521538 locus and TA in individuals with the same genetic background, suggesting a possible functional role of epigenetic modifications associated with tobacco smoking in the modulation of telomerase activity. This result also supports the strength of the design of this study in twins because it could be deduced only through the direct comparison of co-twins with discordant smoking habits. However, further research will be needed to establish a causative role of cg05521538 methylation level and telomerase activity. In addition, the regulation of telomerase activity may occur at various levels, including transcription, post-translational modifications, complex assembly or subcellular localization [reviewed in Wojtyla 2011], and the interaction of smoking with other events regulating TA, different from DNA methylation cannot be ruled out. Indeed, the analysis of telomerase activity performed herein, takes into account all the steps regulating telomerase from transcription to assembly.

Beyond smoking habits, a few other personal and lifestyle factors were considered as covariates in the assessment of telomere maintenance [Rentoukas et al., 2012, Song et al., 2010, Gardner et al., 2005].The results obtained highlighted a significant influence of BMI on TA, consistent with the increased levels of oxidative stress and inflammation associated with obesity and implicated in TA regulation [Chung et al., 2002, Fouquerel et al., 2016]. Also gender was shown to modulate both TA and TL, with significantly higher TA and longer TL in women, in line with the known oestrogen antioxidant capacities [Massafra et al., 2000, Römer et al., 1997, Sack et al., 1994] and positive regulatory effect on TA [Kyo et al., 1999]. On the other hand, no significant influence of age on TA and TL could be disclosed, despite the well-established influence of aging on telomere integrity. The latter results was most likely a consequence of the relatively young study population, which however should not be regarded as a limitation but rather as a mean to minimize the confounding interference of aging on the biological end-points evaluated. Anyway, the overall contribution of other lifestyle factors considered besides smoking habits to TA and TL variability was limited, only explaining a tiny fraction of intra-twin overall variance (intra-class correlation: 0.46 and 0.90, respectively). Conversely, genetic or familiar factors appear to be major determinants of inter-individual variability in TA and TL. However, it has to be pointed out that this study, which only involved MZ twins, was not intended to provide a quantitative measurement of the

influence of genetic determinants on the end-point studied. To this aim a classical twin study, based on the biometric analysis of the results in MZ and DZ twins or siblings, should be conducted.

This study presented some limitations, including the relatively small sample size which call for an independent confirmation of the findings provided. Concerning the impact of tobacco smoke, only few of the study subjects could be considered heavy smokers (i.e. with more than one pack-day), and this limited the possibility to fully appreciate the biological consequence of smoking. Moreover, only MZ twins were enrolled in the study, thus preventing the possibility to disentangle the influence of genetic factors from shared environment through a biometric analysis of results in MZ and DZ twins. However the study design also presented some strengths, as considering each twin couple as experimental unit greatly increased the power of the study, allowing a carefully assessment of the influence of the variable of interest (tobacco smoke in this case), without the disturbance of the noise of inter-individual variability associated to the comparison of genetically heterogeneous study groups.

In conclusion, this is the first study indicating an effect of tobacco smoking on TA in PBMCs from healthy subjects. This effect may result from epigenetic modification induced by the exposure to tobacco smoke which warrant further investigation.

ACKNOWLEDGEMENTS

The authors wish to thank drs. Lorenza Nisticò and Andrea Zijno for critical reading of the manuscript.

This work was supported by the Istituto Superiore di Sanità (ISS) [cap.508 2.1.1.7, fiscal year 2008] and by British American Tobacco (BAT) Italia S.p.A. [contract ISS-BAT Italia S.p.A. n. Q78, years 2008–2010]. The financial support provided to the Istituto Superiore di Sanità (ISS) by British American Tobacco (BAT) Italia S.p.A. to perform this work was preliminary approved by the Scientific Committee of ISS. As stipulated in article 5 of the above-mentioned contract, in view of its role of public health authority ISS retains full and unique responsibility for the interpretation and diffusion of any result with health relevance.

This study was supported by Compagnia di San Paolo, (www.compagniadisanpaolo.it) (G.M.); Human Genetics Foundation, (www.hugef-torino.org) (G.M.)

Conflict of interest statement: The authors declare no conflict of interest.

Statement of Author Contributions

CA, FM and RC designed the study. CA applied for Research Ethics Board approval and recruited the patients. FM, ES performed the experiments and collected the data. SG performed the analysis of DNA methylation. RC, FM, AA, GF and EM analysed the data and prepared draft figures and tables. RC and FM prepared the manuscript draft with critical input from AA, GM, EM and GF.

REFERENCES

Allione A, Marcon F, Fiorito G, Guarrera S, Siniscalchi E, Zijno A, Crebelli R, Matullo G. 2015. Novel epigenetic changes unveiled by monozygotic twins discordant for smoking habits. PLoS One 10(6):e0128265.

Andreoli C, Bassi A, Gregg EO, Nunziata A, Puntoni R, Corsini E. 2015. Effects of cigarette smoking on circulating leukocytes and plasma cytokines in monozygotic twins. Clin Chem Lab Med 53:57-64.

Andreoli C, Gregg EO, Puntoni R, Gobbi V, Nunziata A, Bassi A. 2011. Cross-sectionalstudy of biomarkers of exposure and biological effect on monozygotic twins discordant for smoking. Clin Chem Lab Med 49:137-145.

Artandi SE, DePinho RA. 2000. A critical role for telomeres in suppressing and facilitating carcinogenesis. Curr Opin Genet Dev 10:39-46.

Belinsky SA, Klinge DM, Dekker JD, Smith MW, Bocklage TJ, Gilliland FD, Crowell RE, Karp DD, Stidley CA, Picchi MA. 2005. Gene promoter methylation in plasma and sputum increases with lung cancer risk. Clin Cancer Res 11:6505-6511.

Blackburn EH. 1991. Structure and function of telomeres. Nature 350:569-573.

Breitling LP, Yang R, Korn B, Burwinkel B, Brenner H. 2011. Tobacco-smoking-related differential DNA methylation: 27K discovery and replication. Am J Hum Genet 88:450-457.

Campanella G, Polidoro S, Di Gaetano C, Fiorito G, Guarrera S, Krogh V, Palli D, Panico S, Sacerdote C, Tumino R, Elliott P, Matullo G, Chadeau-Hyam M, Vineis P. 2015. Epigenetic signatures of internal migration in Italy. Int J Epidemiol 44:1442-1449.

Capkova L, Kalinova M, Krskova L, Kodetova D, Petrik F, Trefny M, Musil J, Kodet R. 2007. Loss of heterozygosity and human telomerase reverse transcriptase (*hTERT*) expression in bronchial mucosa of heavy smokers. Cancer 109:2299-2307.

Carlin JB, Gurrin LC, Sterne JA, Morley R, Dwyer T. 2005. Regression models for twin studies: a critical review. Int J Epidemiol 34:1089-1099.

Cech TR. 2004. Beginning to understand the end of the chromosome. Cell 116:273-279.

Chiu CP, Dragowska W, Kim NW, Vaziri H, Yui J, Thomas TE, Harley CB, Lansdorp PM. 1996. Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. Stem Cells 14:239-248.

Chung HY, Kim HJ, Kim KW, Choi JS, Yu BP. 2002. Molecular inflammation hypothesis of aging based on the anti-aging mechanism of calorie restriction. Microsc Res Tech 59:264-272.

Davies MN, Krause L, Bell JT, Gao F, Ward KJ, Wu H, Lu H, Liu Y, Tsai PC, Collier DA, Murphy T, Dempster E, Mill J, UK Brain Expression Consortium, Battle A, Mostafavi S, Zhu X, Henders A, Byrne E, Wray NR, Martin NG, Spector TD, Wang J. 2014. Hypermethylation in the ZBTB20 gene is associated with major depressive disorder. Genome Biol 15:R56. doi: 10.1186/gb-2014-15-4-r56.

Fouquerel E, Lormand J, Bose A, Lee HT, Kim GS, Li J, Sobol RW, Freudenthal BD, Myong S, Opresko PL. 2016. Oxidative guanine base damage regulates human telomerase activity. Nat Struct Mol Biol. doi:10.1038/nsmb.3319. [Epub ahead of print].

Gardner JP, Li S, Srinivasan SR, Chen W, Kimura M, Lu X, Berenson GS, Aviv A. 2005. Rise in insulin resistance is associated with escalated telomere attrition. Circulation 111:2171-2177.

Getliffe KM, Al Dulaimi D, Martin-Ruiz C, Holder RL, von Zglinicki T, Morris A, Nwokolo CU. 2005. Lymphocyte telomere dynamics and telomerase activity in inflammatory bowel disease: effect of drugs and smoking. Aliment Pharmacol Ther 21:121-131.

Greider CW, Blackburn EH. 1996. Telomeres, telomerase and cancer. Sci Am 274:92-97.

Harlid S, Xu Z, Panduri V, Sandler DP, Taylor JA. 2014. CpG sites associated with cigarette smoking: analysis of epigenome-wide data from the Sister Study. Environ Health Perspect 122:673-678.

Herbert BS, Hochreiter AE, Wright WE, Shay JW. 2006. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. Nat Protoc 1:1583-1590.

Hiyama E, Hiyama K. 2003. Telomerase as tumor marker. Cancer Lett 194:221-233.

Hooijberg E, Ruizendaal JJ, Snijders PJ, Kueter EW, Walboomers JM, Spits H. 2000. Immortalization of human CD8+ T cell clones by ectopic expression of telomerase reverse transcriptase. J Immunol 165:4239-4245.

Houben JM, Moonen HJ, van Schooten FJ, Hageman GJ. 2008. Telomere length assessment: biomarker of chronic oxidative stress? Free Radic Biol Med 44:235-246.

Huzen J, Wong LS, van Veldhuisen DJ, Samani NJ, Zwinderman AH, Codd V, Cawthon RM, Benus GF, van der Horst IC, Navis G, Bakker SJ, Gansevoort RT, de Jong PE, Hillege HL, van Gilst WH, de Boer RA, van der Harst P. 2014. Telomere length loss due to smoking and metabolic traits. J Intern Med 275:155-163.

Jeon HS, Choi JE, Jung DK, Choi YY, Kang HG, Lee WK, Yoo SS, Lim JO, Park JY. 2012. Telomerase activity and the risk of lung cancer. J Korean Med Sci 27:141-145.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015.

Kruk PA, Rampino NJ, Bohr VA. 1995. DNA damage and repair in telomeres: relation to aging. Proc Natl Acad Sci U S A 92:258-262.

Kyo S, Takakura M, Kanaya T, Zhuo W, Fujimoto K, Nishio Y, Orimo A, Inoue M. 1999. Estrogen activates telomerase. Cancer Res 59:5917-5921.

Lee MK, Hong Y, Kim SY, London SJ, Kim WJ. 2016. DNA methylation and smoking in Korean adults: epigenome-wide association study. Clin Epigenetics 8:103-119.

.

Lewis KA, Tollefsbol TO. 2016. Regulation of the Telomerase Reverse transcriptase Subunit through Epigenetic Mechanisms. Front Genet 7:83.

Makarov VL, Hirose Y, Langmore JP. 1997. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell 88:657-666.

Massafra C, Gioia D, De Felice C, Picciolini E, De Leo V, Bonifazi M, Bernabei A. 2000. Effects of estrogens and androgens on erythrocyte antioxidant superoxide dismutase, catalase and glutathione peroxidase activities during the menstrual cycle. J Endocrinol 167:447-452.

Matullo G, Guarrera S, Sacerdote C, Polidoro S, Davico L, Gamberini S, Karagas M, Casetta G, Rolle L, Piazza A, Vineis P. 2005. Polymorphisms/haplotypes in DNA repair genes

and smoking: a bladder cancer case-control study. Cancer Epidemiol Biomarkers Prev 14:2569-2578.

McGrath M., Wong J.Y., Michaud D., Hunter D.J., De Vivo I. 2007. Telomere length, cigarette smoking, and bladder cancer risk in men and women. Cancer Epidemiol. Biomarkers Prev 16:815–819.

Morlá M, Busquets X, Pons J, Sauleda J, MacNee W, Agustí AG. 2006. Telomere shortening in smokers with and without COPD. Eur Respir J 27:525–528.

Müezzinler A, Zaineddin AK, Brenner H. 2013. A systematic review of leukocyte telomere length and age in adults. Ageing Res Rev 12:509-519.

Narducci ML, Grasselli A, Biasucci LM, Farsetti A, Mulè A, Liuzzo G, La Torre G, Niccoli G, Mongiardo R, Pontecorvi A, Crea F. 2007. High telomerase activity in neutrophils from unstable coronary plaques. J Am Coll Cardiol 50:2369-2374.

Norrback KF, Hultdin M, Dahlenborg K, Osterman P, Carlsson R, Roos G. 2001. Telomerase regulation and telomere dynamics in germinal centers. Eur J Haematol 67:309-317.

O'Sullivan RJ, Karlseder J. 2010. Telomeres: protecting chromosomes against genome instability. Nat Rev Mol Cell Biol 11:171-181.

Ottini L, Rizzolo P, Siniscalchi E, Zijno A, Silvestri V, Crebelli R, Marcon F. 2015. Gene promoter methylation and DNA repair capacity in monozygotic twins with discordant smoking habits. Mutat Res Genet Toxicol Environ Mutagen 779:57-64.

Palm W, de Lange T. 2008. How shelterin protects mammalian telomeres. Annu Rev Genet 42:301-334.

Rentoukas E, Tsarouhas K, Kaplanis I, Korou E, Nikolaou M, Marathonitis G, Kokkinou S, Haliassos A, Mamalaki A, Kouretas D, Tsitsimpikou C. 2012. Connection between telomerase activity in PBMC and markers of inflammation and endothelial dysfunction in patients with metabolic syndrome. PLoS One 7:e35739. doi: 10.1371/journal.pone.0035739.

Römer W, Oettel M, Menzenbach B, Droescher P, Schwarz S. 1997. Novel estrogens and their radical scavenging effects, iron-chelating, and total antioxidative activities: 17 alpha-substituted analogs of delta 9(11)-dehydro-17 beta-estradiol. Steroids 62:688-694.

Sack MN, Rader DJ, Cannon RO 3rd. 1994. Oestrogen and inhibition of oxidation of low-density lipoproteins in postmenopausal women. Lancet 343:269-270.

Shen XH, Xu SJ, Jin CY, Ding F, Zhou YC, Fu GS. 2013. Interleukin-8 prevents oxidative stress-induced human endothelial cell senescence via telomerase activation. Int Immunopharmacol 16:261-267.

Shenker NS, Polidoro S, van Veldhoven K, Sacerdote C, Ricceri F, Birrell MA, Belvisi MG, Brown R, Vineis P, Flanagan JM. 2013. Epigenome-wide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking. Hum Mol Genet 22:843-851.

Shibuya K, Fujisawa T, Hoshino H, Baba M, Saitoh Y, Iizasa T, Sekine Y, Suzuki M, Hiroshima K, Ohwada H. 2001. Increased telomerase activity and elevated *hTERT* mRNA expression during multistage carcinogenesis of squamous cell carcinoma of the lung. Cancer 92:849-855.

Smith V, Dai F, Spitz M, Peters GJ, Fiebig HH, Hussain A, Burger AM. 2009. Telomerase activity and telomere length in human tumor cells with acquired resistance to anticancer agents. J Chemother 21:542-549.

Song Z, von Figura G, Liu Y, Kraus JM, Torrice C, Dillon P, Rudolph-Watabe M, Ju Z, Kestler HA, Sanoff H, Lenhard Rudolph K. 2010. Lifestyle impacts on the aging-associated expression of biomarkers of DNA damage and telomere dysfunction in human blood. Aging Cell 9:607-615.

Targowski T, Jahnz-Rózyk K, Szkoda T, From S, Rozyńska R, Płusa T. 2005. Influence of nicotine addiction on telomerase activity in malignant non-small cell lung tumors. Przegl Lek 62:1043-1046.

Tsai PC, Bell JT. 2015 Power and sample size estimation for epigenome-wide association scans to detect differential DNA methylation. Int J Epidemiol doi:10.1093/ije/dyv041. [Epub ahead of print].

Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. 2005. Obesity, cigarette smoking, and telomere length in women. Lancet 366:662–664.

von Zglinicki T, Martin-Ruiz CM. 2005. Telomeres as biomarkers for ageing and age-related diseases. Curr Mol Med 5:197-203.

Wang Z, Rhee DB, Lu J, Bohr CT, Zhou F, Vallabhaneni H, de Souza-Pinto NC, Liu Y. 2010. Characterization of oxidative guanine damage and repair in mammalian telomeres. PLoS Genet 6:e1000951.

Weischer M, Bojesen SE, Nordestgaard BG. 2014. Telomere shortening unrelated to smoking, body weight, physical activity, and alcohol intake: 4,576 general population individuals with repeat measurements 10 years apart. PLoS Genet 10:e1004191.

Wojtyla A, Gladych M, Rubis B. 2011. Human telomerase activity regulation. Mol Biol Rep 38:3339-3349.

Yim HW, Slebos RJ, Randell SH, Umbach DM, Parsons AM, Rivera MP, Detterbeck FC, Taylor JA. 2007. Smoking is associated with increased telomerase activity in short-term cultures of human bronchial epithelial cells. Cancer Lett 246:24-33.

Table I. Demographic characteristics of the study population.

	Smokers	Non smokers	All
Subjects (n)	22	22	44
Age (years)			31.5 ± 6.2[a] (23-46)[b]
Gender			
Male	13	13	26 (59.1%)
Female	9	9	18 (40.9%)
Body mass index (kg/m^2)	23.2 ± 3.0	23.6 ± 4.0	23.4 ± 3.5
Smoking habits			
Cigarettes/day	13.7 ± 5.5 (7-30)	-	-
Years of smoking	11.3 ± 6.6 (2-30)	-	-
Pack/year	7.8 ± 5.1 (1-23)	-	-

[a]: mean ± standard deviation; [b]: range min-max

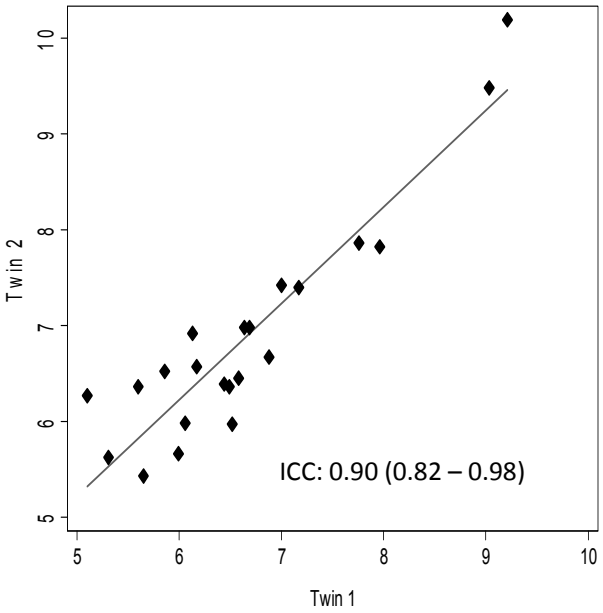
Table II. Values of telomerase activity and telomere length observed in monozygotic twins discordant for smoking habits.

	Relative Telomerase Activity (%)		Telomere Length (kb)	
	Smokers	**Non smokers**	**Smokers**	**Non smokers**
Twin pair				
1	-	-	6.45	6.58
2	51.46	27.74	5.66	5.99
3	20.90	15.15	6.92	6.13
4	24.02	10.89	6.39	6.44
5	12.22	28.90	5.97	6.52
6	15.25	14.29	6.36	6.49
7	-	-	6.52	5.86
8	27.57	19.01	5.62	5.31
9	98.79	40.96	7.86	7.76
10	19.84	46.52	6.98	6.69
11	-	-	7.82	7.96
12	53.03	51.37	7.40	7.17
13	-	-	6.27	5.10
14	55.76	42.59	7.42	7.00
15	-	-	9.48	9.03
16	40.25	35.79	10.19	9.21
17	58.45	17.55	6.57	6.17
18	-	-	6.36	5.60
19	10.14	1.49	5.98	6.06
20	16.09	1.51	5.43	5.65
21	19.29	16.84	6.67	6.88
22	16.99	17.45	6.98	6.64
Mean	33.75	24.25	6.88	6.65
SD	24.09	15.46	1.17	1.07
p value*	**0.03**		**0.04**	

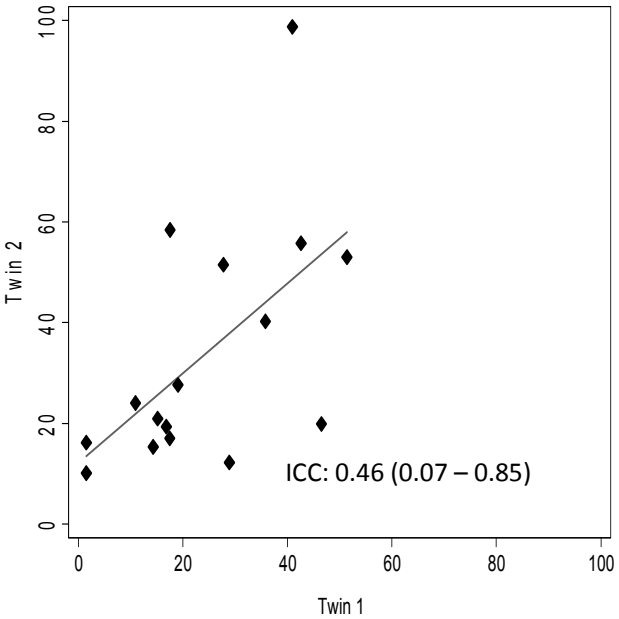
*Wilcoxon signed-rank test; SD: Standard Deviation

Figure 1. Correlation plots of telomerase activity and telomere length within co-twins

A) Telomere length (kb)



B) Relative telomerase activity (%)



ICC: intraclass correlation; in parentheses 95% confidence intervals

Figure 2. Fold increase of relative telomerase activity within twin couples. The black line represents the reference value.

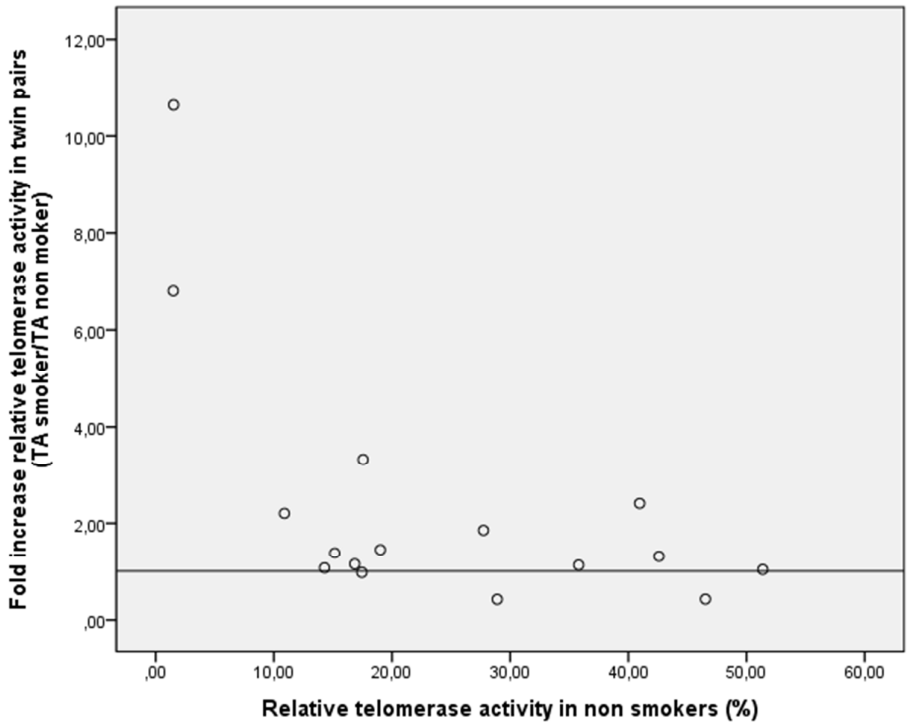


Figure 3. Correlation between telomerase activity and telomere length observed in the study subjects.

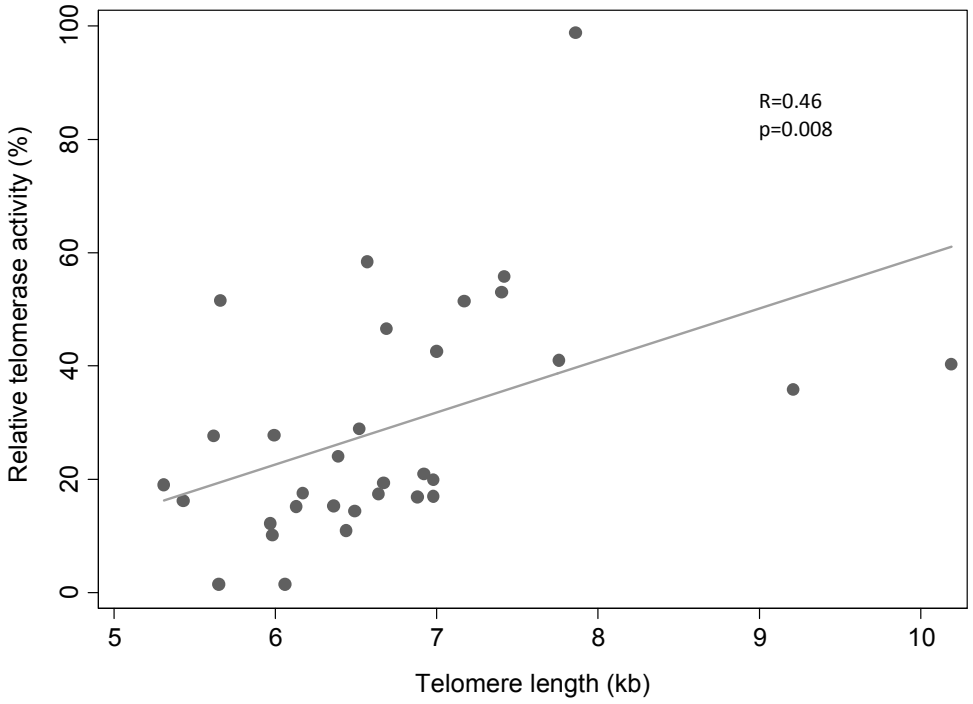


Table III. Multivariate regression analysis: association between telomerase activity, telomere length and twin characteristics.

	Telomerase Activity			Telomere Length		
	β coeff	**P**	**95% CI**	**β coeff**	**P**	**95% CI**
Smoking habits (non smokers)	-11.96	**0.021**	-22.10, -1.82	-0.23	**0.020**	-0.42, -0.04
Age	-0.06	0.890	-0.95, 0.82	0.09	0.093	-0.02, 0.20
Gender (female)	25.91	**0.005**	7.64, 44.18	0.67	0.053	-0.01, 1.34
BMI	2.60	**0.001**	1.02, 4.16	-0.01	0.742	-0.06, 0.04
Costant	-48.67	0.087	-104.39, 7.06	3.45	0.011	0.78, 6.11

BMI: body mass index (kg/m^2); 95%CI: 95% confidence intervals; effect size: estimated from multivariate regression analysis.

Table IV. Differences in mean levels of percentage of *hTERT* DNA methylation (chromosome 5) observed in monozygotic twins discordant for smoking habits.

Probe ID	Position	Mean smokers (β-values)	Mean non smokers (β- values)	Mean Δβ	Effect Size	P-value
cg02048657	1293231	74.83	77.23	-2.40	-2.44	0.0019
cg26221342	1272400	88.91	90.01	-1.09	-1.19	0.0023
cg00675600	1255458	71.63	72.90	-1.27	-2.17	0.0036
cg24881558	1294198	83.60	85.05	-1.45	-1.83	0.0191
cg16429735	1268949	79.03	75.57	3.46	3.30	0.0243
cg05521538	1258994	66.36	68.88	-2.52	-2.11	0.0341
cg22989209	1274847	94.06	92.88	1.18	1.36	0.0401
cg07380026	1296007	74.83	76.46	-1.63	-1.73	0.0493
cg11666982	1293871	84.51	85.51	-1.00	-1.13	0.0528

Figure 4. Correlation between differences in telomerase activity and DNA methylation level of cg05521538 locus observed in each twin pair. Delta is the difference between values recorded in the smoker twin and the non smoker co-twin.