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Short Communication

Effects of cigarette smoking and nicotine metabolite ratio on leukocyte telomere length.

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ABSTRACT

Studies of the effects of smoking on leukocyte telomere length (LTL) using cigarettes smoked per day or pack years smoked (PYS) present limitations. Reported high levels of smoking may not increase toxin exposure levels proportionally. Nicotine metabolism ratio (NMR) predicts total cigarette puff volume and overall exposure based on total N-nitrosamines, is highly reproducible and independent of time since the last cigarette. We hypothesized that smokers with higher NMRs will exhibit increased total puff volume, reflecting efforts to extract more nicotine from their cigarettes and increasing toxin exposure. In addition, higher levels of smoking could cause a gross damage in LTL. The urinary cotinine, 3-OH cotinine and nicotine levels of 147 smokers were analyzed using a LC/MS system Triple-Q6410. LTL and CYP2A6 genotype was determined by PCR in blood samples. We found a significant association between NMR and CYP2A6 genotype. Reduction in LTL was seen in relation to accumulated tobacco consumption and years smoking when we adjusted for age and gender. However, there were no significant differences between NMR values and LTL. In our study the higher exposure was associated with lower number of PYS. Smokers with reduced cigarette consumption may exhibit compensatory smoking behavior that results in no reduced tobacco toxin exposure. Our results suggest that lifetime accumulated smoking exposure could cause a gross damage in LTL rather than NMR or PYS. Nevertheless, a combination of smoking topography (NMR) and consumption (PYS) measures may provide useful information about smoking effects on health outcomes.

1. Introduction

Telomere length is considered a biomarker for aging (Harley et al., 1990; Zakian, 1995). Telomeres are deoxyribonucleic acid (DNA)–protein complexes that cap and stabilize the ends of chromosomes, maintaining genome integrity and protecting from damage. Telomere length is not only related to the basic biology of aging as a trigger of cellular senescence but also reflects the balance between cytotoxic stressors and antioxidant defense mechanisms (Aviv and Susser, 2013; Houben et al., 2008; von Zglinicki et al., 1995). Thus, telomere attrition in circulating white blood cells has been proposed as a marker for cumulative oxidative stress and inflammation and, therefore, biological aging.

Several cross sectional and prospective studies have associated short telomere length with increased risk of cardiovascular disease, pulmonary disease or cancer (Lan et al., 2013; Savale et al., 2009; Wolkowitz et al., 2011). At the same time there are other lifestyle factors such as smoking, obesity, physical inactivity and

Abbreviations: ROS, reactive oxygen species; COT, cotinine; 3´-HCOT, trans-3´-hydroxicotinine; CYP2A6, Cytochrome P450 2A6; NMR, nicotine metabolized ratio; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; LTL, leukocytes telomere length; CPD, cigarettes per day; PYS, pack years smoked; FTND, Fagerstrom Test for Nicotine Dependence; d3-COT, d3-cotinine; LOQ, limit of quantification

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alcohol intake that could be related to short telomere length although some data are conflicting (Chen et al., 2014; Cherkas et al., 2008; Huzen et al., 2014; Weischer et al., 2014).

Human health effects caused by smoking are a potential public health problem. Cigarette smoke contains a large amount of compounds (nicotine, acrolein, formaldehyde, carcinogens), including many oxidants and free radicals that are capable of initiating or promoting oxidative damage (Esterbauer et al., 1991; Voulgaridou et al., 2011). Oxidative damage may result from reactive oxygen species (ROS) generated by the increased and activated phagocytes following cigarette smoke. Cigarette smokers have a higher risk of developing several chronic disorders as cardiovascular diseases, several types of cancer or pulmonary diseases. Therefore, smoking increase levels of oxidative stress, a molecular mechanism for telomere attrition (Huzen et al., 2014; Valdes et al., 2005).

Smokers can extract varying levels of nicotine and other cigarette compounds depending on their smoking topography (puff volume or number of puffs), which reflects the level of toxine exposure (Djordjevic et al., 2000; Strasser et al., 2006).

Nicotine is metabolized to cotinine (COT), and then to trans-3′-hydroxicotinine (3′-HCOT). Mainly, the CYP2A6 enzyme catalyzes the conversion of nicotine to 3′-HCOT. COT has a half-life of 15–20 h compared to nicotine of 0.5–3 h, therefore COT is a better marker to exposure (Benowitz, 1996; Benowitz et al., 2009; Hukkanen et al., 2005a). The ratio of 3′-HCOT/COT, referred as nicotine metabolized ratio (NMR), reflects CYP2A6 activity, environmental factors influencing CYP2A6 and nicotine clearance in vivo (Benowitz et al., 2009; Benowitz, 2010). The ratio has been routinely measured in plasma and urine. The NMR is highly reproducible and independent of time since the last cigarette (Dempsey et al., 2004; Lea et al., 2006). Faster metabolizers of nicotine have higher smoking rates and therefore increased the risk for lung cancer (Boffetta et al., 2006; Derby et al., 2008; Joseph et al., 2005a, 2005b; London et al., 1999; Tyndale and Sellers, 2001; Zhu et al., 2013a, 2013b). Strasser et al., showed that the NMR predicts total cigarette puff volume and carcinogen exposure measured as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) levels (Strasser et al., 2011). Previous studies have shown that telomere length is shorter in peripheral blood leukocytes of smokers compared to nonsmokers (Morla et al., 2006; Rode et al., 2014; Valdes et al., 2005). Most of these assays analyzed leukocytes telomere length (LTL) in lung disease population, reporting an additive association between LTL and smoking on cancer development (Broberg et al., 2005; Wu et al., 2003). On the other hand, the interaction between LTL and smoking history is not clear (Broberg et al., 2005; Walters et al., 2014).

In this study we explored the relationship between accelerating aging in smokers measured as LTL and NMR in addition to cumulative smoking. Telomere length may reflect cumulative exposure to cigarette smoke measured by these biomarkers.

2. Material and methods

2.1. Subject recruitment and sample collection

One hundred and forty-seven healthy smokers (all of Caucasian (Spanish) descent for ≥ 3 generations) between 25 and 65 years of age were recruited from the Occupational Medicine Committee of Banco Popular, Madrid (Spain) from 2010 to 2013. The present genetic analyses were restricted to Caucasian smokers to minimize population stratification as the frequency of these variants varies substantially between populations. Screening of smokers for study inclusion involved a medical history, clinical examination, electrocardiogram, blood and set of lung function tests. Exclusion criteria were not being a smoker and suffer from any illness related to smoking. In addition, we excluded smokers who suffered any disease which affects LTL as cardiovascular disease, cancer, obesity, diabetes, osteoporosis, infectious diseases or chronic psychological stress. Approval was obtained from the local Ethics Committee (Hospital Guadalajara, Guadalajara, Spain) and all patients provided written informed consent in accordance with the principles expressed in Helsinki Declaration.

All participants completed a questionnaire regarding demographic characteristics, smoking habits, self-reported cigarettes per day (CPD), the number of years the person had smoked and cumulative consume as pack years smoked (PYS). Nicotine dependence was assessed with the Fagerstrom Test for Nicotine Dependence (FTND) (Lessov-Schlaggar et al., 2008). In addition, CO levels and lung function (spirometry) were measured in each participant.

On the same day, for the analysis of nicotine metabolites, morning urine samples in sterile bottles were collected and stored at 4 °C until analysis. Peripheral blood samples were obtained by venipuncture and we extracted blood leukocyte DNA from the participants using a standard phenol chloroform protocol. The genotype analyzes were performed in the Biomedicine laboratory of the *Universidad Europea,* Madrid (Spain).

The study followed recent recommendations for replicating genotype–phenotype association studies (NCI-NHGRI Working Group on Replication in Association Studies et al., 2007). Genotyping was performed specifically for research purposes, blood and urine samples were tracked solely with bar-coding and personal identities were only made available to the main study researcher who was not involved in actual genotyping). The DNA samples were diluted with sterile water and stored at −20 °C until analysis.

2.2. Telomere length analysis

Telomere length was measured using a quantitative PCR-based method previously described (Cawthon, 2002; Martinez-Delgado et al., 2011). By this technique telomere length was calculated as a ratio between telomere repeat copy number and a single-copy gene, *36B4*. In order to amplify telomere repeats and the *36B4* gene, we used primers described before (Codd et al., 2010). All samples, for both telomere and *36B4* reactions, were analyzed in triplicate using a Step One Plus (Applied Biosystems, Foster City, CA), in 96 well format. For each sample the relative concentration of both telomere and *36B4* was calculated relative to the calibration sample and PCR efficiency to obtain the ratio, as previously described (Willeit et al., 2010).

2.3. CYP2A6 genotyping

Genotyping of *CYP2A6* (*CYP2A6**1 × 2, *CYP2A6**2 (1799T > A) [rs1801272], *CYP2A6**9 (−48T > G) [rs28399433], *CYP2A6**12) was carried out using genomic DNA isolated from blood samples as previously described (Verde et al., 2011). Subjects were classified into three phenotype groups (very slow, slow and normal-fast metabolizer) according to the *CYP2A6* genotypes, genetically faster metabolizers of nicotine (carriers of two *1 alleles or *1 × 2), slow metabolizers (carriers of *2, *9, and *12) and very slow metabolizers (carriers of two alleles *2, *9, or*12) (Lerman et al., 2007; Verde et al., 2014).

2.4. Biomarkers in urine

Urine (2 ml) was mixed with 2 ml of ammonium acetate buffer (15 mM, pH = 6) followed by the addition of 5 ul of 1000 ng/ml d3-cotinine (d3-COT) and 5 ul of 1000 ng/ml d4-nicotine (as internal

standards). A DSC-MCX SPE cartridge was used for the solid-phase extraction of the subject's urine sample.

SPE cartridges were pre-conditioned with 1 ml each of methanol, ammonium acetate buffer (15 mM, pH=6). The cartridges were then washed with 1 ml of water, 1 ml of 10% methanol and 1 ml of acetic acid (1 M) and analytes were eluted with a mixture of 2 ml of methanol/ammonium hydroxide (98/2) and 2 ml of dichloromethane. The elution product was evaporated to full dryness at 40 °C under a stream of nitrogen. The residue was re-suspended in 500 ul of mobile phase and stored at −20 °C prior to LC/MS/MS analysis.

Nicotine and its metabolites concentrations in urine were measured by a liquid chromatography/mass spectrometry (LC/MS) system TripleQ6410 equipped with MassHunter Workstation software (Agilent USA). A mass analyzer (Agilent USA) was operated in electrospray positive ionization mode and was directly coupled to an Agilent 1200 system (Agilent USA) with a column (Inertsil HILIC, 3 µm, 2.1 mm × 100 mm, GL Sciences, Tokyo, Japan). Typical conditions were as follow: gas temperature, 320 °C; gas flow 11 mL/min, 45 Psi; Capillary voltage 4.0 kV. The gradient mobile phase consisted of 50 mM Ammonium Formate buffer (pH=3.5) (A) and Acetonitrile with 0.1% Formic Acid (B); 0–1.5 min with 77% (B); 1.5–2.0 min with 95% (B); 2–3 min with 90% (B); 3–6 min with 10% (B); 6-6.5 min 10% (B) and 6.6-11 min 77% (B), at a flow rate of 0.25 mL/min.

The fragmentator for precursor ion search and collision energy were set to (90 V and 35 eV) for 3′-HCOT, (100 V and 15 eV) for d3-COT, (100 V and 15 eV) for COT, (80 V and 15 eV) for d4-nicotine and (80 V and 15 eV) for nicotine.

The mass transitions were as follows: for 3′-HCOT (*m/z* 193→80), d3-COT (*m/z* 180→80), COT (*m/z* 177→80), d4-nicotine (*m/z* 167→136) and nicotine (*m/z* 163→136). The limit of quantification (LOQ) with 2 ml of urine was 0.9 ng/ml for cotinine, 1.8 ng/ml for nicotine and 1.5 ng/ml for 3′-HCOT. The limit of detection (LOD) is defined as three standard deviations above the average measured difference between the sample and the black signals (US EPA, 1984). The LOD values were 0.3 ng/ml for cotinine, 0.8 ng/ml for nicotine and 0.75 ng/ml for 3′-HCOT. Recoveries for nicotine and its metabolites were: for COT > 90% and for nicotine and 3′-HCOT > 80%.

2.5. Statistical analysis

Descriptive statistics were presented as mean (SD). ANOVA test was used to compare daily cigarette consumption between the established categories of CO expired and to compare NMR between CYP2A6 genotype. The correlations among exposure variables and LTL were computed using the Pearson's method because most variables followed a normal distribution. ANOVA test was also used to compare smoking habits between telomere length groups. LTL tertiles were used to characterize smokers. We also used logistic regression to analyze interactions between smoking habits and LTL and potential cofounders (age and gender). As categorical variable, telomere length was dichotomized at the 33.3% value in 1st and 2nd–3rd tertiles. The subjects were categorized into two age groups: < 40 and ≥ 40. CPD, years smoking and PYS were also dichotomized. All groups were distributed homogeneously. All statistical analyzes were computed using SPSS 20.0 and statistical significance was set referred to $P < 0.05$ (two-tailed).

3. Results

The study participants smoked on average 12.4 CPD (SD=6.2), 79 (53.7%) of smokers were females and the overall mean age was 43.2 years (SD=11.7). The age distribution did not differ significantly between male's and female's group. They had been smoking for 21.5 years (SD=11.3) with a nicotine dependence score of 2.6 (SD=1.9) and their cumulative smoking mean was 13.5 PYS (SD=11.1).

To verify the number of CPD reported, the levels of CO (ppm) expired were tested in each smoker. As expected, a statistically significant relationship between them was found ($P < 0.001$).

Mean urine NMR value was 1.9 (SD=1.2). Mean values (ng/ml) for nicotine, COT, and 3′-HCOT were 133.7 ng/ml (SD=200.2), 824.7 ng/ml (SD=897.2), 1300.7 ng/ml (SD=1337.7), respectively. Metabolites and NMR values were similar as previously reported (St Helen et al., 2013; Zhu et al., 2013a, 2013b). The relative telomere length ranged from 0.43 and 3.45, with a mean level of 1.33 (SD=0.6).

The analysis of urine metabolites failed in 9 samples due to storage problems. We found significant differences in NMR across *CYP2A6* genotype ($P < 0.01$). Normal-fast metabolizers had higher smoking rates than very slow metabolizers. Mean urine NMR value was 2.37 (SD=1.8) in normal-fast metabolizers, 1.37 (SD=0.6) in slow metabolizers and 0.97 (SD=0.6) in very slow metabolizers (Fig 1). CYP2A6 combined genotypes distributions were: 79.3% normal-fast metabolizers, 13.6% slow metabolizers and 7.1% very slow metabolizers.

The correlations between NMR as continuous variable and various markers in addition to LTL are assessed in Table 1. CPD and PYS correlated to COT levels ($r=0.309$, $P=0.001$; $r=0.266$, $P=0.006$), respectively; the more of each variable, the higher COT levels in urine. As expected, there were a significant negative correlation between COT levels and NMR ($r=-0.288$, $P=0.002$), where higher levels were associated with lower NMR values. Surprisingly, we found a negative correlation between NMR and age, years smoking or PYS ($r=-0.204$, $P=0.028$; $r=-0.278$, $P=0.004$; $r=-0.280$, $P=0.004$), respectively. The higher NMR values were associated with the younger smokers, the lower years smoking and lower number of PYS. On the other hand, there was no significant correlation between relative telomere length as continuous variable and biomarkers. We found a higher NMR in women compared with men ($P=0.003$).

Multicollinearity between several time-related smoking

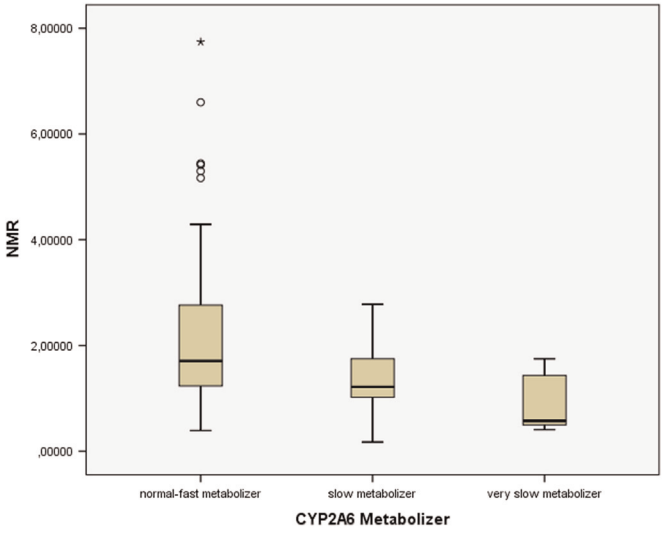

Fig. 1. Association of CYP2A6 genotype and NMR. Note: Subjects were classified into three CYP2A6 metabolizer groups (very slow, slow and normal-fast metabolizer) according to the CYP2A6 genotypes, genetically faster metabolizers of nicotine (carriers of two *1 alleles or *1 × 2), slow metabolizers (carriers of *2, *9, and *12) and very slow metabolizers (carriers of two alleles *2, *9, or*12). Abbreviations: NMR, nicotine metabolized ratio.

Table 1
Pearson correlation coefficients of biomarkers with LTL and other biomarkers.

	Age	Nicotine levels (ng/ml)	Cotinine levels (ng/ml)	Trans-3′-hydroxicotinine levels (ng/ml)	Years smoking	CPD	PYS	FTND	NMR	LTL
Age	1	0.081	0.038	−0.128	0.873	0.109	0.599	−0.013	−0.204	−0.117
	–	0.382	0.680	0.165	< 0.001	0.204	< 0.001	0.875	0.028	0.223
Nicotine levels (ng/ml)		1	0.683	0.535	0.101	0.229	0.203	0.195	−0.204	−0.091
		–	< 0.001	< 0.001	0.304	0.018	0.037	0.044	0.027	0.374
Cotinine levels (ng/ml)			1	0.733	0.079	0.309	0.266	0.223	−0.288	−0.034
			–	< 0.001	0.418	0.001	0.006	0.021	0.002	0.742
Trans-3′-hydroxicotinine (ng/ml)				1	−0.085	0.183	0.068	0.102	0.214	0.018
Years smoking				–	0.388	0.059	0.486	0.249	0.021	0.863
					1	0.152	0.710	0.065	−0.278	−0.087
CPD					–	0.079	< 0.001	0.452	0.004	0.389
						1	0.718	0.747	−0.182	−0.057
						–	< 0.001	< 0.001	0.061	0.571
PYS							1	0.527	−0.280	-0.087
							–	< 0.001	0.004	0.394
FTND								1	−0.181	0.017
								–	0.062	0.863
NMR									1	−0.060
									–	0.558
LTL										1
										–

Note: Each cell contains two values: (a) Pearson correlation coefficient and (b) *P* value of testing if the correlation is significant.
Abbreviations: CPD, cigarettes per day; PYS, pack years smoked; FTND, Fargestrom test for nicotine dependence; NMR, nicotine metabolized ratio; and LTL, leukocyte telomere length.

variables is a common problem. Accordingly, it is necessary to decide which collinear variable should be included. Instead of analyzing only PYS we analyzed PYS and duration, in order to avoid the loss of information. It is proposed that separating intensity and duration may lead to a better fit (Leffondre et al., 2002).

Thereafter, a logistic regression analysis adjusted for age and gender was performed, in order to assess the effect of NMR, PYS and years smoking on the relative LTL. We used 1st tertile value of telomere length as the cutoff between long and short telomeres. The subjects were categorized into two age groups: <40 and ≥ 40. Years smoking were categorized in <20 and ≥ 20. CPD and PYS were also dichotomized at <20, ≥ 20 and <15, ≥ 15, respectively. Analysis showed an association between greater smoking intensity (PYS) and shorter telomere length (OR=2.92, 95% CI=1.09–7.81). Moreover, we observed a significant relationship between years smoking and shorter LTL (OR=3.39, 95% CI=1.28–8.98). On the other hand, there were not association between NMR and shorter telomere length (OR=2.36, 95% CI=0.61–9.16).

4. Discussion

In the present study, we have described variability in the NMR in a Spanish healthy population and have examined factors that affect this ratio, such as CYP2A6 genotype. We have confirmed the association of HCOT/COT ratio with *CYP2A6* genotypes. The rate of nicotine metabolism was lower in smokers with *CYP2A6* alleles associated to decreased activity as previously reported by other authors (Strasser et al., 2007; Strasser et al., 2011). The individuals under study were moderately dependent smokers and women presented higher NMR compared with men ($P=0.003$). This is consistent with previous studies that reported an influence of sex hormones in the metabolism of nicotine and COT (Johnstone et al., 2006). Our study also showed that NMR decreased with age, previous studies have shown that nicotine metabolism is faster in younger smokers (Molander et al., 2001). An impaired renal function and liver blood flow may be expected in older age and thus the NMR level may be expected to vary as the age increases.

In contrast to other findings, we observed a negative correlation between the NMR and PYS (Benowitz et al., 2003). Faster metabolizers presented lower level of nicotine consumption, in opposite, slow metabolizers smoked more quantity. This relationship could be probably due to the joint effect of years smoking and the number of cigarettes smoked per day. Despite of this possible effect, our results are in agreement with previous studies in which individuals slower metabolizers had higher cigarette consumption. Prolonged high levels of nicotine in brain may increase the risk of nicotine dependence due to a tolerance phenomenon (Pomerleau, 1995; Verde et al., 2011).

The NMR is stable biomarker of tobacco smoke exposure and relatively easy to use for assessing metabolizer phenotypes in plasma, serum, saliva or urine (Ho et al., 2009; Rubinstein et al., 2008; Schnoll et al., 2009). Aforementioned biomarker captures genetic and environmental influences. Several approaches have been employed to study the effect of tobacco smoke in LTL (Li et al., 2011; Morla et al., 2006; Savale et al., 2009). Studies have estimated exposure with the reported number of CPD (Kannel, 1981), which has accuracy limitations (Oddoze et al., 1999; Willers et al., 2000). Although asking the number of CPD is actually regarded as the standard measure of exposure, may not be a good indicator. There are many factors that alter the real exposure as individual variability, gender, type of cigarette or the lack of precision reporting the number of CPD (Joseph et al., 2012). In this study, CO levels measurements were used to check CPD reported. Moreover, CPD reported were employed to calculate PYS (accumulated tobacco consumption).

After adjusting for age and gender, reduction in telomere length was seen in relation to accumulated tobacco consumption (PYS) or years smoking. We observed that the smokers with higher accumulated consumption or which have been smoking for more years presented shorter telomere length. LTL shortens with age but it is possible that part of the shortening could be attributed to smoking and no exclusively to ageing. Smoking could lead to more prominent genetic damage in such individuals compared to non-smokers. Moreover long-term smoking could overwhelm telomere shortened in spite of quantity. In view of any relationship between

LTL and cigarettes smoked was not observed, PYS could be related to LTL due to the synergic effect of years smoking and packs smoked.

There is sufficient variability that it may be difficult to predict toxin exposure for an individual based on the number of CPD. Reported high levels of smoking may not increase toxin exposure levels proportionally or at the rate observed at lower levels of smoking. NMR predicts total cigarette puff volume and overall exposure based on total N-nitrosamines 26. N-nitrosamines have been reported to induce oxidative stress and DNA damage (Ahotupa et al., 1987; Clague et al., 2009). The effect of N-nitrosamines on LTL may be due to direct damage on the telomere structure (Li et al., 2011).

Despite of correcting by age and gender, we did not find a statistically significant association between telomere shortening and NMR value in smokers. A possible explanation is the negative correlation between NMR and age that we found. NMR mean was lower in older people. NMR is decreased in the elderly compared to young adults (Molander et al., 2001). However no decrease in CYP2A6 protein levels or nicotine levels in liver microsomes due to age has been detected (Messina et al., 1997). No reduction in telomere length may be attributable to these individual differences between NMR and age. Our study population ranged from 25 to 68 years old, NMR of older smokers could be decreased according to this parameters.

On the other hand, the rate of nicotine metabolism is highly variable between individuals (Hukkanen et al., 2005b). Although there is not a clear relationship between NMR and nicotine dependence or cigarette consumption (Malaiyandi et al., 2006), previous studies showed that smokers maintain plasma nicotine levels by adjusting their smoking behavior (Strasser et al., 2011). We found that smokers with higher NMR values reported lower PYS, suggesting a possible balance between NMR and consumption. This balance could lead to a similar oxidative stress exposure, therefore telomeres could shorten at equivalent rates.

Individuals of the same age have shown a large variability in telomere length, indicating that there are other factors that influence telomere length as well (Martin-Ruiz et al., 2005). Epidemiologic studies have established a strong dose–response relationship between the amount and duration of smoking and various health effects (Alberg et al., 2014; Morla et al., 2006).

According to our results, we consider that lifetime accumulated smoking exposure could cause a gross damage in LTL rather than PYS or NMR. Nevertheless, the number of CPD is not always a reliable measure of toxin exposure and could underestimate tobacco toxin exposure at low levels of smoking or overestimate exposure at high levels of consume (Joseph et al., 2005a, 2005b). CO analysis should be employed to check the number of CPD reported. In addition, NMR analysis is an important goal because even low levels of smoking are associated with significant toxin exposure. For this reason, smoking abstinence is an important goal because even low levels of smoking are associated with significant toxin exposure (Martin-Ruiz et al., 2005).

To our knowledge this is the first study to analyze in smokers a biomarker of nicotine clearance and tobacco exposure, NMR, and its relationship with LTL. Exposure patterns differ significantly from person to person due to differences in how cigarettes are smoked and NMR gives information about toxin exposure. Cigarette smoking causes the majority of lung cancer cases and NMR is associated to lung carcinogens in tobacco (Hecht, 2002; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004; Strasser et al., 2011). Because lung cancer can take years to develop, the identification of practical biomarkers to improve risk assessment would be of great value.

There are some limitations of the study. No reduction in telomere length was seen in relation to NMR, moreover higher exposure (NMR) was associated with lower number of PYS. These findings should also be replicated in larger populations with a narrow age range, moreover including non-smokers and lung tissue samples. In addition, analyzing the influence of smoking-induced DNA-repair genes and telomere length could be helpful to clear the associations. Despite of this, the sample is representative of general population of smokers and we have analyzed NMR and PYS as biomarkers meanwhile other failed studies have analyzed only one measure, CPD.

5. Conclusions

In conclusion, previous studies in general population have reported that LTL shortens physiologically with age but it is possible that part of the shortening could be attributed to smoking and no exclusively to ageing (Morla et al., 2006; Rufer et al., 1999; Samani et al., 2001). In smokers, lifetime accumulated exposure could be more important in telomere shortening than the effect of smoking habits as NMR added to accumulated tobacco consumption.

Nicotine is the major addictive component of tobacco smoke. Smoking behavior affects the tobacco smoke and the related toxic chemicals. Therefore a method for assessing an individual's reliable exposure would facilitate the knowledge of inter-individual differences in risk for tobacco-related diseases. Elucidate these associations could help smokers joining a quit smoking program and/or initiation of preventive treatment. We propose NMR as a biomarker of tobacco exposure in addition to PYS in future designs. Further studies should be carried out to get more information how NMR and PYS influence telomere length, and moreover, how the reduction in LTL affects future disease risk.

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Competing interests

None declared.

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