Original article

Scand J Work Environ Health 2011;37(4):316–324. doi:10.5271/sjweh.3150

N–nitrosamines are associated with shorter telomere length

by Huiqi Li, MSc,[1] Bo AG Jönsson, PhD,[1] Christian H Lindh, PhD,[1] Maria Albin, PhD,[1] Karin Broberg, PhD [1]

Li H, Jönsson BAG, Lindh CH, Albin M, Broberg K. N-nitrosamines are associated with shorter telomere length. *Scand J Work Environ Health.* 2011;37(4):316–324. doi:10.5271/sjweh.3150

Objective Telomeres are critical to maintain the integrity of the chromosomes, and telomere abnormalities are important features of carcinogenesis. Telomere length differs among individuals due to genetic and environmental factors. Aiming to examine the relationship between DNA-damaging agents and average telomere length in peripheral blood, we conducted a cross-sectional study among 157 workers working in the rubber industry in Sweden.

Methods N-nitrosamines were measured in air by personal sampling on Thermosorb/N tubes and analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) for 60 individuals. Based on a similar working situation, the exposure was estimated for all workers. Polycyclic aromatic hydrocarbons (PAH) were measured as the metabolite 1-hydroxypyrene (1-HP) in urine by LC. Carbon disulphide (CS_2) was measured as the metabolite 2-thiothiazolidine-4-carboxylic acid (TTCA) in urine by LC/MS/MS. Toluidines (orto-, meta-, and para-) were measured in urine by gas chromatography (GC)/MS. The average telomere length in peripheral blood was determined by quantitative polymerase chain reaction (PCR).

Results There was a reduction in telomere length with increasing exposure to N-nitrosamines in air [measured (N=60) N-nitrosamines β-coefficient= -10, (95% confidence interval [95% CI] -17– -1.9) P=0.016; estimated (N=157) N-nitrosamines β-coefficient = -5.3, (95% CI -9.5– -0.97) P=0.016]. Also, there were negative associations between para-toluidine [β-coefficient= -0.031 (95% CI -0.055– -0.0063) P=0.014], as well as age [β-coefficient= -0.005 (95% CI -0.007– -0.002) P=0.001] and telomere length. There were no strong associations between other exposures and telomere length nor did smoking modify the effect.

Conclusion N-nitrosamines exposure may lead to telomere shortening.

Key terms aromatic hydrocarbon; carbon disulphide; polycyclic; rubber; toluidine.

Telomeres are specialized DNA-protein structures located at the ends of eukaryotic chromosomes. They consist of a variable number of repeated sequences (TTAGGG) (1). Telomeres are essential regulators of cellular life span (2) and play a key role in maintaining chromosomal stability (3). Short telomere length was related to baseline and mutagen-induced genetic instability (4). Correlations have been observed between short telomeres and human diseases associated with aging, such as cardiovascular diseases (5, 6) and cancer. Short telomere length in peripheral blood appears to be a risk marker for human breast, bladder, head and neck, lung, and renal cell cancers (4, 7–9).

Due to genetic factors, the initial telomere length of a person varies. Telomere length is progressively reduced in most somatic cells due to the end replication problem during cell division (3). Telomeres are successively reduced with age. However, individuals of the same age demonstrate a large variability in telomere length, indicating that there are other factors that influence telomere length as well (10). Apart from progressive reduction in telomere length during cell replication, DNA damage induced by oxidative stress has been suggested as one mechanism involved in accelerated telomere shortening (11, 12). Due to the high content of guanines, telomeres are especially sensitive to the accumulation of reactive oxygen species (ROS)-induced 8-oxo-7, 8-dihydrodeoxyguanosine (8-oxodG) DNA-strand breaks (13, 14). A higher level of 8-oxodG formation in telomeres compared to other non-telomere chromosomes can be induced by exposure to oxidative stress (15). It has also been reported that telomere length shortening can be accelerated by deficient DNA repair capacity (16). Accumulated single-strand breaks, produced by

[1] Department of Laboratory Medicine, Section of Occupational and Environmental Medicine, Lund University, Lund, Sweden.

Correspondence to: Karin Broberg, Section of Occupational and Environmental Medicine, Lund University Hospital, SE-221 85 Lund, Sweden. [E-mail: karin.broberg@med.lu.se]

oxidative stress, have been found to be less efficiently repaired at telomeres than in the rest of the genome. Telomere length could also be shortened by chemicals such as cisplatin, by causing DNA adducts or inhibition of telomerase (17).

It has been described that the workers in the rubber industry have increased risk of developing several diseases, such as coronary heart (18) and airways diseases (19), as well as cancer (20–22). The workers are exposed to a complex mixture of toxic substances. Several of the compounds are known or suspected to be carcinogenic, such as aromatic amines (eg, toluidine), solvents, N-nitrosamines, and polycyclic aromatic hydrocarbons (PAH) (23, 24). Although the exposure levels are reducing year by year, the health effects of these compounds are still worth examining (25). Many compounds in the rubber industry generate oxidative stress and/or DNA adducts [eg, N-nitrosamines, carbon disulphide (CS_2), PAH, and toluidines]. We investigated a cross-sectional study of Swedish rubber workers; this paper reports the relation between exposure to N-nitrosamines, PAH, CS_2, and toluidines, measured in air or by biomarkers of exposure in urine, and mean telomere length in peripheral blood.

Methods

Study subjects

The study comprised 166 exposed workers from 8 different rubber industries in southern Sweden. This was not a random sample from all workers, but a sample of workers from the rubber production industry, who vulcanized with sulfur and agreed to participate in the study. Occupational histories for work in the rubber industry were obtained through interviews and the time of working (months) in the rubber industry was calculated. Individual characteristics of the exposed workers are shown in table 1. Of these workers, 157 workers had information available on telomere length in peripheral blood. For the analysis of urinary biomarkers, urine was collected from exposed workers during the last four hours of an eight-hour work shift. The samples were collected on Tuesdays, Wednesdays, or Thursdays. All biomarkers have short half-lives (26–28) and therefore they can be regarded to be at, or close to, steady-state already on Tuesday afternoon. Thus, the day of sampling will not affect the results. Blood and urine samples were, for the majority of the individuals, obtained within a time period less than half a year from the air measurements, but for 8 individuals samples were obtained 8–12 months after the air measurements. The study subjects gave their informed written consent to take part in the study, and the Regional Ethical Committee of Lund University approved the study.

Table 1. Characteristics of the rubber workers included in the study. [1-HP=1-hydroxypyrene; TTCA=2-thiothiazolidine-4-carboxylic acid.]

	Rubber workers			Telomere length [a]	
	N	Median	Range	Median	Range
Population size	157			0.71	0.16–1.3
Gender					
Male	77			0.70	0.42–1.3
Female	80			0.72	0.16–1.2
Smoking					
Non-smoker	87			0.71	0.16–1.2
Former smoker	19			0.72	0.49–1.1
Smoker	51			0.69	0.42–1.3
Ethnicity					
European	138			0.70	0.16–1.3
Asian	19			0.80	0.42–1.2
Age (year)		38	19–65		
Working time (months)		69	3–408		
N-nitrosamines (µg/m^3)					
Estimated		1.3	0.1–22		
Measured		1.07	0.07–35.5		
1-HP (µmol/mol creatinine)		0.14	0.0020–0.85		
TTCA (µmol/mol creatinine)		24	1.7–690		
Toluidine (ng/ml)					
orto-		0.46	0.025–108		
meta-		0.15	0.025–3.8		
para-		0.090	0.025–4.7		

[a] There were no statistically significant differences between groups.

Exposure assessment

The measured exposure levels and workers' biomarker levels, across different jobs and factories, have been published before for this study population [N-nitrosamines (29), 1-hydroxypyrene (1-HP) (30), and 2-thiothiazolidine-4-carboxylic acid (TTCA) (31)]. The exposure estimates were for N-nitrosamines, based on one air measurement per individual (N=60), and for the biomarkers in urine from one urinary sample per individual (N=157).

N-nitrosamines in the air

N-nitrosamines were measured by personal sampling on Thermosorb/N adsorption tubes (Thermo Fisher Scientific Inc, Walthman, MA, USA) in the breathing zones of 60 rubber workers from 8 companies. The flow rate was 1.5 litre/minute. The work was uniform throughout the 8-hour work shift and, therefore, the sampling was performed only during 3 hours. The samples were stored at +8 °C until analysis. The analyses were performed by liquid chromatography tandem mass spectrometry (LC/MS/MS) as previously described (29). The N-nitrosamines monitored were N-nitrosodimethylamine, N-nitrosodiethylamine,

N-nitrosomorpholine, N-nitrosopiperidine, N-nitrosodi-n-butylamine, N-nitrosopyrrolidine, N-nitrosomethylethylamine and N-nitrosodi-n-propylamine. The sum of N-nitrosamines was used in the calculations.

Biomarkers in urine

Urinary 1-HP is suggested to be the most relevant parameter for estimating individual exposure to PAH (32). The level of 1-HP was analyzed as described (33), by LC (Hewlett-Packard 1050) and fluorescence detection (Hewlett-Packard, Palo Alto, CA, USA) with excitation at 242 nm and emission at 388 nm.

CS_2 is metabolized to TTCA, which has been used as a biomarker of exposure to CS_2 (34). The level of TTCA was analyzed as described (35) by LC/MS/MS.

Orto (o-), meta (m-), and para (p-) toluidine were analyzed by a modified method according to Sennbro et al (36). Aliquots of 1 ml of urine were added with deuterium labeled o-, m- and p-toluidine and 2 ml of 0.3 M sodium hydroxide. The samples were hydrolyzed for 24 hours at 100 °C. Pentane (5 ml) was added and the samples were shaken, centrifuged and then frozen. The pentane phase was derivatized with 20 µl pentafluoropropionic anhydride and then added with 0.5 M phosphate buffer (pH 7.5). The samples were then shaken, frozen and the pentane phase evaporated. The samples were finally dissolved in 200 µl toluene. The levels of toluidine were analyzed by gas chromatography mass spectrometry (GS/MS) in the negative chemical ionization mode using an Agilent 7890A GC (Agilent Technologies, Santa Clara, CA, USA) connected to a 5975C MS and a 7683B auto injector. The column was a J&W DB-5MS (30 m, 0.25 mm ID and 0.25 µm stationary phase). The m/z monitored were for the toluidines 233.2 and for the internal standards 240.2.

It is traditional to adjust 1-HP and TTCA for creatinine content, which was analyzed enzymatically according to Mazzachi et al (37). The concentrations of the toluidines are normally presented unadjusted.

Telomere length analysis

DNA was extracted from peripheral blood with QIAamp 96 DNA blood kit (Qiagen, Hilden, Germany) at the DNA/RNA genotyping Lab, SWEGENE Resource Center for Profiling Polygenic Disease, Lund University, Malmö, Sweden. The DNA samples were then diluted with sterile water to 3.5 ng/µl and stored at -20 °C until analysis.

Relative telomere length quantification was determined by quantitative polymerase chain reaction (PCR) as described in detail (7), based on the method reported by Cawthon (38). The relative length of the telomeres was obtained by calculating the ratio of telomere repeats product and single copy gene product [hemoglobin beta chain (HBG)]. This ratio was then compared with the ratio of a reference DNA. PCR assays for telomere and HBG were always performed in separate 96 wells. An aliquot of 6 µl sample DNA (3 ng/µl) was added to each reaction (end volume 20 µl). A standard curve, a reference DNA and a negative were included in each run. For each standard curve, one calibrator DNA sample was diluted serially by 2-fold per dilution to produce 6 concentrations of 0.625-10 ng/µl. Each sample, standard curve, reference and negative was run in triplicates.

Two master mixes were prepared, one with telomere primers [0.45 µM of each primer (Forward 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTT-GGGTTTGGGTT-3'; Reverse 5'-GGCTTGCCT-TACCCTTACCCTTACCCTTACCCTTACCCT-3'), 0.5U Taq Platina (Invitrogen, Carlsbad, CA, USA), 1×PCR Buffer, 0.8 mM dNTPs, 1.75 mM MgCl2, 0.3mM SybrGreen I (Invitrogen), 1×Rox (Invitrogen)], and one with HBG primers [0.40 µM for each primer (F 5'-TGTGCTGGCCCATCACTTTG-3'; R 5'-ACCAGC-CACCACTTTCTGATAGG-3'), 1×SybrGreen Universal mix (Applied Biosystems; Foster City, CA, USA)].

The PCR was performed on a real-time PCR machine (7900HT, Applied Biosystems). The thermal cycling profile for the telomere amplification was 95 °C for 4 minutes, followed by 30 cycles of 95 °C for 15 seconds and 56 °C for 2 minutes, and for the HBG amplification: 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 54 °C for 1 minute. Standard curves were generated with the Applied Biosystems SDS software 2.3. R^2 for each standard curve was ≥0.99. Standard deviations (for Ct values) were accepted at <0.2.

The ratio (T/S) of the products for telomere repeats and the single copy gene HBG was established by the formula $T/S = 2^{-Ct}$, where $Ct = Ct_{telomere} - Ct_{HBG}$. Included in each run were reference samples that demonstrated a CV of 6.4%, based on 9 runs.

Statistical analysis

In consideration of normally distributed residuals, toluidines (o-, m-, and p-) were converted with the natural logarithm. The first analysis included correlations between age, working time (months), different exposures, and telomere length using Pearson and Spearman's correlation coefficients. Then, all biomarkers were divided into 3 equally sized groups. These exposure categories were used in order to compare the telomere length between low-, medium-, and high-exposure groups, using analysis of variance (ANOVA) and a trend test of Jonckheere Terpstra. However, a data driven categorization of exposure to N-nitrosamines was also performed (figure 1, group 1: <5.0 µg/m^3; group 2: 5.0–20 µg/m^3; group 3: >20µg/m^3).

Thereafter, the effects of the individual characteristics and exposure variables on the telomere length

were estimated as β-coefficients from a general linear model (all exposures were divided by 1000 times to get β-coefficients in a proper scale). Univariate analyses were carried out first, followed by multivariate analyses for adjustments with influential covariates. Potential covariates (gender, ethnicity, age, and working time) were included in multivariate analyses if they were significantly correlated (Spearman's or Pearson correlations) to telomere length.

All statistical analyses were completed by using SPSS 15.0 (SPSS Inc, Chicago, IL, USA) and statistical significance refers to $P<0.05$ (two-tailed).

Results

The relative telomere length ranged from 0.16–1.3, with a median level of 0.71 (table 1). First, the correlations between different variables and telomere length were assessed (table 2). Age, working time, N-nitrosamines, TTCA and p-toluidine correlated to telomere length; the more of each variable, the shorter telomeres. Also, N-nitrosamines were highly correlated with TTCA (positive direction), and weaker with 1-HP (negative); 1-HP and the toluidines were correlated (positive) to each other.

Then, we analyzed the associations between different exposures, categorized in three groups (low-, medium-, and high-exposure) and telomere length. Significant differences between groups (data driven grouping) of estimated N-nitrosamines and measured N-nitrosamines and telomere length were observed (table 3), where higher N-nitrosamines exposure was associated with shorter telomeres. The result of the trend test of Jonckheere Terpstra was also statistically significant for measured N-nitrosamines. There were significant differences between m-toluidine exposure groups as well, but not in a dose-dependent manner.

Thereafter, a univariate linear regression analysis was performed, in order to assess the effect estimates of the different variables on the relative telomere length. Age, working time, N-nitrosamines, TTCA and p-toluidine were all associated with telomere length (table 4). When adjusted for age, only estimated N-nitrosamines, measured N-nitrosamines, and p-toluidine significantly influenced telomere length (table 4). Since working time was strongly correlated to age (Pearson correlation r=0.63), the effect of working time was difficult to disentangle from the age effect on telomere length. To avoid over-adjustment, working time was not included in multivariate analyses.

Discussion

This study demonstrates for the first time an association between exposure to N-nitrosamines and shorter average telomere length in peripheral blood. Also, the results suggest an association between p-toluidine and telomere length.

A strength of this study was the measurement of different markers of DNA-damaging compounds in air and human urine. Also, there was an exposure contrast for the compounds, in particular for N-nitrosamines. Nevertheless, there were some weaknesses to mention. Only 60 individuals were measured for N-nitrosamines in air,

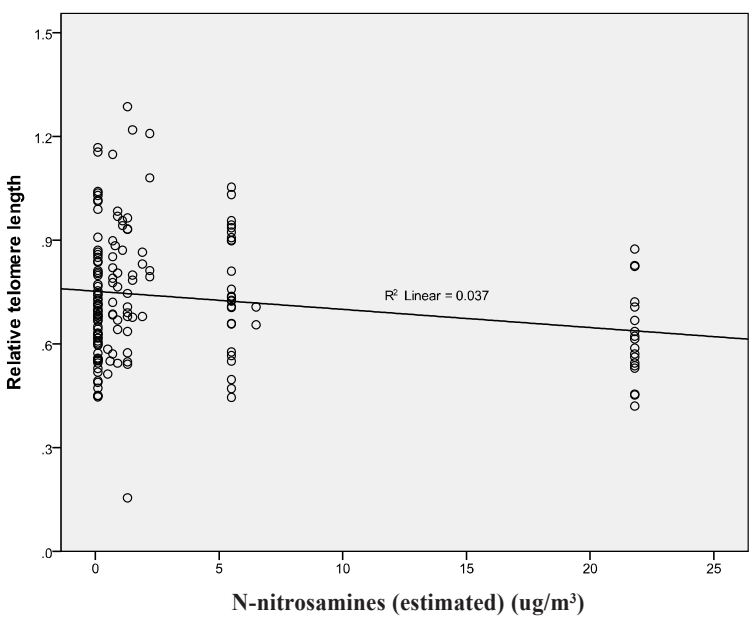

Figure 1. Association between estimated N-nitrosamines and telomere length.

Table 2. Correlations between the telomere length, individual characteristics, and exposure variables. [1-HP=1-hydroxypyrene; TTCA=2-thiothiazolidine-4-carboxylic acid]

	Telomere length	Gender [a]	Smoking [a]	Ethnicity [a]	Age	Working months	N-nitrosamines		1-HP	TTCA	Toluidine [b]		
							Estimated	Measured			orto-	meta-	para-
Telomere length	1.00												
Gender [a]	0.084	1.00											
Smoking [a]	-0.052	0.033	1.00										
Ethnicity [a]	0.0092	0.064	-0.080	1.00									
Age	-0.27 [c]	-0.13	-0.0031	-0.047	1.00								
Working months	-0.18 [d]	-0.11	-0.068	-0.25 [c]	0.63 [c]	1.00							
N-nitrosamines													
Estimated	-0.19 [d]	-0.27[c]	0.11	0.12	0.13	0.12	1.00						
Measured	-0.31 [d]	0.11	-0.074	0.11	0.26 [d]	0.19	0.87 [c]	1.00					
1-HP	-0.011	0.18 [d]	0.284 [c]	-0.096	-0.13	-0.13	-0.26 [c]	-0.21	1.00				
TTCA	-0.17 [d]	-0.12	0.14	-0.0036	0.14	0.20 [d]	0.63 [c]	0.56 [c]	-0.13	1.00			
Toluidine [b]													
orto-	-0.081	-0.19 [d]	0.087	0.20 [d]	0.047	0.023	-0.0054	-0.19	0.24 [c]	-0.0048	1.00		
meta-	-0.11	-0.14	0.33 [c]	-0.13	0.14	0.0074	-0.053	-0.22	0.36 [c]	-0.068	0.47 [c]	1.00	
para-	-0.20 [d]	-0.072	0.26 [c]	0.036	0.067	0.0083	-0.0018	-0.094	0.31 [c]	0.016	0.52 [c]	0.66 [c]	1.00

[a] The correlations between marked and other variables were by Spearman's rho correlation coefficient. The other correlations were based on Pearson correlation coefficient.

[b] orto-, meta-, para-toluidines were converted with the natural logarithm.

[c] Correlation is significant at the 0.01 level (2-tailed).

[d] Correlation is significant at the 0.05 level (2-tailed).

while N-nitrosamines for the remaining individuals were estimated based on work task. However, based on observations on clear and fairly constant differences in exposure conditions for different work tasks during the year of sampling (Ulf Bergendorff, personal communication), we believe that the estimated N-nitrosamines reflect the true levels fairly well. Still, there were quite few individuals with high exposure for N-nitrosamines. We did not have complete information on the occupational history for all workers, apart from time working in the rubber industry. Thus, there is a possibility that there were previous exposures that may influence the telomere length.

Due to the fact that the rubber industry is a complex environment, the association between N-nitrosamines and telomere length may be dependent on exposure to other toxic agents. Of the exposures analyzed in this study, N-nitrosamines correlated to, in particular, the biomarker of CS_2, but the effect of CS_2 was weaker. The biomarkers analyzed were, based on literature search, chosen for representing a group of exposures that compose the main part of the fumes in the Swedish rubber industry. When starting the exposure assessment for vulcanization fumes, we also analyzed toxic compounds (butadiene, styrene, benzene and toluene) in a small subset of samples, but the levels were actually very low (unpublished data). However, we cannot exclude that there may be other exposures in the rubber fumes, not measured here but positively correlating with N-nitrosamines that contribute to telomere shortening. Still, we do not expect historical exposures, such as the established bladder carcinogen beta-naphtylamine (39) to have any strong influence on telomere length in this population. This compound was prohibited in Sweden in 1975 (40), and only a minor fraction of the workers in our study population (11/157) were working before 1975. Furthermore, when excluding those 11 from the analysis, the effects of N-nitrosamines on telomere length was very similar (β= -5.2, P=0.019). Thus, the effect on telomere length from work in the rubber industry probably comes from more recent exposures, such as N-nitrosamines, which can be found in use in rubber industry.

There was a difference in sampling time of blood analyzed for telomere length and air measurements for N-nitrosamines, but the results were in the same direction and level, with and without adjustment for the time difference. The occupational exposure was fairly constant during this period and this is probably not a major bias. The urinary samples were collected on different days (Tuesdays, Wednesdays, or Thursdays). However, all biomarkers measured in this study have short half-lives and therefore they can be regarded to be at, or close to, steady-state already on Tuesday afternoon. Thus, the day of sampling will not substantially affect the results.

The DNA for telomere length determination was extracted from peripheral blood, which is a proxy for the target organ of the effects of the different exposures. The telomeres in whole blood probably mainly reflect telomeres of neutrophils, and to a minor extent lymphocytes, with the former having a lifetime of weeks and the latter weeks to years. Thus, telomere length in blood probably mainly reflects a short term situation. Nevertheless, if the exposures are affecting stem cells of blood, the effect may be pronounced, even after exposure has ceased.

The N-nitrosamines level in this study was higher than previously reported in Netherlands, Germany, UK, Italy,

Table 3. Telomere length in different exposure groups (low, medium and high). [SD=standard deviation; 1-HP=1-hydroxypyrene; TTCA=2-thiothiazolidine-4-carboxylic acid.]

Group	N [a]	Range	Telomere length		P-value [b]	P-trend [c]
			Mean	SD		
N-nitrosamines (µg/m³)						
Estimated					0.04	0.97
Low	64	<0.7	0.72	0.17		
Median	48	0.7–1.5	0.79	0.21		
High	45	>1.5	0.70	0.17		
Measured					0.97	0.97
Low	20	<0.61	0.77	0.22		
Median	20	0.61–2.0	0.76	0.13		
High	20	>2.0	0.75	0.22		
Estimated [d]					0.023	0.075
Low	112	<5.0	0.75	0.19		
Median	27	5.0–20	0.75	0.17		
High	18	>20	0.62	0.13		
Measured [d]					0.028	0.018
Low	52	<5.0	0.78	0.18		
Median	6	5.0–20	0.68	0.20		
High	2	>20	0.45	0.002		
1-HP (µmol/mol creatinine)					0.90	0.98
Low	52	<0.11	0.74	0.19		
Median	52	0.11–0.23	0.72	0.17		
High	51	>0.23	0.74	0.19		
TTCA (µmol/mol creatinine)					0.23	0.22
Low	51	<9.0	0.75	0.20		
Median	51	9.0–40	0.75	0.17		
High	52	>40	0.70	0.17		
Toluidine (ng/ml)						
orto-					0.77	0.70
Low	52	<0.030	0.75	0.18		
Median	50	0.030–0.079	0.72	0.20		
High	50	>0.079	0.73	0.17		
meta-					0.022	0.95
Low	52	<0.010	0.71	0.17		
Median	50	0.010–0.024	0.79	0.19		
High	50	>0.024	0.70	0.16		
para-					0.57	0.15
Low	52	<0.0061	0.75	0.17		
Median	49	0.0061–0.016	0.73	0.18		
High	51	>0.016	0.71	0.19		

[a] The total numbers of individuals included differed between the exposures, since there were different numbers of cases with missing values.
[b] Analysis of variance (ANOVA).
[c] Represents P-trend test of Jonckheere Terpstra.
[d] Data-driven grouping based on the exposure distribution in figure 1.

and Poland (41, 42); and half of the rubber workers in this study exceeded the German target value, the only available exposure limit (31). N-nitrosamines have been reported to induce oxidative stress and DNA damage (43–46), and the main mechanism suggested for N-nitrosamine carcinogenesis is through the formation of DNA adducts that can cause mutations (47, 48). However, most data are derived from tobacco-specific nitrosamines. Also, there is lack of knowledge about the genotoxic effects of N-nitrosamines in vivo for humans. To our knowledge there is only one previous study on the effect of N-nitrosamines on telomere length, in vivo or in vitro. Shimazui et al (49) demonstrated in a rat model of bladder carcinogenesis that exposure to the N-butyl-N-(4-hydroxybutyl) nitrosamine was associated with shorter telomeres. The mechanism for the observation of an association between N-nitrosamines and short telomeres may be through direct damage of the N-nitrosamines on the telomere structure that, if it remains unrepaired, results in shorter telomeres or through N-nitrosamines-induced cell proliferation. However, follow-up studies are warranted for elucidating how the exposure-related reduction in telomere length affects future disease risk. Of note is that shorter average telomere length in peripheral blood is a risk factor for cancer risk (4, 7–9). Also, the shortening of telomeres is associated with an increased number of tumors in mice (50), and telomere length abnormalities have also been implicated in early stages of epithelial carcinogenesis (2).

Table 4. Effect estimates for the associations between individual characteristics, exposures and telomere length. [95% CI= 95% confidence interval; 1-HP=1-hydroxypyrene; TTCA=2-thiothiazolidine-4-carboxylic acid.]

	β-coefficient	95% CI	P-value
Univariate analysis			
Age	-0.0046	-0.0072– -0.0020	0.00068
Working time	-0.00032	-0.00061– -0.000034	0.029
Gender	0.021	-0.037–0.080	0.48
Smoking	-0.0065	-0.039–0.026	0.69
Ethnicity	-0.049	-0.14–0.040	0.28
N-nitrosamines			
Estimated	-5.3	-9.5– -0.97	0.016
Measured	-10	-17– -1.9	0.016
1-HP	-11	-168–146	0.89
TTCA	-0.21	-0.41– -0.0086	0.041
Toluidine [a]			
orto-	-0.010	-0.031–0.010	0.32
meta-	-0.020	-0.048–0.0088	0.17
para-	-0.031	-0.055– -0.0063	0.014
Multivariate analysis [b]			
Working time	-0.000021	-0.00039–0.00034	0.91
N-nitrosamine			
Estimated	-4.4	-8.6– -0.16	0.042
Measured	-8.1	-16– -0.15	0.046
1-HP	-50	-202–102	0.51
TTCA	-0.16	-0.36–0.037	0.11
Toluidine [a]			
orto-	-0.0087	-0.029–0.011	0.39
meta-	-0.013	-0.040–0.015	0.37
para-	-0.028	-0.051– -0.004	0.021

[a] orto-, meta-, para-toluidines were converted with the natural logarithm.
[b] The multivariate analysis was adjusted for age.

There were some indications that the other exposures were associated with reduced telomere length, such as p-toluidine and CS_2 (TTCA). There is little information about p-toluidine and genotoxic effects, as well as cancer risk. Most observations for toluidines concern exposure to o-toluidine, which recently has been associated with an increased risk of bladder cancer (51) and is classified as probable carcinogen (28). CS_2 exposure is associated with increased oxidative stress (52, 53), and, at least at higher exposures, with increased risk of cardiovascular diseases, health effects that also may be mediated by telomere shortening (54, 55). Still, it cannot be ruled out that the effect of CS_2 in our study may be due to the fairly strong correlation to N-nitrosamines. Clearer influence might be found with a larger study population and wider range of exposure. Pavanello and coworkers (56) found an association between PAH exposure (also measured as 1-HP) among coke-oven workers and shorter telomere length. However, the median PAH exposure levels in their study were about 20 times higher (median 3.1, range 0.41–7.5 versus the median of 0.14, range 0.0020–0.85, found in our study) indicating that PAH exposure may shorten telomeres, but at higher exposures than found in the current rubber industry of Sweden. Still, the weaker associations detected may also reflect a true biological difference between CS_2, toluidines, and on the other hand, N-nitrosamines in reducing telomere length.

The effect of estimated working time was also analyzed, which in the univariate analysis was associated with telomere length. However, since working time was strongly correlated to age, the effect of working time was difficult to disentangle from the age effect on telomere length. The lack of effect for working time in the multivariate analysis adjusted for age is probably due to over-adjustment. The association between age and telomere length has been observed in previous studies as well (6, 11). The main ethnic groups among the rubber workers in this study were Europeans and Asians, but we could not detect any difference in telomere length between these groups. We expected to see an association between smoking and telomere length. The lack of association may be due to the fact that the non-smokers are exposed to several substances in the rubber industry that may shorten the telomeres, and therefore, the work-related exposures may blur the effect of the smoking. The effect of smoking should probably rather be analyzed in a referent population with a more non-toxic working environment. However, we cannot rule out that misclassification of smoking status, due to recall bias, could be another possibility for the lack of association.

In conclusion, this study demonstrated that exposure to air N-nitrosamines was associated with reduced telomere length in the rubber industry. Further studies should be carried out to get more information about how other exposures may influence telomere length, and moreover, how the reduction in telomere length affects future occupational disease risk.

Acknowledgements

This work was supported by the Swedish Labor Market Insurance Company (AFA), the Swedish Council for Working Life and Social Research, the Erik Philip-Sörensen Foundation; the Swedish Research Council; and Skane county council's research and development foundation, Sweden. We thank Åsa Amilon, Birgitta Björk, Helene Ottosson, for collecting air samples, Karin Paulsson for telomere length determination, and Ulf Bergendorf for exposure assessment.

References

1. Blackburn EH. Structure and function of telomeres. Nature. 1991;18(350): 569–73. doi:10.1038/350569a0.
2. Raynaud CM, Sabatier L, Philipot O, Olaussen KA, Soria JC. Telomere length, telomeric proteins and genomic instability during the multistep carcinogenic process. Crit Rev Oncol Hematol. 2008;66:99–117.doi:10.1016/j.critrevonc.2007.11.006.

3. Blasco MA. Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet. 2005 Aug; 6: 611–22. doi:10.1038/nrg1656.

4. Wu X, Amos CI, Zhu Y, Zhao H, Grossman BH, Shay JW, et al. Telomere dysfunction: a potential cancer predisposition factor. J Natl Cancer Inst. 2003; 20(95):1211–8. doi:10.1093/jnci/djg011.

5. Balasubramanyam M, Adaikalakoteswari A, Monickaraj SF, Mohan V. Telomere shortening & metabolic/vascular diseases. Indian J Med Res. 2007; 125:441–50.

6. Brouilette SW, Moore JS, McMahon AD, Thompson JR, Ford I, Shepherd J, et al. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study. Lancet. 2007;13(369):107–14. doi:10.1016/S0140-6736(07)60071-3.

7. Broberg K, Bjork J, Paulsson K, Hoglund M, Albin M. Constitutional short telomeres are strong genetic susceptibility markers for bladder cancer. Carcinogenesis. 2005;26:1263–71. doi:10.1093/carcin/bgi063.

8. Jang JS, Choi YY, Lee WK, Choi JE, Cha SI, Kim YJ, et al. Telomere length and the risk of lung cancer. Cancer Sci. 2008;99:1385–9. doi:10.1111/j.1349-7006.2008.00831.x.

9. Shen J, Terry MB, Gurvich I, Liao Y, Senie RT, Santella RM. Short telomere length and breast cancer risk: a study in sister sets. Cancer Res. 2007;1(67):5538–44. doi:10.1158/0008-5472.CAN-06-3490.

10. Martin-Ruiz CM, Gussekloo J, van Heemst D, von Zglinicki T, Westendorp RG. Telomere length in white blood cells is not associated with morbidity or mortality in the oldest old: a population-based study. Aging Cell. 2005;4: 287–90. doi:10.1111/j.1474-9726.2005.00171.x.

11. Shen J, Gammon MD, Terry MB, Wang Q, Bradshaw P, Teitelbaum SL, et al. Telomere length, oxidative damage, antioxidants and breast cancer risk. Int J Cancer. 2009;1(124):1637–43. doi:10.1002/ijc.24105.

12. von Zglinicki T. Oxidative stress shortens telomeres. Trends Biochem Sci. 2002;27:339–44. doi:10.1016/S0968-0004(02)02110-2.

13. Houben JM, Moonen HJ, van Schooten FJ, Hageman GJ. Telomere length assessment: biomarker of chronic oxidative stress? Free Radic Biol Med. 2008; 1(44):235–46. doi:10.1016/j.freeradbiomed.2007.10.001.

14. Kawanishi S, Oikawa S. Mechanism of telomere shortening by oxidative stress. Ann N Y Acad Sci. 2004;1019 278–84. doi:10.1196/annals.1297.047.

15. Oikawa S, Tada-Oikawa S, Kawanishi S. Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening. Biochemistry. 2001;17(40):4763–8. doi:10.1021/bi002721g.

16. Kruk PA, Rampino NJ, Bohr VA. DNA damage and repair in telomeres: relation to aging. Proc Natl Acad Sci U S A. 1995;3;92:258–62.doi:10.1073/pnas.92.1.258.

17. Jamieson ER, Lippard SJ. Structure, Recognition, and Processing of Cisplatin-DNA Adducts. Chem Rev. 1999;8(99):2467–98. doi:10.1021/cr980421n.

18. Gustavsson P, Hogstedt C, Holmberg B. Mortality and incidence of cancer among Swedish rubber workers, 1952-1981. Scand J Work Environ Health. 1986;12(6):538–44.

19. Jonsson LS, Broberg K, Axmon A, Jonsson BA, Littorin M. Symptoms and immunologic markers among vulcanization workers in rubber industries in southern Sweden. Scand J Work Environ Health. 2007;33(4):272–9.

20. Kogevinas M, Sala M, Boffetta P, Kazerouni N, Kromhout H, Hoar-Zahm S. Cancer risk in the rubber industry: a review of the recent epidemiological evidence. Occup Environ Med. 1998;55:1–12. doi:10.1136/oem.55.1.1.

21. Reulen RC, Kellen E, Buntinx F, Brinkman M, Zeegers MP. A meta-analysis on the association between bladder cancer and occupation. Scand J Urol Nephrol Suppl. 2008:64–78. doi:10.1080/03008880802325192.

22. de Vocht F, Sobala W, Wilczynska U, Kromhout H, Szeszenia-Dabrowska N, Peplonska B. Cancer mortality and occupational exposure to aromatic amines and inhalable aerosols in rubber tire manufacturing in Poland. Cancer Epidemiol. 2009;33:94–102. doi:10.1016/j.canep.2009.06.013.

23. International Agency for Research on Cancer (IARC). Rubber industry (The). Lyon:IARC;1998. IARC monographs on the evaluation of carcinogenic risks to humans, volume 28.

24. Peters S, Talaska G, Jonsson BA, Kromhout H, Vermeulen R. Polycyclic aromatic hydrocarbon exposure, urinary mutagenicity, and DNA adducts in rubber manufacturing workers. Cancer Epidemiol Biomarkers Prev. 2008;17: 1452–9. doi:10.1158/1055-9965.EPI-07-2777.

25. de Vocht F, Vermeulen R, Burstyn I, Sobala W, Dost A, Taeger D, et al. Exposure to inhalable dust and its cyclohexane soluble fraction since the 1970s in the rubber manufacturing industry in the European Union. Occup Environ Med. 2008;65:384–91. doi:10.1136/oem.2007.034470.

26. Buchet JP, Gennart JP, Mercado-Calderon F, Delavignette JP, Cupers L, Lauwerys R. Evaluation of exposure to polycyclic aromatic hydrocarbons in a coke production and a graphite electrode manufacturing plant: assessment of urinary excretion of 1-hydroxypyrene as a biological indicator of exposure. Br J Ind Med. 1992;49:761–8.

27. Chang HY, Chou TC, Wang PY, Shih TS. Biological monitoring of carbon disulphide: kinetics of urinary 2-thiothiazolidine-4-carboxylic acid (TTCA) in exposed workers. Toxicol Ind Health. 2002;18:1–14. doi:10.1191/0748233702th125oa.

28. International Agency for Research on Cancer (IARC). ortho-TOLUIDINE. In: Some Aromatic Amines, Organic Dyes, and Related Exposures. Lyon: IARC;2010. IARC monographs on the evaluation of carcinogenic risks to humans, volume 99.

29 Jonsson LS, Lindh CH, Bergendorf U, Axmon A, Littorin M, Jonsson BA. N-nitrosamines in the southern Swedish rubber industries - exposure, health effects, and immunologic markers. Scand J Work Environ Health. 2009;35(3): 203–11.

30. Jonsson LS, Broberg K, Axmon A, Bergendorf U, Littorin M, Jonsson BA. Levels of 1-hydroxypyrene, symptoms and immunologic markers in vulcanization workers in the southern Sweden rubber industries. Int Arch Occup Environ Health. 2008;82:131–7. doi:10.1007/s00420-008-0310-8.

31. Jonsson LS, Broberg K, Bergendorf U, Axmon A, Littorin M, Jonsson BA. Levels of 2-thiothiazolidine-4-carboxylic acid (TTCA) and effect modification of polymorphisms of glutathione-related genes in vulcanization workers in the southern Sweden rubber industries. Int Arch Occup Environ Health. 2007;80: 589–98. doi:10.1007/s00420-007-0171-6.

32. Dor F, Dab W, Empereur-Bissonnet P, Zmirou D. Validity of biomarkers in environmental health studies: the case of PAHs and benzene. Crit Rev Toxicol. 1999;29:129–68. doi:10.1080/10408449991349195.

33. Jongeneelen F, Anzion RBM. 1-Hydroxypyrene. In: Angerer J, Schaller K-H, editors.Analysis of hazardous substances in biological materials. Weinheim: Wiley;1991.

34. Drexler H, Goen T, Angerer J, Abou-el-ela S, Lehnert G. Carbon disulphide. I. External and internal exposure to carbon disulphide of workers in the viscose industry. Int Arch Occup Environ Health. 1994;65:359–65. doi:10.1007/BF00383244.

35. Vermeulen R, Jonsson BA, Lindh CH, Kromhout H. Biological monitoring of carbon disulphide and phthalate exposure in the contemporary rubber industry. Int Arch Occup Environ Health. 2005;78:663–9. doi:10.1007/s00420-005-0017-z.

36. Sennbro CJ, Lindh CH, Tinnerberg H, Gustavsson C, Littorin M, Welinder H, et al. Development, validation and characterization of an analytical method for the quantification of hydrolysable urinary metabolites and plasma protein adducts of 2,4- and 2,6-toluene diisocyanate, 1,5-naphthalene diisocyanate and 4,4'-methylenediphenyl diisocyanate. Biomarkers. 2003;8:204–17. doi:10.1080/1354750031000090660.

37. Mazzachi BC, Peake MJ, Ehrhardt V. Reference range and method comparison studies for enzymatic and Jaffe creatinine assays in plasma and serum and early morning urine. Clin Lab. 2000;46:53–5.

38. Cawthon RM. Telomere measurement by quantitative PCR. Nucleic Acids Res. 200215;30:e47.

39. International Agency for Research on Cancer (IARC). 2-NAPHTHYLAMINE. In: Some aromatic amines, organic dyes, and related exposures. Lyon: IARC;2010. IARC monographs on evaluation of carcinogenic risks to humans, vol 99.

40. Arbetarskyddsstyrelsens anvisningar: Hygieniska gränsvärden. [Swedish Work Environment Authority Provisions] 1974: nr. 100, Stockholm.

41. de Vocht F, Burstyn I, Straif K, Vermeulen R, Jakobsson K, Nichols L, et al. Occupational exposure to NDMA and NMor in the European rubber industry. J Environ Monit. 2007;9:253–9. doi:10.1039/b615472g.

42. Iavicoli I, Carelli G. Evaluation of occupational exposure to N-nitrosamines in a rubber-manufacturing industry. J Occup Environ Med. 2006;48:195–8. doi:10.1097/01.jom.0000200938.96781.64.

43. Ahotupa M, Bussacchini-Griot V, Bereziat JC, Camus AM, Bartsch H. Rapid oxidative stress induced by N-nitrosamines. Biochem Biophys Res Commun. 1987;14(146):1047–54. doi:10.1016/0006-291X(87)90753-4.

44. Robichova S, Slamenova D, Chalupa I, Sebova L. DNA lesions and cytogenetic changes induced by N-nitrosomorpholine in HepG2, V79 and VH10 cells: the protective effects of Vitamins A, C and E. Mutat Res. 2004;13 (560):91–9.

45. Robichova S, Slamenova D, Gabelova A, Sedlak J, Jakubikova J. An investigation of the genotoxic effects of N-nitrosomorpholine in mammalian cells. Chem Biol Interact. 2004;20(148):163–71. doi:10.1016/j.cbi.2004.06.002.

46. Clague J, Shao L, Lin J, Chang S, Zhu Y, Wang W, et al. Sensitivity to NNKOAc is associated with renal cancer risk. Carcinogenesis. 2009;30: 706–10. doi:10.1093/carcin/bgp045.

47. Hecht SS. Recent studies on mechanisms of bioactivation and detoxification of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific lung carcinogen. Crit Rev Toxicol. 1996;26:163–81. doi:10.3109/10408449609017929.

48. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer. 2003;3:733–44. doi:10.1038/nrc1190.

49. Shimazui T, Ami Y, Miyanaga N, Ideyama Y, Nakahara T, Akaza H. Telomerase is upregulated in irreversible preneoplastic lesions during bladder carcinogenesis in rats. Jpn J Cancer Res. 2002;93:495–500.

50. Hande MP, Samper E, Lansdorp P, Blasco MA. Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. J Cell Biol. 1999;22(144):589–601. doi:10.1083/jcb.144.4.589.

51. Carreon T, Hein MJ, Viet SM, Hanley KW, Ruder AM, Ward EM. Increased bladder cancer risk among workers exposed to o-toluidine and aniline: A reanalysis. Occup Environ Med. 2010;67(5):348-50. Epub 2009 Nov 2. doi:10.1136/oem.2009.051136.

52. Wronska-Nofer T, Chojnowska-Jezierska J, Nofer JR, Halatek T, Wisniewska-Knypl J. Increased oxidative stress in subjects exposed to carbon disulfide (CS2)--an occupational coronary risk factor. Arch Toxicol. 2002;76: 152–7. doi:10.1007/s00204-001-0311-9.

53. Wronska-Nofer T, Nofer JR, Stetkiewicz J, Wierzbicka M, Bolinska H, Fobker M, et al. Evidence for oxidative stress at elevated plasma thiol levels in chronic exposure to carbon disulfide (CS2) and coronary heart disease. Nutr Metab Cardiovasc Dis. 2007;17:546–53. doi:10.1016/j.numecd.2006.03.002.

54. Bekaert S, De Meyer T, Rietzschel ER, De Buyzere ML, De Bacquer D, Langlois M, et al. Telomere length and cardiovascular risk factors in a middle-aged population free of overt cardiovascular disease. Aging Cell. 2007; 6:639–47. doi:10.1111/j.1474-9726.2007.00321.x

55. Fitzpatrick AL, Kronmal RA, Gardner JP, Psaty BM, Jenny NS, Tracy RP, et al. Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. Am J Epidemiol. 2007;1(165):14–21.

56. Pavanello S, Pesatori AC, Dioni L, Hoxha M, Bollati V, Siwinska E, et al. Shorter telomere length in peripheral blood lymphocytes of workers exposed to polycyclic aromatic hydrocarbons. Carcinogenesis. 2010;31:216–21. doi:10.1093/carcin/bgp278.

Received for publication: 7 August 2010