Exposure, telomere length, and cancer risk

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2014

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DOCTORAL DISSERTATION
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To be defended at Pufendorf Institute Sölvegatan 2,Lund.

09:00 on 14 February 2014.

Faculty opponent

Göran Roos

Organization
LUND UNIVERSITY
Division of Occupational and Environmental Medicine
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Document name
DOCTORAL DISSERTATION
Date of issue 2014-01-20
Sponsoring organization

Title and subtitle Exposure, telomere length, and cancer risk

Abstract
Telomeres are tandem repeats of TTAGGG at the end of eukaryotic chromosomes. Telomeres play a key role in chromosome stability and regulation of the cellular lifespan. Telomeres are shortened during cell division, and probably, by not yet well characterised factors in the environment. Short telomeres in peripheral blood have repeatedly been associated with increased risk of various types of cancer, as well as with cardiovascular diseases, diabetes, and lung diseases. The aim of this thesis was to explore the effect of exposures on telomere length in different occupational or environmental settings, and also to investigate the association between telomere length and chromosomal aberrations in blood, a biomarker for cancer risk. Different study populations were recruited to elucidate specific hypothesises: In the first study 157 workers in rubber industry were recruited to investigate the effect of exposure to rubber fumes on telomere length; in the second study 101 welders, 34 diesel-exposed workers and 127 controls were examined to elucidate the effect of exposure to particles on telomere length; in the third study 202 women exposed to arsenic via drinking water were analysed for effects of arsenic exposure on telomere length; and finally, in the fourth study 364 male adults were recruited to study the association between telomere length and chromosomal instability. Associations between exposures and telomere length were found: N-nitrosamines were related to shorter telomeres, whereas welding fumes and diesel exhaust showed no significant impact on telomere length. Arsenic in drinking water was related to longer telomeres and the association between arsenic and telomere length was modified by polymorphisms in the main arsenic-metabolizing gene. Although telomere length was associated with chromosome instability, no significant association was found between telomere length and cancer risk in our study, probably due to the limited number of cancer cases. We also found effects of exposure on methylation of DNA, and in turn with chromosome instability, reflecting interactions of the environment with epigenetic processes. The findings of the thesis provide evidence that some exposures, at workplaces or in the general environment, influence the average telomere length in peripheral blood. Since telomeres are key components for genomic stability and often altered during malignant transformation, it is likely that the effect of the exposures on telomeres found here reflect a mechanism of carcinogenesis for the compounds studied.

Key words exposure, telomere, methylation, chromosomal aberrations, cancer.

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language English

ISSN and key title
1652-8220 Lund University, Faculty of Medicine Doctoral Dissertation Series 2014:17

ISBN
978-91-87651-41-0

Recipient's notes

Number of pages 126

Price

Security classification

Signature Huiqi Li Date 2014-01-10

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ISBN 978-91-87651-41-0
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2014

List of papers

This thesis is based on the following papers, which are presented at the end of the thesis.

I. Li H., Jonsson B. A., Lindh C. H., Albin M. and Broberg K., 2011. *N-nitrosamines are associated with shorter telomere length*. Scand J Work Environ Health 37(4): 316-324.

II. Li H., Hedmer M., Wojdacz T., Hossain M. B., Tinnerberg H., Lindh C. H., Albin M., and Broberg K. *Telomere length, 8-oxodG and DNA methylation of tumour suppressor genes in workers exposed to welding fumes or diesel exhaust*. Manuscript

III. Li H., Engstrom K., Vahter M. and Broberg K., 2012. *Arsenic exposure through drinking water is associated with longer telomeres in peripheral blood*. Chem Res Toxicol 25(11): 2333-2339.

IV. Li H., Hilmarsen H. T., Hossain M. B., Bjork J., Hansteen I. L., Albin M., Furu Skjelbred C. and Broberg K., 2013. *Telomere length and LINE1 methylation is associated with chromosomal aberrations in peripheral blood*. Genes Chromosomes Cancer 52(1): 1-10.

Contents

Introduction

Biomarkers for environmental-induced cancer

Various exposures, both occupational and environmental, are known to be carcinogenic. For instance, outdoor air pollution has recently been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC), which is an organization working on identifying the carcinogenicity of exposures based on a review of scientific literature (IARC 2013). It is reasonable to speculate that different mechanisms are involved in the carcinogenesis of different exposures. In order to understand the mechanisms, molecular cancer epidemiology can be a very useful tool to improve our understanding of the pathogenesis of cancer by identifying the pathways, proteins and genes influencing the risk of developing cancer, and thus to find suitable biomarkers for early-stage changes of cancer development.

One good example here is chromosomal aberrations. Chromosomal aberrations are a characteristic of many tumor cells as some have been linked to cancer causation (Mitelman et al. 2007; Ozery-Flato et al. 2011). It was found that a higher frequency of chromosomal aberrations in peripheral lymphocytes was prospectively related to higher cancer risk (Nordenson et al. 1984; Hagmar et al. 1994; Bonassi et al. 2000; Hagmar et al. 2004; Bonassi et al. 2008), and that some genotoxic carcinogens were associated with high frequency of chromosomal aberrations (Andersson et al. 1980; Zapata-Gayon et al. 1982; Rossner et al. 2002; Bocskay et al. 2005; Fucic et al. 2007). Based on the concept that lymphocytes can reflect similar genetic damage as in cells undergoing carcinogenesis, it is reasonable to infer that chromosomal aberrations are involved in the mechanism of carcinogenesis for those exposures.

There are many different types of exposures related to increased cancer risk, but the picture is unclear regarding mechanisms, as well as the dose-effect relationships. In addition, many studies have tried to find biomarkers which appear even earlier than chromosomal aberrations. During the late twentieth century researchers found that the telomeres could be of great importance for chromosome stability.

Telomeres

Telomeres are DNA-protein complexes at the end of eukaryotic chromosomes. They consist of hundreds to thousands of tandem repeats of 5'-TTAGGG-3' (Figure 1) (Blackburn 1991). The average length of human telomeres is usually 10-15 kb in somatic cells, but it can vary substantially between individuals. The protein part, which is known as shelterin, provides protection of the integrity of telomeres (de Lange 2005). Telomeres should be longer than a certain length, together with the integral shelterin complex, to perform proper telomere functioning (Chan and Blackburn 2002). Telomeres play a key role in keeping chromosomal stability by protecting chromosome ends from chromosome fusions or being recognized as strand breaks (Blackburn 2010).

Telomeres are shortened by each cell division, due to the "end-replication problem". Right after understanding the nature of the DNA polymerases, it was predicted that linear DNA molecules could not be completely replicated at the termini by the DNA polymerase. If there would be no protection of the ends of the chromosomes, the ends would be eroded and eventually lead to loss of genetic information and prevent cells from continuing to divide (Olovnikov 1973). Fortunately, there are telomeres to protect the ends of the chromosomes and telomeric repeats are eroded by each DNA replication. In general, during each cell cycle, 50-100 bp of telomeric DNA are deleted from the ends of every chromosome. Therefore, telomere length decreases along with age, which has been observed in telomere length in blood in many population-based studies (Frenck et al. 1998; Svenson et al. 2011).

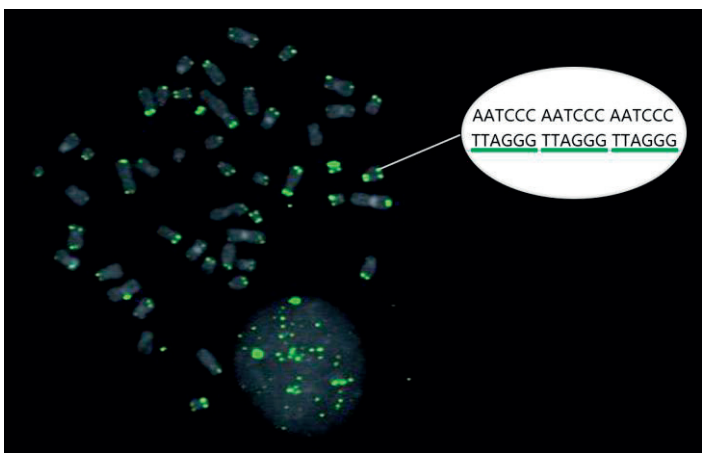

Figure 1. Telomeres are tandem repeats (TTAGGG) at the end of eukaryotic chromosomes. Picture captured by fluorescent *in situ* hybridization.

However, stem and cancer cells need to be able to keep dividing for a large number of cell cycles. Most of the stem cells and 85-90% of cancer cells achieve it by up-regulating expression of telomerase, an enzyme that consists of a reverse transcriptase, encoded by the *TERT* gene, and a RNA template, encoded by the *TERC* gene (Dhaene et al. 2000; Blasco 2005). Telomerase can synthesize hexameric (TTAGGG) repeats and add them to chromosome ends, to compensate for the erosion of telomeres (Chan and Blackburn 2002). On the other hand, in a minor group of cells, telomeres can be maintained or lengthened even when telomerase is absent. The mechanism involved was named as alternative lengthening of telomeres (ALT) (Dunham et al. 2000; Muntoni and Reddel 2005). ALT pathway is generally meditated by homologous recombination. Human ALT-positive cells are usually recognized by the presence of both unusually long and short telomeres and a specific ALT-associated promyelocytic leukemia body (APB) (Nabetani and Ishikawa 2011).

Telomere dysfunction, either with short telomeres or long telomeres, has been associated with diseases. Short telomeres are associated with various age-related diseases, including cardiovascular disease (Brouilette et al. 2007; Willeit et al. 2010; Fyhrquist and Saijonmaa 2012; Khan et al. 2012; Sabatino et al. 2012; Olivieri et al. 2013), diabetes (Zhao et al. 2013), lung diseases (Gansner and Rosas 2013), and different types of cancer such as bladder, lung, breast, colon, head and neck, renal and skin cancer (Broberg et al. 2005; Svenson et al. 2008; Svenson and Roos 2009; Hofer et al. 2011; Ma et al. 2011; Nan et al. 2011; Willeit et al. 2011; Winnikow et al. 2012; Anic et al. 2013). On the other hand, long telomere length has been found to be related to melanoma, lung cancer, pancreatic cancer and soft tissue sarcoma (Anic et al. 2013; Lan et al. 2013; Lynch et al. 2013; Xie et al. 2013). The reason behind this binary association has not been completely understood. Firstly, poor study designs and different telomere measurement techniques may hamper the reliability of the study results. Secondly, it could be the shortest telomere rather than the average telomere length which is critical in the process of cancer development. Moreover, it is also possible that telomeres shall be kept at a proper range to minimize the risk of developing cancer; imbalance of telomere homeostasis, either too short or too long, could lead to increased cancer risk. It has been reported that telomere length in cells with different origin correlates well within the individual (Friedrich et al. 2000; Gadalla et al. 2010). Therefore, telomere length in surrogate tissues such as peripheral blood, sputum, and buccal cells, has been suggested to be a valuable biomarker to evaluate telomere-related cancer risk. Telomere length in surrogate tissues has also been considered as a potential tool for early screening of high cancer risk.

Apart from the natural process of shortening during cell division, telomeres also appear to be sensitive to other factors. A shorter average telomere length as measured in peripheral blood has been associated with increasing age, the male

sex, obesity, dyslipidemia, sedentary lifestyle, mental stress, low socioeconomic status, smoking, and poor diet (Shiels et al. 2011; Fyhrquist and Saijonmaa 2012). The mechanisms of telomere shortening may involve increased oxidative stress, chronic inflammation, DNA damage, and deficient DNA repair capacity (Kruk et al. 1995; Oikawa et al. 2001; von Zglinicki 2002; Kawanishi and Oikawa 2004; Scharrer et al. 2007; Houben et al. 2008; Shen et al. 2009). Since the sequence of telomere repeats is G-rich, it is prone to oxidative modifications of guanine (von Zglinicki 2002; Kawanishi and Oikawa 2004). When this project started in 2009, the relationship between shorter average telomere length in blood and increased risk of various cancers was fairly well studied, but little was known about factors in the environment that induce telomere dysfunction. Rigolin et al. found significantly shorter telomeres in workers exposed to pesticides or organic solvents (Rigolin et al. 2004), but it was only a by-product of a study aiming for association between telomere dysfunction and myelodysplastic syndromes, and the classification of exposure was rough.

Other DNA alterations

There are several types of DNA alterations related to telomere length or cancer risk that have been analyzed in this thesis and some of the their features a presented here.

8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)

Oxidative stress has been suggested to be one of the most important mechanisms of telomere shortening (von Zglinicki 2002; Kawanishi and Oikawa 2004; Valdes et al. 2005). Oxidative stress reflects the imbalance between antioxidants and reactive oxygen species (ROS). At normal conditions, ROS are involved in regulating cell functions due to their roles in several signal transduction cascades. ROS are by-products of the normal metabolism of oxygen and excess amounts of ROS are scavenged by antioxidants. However, many compounds in the environment are able to induce increased ROS levels, and thus, can result in so called oxidative stress. Excessive oxidative stress can cause damage to proteins, lipids, and DNA. If the damages are not repaired efficiently, malfunction of those macromolecules can lead to symptoms. For instance, unrepaired DNA damage by oxidative stress can lead to mutations or chromosomal aberrations, which may result in carcinogenesis.

There are many different markers for oxidative stress, and one of them is 8-oxodG. Hydroxyl radicals and 2'-deoxyguanosine can form a lesion, and when repaired,

the repair products including 8-oxodG is excreted in urine. 8-oxodG is generally considered as a marker for oxidative stress (Cooke et al. 2009), but its relationship with telomere length is not well studied.

DNA methylation

DNA methylation of the cytosine in front of a guanine is a key repressor of gene expression. Although global methylation is generally decreased in carcinogenesis, hypermethylation of tumour suppressor genes is often found as an early change during carcinogenesis (Feinberg and Vogelstein 1983; Feinberg et al. 1988; Robertson and Jones 2000; Gaudet et al. 2003; Chalitchagorn et al. 2004; Deng et al. 2006; Esteller 2008). Repetitive elements, such as long interspersed nuclear elements (*LINE1*) are usually heavily methylated, and genome-wide methylation loss from these sites has been regarded as a common epigenetic event in malignancies cells (Chalitchagorn et al. 2004; Choi et al. 2009). Therefore, DNA methylation could be a biomarker for early detection of cancer-related changes (Ting et al. 2006; Iwamoto et al. 2011; Schneider et al. 2011). There are a few links found between telomeres and DNA methylation. Hypomethylation in subtelomeric region was found to be associated with longer telomeres. The mechanism is probably through activating ALT pathway based on the observed events of telomeric sister chromatid exchanges (Gonzalo et al. 2006; Blasco 2007). Altered DNA methylation has been associated with various environmental exposures (Christensen and Marsit 2011; Hossain et al. 2012; Kippler et al. 2013), and is a new mechanism of toxicities of many exposures.

Chromosomal aberrations

There are several biomarkers suggested to represent intermediate steps in the pathway from exposure to cancer development in humans. Genomic instability is a key feature of tumor cells and in many tumors the instability is represented by numerous chromosomal aberrations including translocations, large deletions, ring chromosomes, single and double strand breaks (Mitelman 1998). Chromosomal aberrations can be divided into two sub-types, chromosome-type and chromatid-type aberrations. Chromosome-type aberrations affect both sister-chromatids whereas chromatid-type aberrations affect only one of the sister-chromatids. Studies based on several European cohorts have shown that chromosomal aberrations are present also in peripheral blood of healthy individuals and that the frequency of chromosomal aberrations is a prospective biomarker for cancer risk, regardless of cancer type or cancer site (Hagmar et al. 1994; Hagmar et al. 1998; Bonassi et al. 2000; Hagmar et al. 2004; Bonassi et al. 2008). Chromosomal

aberrations have been suggested to be induced by different factors, such as exposure to genotoxic agents, deficient DNA repair, reduced folate metabolism, and smoking (Perera et al. 1992; Hagmar et al. 1994; Motykiewicz et al. 1998; Smith et al. 1998; Zeljezic and Garaj-Vrhovac 2001; Bocskay et al. 2005; Skjelbred et al. 2006; Skjelbred et al. 2011), however, these factors only explain a minor part of chromosomal aberrations. The association between chromosomal aberrations and cancer risk in the European cohorts was not modified by exposure to genotoxic compounds (Bonassi et al. 2000).

Occupational and environmental exposures

There are still many carcinogenic exposures present in the general environment as well as at work in Sweden and elsewhere. Despite the fact that some of them are established carcinogens, their mechanisms of action are often not clarified. Below are exposures presented that are studied in the thesis.

Exposure in the rubber industry

Workers in rubber industry are recognized to be at high risk of developing airway diseases (Zuskin et al. 1996; Jonsson et al. 2007), cardiovascular disease (Gustavsson et al. 1986), and several types of cancer (Kogevinas et al. 1998; Reulen et al. 2008; de Vocht et al. 2009; Vlaanderen et al. 2013) due to exposure to a complex mix of compounds that are carcinogenic, allergenic, and irritating. Although improvements have been successfully made in recent years, the health effects of exposed rubber workers are still worth examining (de Vocht et al. 2008; Bolognesi and Moretto 2013). The complexity of exposure in rubber industry results from the industry process: a lot of different compounds are used and additional compounds are formed in different processes. The difficulty of assessing the compositions of rubber fume was partly solved by measuring index substances as has been reported in previous studies (Jonsson et al. 2007; Jonsson et al. 2008; Jonsson et al. 2009). There were no previous studies investigating the effect of rubber fumes on telomere length.

International Agency for Research on Cancer (IARC) has recently published a monograph classifying occupational exposures in rubber industry as “carcinogenic to humans” (Group 1) (IARC 2012).

Exposure to particles in welding fumes and diesel exhaust

As particles, welding fumes and diesel exhaust shared many commonalities. Both types of exposures are derived from combustions, and they consist of gas and particle phases, but with different compositions. The particle phase of welding fumes contains a mixture of metal oxide particles, the composition of which depends on the materials used: mild steel welding mainly generates particles consist of iron and manganese, while stainless steel welding mainly generates particles of iron, manganese, chromium and nickel (Leonard et al. 2010). The particle phase of diesel exhaust consists of elemental carbon, adsorbed organic compounds, sulfate, nitrate, and trace elements (Ris 2007).

Both welding fumes and diesel exhaust have been reported to be carcinogenic, but they were differently classified by IARC: welding fumes are categorized as "possible human carcinogen" (Group 2B) (IARC 1990), while diesel exhaust has been recently categorized as "carcinogenic to humans" (Group 1) (Benbrahim-Tallaa et al. 2012). Recent studies showed 25%-40% increased cancer risk (measured as odds ratio, OR) for lung cancer in welders (Hansen et al. 1996; Ambroise et al. 2006; 't Mannetje et al. 2012; Vallieres et al. 2012; Kendzia et al. 2013). It was previously suggested that the increased cancer risk is due to exposure to nickel and chromium, thus the elevated cancer risk would only be related with welding fumes from stainless steel (Simonato et al. 1991), but recent findings showed increased cancer risk also in welders working with mild steel (Hansen et al. 1996; Ambroise et al. 2006). Epidemiological studies showed that diesel exhaust was associated with elevated risk of developing lung and bladder cancer (Kobeissi et al. 2013; Vermeulen et al. 2013), however, some suggested that the results may suffer from important limitations (Boffetta 2012; Gamble et al. 2012).

Exposure to particles and its effects on telomere length has been studied, but the source and measurement of particles have varied, and the effects seem to differ between short-term and long-term exposures (Hoxha et al. 2009; McCracken et al. 2010; Dioni et al. 2011; Hou et al. 2012).

Exposure to arsenic via drinking water

Arsenic in nature can be oxidized to water-soluble salts and leak into groundwater and thus contaminate the drinking water. In several parts of the world, including Bangladesh, India, China, Argentina, and the United States, arsenic in drinking water exceeds the WHO guideline value of 0.010 mg/L (NRC 2001; IARC 2004). Inorganic arsenic is absorbed in the gastrointestinal tract and eliminated by excretion in urine or by methylation, first to monomethylarsonic acid (MMA) and

then to dimethylarsinic acid (DMA). However, due to the limited capacity of methylation, not all of the inorganic arsenic is methylated to DMA, and some remain as inorganic arsenic or MMA (Vahter 2002). Though all of the three statuses of arsenic can be excreted in urine, the rate of excretion varies. DMA is the most quickly excreted. Arsenic (+3 oxidation state) methyltransferase (AS3MT) is the main methyltransferase, which can methylate both MMA and DMA (Lin et al. 2002; Waters et al. 2004; Thomas et al. 2007; Engstrom et al. 2011).

Arsenic is classified as "carcinogenic to humans" (Group 1) based on sufficient evidences of carcinogenicity of arsenic in humans (IARC 2004). Chronic exposure to arsenic has been reported to increase the risk of cancer in skin, bladder, lung, kidney, liver, and prostate (IARC 2004). Arsenic and its metabolites can induce oxidative stress and in turn cause DNA damage, suppress DNA repair, mitotic arrest, apoptosis, and epigenetic changes (Bhattacharjee et al. 2013). There are also reports showing that arsenic exposure can lead to telomere dysfunction (Liu et al. 2003; Zhang et al. 2003; Ferrario et al. 2009; Mo et al. 2009), and that the direction of effect is dependent on the dose of arsenic.

Aims

General Aims:

To elucidate the effect of occupational and environmental exposures on telomere length in peripheral blood.

To elucidate the association between telomere length and chromosomal aberrations, an established biomarker for cancer risk.

Specific Aims:

To explore the effect of different compounds in rubber fumes on telomere length.

To explore the effect of welding fumes and diesel exhaust on telomere length and other DNA alterations.

To explore the effect of arsenic in drinking water on telomere length, and its mechanism of action.

To explore the relations between telomere length, DNA methylation, chromosome aberrations, and cancer risk.

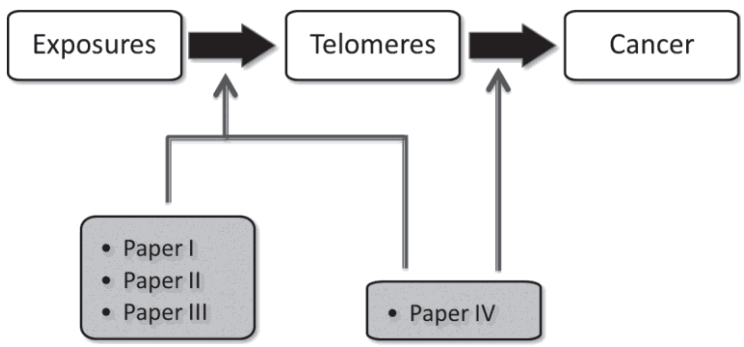

Figure 2. Overview of the papers included in the thesis.

Materials and methods

Study populations

Each study encompassed different study population to test specific hypotheses (Figure 3). The study populations are briefly described below.

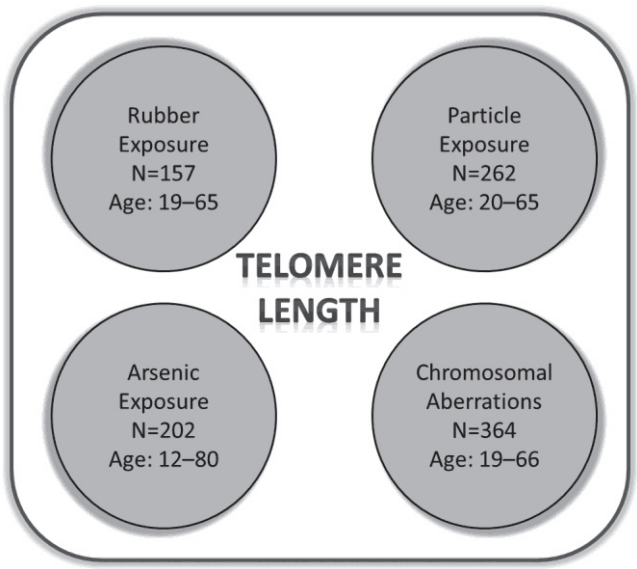

Figure 3. Study populations for each study.

Rubber industry

The study comprised 157 rubber workers from 8 different rubber companies in southern Sweden. Structured interviews were performed by a trained nurse to obtain information about the study participants, including occupational histories for working in the rubber industry. Venous blood samples were collected at the same time as the interview, and urine was collected from workers during the last

four hours of an eight-hour work shift. The study subjects gave their informed written consent to take part in the study, and the study was approved by Regional Ethical Committee of Lund University.

Particle exposure

In the study regarding particle exposure, only nonsmoking (at least the last 12 months) males were included. We recruited 101 welders from 10 different companies in southern Sweden. The number of welders from each company varied a lot depending on the size of the company. To investigate the genotoxic effects of exposure to diesel exhaust, 34 workers in tunnel construction site were recruited. These workers were working on a tunnel drilling platform and they were exposed to diesel exhaust from diesel-driven local trains going in and out of the tunnel. The 127 controls were recruited in storage houses from 7 companies organizing grocery goods. Structured interviews were conducted to obtain information about working environment, working history, disease history, diet, and lifestyle. Questions regarding working environment differed between groups, but all other questions were identical for all study participants. Venous blood and spot urine were collected from each subject. Urine during the last 4 hours of an 8 hour work shift was collected. All study subjects gave their informed written consent to take part in the study and the study was approved by the Regional Ethical Committee of Lund University, Sweden.

Arsenic exposure

In this study, 202 women were recruited (Vahter et al. 1995). The majority of the study participants (N=161) were from the village San Antonio de los Cobres with 0.2 mg/L arsenic in the public drinking water. The other participants (N=41) were from 3 other villages with lower concentrations of arsenic in the water (0.0035, 0.012, 0.073 mg/L for each village) (Concha et al. 2010). Almost all women drank public drinking water exclusively. The women were asked about their disease history and their hands were inspected for arsenic-related skin lesions. Biological samples were collected during daytime as non-fasting spot samples. Blood and urine samples were kept at -20°C before and after transport (with cooling blocks) to Sweden for analysis. For gene expression analysis, 90 individuals matched for age, weight and body mass index (BMI) with a wide range of urinary arsenic (0.010 – 1.3 mg/L) were chosen. Informed consent, both oral and written, was provided by all the study participants. The study was approved by the Ministry of Health in Salta, Argentina, and the Regional Ethical Committee of Karolinska Institutet, Sweden.

Study of chromosomal aberrations

This study was a subgroup of a previous cohort study that aimed to explore the association between chromosomal aberrations in peripheral lymphocytes and cancer risk (Brøgger et al. 1990; Hagmar et al. 1994; Bonassi et al. 1995). The study subjects were 364 healthy male Norwegian adults (>15 years of age) examined cytogenetically during 1980–1999. Subjects with cancer diagnosed before the cytogenetic analysis were excluded from the cohort. Age and information about smoking status at chromosomal aberrations testing was available. Average telomere length and degree of *LINE1* methylation were measured in DNA from the same cells that were cytogenetically analyzed. To investigate the association between telomere length, DNA methylation and cancer risk, a nested case-control study was carried out by matching two controls with similar age for each cancer case. Cancer cases (N=49) diagnosed within the date from chromosomal aberration testing till the end of 2008 were identified according to data from the Cancer Registry of Norway. The tumors were from: skin (N=14, of which one was a malignant melanoma), the gastrointestinal tract (N=6), male sex organs (N=5), the urinary tract (N=4), the respiratory tract (N=3), lymphoid or hematopoietic system (N=3) and the endocrine glands (N=1). Two cases had tumors of uncertain or unknown malignancy potential and 11 cases lacked information about cancer origin. Written informed consent was obtained from all subjects. The Regional Ethics Committee and the Data Inspectorate in Norway approved the study.

Exposure assessment

In order to analyze associations between exposures and telomere length and other DNA alterations, it is critical to perform appropriate exposure measurement to get valid information about exposures. Since the types of exposures were different in each study, different types of measurements were adopted.

Rubber industry

N-nitrosamines were measured in air on 60 rubber workers by personal sampling with Thermosorb/N adsorption tubes in the breathing zone. The flow rate was set to be 1.5 L/min. The working environment was uniform throughout the 8-hour work shift and therefore the sampling was performed for only 3 hours. The samples were stored at +8°C until analysis. The analyses were performed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The N-nitrosamines

measured were N-nitrosodimethylamine (NDMA), N-nitrosomorpholine (NMOR), N-nitrosodi-n-butylamine (NDBA), N-nitrosodiethylamine (NDEA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR), N-nitrosomethylethylamine (NMEA) and N-nitrosodi-n-propylamine (NDPA). The term N-nitrosamines in this thesis refers to the sum of these 8 compounds. Apart from these 60 workers who were measured, we estimated the exposure to N-nitrosamines for the remaining 106 workers based on similar work tasks. Because of the clear and fairly constant differences in exposure conditions for different work tasks during the time period of sampling, the estimated N-nitrosamines probably reflect the true levels fairly well.

Urinary 1-hydroxypyrene (1-HP) was measured in urine as a biomarker for exposure to polycyclic aromatic hydrocarbons (PAH) (Dor et al. 1999). The level of 1-HP was analyzed by LC and fluorescence detection. 2-thiothiazolidine-4-carboxylic acid (TTCA) was measured in urine as a biomarker for carbon disulfide (CS_2) (Drexler et al. 1994). The level of TTCA was analyzed by LC-MS/MS. Orto (o-), meta (m-), and para (p-) toluidine were analyzed by gas chromatography mass spectrometry (GS/MS). The levels of 1-HP and TTCA were adjusted for creatinine content, which was analyzed enzymatically according to Mazzachi et al. (Mazzachi et al. 2000). The concentrations of the toluidines are normally presented unadjusted.

Particle exposures

Welding fumes were measured both personally and stationary as respirable dust, since the typical aerodynamic diameter of the welding particles was around 0.5 μm (Antonini 2003; Hedmer et al. 2013). Personal samples were collected in 70 workers at the breathing zone and the stationary samples once in each welding company. If powered air purifying respirators (PAPRs) were used, the samples collected were from the air outside the PAPRs, and the results were then reduced by a correction factor of 3 to get a better estimate of the exposure inside the PAPRs. The flow rate was 2.2 L/min. Most of the air sampling was performed during full-shift work. The samples were analyzed gravimetrically for respirable dust according to a certified method. The limit of detection (LOD) was 0.05 mg/sample.

Exposure to diesel exhaust was measured by personal sampling of elemental carbon and nitrogen dioxide (NO_2) on 17 tunnel construction workers. Elemental carbon was collected on filter and analyzed gravimetrically. NO_2 was collected by passive samplers at the breathing zone based on molecular diffusion. The samples of NO_2 were sent to the Swedish Environmental Research Institute for analysis.

None of the tunnel construction workers used any respiratory protection. The average sampling time for both elemental carbon and NO_2 was 8 hours.

Respirable dust measurements were performed on 53 welders who participated in the interview and biological sampling. The other 48 welders included in the study had no exposure measurements. Their exposure to respirable dust was estimated according to exposure data from the 53 measured welders plus 17 welders, not included in the study but with similar working tasks. Exposure data from our previous study at the same companies were used in the exposure assessments as well because a number of welders participated in both studies (Hedmer et al. 2013).

Arsenic in urine

The concentrations of arsenic metabolites in urine were measured by high performance liquid chromatography coupled with hydride generation and inductively coupled plasma MS. Urinary arsenic in this thesis refers to the sum concentration of arsenic metabolites. All measured concentrations of elements in urine were adjusted to the mean specific gravity of urine (1.020 g/mL).

Retrospective assessment of exposure for study of chromosomal aberrations

Exposure assessment for the study population was carried out retrospectively (Tinnerberg et al. 2003; Skjelbred et al. 2006). In short, partly structured telephone interviews were performed with subjects or, if deceased, next-of-kin. Other information sources were also used, including contacts with subjects' working companies, former co-workers, company records, and medical records. The individuals were divided as exposed or non-exposed to genotoxic exposures at the time of sampling. The main exposure sources included welding fumes, vinyl chloride, nickel, and styrene.

Biomarkers for DNA alterations

Telomere measurement

Quantitative polymerase chain reaction (qPCR) was adopted to determine relative telomere length based on method reported by Cawthon (Cawthon 2002). Master

mixes for telomere runs were prepared with telomere primers (0.45 µM of each primer, forward 5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3'; reverse 5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3'), 1×PCR Buffer (Life Technologies, Carlsbad, CA, USA), 1.75 mM $MgCl_2$, 0.8 mM dNTPs, 0.3 mM SybrGreen (Life Technologies), 1×Rox (Life Technologies), and 0.5 U Taq Platina (Life Technologies); while the primers for hemoglobin beta chain (*HBG*) gene, the single copy gene, was 0.40 µM for each one, forward 5'-TGT GCT GGC CCA TCA CTT TG-3', reverse 5'-ACC AGC CAC CAC TTT CTG ATA GG-3'. Different master mixes were used in different studies, including 1×SybrGreen Universal mix (Applied Biosysterms, Foster City, CA, USA) and KAPA SYBR FAST qPCR Kit Master Mix (2X) ABI Prism (Kapa Biosystems, Woburn, MA, USA). The PCR was performed on a real-time PCR machine (7900HT, Applied Biosystems). Approximately 20 ng of sample DNA was added to each reaction (end volume 20 µl). A standard curve, a reference DNA and a blank were also included in each run. For the standard curve, one calibrator DNA sample was diluted serially by 2-fold per dilution to produce 5 concentrations of 1-16 ng/µl to cover the range in which all samples should fall. Each sample, standard curve, reference and blank was run in triplicates. The thermo cycling profile for the telomere amplification was 95°C for 3 minutes, flowed by 25 cycles of 95°C for 15 seconds and 56°C for 2 minutes. The thermo cycles for *HBG* was 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 54°C for 1 minute. The standard curves were generated by the Applied Biosystems SDS software 2.3. R^2 for each standard curve was >0.99. Standard deviation of Ct values for the triplicates of each sample was accepted at <0.1. The relative length of the telomeres was obtained through calculating the ratio (T/S) of Ct of telomere repeats product (T) and single copy gene product (S, here *HBG*) for each subject, by the formula $T/S = 2^{-\Delta Ct}$, where ΔCt = Ct(telomere) – Ct(*HBG*). This ratio was then compared with the ratio of the reference DNA to adjust for differences between runs. The relative telomere length is an arbitrary value. The coefficients of variance (CV) in the 4 studies were 6.4% (Paper I), 7% (Paper II), 10% (Paper III), and 7.2% (Paper IV).

8-oxodG measurement

Urinary 8-oxodG was measured in the study of particle exposures. The concentration was measured by a simplified method with LC-MS/MS. The urine samples were processed and analyzed in duplicates at 2 occasions, and CV was 10%. Three internal controls (urine samples from 2 Swedish adults) were included in each batch and they presented CVs of 12%, 9%, and 4%. The LOD for the concentration of 8-oxodG was 0.5 nmol/L. The concentration of urinary 8-oxodG was adjusted to the specific gravity.

DNA methylation

Methylation sensitive high resolution melting (MS-HRM)

In the study of particle exposure, 10 genes that have been reported to be hypermethylated in patients with lung cancer were chosen to measure the status of methylation: homeobox A9 (*HOXA9*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), short stature homeobox 2 (*SHOX2*), adenomatous polyposis coli (*APC*), O-6-methylguanine-DNA methyltransferase (*MGMT*), cadherin 1, type 1, E-cadherin (*CDH1*), Dorsocross1 (*DOC1*), Ras association (RalGDS/AF-6) domain family member 1A (*RASSF1A*), homeobox B13 (*HOXB13*), and BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*).

DNA samples (500 ng/sample) were bisulfite-treated. Control DNA consisted of 0.1%, 1%, 10%, and 100% of methylated DNA and was bisulfite-treated in the same way as the samples. PCR amplification was performed on a real-time PCR machine. MS-HRM results were aligned according to the melting curve of the controls. MS-HRM is a qualitative method, so result for each sample was categorized as methylated or nonmethylated, but the degree of methylation for each sample was unknown.

Pyrosequencing

For study of chromosomal aberrations, the degree of methylation of *LINE1* was measured with 400 ng DNA (20 ng/μL) for each subject. DNA was extracted from fixative or unstained slides, and then bisulfite-treated. The degrees of methylation at 4 CpG sites were measured by a commercial kit (Qiagen) following manufacturer's instruction. The degree of methylation was expressed as percentage of methylated cytosine over total cytosine. The repeatability of the method was tested in previous study, and the CV was 2.0% (Hossain et al. 2012).

Gene expression analysis

Gene expression analysis was performed in the study investigating the effect of arsenic exposure via drinking water. RNA was extracted from whole blood with the PAXgene Blood RNA kit (PreAnalytiX) and stored in -80°C. DirectHyb HumanHT-12 v4.0 (Illumina, San Diego, CA, USA) was used according to the manufacturer's instructions and the analysis was performed at SCIBLU core facility at Lund University. Gene expression data for telomere-related genes selected based on literature searches were included in the analyses (120 transcripts from 62 genes). Eighty-five of the 90 individuals had data on telomere length.

Cytogenetic analysis of chromosomal aberrations

Phytohaemagglutinin-stimulated lymphocyte cultures from heparinized whole blood were used for cytogenetic analysis. The same 3 microscopists carried out the scoring of chromosomal aberrations in a double-blind fashion on coded slides from 100 cells (in early studies) or 200 cells (in more recent studies) per subject. Different types of chromosomal aberrations were recorded and harmonized among the scorers using criteria described earlier (Savage 1976; Brøgger et al. 1990). Frequency of chromosomal aberrations was defined as the percentage of cells with aberrations excluding gaps. Chromosome-type aberrations included chromosome-type breaks, ring chromosomes, marker chromosomes, and dicentrics; chromatid-type aberrations included chromatid-type breaks and chromatid exchanges.

Statistical analyses

Spearman's ρ correlations were investigated between various variables including characteristics, exposure status, and biomarkers. In all 4 studies, telomere length showed a normal distribution. Data-driven categorization was carried out in the study of rubber exposure due to observed clear difference of exposure levels. The mean differences between several groups were generally tested by analysis of variance (ANOVA) or analysis of covariance (ANCOVA) if adjustments were needed. The distributions of categorized variables between groups were examined by Mann-Whitney test or Kruskal-Wallis H test. In Paper I, the trends of biomarkers in 3 groups were examined by trend test of Jonckheere-Terpstra.

In order to investigate associations between exposures and biomarkers, the general linear model was used when the dependent variable was continuous. Beta coefficients (β), 95% confidence intervals (CI), and P-values were derived from the models to estimate the effects of each factor on the dependent variable. The residuals were always tested for normal distribution. Logistic regression was adopted when the dependent variable was binary and ORs were obtained as effect estimates. The frequency of chromosomal aberrations were rounded to the upper integer and used as ordinal outcome variables in ordinal regression. Age was considered as a confounder and included into the models for adjustment. Other possible confounders were also considered. In Paper I, possible confounders were included if they were significantly correlated with the dependent variable. Since working time was strongly correlated to age, working time was not included in multivariate analyses in order to avoid over-adjustment. In Paper II, factors were included if they remain significant in the final model or the effect estimates for other independent variables changed more than 10% when included. In Paper III,

chewing coca leaves were considered as possible confounder, but it did not correlate with the outcomes, and thus, was not included in the model. Since age and BMI were significantly correlated, only age was included into the model to avoid over-adjustment. In Paper IV, models were adjusted for age. The effect of occupational exposure and smoking status were more thoroughly investigated. They were first each introduced into the model to investigate their effect size. Then they were stratified and in each stratify the associations between telomere length and DNA methylation versus chromosomal aberrations were analyzed.

In Papers III and IV, sub-cohorts were studied. In Paper III, 90 individuals were chosen for gene expression analysis, and these 90 subjects were matched for age, weight and BMI, with a wide range of urinary arsenic (0.010 – 1.3 mg/L). The correlations between urinary arsenic and expression levels of telomere-related genes, as well as the associations between telomere length and expression levels of telomere-related genes were analyzed by Spearman's correlation. To avoid making Type I error, P-values were adjusted for false discovery rate (FDR). The genes were first ranked by their P-values of Spearman's correlation. The expected P-values were calculated as the preset FDR (0.05 in this study) times the corresponding rank divided by the total number of genes. The gene with observed P-value lower than expected P-value of the lowest rank was then considered as significant, along with other genes of higher ranks. In Paper IV, a nested case-control study was performed to investigate associations of telomere length and chromosomal aberrations versus cancer risk. Two controls (total N=98) with similar age for each case with malignant cancer at follow-up (N=49) were matched. Logistic regression analysis with case/control-status as outcome was performed, and risk estimates were measured as OR. Telomere length, *LINE1* methylation and chromosomal aberrations were evenly trichotomized in the control group respectively, and then these cut points were applied to the case group as well. To notice, since there were many ties in chromosomal aberrations value, numbers of each group are not exactly the same. Risk estimates were derived for different telomere length, *LINE1* methylation or chromosomal aberrations groups. Age was introduced for adjustment since the matching was not perfect.

All statistical analyses were completed by using SPSS (SPSS Inc, Chicago, IL USA), except for the FDR adjustments which were made using R (2.15.1). Statistical significance refers to $P<0.05$ (two-tailed).

Results

Rubber exposure and telomere length

In this study, we aimed to elucidate associations between markers of different rubber exposures and telomere length. Of the 8 types of N-nitrosamines analyzed, NMEA and NDPA were not detected in any subject. NDMA was the predominant N-nitrosamine. The median of relative telomere length was 0.71, with a range of 0.16-1.3. Age, working time, total N-nitrosamines, NDMA, NDBA, NDEA, NPIP, TTCA and p-toluidine significantly correlated inversely with telomere length. Between the markers of exposures, N-nitrosamines were strongly correlated with TTCA (positively), and weak with 1-HP (inversely). 1-HP and the toluidines were positively correlated to each other. NDMA, NDBA, NDEA, and NPIP were positively correlated to each other (r_s ranged 0.40–0.94).

The associations between exposure groups (low, medium, and high exposure) and telomere length were analyzed by analysis of covariance with age adjustment. Borderline significances of differences between groups of estimated and measured N-nitrosamines and telomere length were observed (Table 2), where high level of N-nitrosamines exposure was associated with short telomeres. There were significant differences between m-toluidine exposure groups as well, but not in a dose-dependent manner.

Table 1. Exposures of the rubber workers included in the study.

	Median	Range
Estimated N-nitrosamines, μg/m^3	0.90	0.10–22
Measured N-nitrosamines, μg/m^3	1.3	0.10–22
NDMA μg/m^3	1.1	0.090–28
NMOR μg/m^3	0.10	0.080–1.7
NDBA μg/m^3	0.080	0.010–0.053
NDEA μg/m^3	0.040	0.030–4.6
NPIP μg/m^3	0.010	0.010–2.8
NPYR μg/m^3	0.010	0.010–1.9
1-HP, μmol/mol creatinine	0.14	0.0020–0.89
TTCA, μmol/mol creatinine	19	0.20–950
o-toluidine, ng/ml	0.46	0.025–108
m-toluidine, ng/ml	0.15	0.025–3.8
p-toluidine, ng/ml	0.090	0.025–4.7

Table 2. Relative telomere length of the rubber workers and in subgroups. P-values were from analysis of covariance (ANCOVA) with age adjustment.

	Group	N	Mean±SD	P (ANCOVA)
Total population		157	0.73±0.19	
Estimated	Low	112	0.75±0.19	0.061
N-nitrosamines	Median	27	0.75±0.17	
($\mu g/m^3$)	High	18	0.62±0.13	
Measured	Low	46	0.78±0.19	0.092
N-nitrosamines	Median	11	0.77±0.19	
($\mu g/m^3$)	High	3	0.50±0.08	
1-HP	Low	52	0.74±0.19	0.70
(µmol/mol	Median	52	0.72±0.17	
creatinine)	High	51	0.74±0.19	
TTCA	Low	51	0.75±0.20	0.22
(µmol/mol	Median	51	0.75±0.17	
creatinine)	High	52	0.70±0.17	
o-toluidine	Low	52	0.75±0.18	0.82
(ng/mL)	Median	50	0.72±0.20	
	High	50	0.73±0.17	
m-toluidine	Low	52	0.71±0.17	0.0066
(ng/mL)	Median	50	0.79±0.19	
	High	50	0.70±0.16	
p-toluidine	Low	52	0.75±0.17	0.49
(ng/mL)	Median	49	0.73±0.18	
	High	51	0.71±0.19	

General linear model was performed in order to assess the effect estimates of the different variables on the relative telomere length with age adjustment. Estimated N-nitrosamines were significantly associated with short telomere length, and measured N-nitrosamines showed a borderline significance. Among the six N-nitrosamines that were present in the rubber industry, NDMA, NDEA, and NPIP were inversely associated with telomere length (Table 3, Figure 4). NDBA, NMOR, and NPYR were not significantly associated with telomere length.

Table 3. Effect estimates for the associations between individual characteristics, exposures and telomere length with age adjustment.

	β	95% CI	P
Estimated N-nitrosamine	-0.0044	-0.0086– -0.00016	0.042
Measured N-nitrosamine	-0.0088	-0.018–0.000072	0.052
NDMA	-0.013	-0.024– -0.0019	0.023
NMOR	0.047	-0.055–0.15	0.36
NDBA	-0.44	-0.90–0.024	0.062
NDEA	-0.075	-0.14– -0.011	0.023
NPIP	-0.12	-0.22– -0.026	0.014
NPYR	0.030	-0.17–0.23	0.76
1-HP	-0.050	-0.20– -0.10	0.51
TTCA	-0.00016	-0.00036–0.000037	0.11
o-toluidine	0.0016	-0.0014–0.0047	0.30
m-toluidine	-0.060	-0.14–0.016	0.12
p-toluidine	-0.030	-0.080–0.020	0.24

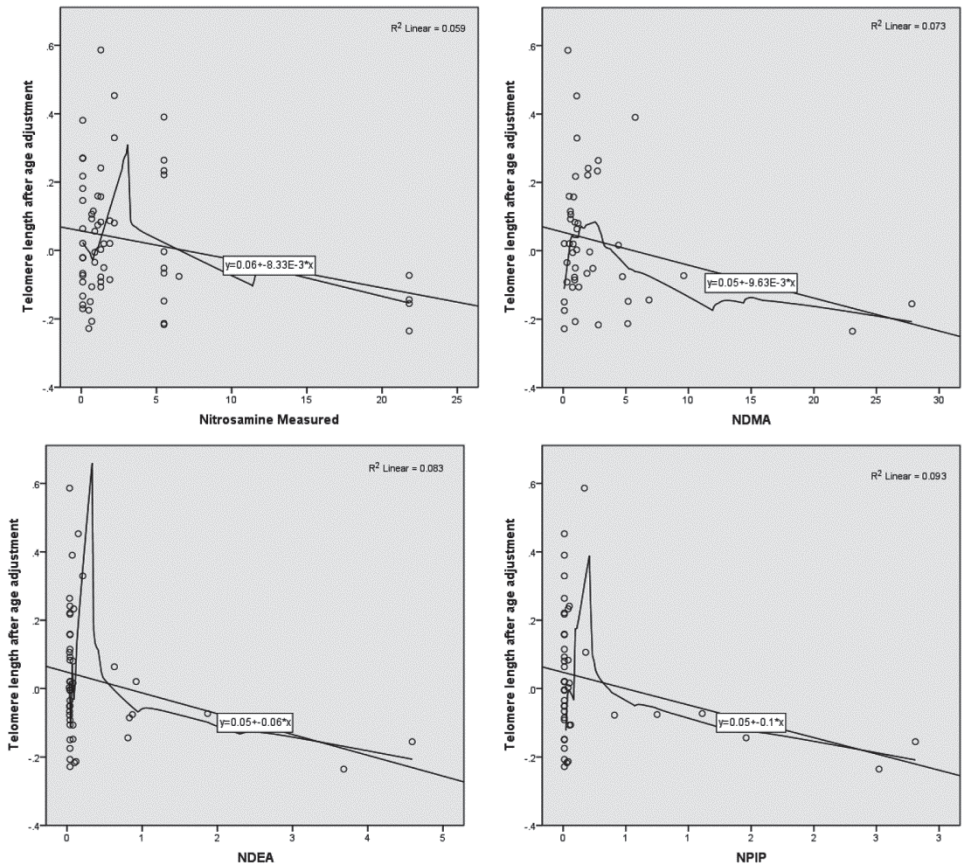

Figure 4. Scatterplots with linear and loess fit lines for the associations between age-adjusted telomere length and measured N-nitrosamines, NDMA, NDEA, and NPIP.

Particle exposure and telomere length

In this study we aimed at clarifying the relations between welding fumes and diesel exhaust and telomere length. The different exposure groups were rather similar for characteristics of disease history and life style. However, more welders reported to use wood burning stove/boiler at home. The diesel-exposed workers had higher level of education and more exposure to passive smoking compared to

the welders and controls. A larger proportion of the controls lived in cities and less in small towns.

Most welders worked with mild steel apart from 11 welders who had work tasks related to stainless steel. For most welders the measured exposure to welding fumes was not high: out of 101, only 4 had exposure exceeding the occupational exposure limit (5 mg/m^3) (Swedish Work Environment Authority 2011). For tunnel workers the exposures to NO_2 and elemental carbon were of a wide range, mainly because the diesel-driven trains and the workers themselves moved frequently during the work shift. The levels of NO_2 and elemental carbon measured in this study was relatively low compared to other studies of tunnel workers (Bakke et al. 2001; Sauvain et al. 2003), and the level of NO_2 was lower than the occupational exposure limit (2 mg/m^3) (Swedish Work Environment Authority 2011). The controls had no occupational exposure to welding fumes or diesel exhaust.

The mean level of urinary 8-oxodG was 14 nmol/L (range 3.6-51). The mean relative telomere length was 0.90 (0.37-1.8). *HOXA9* were methylated in the majority of the subjects (95%); *CDKN2A* (37%) and *SHOX2* (45%) in about one third to a half of the subjects; *MGMT* (17%) and *APC* (4.3%) in the minority of the subjects. *BNIP3* failed in our analysis; and 3 genes (*DOC1, RASSF1A, HOXB13*) were nonmethylated in all subjects; *CDH1* was methylated only in one subject. Therefore, only the first 5 genes were included in the statistical analysis.

Linear regression analysis showed that 8-oxodG increased in both welders and diesel-exposed workers compared to controls, by 1.4 nmol/L and 3.3 nmol/L, respectively. Relative telomere length changed in the welders and diesel-exposed workers in different directions compared with the controls. The telomeres were 0.048 units shorter in the welders and 0.073 units longer in diesel-exposed workers. Though current residence was correlated with 8-oxodG and passive smoking with telomere length by Spearman's correlation, these 2 factors were not included in the final models since they did not meet the criteria of inclusion. *APC* methylation was significantly different between the welders and the controls, where the welders had a much higher risk of methylation (OR=9.8, 95% CI 1.2–81). Welders also showed a nonsignificant higher risk of HOXA9 methylation than controls (OR=4.1, 95% CI 0.88–19). Since all subjects in the diesel-exposed group had methylation of *HOXA9*, the difference in methylation between diesel-exposed workers and controls was not calculable.

Welders and controls were analyzed for effects of ongoing exposure (based on results from exposure measurement and assessment) on 8-oxodG, telomere length and DNA methylation. No significant association was found.

Arsenic exposure and telomere length

The aim of this study was to evaluate the effect of arsenic on telomere length, and the modifying effect of polymorphisms in *AS3MT*. Study subjects from different villages shared ethnical background and socioeconomic status, and their diets did not differ substantially. Four women reported cigarette smoking, one reported alcohol consumption, and almost half of the women (46.5%) reported coca leaves chewing on a regular basis. Only 3 women reported medicine intake at the time of the study; one was being treated for gastritis and 2 for hypertension. There was a wide range in urinary arsenic concentrations (median 0.23 mg/L, range 0.010–1.3 mg/L), and arsenic metabolites (Table 4). However, despite of the high level of arsenic exposure, none of the women showed any sign of arsenic-related hyperkeratosis on their hands or reported any history of malignancy. Relative telomere length showed a median level of 0.37 with a range of 0.18–0.67. Telomere length significantly correlated with age and BMI in an inverse direction, and non-significantly to urinary arsenic in a positive direction. BMI was positively correlated with age.

Linear regression model showed that telomere length was positively associated with urinary arsenic (β=0.066, 95% CI 0.0034–0.13, Table 5, Figure 5), inorganic arsenic (β=0.53, 95% CI 0.15–0.91), and MMA (β=0.50, 95% CI 0.014–0.99), while DMA showed a borderline significance (β=0.073, 95% CI -0.0078–0.15). When stratified by median of the fraction of metabolites, the associations between urinary arsenic and telomere length were only significant in the group with a high fraction of inorganic arsenic and the group with a high fraction of MMA, but not in the groups with a low fraction of inorganic arsenic or MMA. All analyses were adjusted for age.

The association between urinary arsenic and telomere length was analyzed with two major haplotypes of *AS3MT* to evaluate genetic modification. Both the slow and more toxic-metabolizing *AS3MT* haplotype 1 and the fast and less toxic-metabolizing haplotype 2 interacted with the effect of arsenic on telomere length. When the effect of urinary arsenic versus telomere length was analyzed in data stratified for *AS3MT* haplotypes, it was observed that with increasing copy number of haplotype 1, there was a trend for stronger effect of urinary arsenic on telomere length, and a trend in the opposite direction was found with increasing copy number of haplotype 2. However, the associations were not significant in groups with two copies of haplotype 1 or two copies of haplotype 2.

Table 4. Concentrations of total urinary arsenic and arsenic metabolites (adjusted for specific gravity) and fractions of arsenic metabolites. [a]

	N=202[b]		N=90[b]	
	Median	Range	Median	Range
Total arsenic (mg/L)	0.23	0.010–1.3	0.20	0.010–1.3
Urinary iAs (mg/L)	0.026	0.00058 – 0.18	0.025	0.00058 – 0.18
Fraction of iAs (%)	12	2.3–34.2	13	3.3–33
Urinary MMA (mg/L)	0.017	0.00060 – 0.20	0.015	0.00060 – 0.20
Fraction of MMA (%)	7.7	2.4–22.1	7.7	2.4–18
Urinary DMA (mg/L)	0.17	0.0071 – 0.87	0.15	0.0071 – 0.87
Fraction of DMA (%)	80	56–95	79	56–94

[a.] iAs = inorganic arsenic, MMA = monomethylarsonic acid, DMA = dimethylarsinic acid

[b] N=202 whole study population, N=90 subgroup analyzed for expression of telomere-related genes in whole blood.

Table 5. Associations between total urinary arsenic and arsenic metabolites (mg/L) and telomere length with adjustment for age. [a]

	β	95% CI	P
Total arsenic	0.066	0.0034–0.13	0.039
iAs	0.53	0.15–0.91	0.0064
MMA	0.50	0.014–0.99	0.044
DMA	0.073	-0.0078–0.15	0.076

[a.] iAs = inorganic arsenic, MMA = monomethylarsonic acid, DMA = dimethylarsinic acid

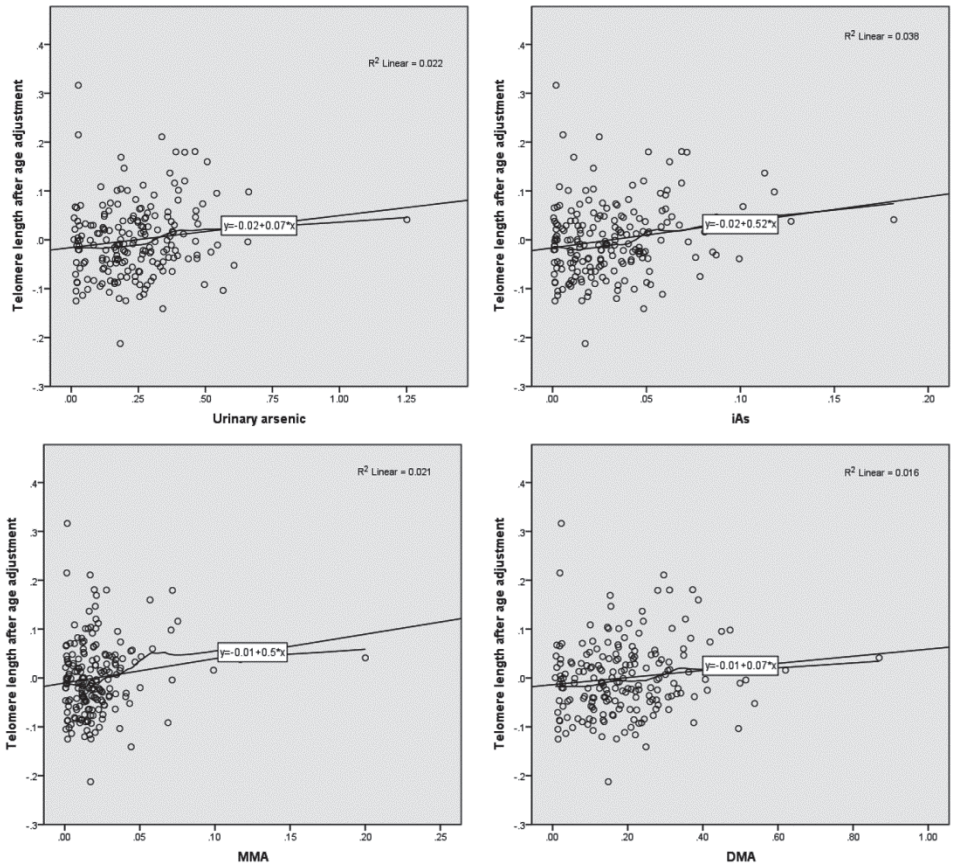

Figure 5. Scatterplot with linear and loess fit lines showing the associations between age-adjusted telomere length and total urinary arsenic and arsenic metabolites.

In order to elucidate the mechanism of action of arsenic on telomere length, gene expression analysis was performed in a subfraction of the women. Spearman's correlation between urinary arsenic and gene expression in peripheral blood, as well as telomere length and gene expression showed some significant correlations. Expression of one *TERT* transcript (encodes the longer isoform, isoform 1) was positively correlated with urinary arsenic, but it was not correlated with telomere length. There were 3 genes that positively correlated in expression with telomere length [SCY1-like 1 (*SCYL1*), replication protein A1 (*RPA1*), and RAD1 homolog (*RAD1*)], while there were 8 genes inversely associated [MUS81 endonuclease homolog (*MUS81)*, RAP1 interacting factor homolog (*RIF1*), HUS1 checkpoint homolog (*HUS1*), SCY1-like 3 (*SCYL3*), cyclin-dependent kinase 2 (*CDK2*), RAP1B, member of RAS oncogene family (*RAP1B*), golgin, RAB6-interacting (*GORAB*), and topoisomerase (DNA) II alpha (*TOP2A*)]. However, none of them remained significant after FDR adjustments. There was a weak positive association between urinary arsenic and *RAP1B*, but none of the other genes listed above showed any associations with urinary arsenic.

Chromosome instability and telomere length

This study was originally aimed for analysis of chromosomal aberrations and cancer risk, but was here used to explore the hypothesis that short telomeres induce chromosomal aberrations, and in turn, higher cancer risk. Analysis of *LINE1* methylation was evaluated as well. Controls and cases of this study did not differ much in smoking status or levels of exposure. The case group was older at cohort enrolment. Relative telomere length in this study population demonstrated a median value of 0.88, and the percentage of *LINE1* methylation a median value of 80%.

Spearman's correlation showed an inverse correlation between telomere length and total chromosomal aberrations. Telomere length was significantly correlated with chromosome-type aberrations but not with chromatid-type aberrations. *LINE1* methylation was positively correlated with chromatid-type aberrations, but not with chromosomal aberrations, chromosome-type aberrations, or telomere length. Age was a major factor in this study, since it was significantly correlated with telomere length (inversely), *LINE1* methylation (inversely), chromosomal aberrations (positively), and chromosome-type aberrations (positively), but not with chromatid-type aberrations. Therefore, all analyses presented below were adjusted for age.

Telomere length and *LINE1* methylation in groups of different exposure and smoking status are presented in Table 6. A weak effect of smoking on telomere

length was found in former smokers compared with nonsmokers (P=0.034). Exposure status had no effect on telomere length. *LINE1* methylation was influenced by smoking and exposure status. Compared with nonsmokers, higher degree of *LINE1* methylation was found in smokers and former smokers. Higher degree of *LINE1* methylation was found in exposed subjects compared with non-exposed.

Table 6. Telomere length and *LINE1* methylation in all subjects as well as stratified for groups of exposure and smoking status (mean±SD).

	Telomere length	P[a]	*LINE1* methylation	P[a]
Total	0.89±0.30		79±5.3	
Exposed	0.86±0.25	0.13	80±4.0	0.017
Nonexposed	0.91±0.33		79±5.3	
Smokers	0.90±0.26	0.10	80±4.5	0.029
Former smokers	0.80±0.25		80±4.2	
Nonsmokers	0.92±0.36		79±5.4	

[a] P-values were derived from ANCOVA.

Ordinal regression showed that a short telomere length was associated with a high frequency of chromosomal aberrations, in particular chromosome-type aberrations. *LINE1* methylation was positively associated with chromosomal aberrations, in particular chromatid-type aberrations. When telomere length and *LINE1* methylation were simultaneously included in the model, both were associated with chromosomal aberrations but in opposite directions. Telomere length was significantly associated with chromosome-type aberrations while *LINE1* methylation with chromatid-type aberrations. A significant interaction was found between *LINE1* methylation and occupational exposure for chromosomal aberrations. Results of stratified analysis showed that *LINE1* methylation was positively associated with chromosomal aberrations only in the nonexposed group (P=0.044).

In the nested case-control study, the age matching was fairly good but not perfect. None of telomere length, *LINE1* methylation or chromosomal aberrations was significantly associated with cancer risk. The results were similar when telomere length, *LINE1* methylation, chromosomal aberrations and age were all included in the model.

Other factors and telomere length

In all 4 studies of this thesis, the association between age and telomere length showed an inverse direction. The associations were significant in studies of rubber exposure, arsenic exposures, and chromosomal aberrations, but not significant in the study of particle exposure (Table 7).

Information about BMI was available in studies of particles and arsenic. A significant inverse association was found between telomere length and BMI in the study of arsenic exposure, and nonsignificant inverse effect was observed between telomere length and BMI in the study of particle exposure (Table 7).

Table 7. The effects of age and BMI on telomere length in each study.

Study	Factor	β	95% CI	P
Rubber exposure	age	-0.0046	-0.0072– -0.0020	0.00068
	BMI	---	---	---
Particle exposure	age	-0.0020	-0.0045–0.00043	0.11
	BMI	-0.0048	-0.11–0.0016	0.14
Arsenic exposure	age	-0.0017	-0.0024– -0.00092	0.000016
	BMI	-0.0041	-0.0063– -0.0018	0.00048
Chromosomal aberrations	age	-0.0036	-0.0062– -0.00099	0.0069
	BMI	---	---	---

We were not able to analyze the effect of sex on telomere length due to the imbalance of gender distribution. There were only women in the study of arsenic, and only men in studies of particle exposure and chromosomal aberrations. There were 77 men and 80 women in the study of rubber exposure, but telomere length was not significantly different between sexes (P=0.48).

We also evaluated the effect of smoking on telomere length in the studies of rubber exposure and chromosomal aberrations. However, we did not observe any significant effect of smoking status on telomeres. In the studies of rubber exposure, particle exposure, and chromosomal aberrations, information about former smoking was available. Telomere length did not differ in former smokers in the rubber industry or in relation to particles, but former smokers in the study of chromosomal aberrations showed somewhat shorter telomeres than subjects who never smoked (P=0.062, Bonferroni adjustment for multiple comparison). It was

not possible to investigate the effect of smoking on telomere length in the studies of particle and arsenic exposures. Only nonsmoking workers for at least the last 12 months were included in the study of particle exposure. Only 4 women in study of arsenic exposure reported cigarette smoking.

Discussion

Key findings

The results of this thesis strengthen the notion that the ends of the chromosomes, i.e., the telomeres, are DNA structures that are susceptible to exposure to compounds in our environment. However, the effect on the telomeres varies depending on type of compound. Shorter telomere length was found in association with exposure to N-nitrosamines whereas longer telomeres were found in relation to exposure to arsenic. In contrast, exposure to particles did not show any strong significant impact on telomere length. These findings help to better understand the carcinogenesis of occupational and environmental exposures. N-nitrosamines are likely to shorten the telomere length, and possibly leading to more chromosome instability and eventually, cancer. On the other hand, environmental arsenic exposure is related with longer telomeres, by activating *TERT* or ALT pathway, and then premalignant tumor cells can more easily become immortal. The results of the thesis also showed that a high frequency of chromosomal aberrations, a biomarker for cancer risk, is linked to short telomere length.

Strength and Limitations

Aspects of exposures

It is essential to perform appropriate exposure measurements to truly identify the effect of exposures. This is critical in the rubber industry where the exposure is a mixture and the different compositions vary in toxicity. Measurements for different compounds representative of group of compounds were carried out in air and in urine, either directly or by measuring metabolites. These measurements provided the opportunity to disentangle the effects of different chemicals. In the study about particles, respirable dust was measured in the majority of the welders, and the fact that not all individuals were included in respirable dust measurement due to practical reasons did somewhat hamper our ability of elucidating the effect of particles on telomere length. Furthermore, no analysis of chemical composition

was performed, excluding the possibility to analyze the effects of the metal oxides and other potential chemicals in relation to telomere length or other DNA alterations.

In the studies of rubber, arsenic, and particle exposures, wide ranges of exposure metrics were observed. In the rubber study, concentrations of N-nitrosamines covered a 220-fold range; for the study on particles, respirable dust concentrations covered a 190-fold range; in the study of arsenic total urinary arsenic concentrations covered a 120-fold range, and inorganic arsenic a 300-fold range. The big contrast of exposure provided better chance to investigate the toxic effect of these exposures, and to explore the dose-response relationship.

The exposures in these studies were all complex. Subjects were facing a mixture of compounds, for instance, workers in rubber industry were also exposed to PAH, CS_2, toluidines, and more compounds that we did not measure or were not possible to measure; welders were also exposed to various concentrations of metal oxides, alloys and flux compounds; women in Argentina were also exposed to lithium, boron, and other elements. This made it more difficult to ascertain the effect of specific compound. We could not rule out the possibility that there may be other exposures in the population, not measured but positively correlated with the measured ones that really contribute to telomere length alteration.

There was a time gap between biological sampling and exposure measurement in studies of the rubber industry and particle exposure. However, since the exposure levels were basically consistent during the time gap based on the information from questionnaire data and from the companies, it was not considered as a major bias.

Telomere length in peripheral blood

The DNA for telomere length determination was extracted from peripheral blood, which is a proxy for the target organ of effects of different exposures. The telomeres in whole blood probably mainly reflect telomeres of neutrophils and lymphocytes, with the former composing approximately 60% of white blood cells with a lifetime of weeks and the latter 30% and a lifetime of weeks to years. Thus, the effect on telomere length in blood is probably a mixture of short-term and long-term effects. It has been shown that telomere length in peripheral blood is a dynamic feature (Svenson et al. 2011). Despite of the repeatedly found inverse association between shorter telomeres and older age, telomere length in blood can be shortened or lengthened during a rather short period of time (months). This dynamic pattern increases the difficulty of interpreting the results from our cross-sectional studies.

Although the possibility to identify effects of exposures on the telomere length was hampered by the nature of blood as a complex mixture and the fluctuation of telomere length in blood cells, clear associations between exposures and telomere length in peripheral blood were observed in two of our studies. These associations demonstrate that, despite of the uncertainty of average telomere length in peripheral blood, it is still a fairly good biomarker for DNA damage induced by some of the environmental factors.

Telomere length alterations

There are several studies published by other research groups focusing on the effect of exposures on telomere length in human peripheral blood during the period 2009-2013. Aberrant telomere length was found in relation to different exposures including traffic pollution (Hoxha et al. 2009; Hou et al. 2012), black carbon (McCracken et al. 2010), polycyclic aromatic hydrocarbons (Pavanello et al. 2010), persistent organic pollutants (Shin et al. 2010), short-term exposure to particulate matter (Dioni et al. 2011), pesticides (Rollison et al. 2011), hazardous waste (De Felice et al. 2012), lead (Wu et al. 2012), and cadmium (Lin et al. 2013) (Table 8). The effects found were dependent on specific compound, and also the time period of exposure, but in most cases exposure to toxicants were associated with telomere shortening. Our work contributed to the growth of knowledge as well, and the results by others are partly commented below in relation to our results.

Table 8. Studies reporting associations between exposures and telomere length in peripheral blood.

Publish year	Exposure	Sample size	Telomere in exposed subjects
2004	Pesticides or organic solvents	20 exposed 35 controls	Shorter
2009	Traffic pollution	77 exposed 57 controls	Shorter
2010	Black carbon	165 never-smoking men, repeated measurement	Shorter
2010	Polycyclic aromatic hydrocarbons	48 coke-oven workers 44 controls	Shorter

Table 8. Studies reporting associations between exposures and telomere length in peripheral blood.

2010	Persistent organic pollutants	84 healthy subjects	Longer
2011	Short-term exposure to particulate matter	63 workers repeated measurement	Longer
2011	Pesticides	13 exposed MDS patients 47 nonexposed MDS patients	Shorter
2012	Hazardous waste	50 exposed pregnant women 50 non-exposed pregnant women	Shorter
2012	Air pollution	60 truck drivers 60 office workers repeated measurements	Longer (short-term) Shorter (long-term)
2012	Lead	60 battery workers with normal lead level 84 battery workers with abnormal lead level	Shorter
2013	Omega-3 fatty acids	31 with no extra omega-3 fatty acids 40 with 1.25 g/day omega-3 fatty acids 35 with 2.5 g/day omega-3 fatty acids	Longer
2013	Cadmium	227 exposed 93 controls	Shorter

* MDS stands for myelodysplastic syndromes

Rubber exposure induced DNA alterations

Short telomere length was found in relation to occupational exposures to N-nitrosamines (Li et al. 2011). The relation was especially significant with NDMA, NDEA and NPIP. NDMA and NDEA have been classified as probable carcinogens to humans (Group 2A) while the other 4 (NDBA, NMOR, NPIP, and NPYR) were classified as possible carcinogens to humans (Group 2B) (IARC 1978). However, these classifications were mostly based on studies focusing on N-nitrosamines from tobacco smoking or from contamination in water. Our finding provided some supporting evidence for NDMA and NDEA carcinogenicity and some new evidence for NPIP in the occupational setting of rubber industry. N-nitrosamines have been reported to induce oxidative stress, DNA adducts, strand breaks, and alkylation of DNA (Ahotupa et al. 1987; Robichova et al. 2004; Robichova et al. 2004; Cheng et al. 2008), and these effects are known to cause telomere shortening.

Particle exposure induced DNA alterations

There was a non-significant decrease of telomere length in welders compared to controls, and the effect estimate was quite small. There are no reports regarding the effect on telomere length by long-term exposure to welding fumes, but earlier studies showed shorter telomere length with long-term exposure to particulate matters (Hoxha et al. 2009; McCracken et al. 2010; Hou et al. 2012). The lack of significant association in our study may be due to the healthy worker effect, i.e. welders who suffer from short telomeres, and in turn diseases, have left the work as welders, and only more healthy ones have remained in the industry. In this study, oxidative stress was measured in the form of urinary 8-oxodG. No significant increase of 8-oxodG was observed in the welders, and no significant association between 8-oxodG and telomere length either. This could further lend support to the idea of a healthy worker effect. However, there were some DNA alternations observed in welders versus controls: welders showed a higher probability of *APC* methylation, which is in line with a previous study showing hypermethlation of *APC* in post-exposure samples compared with baseline samples from 63 male healthy steel workers (Hou et al. 2011). This pattern of methylation change may indicate epigenetic changes related with cancer development induced by welding fumes.

Longer telomeres were found in the diesel-exposed group, with borderline significance compared with the controls. There are some studies showing that short-term exposure to particles is actually associated with longer telomeres (Dioni et al. 2011; Hou et al. 2012). In our study, the diesel-exposed workers took 12-hour work shift for continuous 6 days and then a break for 9 days. It is reasonable

to speculate that they were under short-term exposure within these 6 days and the effect could be washed out during the break for 9 days. On the other hand, increased level of 8-oxodG was found in the diesel-exposed workers. This result is in line with previous studies (Han et al. 2010; Wei et al. 2010; Lee et al. 2012). The change of 8-oxodG concentrations probably reflected effect of short-term exposure, since 8-oxodG was suggested to have a short half-life (Nuernberg et al. 2008). The mechanism for the association between short-term exposure to particles and longer telomeres may be through triggering or sustaining inflammation by renewing immune cells in peripheral blood. *HOXA9* methylation was observed in all diesel-exposed workers, but the P-value was incalculable due to the observed number of nonmethylated diesel-exposed worker was zero. Such change of methylation pattern may indicate processes related with diesel exhaust induced lung cancer and needs to be followed up in a larger study population.

Arsenic induced DNA alterations

Longer telomeres were also found in the population chronically exposed to arsenic (Li et al. 2012). Our findings suggest that long-term exposure to arsenic can extend the life span of cells by elongation of the telomeres and activation of *TERT*. Our finding was in line with previous reports, *in vitro* or *in vivo*. An association between arsenic exposure and expression of *TERT* was found in a population in Inner Mongolia, as well as an association between expression of *TERT* and the severity of arsenic-related hyperkeratosis (Mo et al. 2009). Similar arsenic-related increase in *TERT* expression was found in our study, and longer telomeres were related to arsenic exposure. However, none of the Argentinean women in this study demonstrated hyperkeratosis. This may partly be explained by the genetic background for arsenic metabolism efficiency, as a high frequency of fast arsenic-metabolizing *AS3MT* haplotype was found in this population. The arsenic metabolism efficiency modified the effect of arsenic on telomere length, and individuals lacking the fast arsenic metabolizing *AS3MT* haplotype had stronger effect of long telomeres related with arsenic exposure compared with individuals carrying this haplotype.

Although increased *TERT* expression associated with arsenic exposure was found, no association between *TERT* expression and telomere length was observed in the study. Results of gene expression may provide some possible explanations. Among the genes that were associated with telomere length, *SCYL1* was involved in DNA damage response (Kim et al. 1994); *RAD1* and *HUS1* encode components of 9-1-1 complex, which promotes DNA damage response signaling (Francia et al. 2006; Francia et al. 2007); *MUS81* plays a key role in ALT pathway (Zeng et al. 2009; Zeng and Yang 2009). These findings suggested that the mechanism of

arsenic-induced telomere elongation may also be through increased DNA repair and ALT pathway, but it needs to be verified by future studies.

Telomere length, DNA methylation, chromosomal aberrations and cancer risk

No association between occupational genotoxic exposure and telomere length was found in this study. The main reason is likely that the exposure assessment was relatively rough. It was carried out retrospectively, and some of the study participants were not reachable. The data of exposure was qualitative, as exposed or nonexposed, and no information about exposure dose was available.

Shorter telomere length was found to be more related with chromosome-type than chromatid-type aberrations (Li et al. 2013), which is in line with chromosomal aberration patterns observed in tumor cells with short telomeres (Gisselsson et al. 2001; Swiggers et al. 2006). This finding stressed the notion that short telomere length can induce chromosomal instability. Chromosome-type aberrations arise from double-strand breaks in resting lymphocytes *in vivo* in the G_0-phase, and previous studies showed that short telomeres could induce sister chromatid fusion followed by breakage/fusion/bridge cycles which can last for many cell generations (Lo et al. 2002), and finally lead to dicentric chromosomes and ring chromosomes (Counter et al. 1992; Bailey and Murnane 2006). Our result provided supporting evidence that telomere dysfunction is involved in development of chromosome-type aberrations.

LINE1 methylation was associated with chromatid-type aberrations. Chromatid-type arise from DNA double strand breaks formed from initial DNA lesions *in vitro* in the S-phase, and our result suggests that epigenetic alterations are involved in chromatid-type aberrations.

In this study, no DNA alteration, including telomere length, *LINE1* methylation, and chromosomal aberrations, was found to be significantly associated with cancer risk. The nonsignificant results probably resulted from the limited number of cancer cases. Larger cohorts or longer follow ups should be carried out to better reveal the associations between the DNA alterations and cancer risk.

Conclusions

Telomere length in peripheral blood is associated with occupational and environmental exposures:

- Occupational exposure to compounds in rubber fume, especially N-nitrosamines, was associated with shorter telomere length. This might be part of mechanism of carcinogenesis of N-nitrosamines.
- Occupational exposure to welding fumes or diesel exhaust did not significantly affect telomere length.
- Chronic exposure to arsenic via drinking water was associated with longer telomeres, and haplotypes of the arsenic-metabolizing gene *AS3MT* modified the association. Expression of the telomere-elongating gene *TERT* was correlated to arsenic exposure.

Shorter telomere length was associated with higher frequency of chromosomal aberrations, especially with chromosome-type aberrations. This finding is likely to explain part of the mechanism for how chromosomal aberrations arise.

Acknowledgements

It is my honor to be granted the position and to work with various projects. Along this journey, I received countless warm helps from all people I met, and I'm so grateful to all of you. I would like to express my sincere gratitude to everyone who has supported me and in particular to the followings:

My supervisor Karin Broberg who made all of this possible, who taught me so much in academic research, who helped with my life and my family, who was always kind and understanding.

My co-supervisor Maria Albin who contributed so much to every paper and also encouraged me a lot with her warm smile.

Eva Assarsson who did so much in the field work and also made me not only survive in Sweden but also enjoy the beauty of it.

Karin Paulsson who taught me the method of telomere measuring and kept the lab in perfect order.

Maria Hedmer who did the exposure measurements and endured my endless interruption.

Håkan Tinnerberg, Christian Lindh, and Bo Jönsson for all precious comments for my papers.

Jonas Björk for helps with statistics.

Lars Rylander and David Gisselsson for valuable comments on my half-time seminar.

Staffan Skerfving for valuable comments on my final seminar.

Yulia Lindholm for all my questions regarding economics and papa-ledig.

Helen Åkesson for all the reagents ordering that never ends.

Ralf Rittner for the computer and technological support.

Giovanni Ferrari and Berit Holmskov for the happy mood in the lab.

Karin Engström for helps with papers and also for sharing the experience of raising kid.

Bakhtiar Hossain and Shegufta Ameer for a nice working atomsphere in the shared office room and also the help with academic and living suggestions, and of course the great food.

Harriet Mörtstedt, Gabriella Johansson and Ayman Alhamdow for great experience of sharing room with.

Former and present Doktorand fellows for all the great time and inspiring conversations we have had together.

My wife Yiyi Xu for being my strongest support all these years. I realize more and more that our marriage is my wise decision, and hope you feel the same. Moreover, thank you for bringing Aaron to us. However, I'm not going to thank you, Aaron, for reducing my energy and time in research. Probably you will not understand it by the time you can read it, but I hope you do when you get your first kid. Papa loves you.

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Paper I

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

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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

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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 ($\mu g/m^3$)					
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/m³; group 2: 5.0–20 µg/m³; group 3: >20µg/m³).

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,

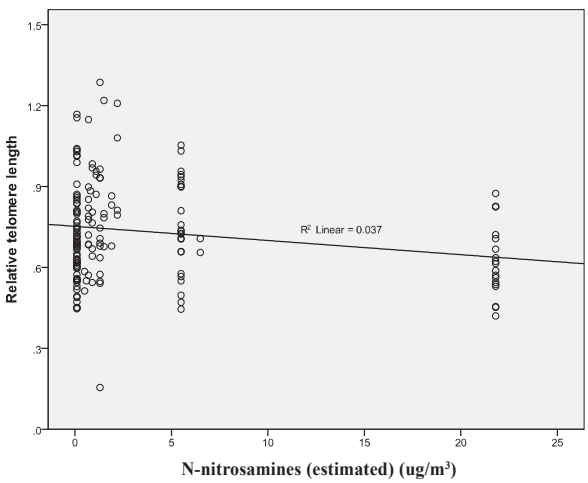

Figure 1. Association between estimated N-nitrosamines and telomere length.

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.

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Received for publication: 7 August 2010

Paper II

Telomere length, 8-oxodG and DNA methylation of tumour suppressor genes in workers exposed to welding fumes or diesel exhaust

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Key words: particle, welding fume, diesel exhaust, 8-oxodG, epigenetic, telomere

Abstract

Objectives To elucidate relations between occupational exposure to particles such as welding fumes and diesel exhaust and oxidative stress and cancer-related DNA alterations.
Methods There were 101 welders, 34 diesel-exposed workers and 127 controls (all non-smoking) recruited from southern Sweden. A structured interview and biological sampling were conducted for each subject. Personal sampling of respirable dust was performed for the welders and elemental carbon and nitrogen dioxide were measured in the diesel-exposed workers. 8-oxodG was measured in urine by a simplified method with LC-MS/MS and telomere length in peripheral blood by quantitative PCR. DNA methylation of 10 tumor suppressor genes was analyzed in peripheral blood by methylation sensitive high resolution melting.
Results The average mass concentration of respirable dust for welders was 2.3 mg/m^3 (range 0.1-19.3). Diesel-exposed workers were exposed to NO_2 84 µg/m^3 (27-222) and elemental carbon 3.3 µg/m^3 (<0.4-10.3). Urinary concentrations of 8-oxodG were higher in the welders and diesel-exposed workers compared with the controls (P=0.070 and P=0.0039, respectively). The telomere length in the welders group was 0.048 units shorter (P=0.090), in the diesel-exposed group it was 0.073 units longer (P=0.077) than the control group. The fraction of individuals with methylation of *APC* was significantly higher in the welders than the controls group (P=0.014). There was a non-significant increase of methylation of *HOXA9* in both welders and diesel-exposed workers. No clear associations with years working with particle exposure or ongoing exposure and DNA alterations could be found.
Conclusions There were weak associations between cancer-related DNA alterations and occupational exposure to particles in current workers.

Introduction

Approximately 16,000 welders in Sweden and several million people around the world are occupationally exposed to welding fumes (Antonini 2003; IVL 2013). Welding fume has been categorized as possible human carcinogen (group 2B) (IARC 1990). There are reports showing 25-40% increase in risk (measured as odds ratio, OR) for lung cancer in welders ('t Mannetje et al. 2012; Ambroise et al. 2006; Hansen et al. 1996; Kendzia et al. 2013; Vallieres et al. 2012). However, negative result has also been reported in large cohorts from Sweden and other countries (Danielsen et al. 2000; Gustavsson et al. 2000; Sjogren et al. 1987), and it is not clear if the excessive lung cancer risk is merely due to exposure to welding fumes. Welding fumes are derived from combustion and contain a mixture of metal oxide particles; mild steel generate welding fumes mainly consisting of iron and manganese, stainless steel also generate fumes with chromium and nickel (Leonard et al. 2010). It has been suggested that the increased cancer risk is only related to welding with stainless steel (Simonato et al. 1991), but recent findings showed that even welding in mild steel is associated to increased cancer risk (Ambroise et al. 2006; Hansen et al. 1996). Other particle exposures are related to lung cancer risk, one is diesel exhaust that recently has been classified as a group 1 human carcinogen (Benbrahim-

Tallaa et al. 2012). Diesel exhaust is emitted from diesel-driven engines and is a complex mixture of elemental carbon, adsorbed organic compounds, and small amounts of sulfate, nitrate, and trace elements (Ris 2007).

Increased oxidative stress in relation to exposure to welding fumes or diesel exposure has been found with different biomarkers (Fidan et al. 2005; Han et al. 2005; Li et al. 2004). Welding fumes and diesel exhaust can induce free radical activity on the surface of the particles (Dellinger et al. 2001; Leonard et al. 2010; Liu et al. 2013; Møller et al. 2008; Wauters et al. 2013). In the human body, free radicals can target the DNA and lead to formation of lesions as 8-oxo-deoxyguanosine, 8-oxo-deoxyadenosine, and deoxythymidine glycol. If DNA damage accumulates and is not repaired in time, it could lead to chromosomal aberrations or mutations. This has been suggested as a possible mechanism for welding fume- or diesel-induced cancer development (Chuang et al. 2010; du Plessis et al. 2010). Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is mainly produced by an interaction between hydroxyl radicals and the 2'-deoxyguanosine. This lesion is usually removed from the body and excreted in urine, and 8-oxodG is generally considered as a marker of oxidative stress (Cooke et al. 2009). In one study it has been shown that urinary 8-oxodG concentrations predict lung cancer risk among nonsmokers (Loft et al. 2006).

Telomere is a DNA-protein structure that consists of tandem repeats of TTAGGG at the end of eukaryotic chromosomes (Blackburn 1991). Telomeres play a key role in chromosome stability (Blasco 2005) and short telomeres have been reported to be associated with various types of cancer, including bladder, lung, breast, colon, head and neck, renal and skin cancer (Broberg et al. 2005; Hofer et al. 2011; Ma et al. 2011; Nan et al. 2011; Willeit et al. 2011; Winnikow et al. 2012). One study showed that telomere length in peripheral blood increased after 3 days of exposure to metal-rich particles (Dioni et al. 2011), but there are no reports regarding the effect of long-term exposure to welding fumes on telomere length. In parallel, studies on exposure to air pollution suggested that telomere length increased with short-term exposure but decreased with long-term exposure (Hou et al. 2012; Hoxha et al. 2009; McCracken et al. 2010).

One key regulator of gene expression is 5-methylcytosine DNA methylation. Hypermethylation of tumor suppressor genes, which turn off their gene expression, is often found as an early change during carcinogenesis, both in tumour tissue and body fluids including peripheral blood (Balgkouranidou et al. 2013; Esteller 2008). The methylation of these genes can therefore be used as a biomarker for early detection of cancer-related changes (Iwamoto et al. 2011; Schneider et al. 2011; Ting et al. 2006).

The major aim of this study was to elucidate if current welders in Sweden show signs of cancer-related DNA alterations. We also included a smaller diesel-exposed group for comparison, as diesel particles is often considered to be more genotoxic than welding fume particles (Benbrahim-Tallaa et al. 2012; IARC 1990).

Material and Methods

Study population

We recruited 101 welders from 10 different companies in Southern Sweden, and the characteristics of 8 of the companies with welders have recently been described (Hedmer et al. 2013). In brief, the 10 companies produced heavy vehicles (dumper trucks, fork-lift trucks, wheel loaders, asphalt rollers, and railway wagons), lifting tables, stoves, heating boilers and pumps, and equipment for the mining industry (Table 1). The number of welders from each company ranged 4-24. We also recruited 34 tunnel construction workers to investigate the genotoxic effects of exposure to diesel exhaust. These workers were working on a tunnel drilling platform and exposed to diesel exhaust from diesel-driven local trains going in and out of the tunnel. The 127 controls were recruited in storage houses from 7 companies organizing grocery goods. For inclusion in the study, the subjects should be male and non-smokers at least for the last twelve months. Structured interviews with a questionnaire were conducted by a trained nurse to obtain information about ethnicity, education, medical history, personal and family disease history, diet, activity, smoking, alcohol consumption, residence history, wood burning stove/boiler at home, wood smoke from the neighborhood, exposure to traffic, working environment, occupational history, and hobbies. All study participants answered the same questionnaire, apart from questions regarding work tasks that differed between the exposure groups.

Venous blood and spot urine samples were collected from each subject. Urine was collected during the last 4 hours of an 8 hour work shift throughout the week. All study subjects gave their informed written consent to take part in the study and the study was approved by the Regional Ethical Committee of Lund University, Sweden.

Exposure assessment

Measurement of ongoing exposure to welding fumes

Welding fumes consist of metal and metal oxide particles typically of 0.5 µm, and the particles can therefore be considered to be in the respirable size range (Antonini 2003; Hedmer et al. 2013). Measurements of respirable dust were performed once in each of the welding companies and samples were collected in the workers' breathing zones of 70 welders. If powered air purifying respirators (PAPRs) were used the air outside the PAPRs was sampled. Exposure to welding fumes was measured by air sampling on preweighed 37 mm mixed cellulose ester filters (0.8 µm pore size) fitted in leak free cassettes (Sure-Seal, USA) made of conductive polypropylene. Respirable dust cyclones of nickel-plated aluminium (IL-4, BGI Inc., USA) were attached to the filter cassettes. Battery powered sampling pumps (MSA Escort Elf, Pennsylvania, USA) were operated at a flow rate of 2.2 L/min. The air flow was before, during and after the sampling regularly checked with a primary calibrator (TSI Model 4199, TSI Inc., USA). Most of the air sampling was performed during full-shift work; the average sampling time was 6.8 h (range 2.4-8.6 h). The filter samples were analysed gravimetrically for respirable dust according to a certified method. The LOD was set to 0.05 mg/sample.

Parallel measurements of respirable dust were performed for assessing the workplace protection factor (WPF) for the PAPRs. A set-up consisting of two parallel sampling systems for respirable dust was used: one for sampling inside and one for sampling outside the PAPR — on the shoulder in the breathing zone. Parallel samplings were performed on 3 workers at different companies and the respirable dust concentrations were between 3 to 7 times lower inside the PAPRs compared with concentrations in the breathing zones.

Assessment of ongoing exposure to respirable dust

Respirable dust measurements were performed on 53/101 (26/53 used PAPRs) welders with blood and urine samples. Since the respirable dust was measured outside the PAPRs the concentrations were then reduced by a correction factor of 3 to get a better estimate of the exposure inside the PAPRs. The correction factor was based on the results from our parallel respirable dust measurements and literature data of WPFs of PAPRs (Goller and Paik 1985; Han 2002; Hedmer et al. 2013; Janssen et al. 2007).

Forty-eight welders were included in the medical analyses but had no exposure measurements. Their exposure to respirable dust was assessed by exposure data from the 53 measurements mentioned above and plus 17 welders not included in the study, but working with similar tasks. Exposure data from our previous study at the same companies were used in the exposure assessments because a number of welders participated in both (Hedmer et al. 2013).

Measurements of ongoing exposure to diesel exhaust

Exposure to diesel exhaust for 17 tunnel construction workers was measured by personal sampling of elemental carbon and nitrogen dioxide (NO_2). Sampling of elemental carbon were conducted by using size-selective cyclones (BGI4L, BGI Inc., USA) on 37-mm quartz filters (SKC Inc., USA) mounted in plastic 3 pieces filter cassettes (Sure-Seal). An Escort ELF pump (MSA, USA) set at 2.2 L/min provided sample flow. The air flow was before, during and after the sampling regularly checked with a primary calibrator (TSI Model 4199). The filters were analysed according to NIOSH NMAM 5040 with thermal-optical analysis (DRI Model 2001 OC/EC Carbon Analyzer, Atmoslytic, USA) (Birch and Cary 1996).

NO_2 was measured in breathing zone of the workers with passive samplers based on molecular diffusion. The NO_2 samplers were sealed with caps and stored in plastic containers before and after sampling. The analysis of NO_2 was performed by the Swedish Environmental Research Institute. None of the tunnel construction workers used any respiratory protection. The average sampling time for both elemental carbon and NO_2 was 8.0 h (range 5.6-9.7 h).

8-oxodG measurement

Concentrations of urinary 8-oxodG were measured with a simplified method by the use of liquid chromatography tandem mass spectrometry (LC–MS/MS). Five pmol of internal standard, 15N5-8-oxodG, was added directly to 100 µl of urine and then ultrapure water was added to make a final volume of 1 mL, prior to quantitative analysis by a triple

quadruple linear iron trap mass spectrophotometer (QTRAP® 5500; AB SCIEX, Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion-Spray source coupled to a high-speed LC system with four pumps (Prominence UFLC, UFLCXR; Shimadzu Corporation, Kyoto, Japan). The MS analyses were carried out using selected reaction monitoring in the positive ion mode. The 8-oxodG concentrations were determined by peak area ratios between 8-oxodG and the internal standard 15N5-8-oxodG. The urine samples were processed and analyzed in duplicates at 2 occasions, and the repeatability of the method, expressed as the coefficient of variation (CV), was 10%. Three internal controls (urine samples from 2 Swedish adults) were included in each batch for which the CVs were 12%, 9%, and 4%, respectively. The 8-oxodG concentrations were above the LOD (0.5 nmol/L) in all urine samples. The concentration of urinary 8-oxodG was adjusted to the specific gravity.

Telomere length

DNA was isolated from whole peripheral blood by Qiagen DNA Blood Midi kit (Qiagen, Heidelberg, Germany). Quantitative PCR was adopted to determine relative telomere length as described previously (Li et al. 2012). Briefly, master mixes for telomere runs were prepared with telomere primers (0.45 μM of each primer), 1×PCR Buffer (Life Technologies, Carlsbad, CA, USA), 1.75 mM $MgCl_2$, 0.8 mM dNTPs, 0.3 mM SybrGreen (Life Technologies), 1×Rox (Life Technologies), and 0.5 U *Taq* Platina (Life Technologies); while hemoglobin beta chain (*HBG*) gene runs were prepared with *HBG* primers (0.40 μM for each primer) and KAPA SYBR FAST qPCR Kit Master Mix (2X) ABI Prism (Kapa Biosystems, Woburn, MA, USA). The PCR was performed on a real-time PCR machine (7900HT, Applied Biosystems, Foster City, CA, USA). Five microliter of sample DNA (4 ng/μl) was added to each reaction (end volume 20 μl). A standard curve, a reference DNA and a negative control were also included in each run, all run in triplicates. For the standard curve, one calibrator DNA sample was diluted serially by 2-fold per dilution to produce 5 concentrations of 1-16 ng/μl. R^2 for each standard curve was >0.99. Standard deviations (for C_t values) were accepted at <0.1. The relative length of the telomeres was obtained through calculating the ratio (T/S) of telomere repeats product and single copy gene product (S, here *HBG*) for each individual, by the formula $T/S = 2^{-\Delta Ct}$, where $\Delta Ct = Ct_{telomere} - Ct_{HBG}$. This ratio was then compared with the ratio of the reference DNA. The telomere length ratio is an arbitrary value. The CV of 12 runs was 7%.

DNA methylation

We chose 10 genes that have been reported aberrantly methylated in patients with lung cancer to measure the status of methylation: homeobox A9 (*HOXA9*) (sputum specimens) (Hwang et al. 2011), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) (peripheral blood and tumour) (Tan et al. 2013; Vaissiere et al. 2009), short stature homeobox 2 (*SHOX2*) (plasma) (Kneip et al. 2011), adenomatous polyposis coli (*APC*) (sputum specimens) (Konno et al. 2004), O-6-methylguanine-DNA methyltransferase (*MGMT*) (serum and sputum specimens) (Esteller et al. 1999; Guzman et al. 2012), cadherin 1, type 1, E-cadherin (*CDH1*) (sputum specimens) (Guzman et al. 2012), Dorsocross1 (*DOC1*), Ras association (RalGDS/AF-6) domain family member 1A (*RASSF1A*) (peripheral blood and tumour tissue) (Tan et al. 2013; Vaissiere et al. 2009), homeobox B13 (*HOXB13*) (cell

culture) (Rauch et al. 2006), and BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*) (tumour) (Castro et al. 2010).

Bisulfite modification was performed on 500 ng/sample of the template DNA with EZ-96 DNA Methylation-Gold™ kit (Zymoresearch, CA, USA) according to manufacturer's protocol. Controls included methylated (Universal Methylated Human DNA Standard, Zymoresearch) and unmethylated (EpiTect Control DNA, Qiagen) DNA and they were prepared following the same procedure as the DNA samples from blood. Methylation Sensitive High Resolution Melting (MS-HRM) assays were designed according to the guidelines described earlier (Wojdacz et al. 2008b). Each PCR reaction was performed in triplicates with PCR mix containing 1x MeltDoctor™ HRM Master Mix (Life Technologies), 500 nM of each primer and 15 ng of the template (theoretical calculation after bisulfite modification). All MS-HRM results were aligned according to the melting curve of the controls. The controls consisted of mixtures of the reference samples: 0.1%, 1%, and 10% of methylated template in an unmethylated background, and 100% fully methylated template. PCR amplification was performed on a 7900HT with the parameters for amplification and HRM analyses: hot start for 10 min at 95°C; 50 cycles of 95°C for 15 s; annealing at optimal primer annealing temperature for 10 s; elongation at 70°C for 20 s. The HRM analysis consisted of denaturation at 95°C for 15 s at ramp rate 100; re-annealing at 60°C for 1 min at ramp rate 100; melting from 60 to 95°C at ramp rate 1. The MS-HRM assays were calibrated to detect methylated alleles at the level of 15 pg in the background of unmethylated alleles. We were able to detect around 3 cells in our experimental set up, based on calculations not including losses of the template during sample processing or degradation during bisulfite modification. HRM is a quantitative method, which can only tell if there is any or no methylation in each sample, so that samples can be categorized as methylated but the degree of methylation between samples can differ. HRM data were analysed using the SDS2.4.1 software (Life Technologies).

Statistical analyses

Medians and 5-95 percentiles of age, BMI, and levels of biomarkers were calculated and are listed in Table 2. The differences among groups were compared by analysis of variance (ANOVA). Spearman's rho correlations between 8-oxodG, telomere length, DNA methylation, and information regarding health history, life style and hobbies were examined to determine which factors should be adjusted for in the multivariate analysis.

The general linear model was used to identify associations between 8-oxodG and telomere length versus exposure. Logistic regression was used for associations between DNA methylation and exposure. Associations between telomere length, 8-oxodG and DNA methylation were investigated by linear regression models. All models were adjusted for age as a general confounder for DNA alterations (Vijgt and Suh 2013). Other possible confounders were considered as well, and the criteria of including them in the models were that their effect estimate should be significant in the final model or the effect estimates for other independent variables changed more than 10% when the covariate were included. All statistical analyses were completed by using SPSS 21.0 (SPSS Inc, Chicago, IL, USA) and statistical significance refers to $P<0.05$ (two-tailed).

Results

General characteristics of the study subjects

The study subjects in different exposure groups were similar for the majority of characteristics (Table 2). The majority (74%) reported to have Swedish ancestry, and the distributions in the exposure groups were not significantly different (P=0.49). Most subjects had training school as the highest achieved level of education (72%), but diesel-exposed workers reported higher level of education (P=0.0015 for difference between groups). Subjects across groups did not differ regarding cancer incidence, prescriptive medication, vegetable intake, fruit intake, fish intake, snuffing, wine consumption, or daily time spent in traffic. All subjects were non-smokers, and the percentages of former smokers in different groups did not differ (P=0.40), but the patterns of exposure to passive smoking did (P<0.001): diesel-exposed subjects were more exposed than the other groups. Performing wood burning with stove/boiler at home was more common in welders (P<0.001). The current residence differed: a larger proportion of the controls lived in cities and less in small towns (P=0.017, Fisher's Exact test).

Occupational exposure

Welders had been working with welding for an average of 7.0 years (range 0 – 45 years) and only 9 welders less than 12 months. Most welders were working with mild steel: 11 had work tasks related to stainless steel. The welders' ongoing exposure to welding fumes and the tunnel construction workers ongoing exposures to diesel exhausts are presented in Table 1. Out of 101 welders, 97 had exposure lower than the occupational exposure limit (5 mg/m^3) (Swedish Work Environment Authority 2011). The tunnel workers had a wide range of exposure for NO_2 and elemental carbon, reflecting that the diesel-driven train and the workers themselves moved during the working shift. In addition, the levels of NO_2 and elemental carbon in this study is relatively low compared with other studies focusing on tunnel workers (Bakke et al. 2001; Sauvain et al. 2003), and the level of NO_2 is quite low compared with occupational exposure limit (2 mg/m^3) (Swedish Work Environment Authority 2011). The controls had, based on questionnaire data, no occupational contact with welding fumes or diesel exhaust. The personal measurement on 19 workers from two companies in the control group showed that average and median exposure to respirable dust was 0.1 mg/m^3. Stationed measurement of respirable dust was carried out in the four other control companies and the results were similar to personal samplings in those two companies. NO_2 and elemental carbon were not measured in control companies since there was no emitting source of diesel exhaust.

Oxidative stress and DNA alterations

The average of urinary 8-oxodG was 14 nmol/L (range 3.6-51). The mean relative telomere length was 0.90 (0.37-1.8). *HOXA9* showed methylation in the majority of the subjects (95%, Table 2); *CDKN2A* and *SHOX2* were methylated in 37% and 45%, *MGMT* and *APC* in 17% and 4.3% of the subjects. *BNIP3* failed in our analysis; 3 genes (*DOC1, RASSF1A, HOXB13*) were unmethylated in all subjects, and *CDH1* was methylated only in one subject; thus, these latter 5 genes were not included in the statistical analysis.

Levels of 8-oxodG and the relative telomere length were significantly different between exposure groups (P=0.0090 and 0.013, Table 2). 8-oxodG concentrations were increased in the diesel-exposed group compared with controls (P=0.0029); the telomere length was in average longer in the diesel-exposed workers compared with the controls (P=0.077) or with the welders (P=0.0039). Proportions of DNA methylation were different for *APC* (P=0.0040), and post-hoc tests showed that more welders showed methylation of *APC* compared with controls (P=0.0022).

Linear regression analysis between biomarkers of DNA alterations and exposure showed that urinary 8-oxodG in the welders group were in average 1.4 nmol/L higher than the controls (P=0.070); while 8-oxodG in the diesel-exposed group was 3.3 nmol/L higher than the controls (P=0.0029, Table 3). Relative telomere length in the welders group was 0.048 units shorter (P=0.090), and in the diesel-exposed group it was 0.073 units longer (P=0.077) than the controls. Based on the Spearman's correlation, current residence was correlated with 8-oxodG and passive smoking with telomere length (Supplement Tables 1 and 2). When including current residence in the model for 8-oxodG or passive smoking for telomere length, the effect estimates did not change substantially. DNA methylation of *APC* showed significant difference between the welders and the controls groups, where welders group showed a higher probability of methylation [odds ratio OR=9.8, 95% confidence interval (CI): 1.2 – 81]. The welders also showed a higher probability of methylation of *HOXA9* than the controls (OR=4.2, 95% CI: 0.89 – 20), while the difference between the diesel-exposed group and the controls was not calculable due to the fact that all subjects in the diesel-exposed group were categorized as methylated.

Welders and controls were analysed for associations between 8-oxodG, telomere length and DNA methylation and ongoing exposure based on actual measurements and exposure assessment (Table 4). However, there was no significant association between ongoing exposure and any of the biomarkers.

Discussion

We analysed different types of cancer-related DNA alterations in blood, as a proxy for early carcinogenic changes, in relation to occupational exposure to particles such as welding fumes and diesel exhaust. However, there were no strong genotoxic effects of the exposures. More methylation of the tumour-suppressor genes, *APC*, as well as non-significantly increased 8-oxodG in urine and shorter telomeres, was found in welders compared with controls, but the effects could not be linked to ongoing exposure. There was no clear evidence for the notion that the diesel exhaust was more genotoxic than welding fumes at levels measured in this study. The diesel-exposed workers had somewhat higher concentrations of 8-oxodG and longer telomeres compared with the other groups. However, due to limited exposure data we could not conclude if this was a long-term or short-term effect of diesel exposure.

There are advantages with this study. Only nonsmokers were recruited, as smoking is a known confounder for associations between DNA damage and exposure to

fine particles and organic hydrocarbons. We carefully evaluated the exposure by questionnaire data as well as direct measurements for particle exposure at the different companies involved, even in controls. MS-HRM is a very sensitive and specific method for detection of methylation (Wojdacz and Dobrovic 2007; Wojdacz et al. 2008a) and the 8-oxodG and telomere length analysis showed low level of methodological variation. Although the number of diesel-exposed subjects was smaller, we still found significant differences between groups showing that our measurements of DNA alterations were reliable. There are nevertheless some weaknesses to mention. Exposure measurement could not be done on all study participants and they were not performed on the same day as interview and sampling. We analysed DNA methylation in peripheral blood and not in lung tissue or induced sputum, which can be better surrogate for lung tissue than peripheral blood. Methylation changes in peripheral blood could reflect an alteration of cell composition with exposure, or alternatively methylation changes related to the exposure in different cell types. In this study we could not tell the difference.

We did not observe a significant increase of 8-oxodG in the welders. Higher oxidative stress and lower antioxidant capacity, measured as oxidized lipoproteins, total antioxidant status, and superoxide dismutase levels, have been reported in welders (Han et al. 2005; Liu et al. 2013; Stepnewski et al. 2003), but results regarding urinary 8-oxodG and exposure to welding fumes were not consistent. Nuernberg et al. reported a significant increase of urinary 8-oxodG after 6 hours' exposure to welding fumes (Nuernberg et al. 2008), whereas no significant difference of 8-oxodG between welders and controls was found by Liu et al. (Liu et al. 2013). It was suggested that urinary 8-oxodG might have a short half-life, and may normalize to baseline within 24 hours (Nuernberg et al. 2008). In our study the urine samples collected were from the last 4 hours of an 8 hour work shift, and it should thus be able to reflect changes induced by short-term exposure. Increased oxidative stress was found in the diesel-exposed group, which was in line with previous studies (Han et al. 2010; Lee et al. 2012; Wei et al. 2010). However, studies showing no association between 8-oxodG and diesel exposure are also found in the literature (Allen et al. 2009; Harri et al. 2005).

The telomere length in blood was marginally shorter in welders compared with the controls and somewhat longer telomeres were found in the diesel-exposed group. The different direction in findings between the groups could reflect long-term versus short-term effects: Studies on telomere length and particulate matter (Dioni et al. 2011; Hou et al. 2012; Hoxha et al. 2009; McCracken et al. 2010) suggest that short-term exposure induce longer telomeres and long-term exposure shorter telomeres (Zhang et al. 2013). In our study, diesel-exposed workers took 12-hour work shift for continuous 6 days and then took 9 days break. One could speculate that they were under short-term exposure within these 6 days and the effect could be washed out in the 9 days break.

APC encodes a tumour suppressor protein that regulates the Wnt signaling pathway, which is very important in control of cell growth (Sparks et al. 1998). Inactivating mutations of *APC* are one of the most characteristic genetic changes in relation to adenomas and carcinomas and it can occur very early in the sequence (Arends 2013). Hypermethylation of the *APC* promoter has been found in serum and plasma of patients with lung cancer and in patients with other types of epithelial cancer compared

with healthy controls (Usadel et al. 2002). The result from our study are in line with a previous study that showed an increase in *APC* methylation in workers in an electric steel plant after 3 days of occupational exposure to metal-rich particles (Hou et al. 2011). Such change of methylation pattern may indicate processes related with particle-induced lung cancer and stresses that this finding needs to be followed up. A non-significant increase of methylation of *HOXA9* was also observed in both welders and diesel-exposed workers. *HOXA9* has been reported to be hypermethylated in lung cancer tissue and induced sputum specimens, especially in early stage of lung cancer (Hwang 2011), and also in bladder cancer (Reinert 2011). In this study, the increase of methylation in the welders group was not significant, and the difference between the diesel-exposed group and the controls were not calculable due to that all subjects in the diesel-exposed group were categorized as methylated. Still, this pattern could reflect that the diesel-exposed group may be going through certain processes related to carcinogenesis.

We did not find any associations between DNA alterations and ongoing exposure, suggesting that effects of methylation could be a long-term effect. We believe that the average concentration of welding fume within the workshop is stable over time based on data from this study and previous studies (Hedmer et al. 2013). Still, as mentioned in the weakness of the study, the fact that the exposure measurement was not done on the same day as investigation and sampling may limit the analysis of association between exposure measurement and biomarkers.

In summary, our study shows that current workers occupationally exposed to welding fume or diesel-exhaust show a small increment in levels of DNA alterations. If these findings are related to higher risk of future particle-induced lung cancer needs to be followed up.

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Tables

Table 1. A) Respirable dust concentrations for Swedish welders measured in 10 companies in the manufacturing industry. B) Exposure to diesel exhaust among tunnel construction workers.

Welders					
			Mass concentration of respirable dust (mg/m^3)		
Company	Manufactured product	No. of workers	GM[a]	GSD[a]	Range
1	Hydraulic lifting tables	5	1.2	2.6	0.5-3.6
2	Containers	6	0.9	2.8	0.2-4.3
3	Stoves	6	0.6	2.9	0.2-2.3
4	Heating boilers and pumps	10	0.3	2.9	0.1-2.2
5	Fork-lift trucks	9	3.6	2.8	0.5-11.8
6	Dumper-trucks	9	1.9	2.0	0.5-5.8
7	Wheel loaders	9	1.7	2.7	0.5-9.1
8	Equipment for the mining industry	4	1.0	2.0	0.5-2.3
9	Railway wagons	7	2.2	2.9	0.8-19.3
10	Asphalt rollers	5	1.2	2.3	0.6-3.3
Total		70	1.2	3.3	0.1-19.3
Tunnel workers					
			Mass concentration ($\mu g/m^3$)		
Exposure metric		No. of workers	GM[a]	GSD[a]	Range
NO_2		16[b]	72	2	27-222
Elemental carbon		17	2.2	3.2	<0.4-10.3

[a] GM= geometric mean; GSD= geometric standard deviation
[b] One worker lost his passive sampler during the sampling

Table 2. Basic characteristics including biomarkers of DNA alterations for groups of controls, welders and diesel-exposed workers. P-value for differences between groups.

	Controls	Welders	Diesel-exposed	Total	P
Age[a]	43 (23-56)	41 (23-60)	39 (26-59)	42 (23-59)	0.59[f]
BMI[a]	27 (22-34)	28 (22-34)	27 (21-33)	27 (22-34)	0.34[f]
Ethnicity[b]	122/5 (3.9%)	96/5 (5.0%)	31/3 (8.8%)	249/13 (5.0%)	0.49[g]
Education[c]	109/17 (87%)	94/7 (93%)	23/11 (68%)	226/35 (87%)	0.0015[g]
Years working in current occupation[a]	6.0 (0.83-24)	7.0 (0.50-24)	4.0 (0.33-26)	6.0 (0.5-24)	0.058[f]
History of cancer (yes/no)	2/125	0/101	0/33	2/259	0.62[g]
Family history cancer[d]	26/95/5	16/80/5	8/26/0	50/201/10	0.65[g]
Smoking history (yes/no)	43/83 (34%)	43/58 (43%)	11/23 (32%)	97/164 (37%)	0.40[g]
Passive smoking (yes/no)	16/111 (13%)	29/72 (29%)	20/13 (61%)	65/196 (25%)	<0.001[g]
Wood burning stove/boiler at home (yes/no)	27/100 (21%)	47/54 (47%)	13/21 (38%)	87/175 (33%)	<0.001[g]
8-oxodG[a] (nmol/L)	13 (6.2-20)	14 (7.0-28)	15 (7.7-30)	14 (6.8-23)	0.0090[f]
Relative telomere length[a]	0.88 (0.57-1.3)	0.86 (0.55-1.2)	0.93 (0.66-1.5)	0.88 (0.56-1.3)	0.013[f]
Methylation *HOXA9*[e]	116/10 (92%)	95/2 (98%)	33/0 (100%)	244/12 (95%)	0.070[g]
Methylation *CDKN2A*[e]	46/80 (37%)	43/58 (43%)	8/26 (24%)	97/164 (37%)	0.14[g]
Methylation *SHOX2*[e]	59/63 (48%)	42/56 (43%)	14/20 (41%)	115/139 (45%)	0.64[g]
Methylation *APC*[e]	1/126 (0.79%)	9/84 (9.7%)	1/33 (2.9%)	11/243 (4.3%)	0.0040[g]
Methylation *MGMT*[e]	20/107 (16%)	16/85 (16%)	9/25 (26%)	45/217 (17%)	0.31[g]

a Median (5% - 95%).
b Subjects from Europe/others (%).
c Subjects attained education as high school or lower/university or higher (% of lower educated).
d Subjects with/without/do not know about parents' history of cancer.
e Subjects with/without (%) methylation.
f The P-values were from ANOVA.
g The P-values were from Fisher's Exact tests.

Table 3. Associations between DNA alterations and occupational exposure to welding fumes and diesel exhaust with adjustment for age.

DNA alterations	Variables	Beta/OR	95%CI	P
8-oxodG[a]	Exposure group			
	Welder	1.4	-0.15 – 2.9	0.076
	Diesel-exposed	3.4	1.1 – 5.6	0.0031
	Age	0.026	-0.042 – 0.094	0.45
Telomere length[a]	Exposure group			
	Welder	-0.047	-0.10 – 0.0075	0.090
	Diesel-exposed	0.070	-0.010 – 0.15	0.086
	Age	-0.0018	-0.0043 –0.00060	0.14
Methylation *HOXA9*[b]	Exposure group			
	Welder	4.1	0.88 – 19	0.073
	Diesel-exposed	---	---	---
	Age	1.0	0.95 – 1.1	0.76
Methylation *CDKN2A*[b]	Exposure group			
	Welder	1.3	0.75 – 2.2	0.36
	Diesel-exposed	0.55	0.23 – 1.3	0.18
	Age	1.0	0.99 – 1.0	0.27
Methylation *SHOX2*[b]	Exposure group			
	Welder	0.78	0.46 – 1.3	0.37
	Diesel-exposed	0.78	0.36 – 1.7	0.54
	Age	1.0	1.0 – 1.1	0.018
Methylation *APC*[b]	Exposure group			
	Welder	9.8	1.2 – 81	0.034
	Diesel-exposed	2.9	0.17 – 49	0.46
	Age	1.0	0.95 – 1.1	0.86
	Wood burn at home	4.5	1.1 – 18	0.034
Methylation *MGMT*[b]	Exposure group			
	Welder	1.0	0.49 – 2.0	0.99
	Diesel-exposed	2.0	0.82 – 5.1	0.12
	Age	1.0	1.0 – 1.1	0.088

[a] General linear model.
[b] Logistic regression.

Table 4. Associations between DNA alterations and current occupational exposure to welding fumes (measured or estimated) with adjustment for age.

DNA alterations	Variables	Beta/OR	95%CI	P
8-oxodG[a]	Ongoing exposure	0.47	-0.15 – 1.1	0.14
	Age	0.0014	-0.072 – 0.075	0.97
Telomere length[a]	Ongoing exposure	-0.011	-0.033 – 0.011	0.34
	Age	-0.0010	-0.0036 – 0.0016	0.46
Methylation *HOXA9*[b]	Ongoing exposure	8.6	0.49 – 152	0.14
	Age	1.0	0.95 – 1.1	0.75
Methylation *CDKN2A*[b]	Ongoing exposure	1.1	0.92 – 1.4	0.24
	Age	1.0	0.98 – 1.0	0.61
Methylation *SHOX2*[b]	Ongoing exposure	0.95	0.77 – 1.2	0.63
	Age	1.0	0.99 – 1.0	0.20
Methylation *APC*[b]	Ongoing exposure	0.85	0.45 – 1.6	0.62
	Age	1.0	0.94 – 1.1	0.78
	Wood burn at home	6.0	1.5 – 24	0.013
Methylation MGMTb	Ongoing exposure	0.91	0.67 – 1.2	0.56
	Age	1.0	1.0 – 1.1	0.095

[a] General linear model.
[b] Logistic regression.

Supplementary Material

Supplementary Table 1. Spearman's rho correlation among markers.

	Age	BMI	Ongoing exposure	Current residence	Ethnicity	Education	Cancer	Cancer family	Vegetable intake	Fruit intake	Fish intake
8-oxodG	0.023	-0.090	0.11	0.14a	0.079	0.060	-0.10	-0.028	-0.0072	0.025	0.041
Telomere length	-0.10	-0.11	-0.11	-0.096	-0.025	0.086	-0.069	0.067	0.032	0.030	0.039
Methylation *HOXA9*	0.018	-0.11	0.13a	0.13a	0.13a	-0.0077	0.020	-0.049	0.092	-0.0010	0.049
Methylation *CDKN2A*	0.063	0.029	0.11	0.12	-0.035	-0.0049	-0.068	0.035	-0.037	0.017	-0.059
Methylation *SHOX2*	0.15a	0.091	-0.055	-0.073	-0.0060	-0.033	-0.081	0.032	-0.040	0.022	-0.056
Methylation *APC*	0.014	0.055	0.16a	0.14a	-0.038	-0.14a	-0.019	-0.072	0.098	0.10	0.017
Methylation *MGMT*	0.10	0.021	-0.016	-0.0032	0.081	0.028	-0.040	-0.0064	-0.0030	0.072	0.086

[a] P<0.05 [b] P<0.01

Supplementary Table 2. Spearman's rho correlation for different types of DNA alterations.

	8-oxodG	Telomere length	*HOXA9*	*CDKN2A*	*SHOX2*	*APC*	*MGMT*
8-oxodG	1.0						
Telomere length	0.00069	1.0					
Methylation *HOXA9*	0.11	0.013	1.0				
Methylation *CDKN2A*	0.066	0.056	0.084	1.0			
Methylation *SHOX2*	-0.014	-0.11	-0.17b	0.068	1.0		
Methylation *APC*	0.079	-0.11	0.044	-0.16a	0.042	1.0	
Methylation *MGMT*	0.069	-0.10	0.0031	-0.099	-0.029	0.0094	1.0

[a] P<0.05 [b] P<0.01

Physical activity	Snuff	Smoking history	Passive smoking	Wine	other Alcohol	Wood Burn at home?	Wood Smoke in neighbo-urhood	Traffic intensity out of house window	Time In Traffic everyday	Hobby expose to welding fume	hobby expose to engine exhaust	Hobby expose to dust
-0.029	0.048	-0.056	0.075	-0.011	-0.026	0.081	-0.083	0.062	-0.0085	-0.10	-0.019	0.11
0.035	-0.0071	-0.0089	0.13a	-0.050	0.020	-0.078	-0.014	-0.052	0.034	-0.020	-0.10	0.055
0.030	0.081	0.053	-0.0015	0.045	-0.035	0.11	0.072	0.0032	-0.062	0.053	-0.099	0.016
-0.052	-0.013	0.054	-0.055	-0.091	0.020	0.028	0.078	-0.0025	0.00050	0.098	0.051	0.015
-0.13a	-0.088	0.017	-0.012	-0.057	0.034	-0.10	-0.051	0.065	-0.065	-0.12	-0.15	-0.068
-0.085	-0.029	0.081	-0.035	0.12	0.018	0.19b	-0.018	0.0000	-0.11	-0.049	0.16	-0.073
-0.0036	-0.059	-0.040	-0.075	-0.073	-0.11	-0.042	-0.025	-0.044	-0.052	-0.063	0.020	0.039

Paper III

Chemical Research in Toxicology

Article

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Arsenic Exposure through Drinking Water Is Associated with Longer Telomeres in Peripheral Blood

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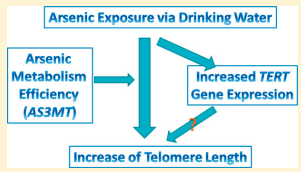
ABSTRACT: Inorganic arsenic is a strong carcinogen, possibly by interaction with the telomere length. The aim of the study was to evaluate how chronic arsenic exposure from drinking water as well as the arsenic metabolism efficiency affect the individual telomere length and the expression of telomere-related genes. Two hundred two women with a wide range in exposure to arsenic via drinking water (3.5−200 μg/L) were recruited. Concentrations of arsenic metabolites in urine [inorganic arsenic (iAs), methylarsonic acid (MMA), and dimethylarsinic acid (DMA)] were measured. The relative telomere length in blood was measured by quantitative real-time polymerase chain reaction. Genotyping (N = 172) for eight SNPs in *AS3MT* and gene expression of telomere-related genes (in blood; N = 90) were performed. Urinary arsenic (sum of metabolites) was positively associated with telomere length ($\beta = 0.65 \times 10^{-4}$, 95% CI = 0.031×10^{-4}−1.3×10^{-4}, adjusted for age and BMI). Individuals with above median fractions of iAs and MMA showed significantly longer telomeres by increasing urinary arsenic ($\beta = 1.0 \times 10^{-4}$, 95% CI = 0.21×10^{-4}−1.8×10^{-4} at high % iAs; $\beta = 0.88 \times 10^{-4}$ 95% CI = 0.12×10^{-4}−1.6×10^{-4} at high % MMA) than those below the median (p = 0.80 and 0.44, respectively). Similarly, carriers of the slow and more toxic metabolizing *AS3MT* haplotype showed stronger positive associations between arsenic exposure and telomere length, as compared to noncarriers (interaction urinary arsenic and haplotype p = 0.025). Urinary arsenic was positively correlated with the expression of telomerase reverse transcriptase (*TERT*, Spearman r = 0.22, p = 0.037), but no association was found between *TERT* expression and telomere length. Arsenic in drinking water influences the telomere length, and this may be a mechanism for its carcinogenicity. A faster and less toxic arsenic metabolism diminishes arsenic-related telomere elongation.



INTRODUCTION

Telomeres are formed by tandem repeats (TTAGGG) located at the end of each eukaryotic chromosome. They are shortened for each cell division due to "the end replication problem".[1] Telomeres are responsible for regulation of cellular life span. At a certain length, the telomeres signal the cell to stop dividing, since too short telomeres may cause genomic instability that accelerates the accumulation of genetic changes responsible for tumorigenesis.[2−5] Short telomeres in peripheral blood have repeatedly been reported as a risk marker for several cancer types.[6−9] Telomere maintenance is the primary mechanism by which cancer cells overcome mortality and extend their life span,[2,4] which is mainly sustained by the activation of the telomere-elongating protein telomerase reverse transcriptase (encoded by the gene *TERT*).

Environmental carcinogens may influence the telomere length,[10,11] but there are few human studies on arsenic exposure. Arsenic in drinking water is a major public health problem in several parts of the world, in particular Bangladesh, India, China, Argentina, and the United States.[12] Arsenic is a potent carcinogen, and chronic exposure increases the risk of cancer in the skin, urinary bladder, lung, and possibly in the kidney, liver, and the prostate. However, arsenic is not a strong direct mutagen, and several other modes of action for arsenic carcinogenesis have been suggested.[13−16] There is some support that arsenic alters the telomeres: arsenic increased telomere attrition, chromosomal rearrangements, and apoptotic cell death in mouse embryos with short telomeres.[17] On the other hand, in vitro studies showed that arsenic increased the activity of *TERT*.[18−20] Also, in people exposed to arsenic via drinking water (1−1000 μg/L) in Inner Mongolia, *TERT* expression was positively associated with both arsenic concentrations in water and in nails and the severity of hyperkeratosis, a common arsenic-related skin lesion.[19] These apparently contrasting effects of arsenic on telomere length may be related to the arsenic dose. In human cord blood cells, sub-nanomolar arsenite was found to increase *TERT* gene and protein expression in vitro, resulting in maintained telomere length, while at 1 μM concentrations, the *TERT* expression and telomere length decreased.[18]

Also, the form of arsenic and the metabolism in the body may be influential. Inorganic arsenic (iAs) is metabolized in humans by a series of reduction and methylation reactions that produce methylarsonic acid (MMA) and dimethylarsinic acid (DMA). The addition of two methyl groups to form DMA from the highly reactive arsenic form arsenite (AsIII), via the even more reactive MMAIII, results in the formation of a much less reactive compound, which is rapidly excreted in urine.[21] Thus, a higher fraction of MMA and a lower fraction of DMA

Received: May 16, 2012
Published: August 23, 2012

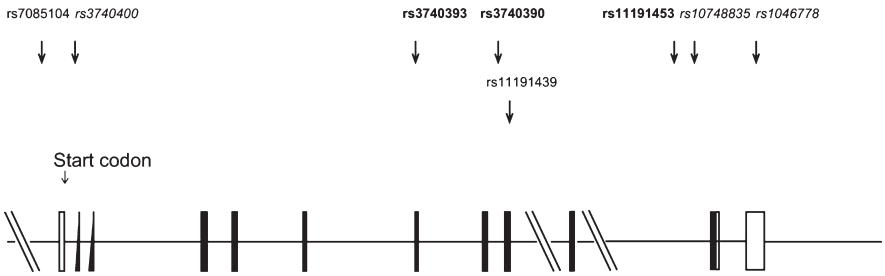


Figure 1. *AS3MT* gene and polymorphisms under study. The black line shows the 5′- and 3′-flanking regions and the intronic regions. Translated exons are illustrated as black boxes, while untranslated regions are illustrated as white boxes. Polymorphisms are illustrated by arrows pointing at their positions in the gene. Inclined lines are shown when parts of the intronic or 5′-flanking regions are shortened for the sake of simplicity.

in urine are associated with a lower rate of arsenic excretion in urine[21,22] and more toxic effects.[23,24] The main methyltransferase in arsenic metabolism is arsenic (+3 oxidation state) methyltransferase (AS3MT),[25] which can methylate both iAs and MMA. We have previously shown that the *AS3MT* genotype is the main determinant of the efficiency of the metabolism of arsenic.[26]

The aim of present study was to clarify how human exposure to arsenic in drinking water affects the telomere length and expression of telomere-related genes and whether the effect is modified by the arsenic metabolism efficiency. Our main hypothesis was that arsenic influences the telomere length through action on expression of *TERT*. However, arsenic may also act through other mechanisms; one plausible is that arsenic acts on expression of telomere binding proteins that stabilize the telomere structure.

MATERIALS AND METHODS

Study Site and Population. We studied 202 women living on the Andean plateau (3800 m above sea level) in Northern Argentina, an area that has minimal industrial and vehicle-derived environmental pollution. However, some villages have elevated concentrations of arsenic in drinking water.[27] The recruitment of the study participants and their characteristics have been described previously in detail.[26,28] In short, the study participants were recruited with the help of the local health clinics. Most of the study participants (N = 161) were from the village San Antonio de los Cobres with about 5000 inhabitants and 200 μg/L arsenic in the public drinking water. The other participants (N = 41) were from three small surrounding villages with lower concentrations of arsenic in the water (3.5−73 μg/L).

The interviews revealed that almost all women drank public drinking water exclusively and that their diets consisted mainly of corn, beans, chicken, and pork. Four women reported that they smoked cigarettes, one reported drinking alcohol, and almost half of the women (46.5%) reported that they often chewed coca leaves. Only three women reported taking any medication at the time of the study; one was being treated for gastritis, and two were being treated for hypertension. The women were asked if they had had any diseases, and their hands were inspected for arsenic-related skin lesions.

Biological samples were collected during the daytime as nonfasting spot samples. Peripheral blood for DNA extraction (N = 202) was collected in K_2EDTA tubes (Vacuette; Greiner, Germany), and blood for RNA extraction (N = 122; the first women recruited to the study) was collected in PAX tubes (Beckton Dickinson, Franklin Lakes, NJ). Spot urine samples were collected and processed as described previously.[29] Blood and urine samples were kept at −20 °C before and after transport (with cooling blocks) to Sweden for analysis. Informed consent, both oral and written, was provided by all of the study participants. The study was approved by the Ministry of Health in Salta, Argentina, and the Regional Ethical Committee of Karolinska Institutet, Sweden.

Analysis of Arsenic in Urine. The concentrations of arsenic metabolites, that is, iAs, MMA, and DMA, were measured using high-performance liquid chromatography coupled with hydride generation and inductively coupled plasma mass spectrometry (Agilent 7500ce; Agilent Technologies, Tokyo, Japan), employing appropriate quality control.[30] The sum of metabolite concentrations (hereafter called urinary arsenic) was used to assess arsenic exposure. The fractions of the different metabolites in urine (percentages of urinary arsenic) were used to assess the efficiency of arsenic metabolism. All measured concentrations of elements in urine were adjusted to the mean specific gravity of urine (1.020 g/mL), determined by a digital refractometer (EUROMEX RD 712 clinical refractometer; EUROMEX, Arnhem, The Netherlands).[17]

Telomere Length Determination and Genotyping. DNA was isolated from whole blood by Qiagen DNA Blood Mini kit (QIAGEN, Heidelberg, Germany). Genotyping for eight *AS3MT* polymorphisms that have been associated with arsenic metabolism[26] (Figure 1 shows the *AS3MT* structure and the positions of the polymorphisms genotyped) was performed using Sequenom (San Diego, CA) technology by Swegenés DNA Facility at Malmö University Hospital, Malmö, Sweden. Haplotypes from the genotypes of these polymorphisms were inferred using PHASE software.[31] The two major *AS3MT* haplotypes in this population were evaluated here: (1) AAGGTTGT (high fraction of MMA and low fraction of DMA, i.e., a slow and more toxic metabolism, haplotype frequency = 26%), and (2) GCCATCAC (low % MMA and high DMA, i.e., a faster and less toxic metabolism, haplotype frequency = 70%), where the order of the polymorphisms in *AS3MT* is (5′-3′): rs7085104, rs3740400, rs3740393, rs3740390, rs11191439, rs11191453, rs10748835, and rs1046778.[26]

For nine individuals, there was not enough DNA for telomere length determination. Relative telomere length quantification was determined by quantitative polymerase chain reaction (PCR) as described in detail,[32] based on the method reported by Cawthon.[33] In short, an aliquot of 6 μL of sample DNA (3 ng/μL) was added to each reaction (end volume, 20 μL). A standard curve, a reference DNA, and a negative control were included in each run. For each standard curve, one calibrator DNA sample was diluted serially by 2-fold per dilution to produce five concentrations of 0.625−10 ng/μL. Each sample, standard curve, reference, and negative was run in triplicate. Master mixes were prepared, containing 0.5U *Taq* Platina (Invitrogen, Carlsbad, CA), 1 × PCR buffer, 0.8 mM dNTPs, 1.75 mM $MgCl_2$, 0.3 mM SybrGreen I (Invitrogen), 1 × Rox (Invitrogen), and either telomere primers (0.45 μM of each primer), or hemoglobin β chain (*HBG*) primers (0.45 μM for each primer). The PCR was performed on a real-time PCR machine (7900HT, Applied Biosystems). The R^2 for each standard curve was >0.99. Standard deviations (for Ct values) were accepted at <0.2. The relative length of the telomeres was obtained through calculating the ratio (T/S) of telomere repeats

product and single copy gene product (*HBG*) for each individual, by the formula $T/S = 2^{-Ct}$, where $Ct = Ct_{telomere} - Ct_{HBG}$. This ratio was then compared with the ratio of the reference DNA. The telomere length ratio is unitless. The reference samples were included in each run and demonstrated a CV of 10%, based on eight runs.

Gene Expression Analysis. RNA was extracted from whole blood with the PAXgene Blood RNA kit (PreAnalytiX) and stored in −80 °C. The RNA concentration and purity were measured by a Nanodrop spectrophotometer (Wilmington, DE), and the RNA integrity (RIN) was evaluated on a Bioanalyzer 2100 (Agilent, CA), showing a good quality of the RNA (RIN > 7.5). For the gene expression analysis, 90 individuals were chosen with a wide range of urinary arsenic (10−1251 μg/L), which were matched for age, weight, and body mass index (BMI). For the whole genome gene expression analysis, DirectHyb HumanHT-12 v4.0 (Illumina, San Diego, CA) was used according to the manufacturer's instructions, and the analysis was performed at SCIBLU core facility at Lund University. Background signals were filtered from the gene expression data by BioArray Software Environment (BASE).[34] Gene expression data for telomere-related genes selected based on literature searches were included in the analyses (120 transcripts from 62 genes). Eighty-five of the 90 individuals had data on telomere length.

Statistical Analysis. Correlations between variables were analyzed by Spearman's ρ correlation (r_s). The linearity of the relationship of arsenic exposure (measured as urinary arsenic) and telomere length was evaluated by visual inspection of a scatter plot (Figure 2), quintiles of urinary arsenic, and analyzing the residuals by P−P plots. Association between telomere length and urinary arsenic was then analyzed by linear regression analysis. The models were adjusted for age and BMI, which were statistically significantly associated with the telomere length in the Spearman's correlation analysis.

To evaluate potential effects of arsenic metabolism on the telomere length, we stratified the associations between arsenic in urine and telomere length for the metabolite fraction (median split). We also evaluated the effect of the major arsenic metabolizing gene *AS3MT* on the association between arsenic and telomere length (first-degree relatives were excluded, total $N = 166$ with telomere length data) by multivariate regression analysis where we included haplotype (as categorical variable), urinary arsenic, and an interaction term between haplotype and urinary arsenic. To obtain an effect estimate in carriers with different numbers of haplotype copies, we performed regression analysis of urinary arsenic and telomere length, stratified by *AS3MT* haplotype. All analyses were adjusted for age and BMI.

Spearman's correlation was adopted to investigate the correlation between urinary arsenic and expression levels of telomere-related genes as well as the association between telomere length and expression levels of telomere-related genes. The p values were adjusted for false discovery rate (FDR) by the approach reported by Benjamini and Hochberg.[35] All statistical analyses were completed using SPSS 18.0 (SPSS Inc., Chicago, IL), except for the FDR adjustments, which were made using R (2.15.1). Statistical significance refers to $P < 0.05$ (two-tailed).

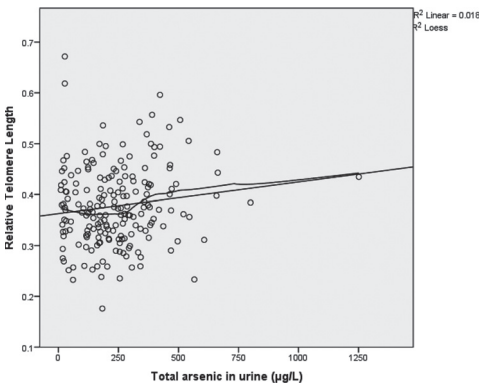


Figure 2. Scatter plot that shows the association between arsenic concentration in urine (μg/L, adjusted to specific gravity 1.020 g/mL) and the telomere length in blood (arbitrary unit). The linear fit line and loess fit line are presented.

RESULTS

The participant characteristics, the concentrations of arsenic metabolites in urine, and relative telomere length are listed in Table 1. None of the women showed any sign of arsenic-related hyperkeratosis on their hands or reported any history of malignancy. There was a wide range in urinary arsenic concentrations (median = 230 μg/L, range = 0.1−1251 μg/L), whereas the relative telomere length varied more than 3-fold (median = 0.37, range = 0.18−0.67). The telomere length was inversely correlated with age and BMI and nonsignificantly positively related with urinary arsenic ($p = 0.21$; Table 1). However, it was positively associated with the fraction of iAs in urine and inversely associated with the fraction of DMA ($p = 0.030$ and 0.013, respectively). BMI increased with increasing age ($r_s = 0.46$, $p < 0.01$). In the subgroup of women analyzed for gene expression in peripheral blood, the correlations between the telomere length and the participant characteristics were similar; however, the correlations with urinary arsenic metabolite fractions were weaker and nonsignificant.

A linear relationship was found between arsenic in urine and telomere length. In the linear regression model, urinary arsenic showed significant positive association with telomere length [$\beta = 0.65 \times 10^{-4}$, 95% confidence interval (CI) 0.031×10^{-4}−1.3

Table 1. Characteristics of the Study Participants, Concentrations of Arsenic in Urine, And Relative Telomere Length in Blood[a]

	$N = 202^b$			$N = 90^b$		
variables	median	range	r_s^c	median	range	r_s^c
age (years)	34	12−80	−0.34**	32	12−65	−0.28**
BMI (kg/m^2)	24.7	16.4−40	−0.28**	24.0	16.4−35.7	−0.32**
urinary arsenic (μg/L)[d]	230	10.1−1251	0.13	203	10.1−1251	0.14
fraction of iAs (%)	11.6	2.3−34.2	0.16*	13.0	3.3−32.9	0.096
fraction of MMA (%)	7.7	2.4−22.1	0.020	7.7	2.4−18.5	0.021
fraction of DMA (%)	80.2	56.4−95.1	−0.18*	78.8	56.4−93.5	−0.13
relative telomere length	0.37	0.18−0.67		0.38	0.24−0.62	

[a]Also, associations of each variable (Spearman correlation, r_s) with the telomere length are shown. [b]$N = 202$ whole study population, and $N = 90$ subgroup analyzed for expression of telomere-related genes in whole blood. [c]*$p < 0.05$; **$p < 0.01$. [d]Adjusted for the specific gravity of urine (1.020 g/mL).

$\times 10^{-4}$; Table 2 and Figure 2]. There were two outliers with long telomere length and low arsenic exposure, and when excluding them, the associations between urinary arsenic and telomere length became stronger ($\beta = 0.87 \times 10^{-4}$, 95% CI 0.30×10^{-4}–1.4×10^{-4}); there was also one outlier with high urinary arsenic (urinary arsenic = 1251 μg/L), but the effect estimate was similar when excluding this individual ($\beta = 0.72 \times 10^{-4}$, 95% CI 0.039×10^{-4}–1.4×10^{-4}). These three outliers did not differ markedly from the rest of the population with respect to the characteristics evaluated. When stratified by the median of metabolites fraction, significant associations between urinary arsenic and telomere length were only found in the group with a high fraction of iAs ($\beta = 1.0 \times 10^{-4}$, 95% CI 0.21×10^{-4}–1.8×10^{-4}) and group with a high fraction of MMA ($\beta = 0.88 \times 10^{-4}$, 95% CI 0.12×10^{-4}–1.6×10^{-4}; Table 2) than those below the median ($\beta = 0.13 \times 10^{-4}$, 95% CI -0.85×10^{-4}–1.1×10^{-4} and $\beta = 0.41 \times 10^{-4}$, 95% CI -0.64×10^{-4}–1.5×10^{-4}). All analyses were adjusted for age and BMI.

Table 2. Associations of Urinary Arsenic Concentrations (Sum of Arsenic Metabolites; μg/L) or Fractions (%) of the Different Arsenic Metabolites in Urine and Telomere Length by Linear Regression Analysis

		relative telomere length		
		β-coefficient	95% CI	p value
urinary arsenic[a]				
		0.65×10^{-4}	0.031×10^{-4}–1.3×10^{-4}	0.040
urinary arsenic				
iAs %[b]	low	0.13×10^{-4}	-0.85×10^{-4}–1.1×10^{-4}	0.80
	high	1.0×10^{-4}	0.21×10^{-4}–1.8×10^{-4}	0.014
MMA %[b]	low	0.41×10^{-4}	-0.64×10^{-4}–1.4×10^{-4}	0.44
	high	0.88×10^{-4}	0.12×10^{-4}–1.6×10^{-4}	0.024
DMA %[b]	low	0.63×10^{-4}	-0.13×10^{-4}–1.4×10^{-4}	0.10
	high	0.29×10^{-4}	-0.78×10^{-4}–1.4×10^{-4}	0.59

[a]Linear regression analysis of urinary arsenic (μg/L) vs telomere length, adjusted for age and BMI. [b]Linear regression analysis of urinary arsenic vs telomere length, stratified for the median of fractions of iAs, MMA, or DMA. The analyses were adjusted for age and BMI.

Genetic modification by the two major haplotypes of *AS3MT* was evaluated for the association between urinary arsenic and telomere length. Interactions were found both for the slow and the more toxic-metabolizing *AS3MT* haplotype 1 ($p = 0.036$) and the fast and less toxic-metabolizing haplotype 2 ($p = 0.025$) with arsenic on telomere length. When the analysis of urinary arsenic versus telomere length was stratified for *AS3MT* haplotypes, there was a trend for longer telomere length by arsenic exposure with increasing copy number of haplotype 1 and a trend for longer telomere length for decreasing copy number of haplotype 2 (Table 3). However, these associations were not significant in the groups with two copies of haplotype 1 or haplotype 2.

Urinary arsenic was analyzed in relation to expression of *TERT*, since our main hypothesis was that arsenic affects the telomeres by regulation of expression of this gene. One *TERT* transcript (encodes the longer isoform, isoform 1) was positively associated with arsenic in urine (in the unadjusted analyses, $p = 0.037$, after FDR adjustments $p = 0.38$; Table 4) but not with the telomere length. Urinary arsenic was further evaluated in relation to other telomere-related genes and showed a weak positive association with *RAP1B*. The telomere length was positively associated with expression of three genes [SCY1-like 1 (*SCYL1*; Figure 3), replication protein A1 (*RPA1*), and RAD1 homologue (*RAD1*)], while the telomere length was inversely associated with expression of eight genes [MUS81 endonuclease homologue (*MUS81*), RAP1 interacting factor homologue (*RIF1*), HUS1 checkpoint homologue (*HUS1*), SCY1-like 3 (*SCYL3*), cyclin-dependent kinase 2 (*CDK2*), RAP1B, member of RAS oncogene family (*RAP1B*), golgin, RAB6-interacting (*GORAB*), and topoisomerase (DNA) II α (*TOP2A*)]. After FDR adjustments, none of these associations remained significant.

DISCUSSION

The study showed for the first time that arsenic exposure through drinking water is associated with longer telomeres in vivo. The mechanism for the action of arsenic on the telomere length may probably mediated through up-regulation of telomerase (*TERT*), which was positively associated with arsenic in urine. It is well-known that cancer cells overcome mortality and extend their life span[2] by maintenance of the telomere length, which is often achieved by up-regulation of telomerase.[4] The findings of this study suggest that part of the carcinogenicity of arsenic may be through extending the life span of premalignant cells by elongation of the telomeres. Additionally, the arsenic metabolism efficiency markedly modified the effect of arsenic on telomere length, and individuals carrying the *AS3MT* haplotype associated with a slower and more toxic metabolism of arsenic had longer telomeres as compared to individuals that lack this haplotype. Thus, individuals with a slower arsenic metabolism are probably at increased risk for effects of arsenic on the telomeres and possibly for arsenic-related carcinogenesis.

Table 3. Association (β) between Arsenic Concentrations in Urine and Telomere Length, Stratified for the Number of *AS3MT* Haplotype Copies (Linear Regression Analysis Adjusted for Age and BMI)

haplotype[a]	N copies	N ind.	β	95% CI	p value
haplotype 1	0	92	0.54×10^{-4}	-0.43×10^{-4}–1.5×10^{-4}	0.27
AAGGTTGT	1	58	1.6×10^{-4}	0.52×10^{-4}–2.7×10^{-4}	0.004
	2	16	4.8×10^{-4}	-1.3×10^{-4}–0.0010	0.12
haplotype 2	0	18	4.9×10^{-4}	0.44×10^{-4}–9.4×10^{-4}	0.034
GCCATCAC	1	67	1.1×10^{-4}	0.19×10^{-4}–2.1×10^{-4}	0.019
	2	81	0.69×10^{-4}	-0.45×10^{-4}–1.8×10^{-4}	0.23

[a]Two major *AS3MT* haplotypes are present in this population: (1) AAGGTTGT (associated with a high fraction of MMA and a low fraction of DMA, that is, a slower and more toxic metabolism and (2) GCCATCAC (i.e., low % MMA and high DMA). The order of the polymorphisms in the haplotypes is according to the 5′ to 3′ direction of *AS3MT*: rs7085104, rs3740400, rs3740393, rs3740390, rs11191439, rs11191453, rs10748835, and rs1046778.

Table 4. Correlations (Spearman, r_s) between Gene Expression and Telomere Length as Well as between Gene Expression and Total Concentrations of Urinary Arsenic Metabolites[a]

		telomere			urinary arsenic		
gene[b]	transcript	r_s	*P* value, unadjusted	*P* value, FDR adjusted	r_s	*P*	*P* value, FDR adjusted
SCYL1	ILMN_2400874	0.35	0.001	0.11	0.03	0.80	0.95
SCYL1	ILMN_1731991	0.32	0.002	0.11	−0.05	0.62	0.92
RPA1	ILMN_1795719	0.29	0.007	0.17	−0.06	0.59	0.91
RAD1	ILMN_1763765	0.23	0.036	0.43	0.04	0.71	0.95
MUS81	ILMN_1780937	−0.32	0.003	0.11	−0.10	0.37	0.74
RIF1	ILMN_2149053	−0.31	0.004	0.11	0.01	0.95	0.97
HUS1	ILMN_2136615	−0.26	0.017	0.35	−0.12	0.28	0.68
SCYL3	ILMN_1743427	−0.25	0.021	0.36	0.11	0.29	0.68
CDK2	ILMN_1665559	−0.23	0.031	0.43	−0.01	0.95	0.97
RAP1B	ILMN_1701434	−0.23	0.033	0.43	0.20	0.065	0.41
GORAB	ILMN_2121316	−0.22	0.043	0.47	0.08	0.47	0.83
TOP2A	ILMN_1686097	−0.21	0.050	0.50	−0.03	0.81	0.95
TERT	ILMN_2373119	0.06	0.61	0.92	0.22	0.037	0.38
TERT	ILMN_1796005	−0.12	0.26	0.92	−0.07	0.53	0.88

[a]Note: The ILMN_2373119 was a component of the *TERT* transcript NM_198253.2, which represents the longer transcript and encodes the longer isoform (isoform 1), while ILMN_1796005 was a component of the *TERT* transcript NM_001193376.1, which lacks an alternate in-frame exon in the middle portion of the coding region as compared to NM_198253.2. This results in a shorter protein (isoform 2) as compared to isoform 1 (ref: www.ncbi/nih/gov/gene). [b]Abbreviations: FDR, false discovery rate; *SCYL1*, SCY1-like 1; *RPA1*, replication protein A1; *RAD1*, RAD1 homologue; *MUS81*, replication protein A2; *RIF1*, RAP1 interacting factor homologue; *HUS1*, HUS1 checkpoint homologue; *SCYL3*, SCY1-like 3; *CDK2*, cyclin-dependent kinase 2; *RAP1B*, RAP1B, member of RAS oncogene family; *GORAB*, golgin, RAB6-interacting; *TOP2A*, topoisomerase (DNA) II α; and *TERT*, telomerase reverse transcriptase.

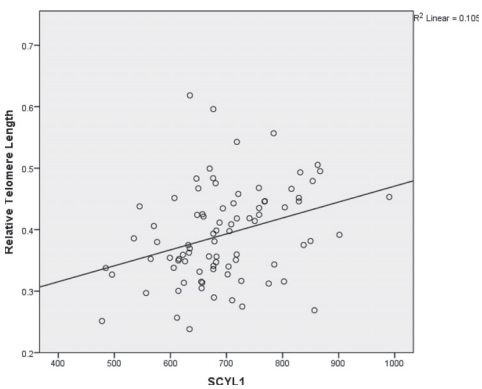


Figure 3. Scatter plot depicting the relationship between the gene expression of *SCYL1* (fluorescence intensity) and the relative telomere length (arbitrary unit) in blood.

There is some support for our results from in vitro and in vivo studies. Arsenite treatment in vitro of keratinocytes or cord blood lymphocytes induced telomerase and telomere length and was also associated with increased cell viability and cell proliferation.[18−20] In a population in Inner Mongolia who were exposed to arsenic via drinking water, an association between expression of *TERT* and arsenic exposure was found, as well as an association between expression of *TERT* and the severity of arsenic-related hyperkeratosis.[19] We found similar arsenic-related increase in *TERT* expression as in the study from Inner Mongolia, and we could also show longer telomeres in relation to arsenic exposure. However, none of the Argentinean women demonstrated hyperkeratosis, whereas in the study from Mongolia, 30% of the individuals had signs of hyperkeratosis. Actually, among the approximately 400 women from this area examined for hyperkeratosis,[26,30] we have not identified one single individual with thickening of the skin. This partly may be explained by the high frequency of the fast arsenic metabolizing haplotype of *AS3MT* in this area, as the *AS3MT* haplotype modified the effect of arsenic on the telomere length.

The expression of telomerase (*TERT*) was positively associated with arsenic in urine, but there was no association between telomere length and *TERT*, which may reflect that these biomarkers are different in time perspective: the effect on telomere length is probably a chronic effect, whereas *TERT* expression reflects a short-term effect of arsenic. The lack of association may also indicate that other processes than telomerase activity are involved in the arsenic-related telomere lengthening. We therefore analyzed expression of genes that influence the telomere stability in vitro in relation to arsenic exposure.

We did not find any evidence that arsenic interacted with their expression, but some of the genes were correlated to telomere length per se, although none of them were significant after FDR adjustments and needs to be cautiously interpreted. Nevertheless, our data indicate a possible role of some telomere-related genes for telomere stability in vivo, and we comment on some of the findings. To our knowledge, this is the first time expression of these genes has been analyzed in relation to human telomere length in vivo. *SCYL1* encodes a protein that can bind specific DNA sequences and can activate transcription of *TERT*, and SCYL1 is involved in DNA damage response when telomeres are shortened in vitro.[36] The positive correlation between *SCYL1* expression and telomere length could indicate that this protein also is up-regulated in vivo and promote longer telomeres or stabilizes the telomere structure. Also, *RAD1* expression, which has a critical role in maintaining telomeres in human cells,[13] was found to be positively associated with telomere length. *RAD1* encodes a component of a heterotrimeric cell cycle checkpoint complex (also known

as the 9−1−1 complex), which localizes to sites of genome damage and promotes DNA damage response signaling.[13,37] Another component of this complex, *HUS1*, was associated with telomere length but in the opposite direction. Telomerase-negative cancer cells can maintain their telomeres via the alternative lengthening of telomeres (ALT) pathway.[38] *MUS81* is an endonuclease that plays a key role in the maintenance of telomeres in cells proficient for ALT,[39] and depletion of *MUS81* in vitro leads to reduced telomere recombination and with growth arrest. *RIF1* encodes a protein that shares homology with the telomere binding protein, Rap1 interacting factor 1, in yeast. *RIF1* is involved in intra-S-phase checkpoint[40,41] and is considered as a negative regulator of telomere length, supported by the inverse correlation effect seen in our study.

Telomere length was inversely associated with age, which has been shown earlier from studies of ours and others.[32,42,43] Also, an inverse association between BMI and telomere length was shown, as reported by others,[5,44−46] supporting the notion that obesity accelerates human aging. Induction of longer telomeres may reflect a difference in the cell population in blood, with younger cells in arsenic-exposed individuals. It was shown by an in vitro study that the telomere length increased in inflammatory cells during acute inflammation.[47] However, subjects in this study were chronically (98% of them had lived in their current village for 2 years or more) exposed to arsenic in drinking water, and arsenic-related elongation of telomeres probably is not reflecting a short-term response.

In conclusion, this study found that urinary arsenic is associated with longer telomeres, in particular in individuals with a genotype associated with a slow arsenic metabolism. The effect of arsenic on telomeres may be through up-regulation of *TERT*, and as well by alteration of other genes that stabilize the telomere structure. Future studies are needed to follow-up if the arsenic-related telomere elongation is associated with increased cancer risk.

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Funding

This study was supported by the Swedish Council for Working Life and Social Research, The European Union within the Sixth Framework Programme for RTD ("PHIME" contract no. FOOD-CT-2006-016253), the Kungliga Fysiografiska sällskapet, and Erik Philip Sörensens Foundation.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Alejandro Cardozo, Plácida Mamaní, Esperanza Casimiro, and Eva Assarsson for recruiting the volunteers and Margaretha Grandér and Brita Palm for arsenic speciation.

ABBREVIATIONS

AS3MT, arsenic (+3 oxidation state) methyltransferase; BMI, body mass index; CI, confidence interval; DMA, dimethylarsinic acid; FDR, false discovery rate; iAs, inorganic arsenic; MMA, methylarsonic acid; SNP, single nucleotide polymorphisms; TERT, telomerase reverse transcriptase

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Paper IV

RESEARCH ARTICLES

Telomere Length and *LINE1* Methylation is Associated with Chromosomal Aberrations in Peripheral Blood

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The frequency of chromosomal aberrations in peripheral blood predicts a probable cancer risk. The individual telomere length and methylation of repetitive elements may be susceptibility factors for chromosomal aberrations. A cohort of healthy Norwegian men ($N = 364$) recruited during 1980–1999 were analyzed for chromosomal aberrations in phytohemagglutinin-stimulated lymphocytes from peripheral blood. Chromosome-type or chromatid-type aberrations were scored. DNA was extracted from slides cytogenetically analyzed and relative average telomere length and methylation of *LINE1* repeats were determined by quantitative polymerase chain reaction and bisulfite pyrosequencing, respectively. Information about individuals with malignant tumors ($N = 49$) diagnosed after chromosomal aberrations testing until end of 2008 was obtained and two matched controls per case were used in a nested case–control analysis. Shorter relative telomere length and higher methylation of *LINE1* were associated with higher frequency of total chromosomal aberrations ($\beta = -0.76$, $P = 0.022$; and $\beta = 0.042$, $P = 0.048$, respectively; age-adjusted ordinal regression). The telomere length was stronger associated with chromosome-type ($\beta = -1.00$, $P = 0.006$) than with chromatid-type aberrations ($\beta = -0.49$, $P = 0.115$). The *LINE1* methylation was stronger associated with chromatid-type ($\beta = 0.062$, $P = 0.003$) than with chromosome-type aberrations ($\beta = 0.018$, $P = 0.41$). Telomere length [individuals with short telomeres odds ratio (OR) = 0.87, 95% confidence interval (CI) 0.38–2.0], *LINE1* (individuals with high methylation OR = 1.04, 95% CI 0.43–2.5) and chromosomal aberrations (individuals with high frequency OR = 1.6, 95% CI 0.63–3.9) at baseline did not predict cancer risk, but the conclusions were hampered by low statistical precision. The results suggest that shorter telomere length and higher *LINE1* methylation in peripheral blood lymphocytes are predisposition factors for increased frequency of chromosomal aberrations.

INTRODUCTION

Studies based on several European cohorts have shown that the frequency of chromosomal aberrations in peripheral blood is a predictive risk marker for cancer, regardless of cancer type or cancer site (Bonassi et al., 2000; Hagmar et al., 2004). Chromosomal aberrations have been associated with occupational exposure to (clastogenic) genotoxic agents as well as smoking (Zeljezic and Garaj-Vrhovac, 2001; Bocskay et al., 2005), but the association between chromosomal aberrations and cancer risk was not modified by exposure to genotoxic compounds (Bonassi et al., 2000). These findings suggest that individual susceptibility factors may play a major role for the occurrence of chromosomal aberrations. Deficient DNA repair of strand breaks, reduced folate metabolism, genetic variation in xenobiotic metabolism that affect susceptibility to genotoxic agents, factors that all may result in chromosomal instability. Indeed, recent studies indicate that genetic variants in DNA repair, xenobiotic-metabolizing, and folate-metabolizing genes affect the frequency of chromosomal aberrations (Skjelbred et al., 2006; Skjelbred et al., 2011). However, the genetic variation in those genes could only explain a minor fraction of the individual variation in chromosomal aberrations, and thus, other not yet known factors are probably influential.

The constitutional telomere length may be such a factor. Telomeres are a variable number of nucleotide repeated sequences (TTAGGG) with proteins located at the ends of eukaryotic

Additional Supporting Information may be found in the online version of this article.

Supported by the Swedish Council for Working Life and Social Research, the County Skåne in Sweden, the Kungliga Fysiografiska sällskapet, and the Research Unit at Telemark Hospital, Skien, Norway.

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Received 2 April 2012; Accepted 31 July 2012

DOI 10.1002/gcc.22000

Published online 21 September 2012 in Wiley Online Library (wileyonlinelibrary.com).

chromosomes (Blackburn, 1991). Telomeres are of great importance in maintaining chromosomal stability and cellular DNA damage and repair (Hahn, 2003; Callen and Surralles, 2004; Blasco, 2005; Rodier et al., 2005). Short average telomere length in peripheral blood appears to be a susceptibility marker for human breast, bladder, head and neck, lung, and renal cell cancers (Wu et al., 2003; Broberg et al., 2005; Shen et al., 2007; Jang et al., 2008) and meta studies show that the presence of shortened telomeres is a marker for susceptibility to human cancer, but for which cancer types is not clearly established (Ma et al., 2011; Wentzensen et al., 2011). Short telomere length caused chromosomal instability and epithelial carcinogenesis in mice (Artandi et al., 2000). In humans, correlations between short telomeres and chromosomal aberrations have been observed in solid tumor cells (Gisselsson et al., 2001; Stewenius, 2005) and in patients with different types of leukemia (Hartmann et al., 2005; Swiggers et al., 2006). Taken together, these observations indicate that telomere length might be a susceptibility marker for chromosomal aberrations in peripheral blood in individuals without malignancy.

It has been known for a long time that tumor cell DNA is hypomethylated in comparison with wild-type cells (Feinberg and Vogelstein, 1983; Feinberg et al., 1988). This has been demonstrated for repetitive elements, such as long interspersed nuclear elements (*LINE1*): CpG sites in *LINE1* are usually heavily methylated but genome-wide loss of methylation in these sites is a common epigenetic event in malignant cells (Chalitchagorn et al., 2004; Choi et al., 2009). This pattern of DNA methylation has led to the suggestion that the hypomethylation of non-promoter regions of DNA and of structural elements might cause enhanced genomic instability, including chromosomal instability, in cancer, however, the relationship between the two processes is not yet clear (Baylin and Ohm, 2006).

The aim of this study was to investigate the association between telomere length, *LINE1* methylation, and chromosomal aberrations in peripheral blood, and to compare their predictivity for future cancer risk.

MATERIALS AND METHODS

Study Population

We measured the average telomere length and degree of *LINE1* methylation in a cohort previously analyzed for chromosomal aberrations. The study cohort consisted of 364 healthy Norwegian male adult individuals (>15 years of age) examined cytogenetically during 1980–1999. The subjects were originally selected for cytogenetic studies because of various, mainly occupational, exposures to mutagens or carcinogens or as unexposed referents (Brøgger et al., 1990; Hagmar et al., 1994; Bonassi et al., 1995). Exposure assessment for the study population has been described previously (Tinnerberg et al., 2003; Skjelbred et al., 2006). In short, occupational hygienists performed partly structured telephone interviews with subjects or, if deceased, with next-of-kin. Other information sources included contacts with companies at which the subjects had been employed, former coworkers, company records, and medical records. The individuals were divided into two groups based on genotoxic exposure status at the time when the sample was taken for cytogenetic analysis. The main exposure sources included welding fumes, vinyl chloride, nickel, and styrene. This cohort has been followed up to analyze if chromosomal aberrations were predictive with regard to cancer risk (Brøgger et al., 1990; Hagmar et al., 1994). Subjects with cancer diagnosed before the cytogenetic analysis were not included in the cohort. Age and information about smoking status at chromosomal aberrations testing was available. Information about cases in the cohort with malignant tumors ($N = 49$) diagnosed from the date of chromosomal aberration testing until the end of 2008 was obtained from The Cancer Registry of Norway. The tumors were from: skin ($N = 14$, of which one was a malignant melanoma), the gastrointestinal tract ($N = 6$), male sex organs ($N = 5$), the urinary tract ($N = 4$), the respiratory tract ($N = 3$), lymphoid or hematopoietic system ($N = 3$), and the endocrine glands ($N = 1$). Two cases had tumors of uncertain or unknown malignancy potential and eleven cases lacked information about cancer origin. A nested case–control study was performed by matching two controls from the cohort with similar age for each case with malignant tumors. Written informed consent was obtained from all subjects. The Regional Ethics Committee and the Data Inspectorate in Norway approved the study.

Cytogenetic Analysis

Phytohemagglutinin-stimulated lymphocyte cultures from heparinized whole blood were used

for cytogenetic analysis according to methods described earlier (Skyberg et al., 1989). Chromosomal aberrations were scored by the same three microscopists in a double-blind fashion on coded slides from 100 cells per subject in the earliest studies and from 200 cells per subject in more recent studies. Different types of chromosomal aberrations were recorded, using scoring criteria described by Brøgger et al. (1990) and Savage (1976) and harmonized among the scorers. Frequency of chromosomal aberrations was defined as the number of cells with aberrations, excluding gaps, per hundred cells. Chromosome-type aberrations included chromosome-type breaks, ring chromosomes, marker chromosomes, and dicentrics; and chromatid-type aberrations included chromatid-type breaks and chromatid exchanges. Fixed cell suspensions and slides were stored from all subjects.

DNA Isolation

Cells that had been stored in methanol-acetic acid fixative at −20°C for 3–22 years were used for DNA extraction, as described (Skjelbred et al., 2006). There were seven samples that were negative in the telomere analysis and those were sampled between 1992 and 1999. There were 29 samples that either did not have DNA left or were negative in the *LINE1* analysis and those were sampled between 1980 and 1998.

Telomere Length Analysis

The DNA samples were diluted with sterile water to 0.8 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 (Li et al., 2011), based on the method reported by Cawthon (2002). In short, an aliquot of 5 μl sample DNA was added to each reaction (end volume 20 μl). A standard curve, a reference DNA and a negative control sample were included in each run. For each standard curve, one calibrator DNA sample was diluted serially by two-fold per dilution to produce seven concentrations of 0.25–16 ng/μl. Each sample, standard curve, reference, and negative was run in duplicates. Master mixes were prepared, containing 0.5U *Taq* Platina (Invitrogen, Carlsbad, CA), 1 × PCR Buffer, 0.8 mM dNTPs, 1.75 mM $MgCl_2$, 0.3 mM SybrGreen I (Invitrogen), 1 × Rox (Invitrogen), and either telomere primers (0.45 μM of each primer) or hemoglobin beta chain (*HBG*) primers (0.45 μM for each primer). The PCR was performed on a real-time PCR machine (7900HT, Applied Biosystems). R^2 for each standard curve was >0.99. The PCR efficiency ranged between 1.8 and 2.1, with one run of HBG showing 1.7. If the duplicates differed in Ct with more than 0.2 standard deviations, the sample was diluted and reanalyzed.

The relative length of the telomeres was obtained through calculating the ratio (T/S) of telomere repeats product and single copy gene product (*HBG*) for each individual, by the formula $T/S = 2^{-\Delta Ct}$, where $\Delta Ct = Ct_{telomere} - Ct_{HBG}$. This ratio was then compared with the ratio of the reference DNA. The reference samples were included in each run and demonstrated a coefficient of variance of 7.2%, based on 11 runs.

Bisulfite Treatment and *LINE1* Pyrosequencing

Four hundred nanograms DNA (20 ng/μL) from fixative was bisulfite-treated using EpiTect® kit (Qiagen, Heidelberg, Germany). Commercially available kit (Qiagen) was used to measure *LINE1* methylation at four CpG sites (Pos 305 to 331 of GenBank accession no X58075) following manufacturer's instruction. Briefly, 1 μL of bisulfite-treated DNA was used in a 25 μL PCR reaction using Pyromark PCR kit (Qiagen). The reverse PCR primer was biotinylated. A single PCR fragment spanning a part the genetic element was amplified and the degree of methylation was analyzed in a single pyrosequencing reaction using 20 μL of PCR product. The PCR product was purified using Streptavidin Sepharose High Performance beads (Amersham Biosciences, Uppsala, Sweden). The sepharose beads containing immobilized PCR products were purified, washed and denatured using 0.2 M NaOH, and washed again using a vacuum prep tool (Pyrosequencing Inc., Westborough, MA). Twelve microliters of 0.3 μmol/L of pyrosequencing primer was annealed to the purified single-stranded PCR product and pyrosequencing was done using the PSQ HS96 Pyrosequencing System (Qiagen). The degree of methylation was expressed as percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Bisulfite conversion was verified using non-CpG cytosine residues as built-in controls and complete conversion of cytosine at a non-CpG site ensured successful bisulfite conversion. The repeatability of the method was tested in previous study, and the

TABLE 1. Characteristics of the Study Subjects at Cohort Enrollment

			All	Incident cancer[a]	Matched control
			$N = 364$	$N = 49$	$N = 98$
Age			39 (22–62)	54 (27–65)	51 (25–63)
Smoking status (smoker/former/nonsmoker)			190/43/129	26/6/17	54/12/30
Occupational genotoxic exposure (Non-exposed/exposed)			206/158	32/17	52/46
Total chromosomal aberrations (%)[b]			1 (0–7)	1.5 (0–5)	1 (0–4.5)
Chromosome-type aberrations (%)[b]			0.5 (0–9)	1 (0–4)	0.5 (0–7)
Chromatid-type aberrations (%)[b]			4.5 (0–18.5)	4.5 (0–18.5)	4.5 (0–17)
Telomere length[c]	Total		0.88 (0.48–1.31)	0.81 (0.54–1.57)	0.84 (0.43–1.26)
	Exposure	Non-exposed	0.88 (0.51–1.31)	0.84 (0.39–1.61)	0.84 (0.60–1.27)
		Exposed	0.87 (0.42–1.27)	0.78 (0.66–1.20)	0.85 (0.22–1.19)
	Smoking	Smokers	0.88 (0.51–1.26)	0.81 (0.67–1.20)	0.86 (0.43–1.20)
		Former smokers	0.82 (0.32–1.20)	0.92 (0.39–1.20)	0.84 (0.61–1.31)
		Non-smokers	0.89 (0.54–1.55)	0.80 (0.39–1.61)	0.84 (0.57–1.27)
LINE1 methylation[c]	Total		80.4 (70.6–86.1)	79.3 (65.8–84.5)	79.8 (71.2–85.8)
	Exposure	Non-exposed	79.8 (69.2–86.1)	80.1 (70.9–86.2)	78.1 (62.1–84.3)
		Exposed	81.2 (72.8–85.8)	81.2 (72.8–85.8)	82.5 (70.5–86.7)
	Smoking	Smokers	81.0 (71.0–86.3)	81.0 (72.1–87.0)	80.9 (69.2–84.5)
		Former smokers	80.0 (71.9–86.1)	79.9 (73.2–86.2)	83.1 (70.5–84.5)
		Non-smokers	80.0 (70.0–85.8)	80.3 (71.2–85.8)	76.1 (57.8–86.7)

[a]All subjects were healthy at the time of cytogenetic analysis. Incident cancer refers to cancers diagnosed after the cytogenetic analysis (baseline).
[b]Frequency of chromosomal aberrations was defined as the number of cells with aberrations, excluding gaps, per 100 cells. Chromosome-type aberrations included chromosome-type breaks, ring chromosomes, marker chromosomes, and dicentrics; and chromatid-type aberrations included chromatid-type breaks and chromatid exchanges. Median (range) was given.
[c]Number of individuals with telomere length data = 357; with *LINE1* data = 335. Median (5/95th percentile) was given.

variation coefficient was 2.0% (Hossain et al., 2012).

Statistical Analysis

Spearman's rho correlation test was carried out between age, telomere length, percentage of average *LINE1* methylation and chromosomal aberrations and its subtypes among all subjects. Age was always treated as a continuous variable. The effects of smoking or occupational exposure on *LINE1* methylation and telomere length were analyzed with linear regression (age adjusted). Ordinal regression was used to analyze the association between telomere length and *LINE1* methylation versus chromosomal aberrations (and its subtypes). Chromosomal aberrations, chromosome-type aberrations, and chromatid-type aberrations values were rounded to the upper integer and used as ordinal outcome variables in the regression analysis. Interaction terms between biomarkers (telomere length or *LINE1* methylation) and occupational exposure or smoking status were introduced into the ordinal regression model with chromosomal aberrations (or its subtypes) as outcome variable. Data were also stratified according to smoking status or occupational exposure, and similar ordinal regressions were completed in stratified data. Due to the strong correlation between age and biomarkers (telomere length, *LINE1* methylation, and chromosomal aberrations), age was introduced into the model for adjustment.

A nested case–control study for the associations of telomere length and chromosomal aberrations versus cancer risk was performed by matching two controls (total $N = 98$) with similar age for each case with malignant cancer at follow-up ($N = 49$). Logistic regression analysis was performed with case/control-status as outcome and with odds ratio (OR) as effect estimates. Here, telomere length, *LINE1* methylation, and chromosomal aberrations were trichotomized in equal parts in the control group respectively, and then these cut points were applied to the case group as well (cut points for chromosomal aberrations were low <1%, medium = 1% and high >1%; for telomere length 0.77 and 0.91; for *LINE1* methylation 78.02 and 81.83). To notice, since there were many ties in chromosomal aberrations value, numbers of each group are not exactly the same. Risk estimates were derived for different telomere length, *LINE1* methylation or chromosomal aberrations groups. Age was introduced for adjustment since the matching was not perfect and unconditional regression analysis hence conducted.

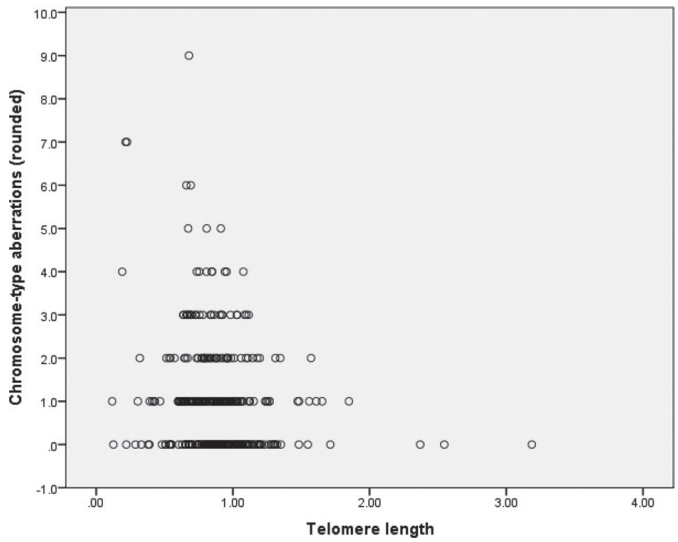

Figure 1. Graph depicting the association between chromosome-type aberrations (values were rounded to the upper integer resulting in eight groups) and telomere length in peripheral blood.

All statistical analyses were completed by using SPSS 18.0. Statistical significance refers to $P < 0.05$ (two-tailed).

RESULTS

The characteristics of the study population are shown in Table 1. The proportions of smokers were not significantly different between controls and cases (P-value = 0.96), neither differed number of exposed and non-exposed individuals ($P = 0.90$). The case group was older at cohort enrolment than the group of all individuals ($P < 0.001$). The median of relative telomere length was 0.81 for the cases and 0.88 for the controls ($P = 0.41$); 0.88 in the non-exposed group and 0.87 in the exposed group ($P = 0.55$); 0.88 in smokers, 0.82 in former smokers, and 0.89 in non-smokers ($P = 0.097$). The median of percentage of average *LINE1* methylation was 79.3 for the cases and 79.8 for the controls ($P = 0.026$); 81.2 in the exposed group and 79.8 in the non-exposed group ($P = 0.009$); 81.0 in smokers, 80.0 in former smokers, and 80.0 in non-smokers ($P = 0.13$).

Telomere length and total chromosomal aberrations were negatively correlated with each other ($r_s = -0.16$, $P = 0.003$), but telomere length was stronger correlated with the subtype chromosome-type aberrations ($r_s = -0.16$, $P = 0.002$, Fig. 1) than with chromatid-type aberrations ($r_s = -0.090$, $P = 0.090$). When two outliers with telomere length >2.0 were excluded, the correlations were very similar (chromosomal aberrations $r_s = -0.15$, $P = 0.006$; chromosome-type aberrations $r_s = -0.15$, $P = 0.005$; and chromatide-type aberrations $r_s = -0.081$, $P = 0.13$). *LINE1* methylation was positively correlated with chromatid-type aberrations ($r_s = 0.12$, $P = 0.033$), but not with telomere length ($r_s = 0.062$, $P = 0.26$), chromosomal aberrations ($r_s = 0.002$, $P = 0.98$), or chromosome-type aberrations ($r_s = -0.10$, $P = 0.85$). Age was negatively correlated with telomere length ($r_s = -0.16$, $P = 0.003$) and *LINE1* methylation ($r_s = -0.26$, $P < 0.001$), and positively correlated with total chromosomal ($r_s = 0.15$, $P = 0.005$) and chromosome-type ($r_s = 0.16$, $P = 0.002$), but not with chromatid-type aberrations ($r_s = -0.002$, $P = 0.969$). Given the correlation between age and other biomarkers, all analyses presented below were adjusted for age.

Smoking and exposure status influenced *LINE1* methylation (Table 2). Former smokers ($\beta = -1.8$, $P = 0.041$) and smokers ($\beta = -1.3$, $P = 0.018$) had higher degree of *LINE1* methylation compared with non-smokers. Exposed subjects had higher degree of *LINE1* methylation

TABLE 2. Effects (as Beta Estimates from Linear Regression) of Smoking and Occupational Exposure on Telomere Length (TL) and *LINE1* Methylation

Dependent	Predictor	β-Coefficient	95% CI	*P*-value
TL	Smoking			
	Nonsmoker	–	–	–
	Former smoker	−0.11	−0.22 to −0.0083	0.034
	Smoker	−0.022	−0.088–0.045	0.53
	Age	−0.0028	−0.0054 to −0.00022	0.034
TL	Exposure			
	Nonexposed	–	–	–
	Exposed	−0.049	−0.11–0.014	0.13
	Age	−0.0037	−0.0063 to −0.0011	0.0058
LINE1	Smoking			
	Nonsmoker	–	–	–
	Former smoker	1.8	0.07–3.4	0.041
	Smoker	1.3	0.22–2.4	0.018
	Age	−0.11	−0.15 to −0.071	<0.0001
LINE1	Exposure			
	Nonexposed	–	–	–
	Exposed	1.3	0.23–2.3	0.017
	Age	−0.10	−0.14 to −0.061	<0.0001

TABLE 3. Effects (as Beta Estimates from Ordinal Regression) of Telomere Length (TL), *LINE1* Methylation, and Age on Frequency of Total Chromosomal Aberrations (CA), Chromosome-type Aberrations (CSA) and Chromatid-type Aberrations (CTA) in Peripheral Blood

Dependent	Predictor	β-Coefficient	95% CI	*P*-value
CA	TL	−0.88	−1.5 to −0.23	0.008
CA	TL	−0.76	−1.4 to −0.11	0.022
	Age	0.021	0.005–0.037	0.010
CSA	TL	−1.00	−1.73 to −0.28	0.006
	Age	0.022	0.006–0.039	0.008
CTA	TL	−0.49	−1.10– 0.12	0.115
	Age	0.002	−0.014–0.017	0.830
CA	*LINE1*	0.025	−0.015–0.065	0.222
CA	*LINE1*	0.042	0.00032–0.084	0.048
	Age	0.027	0.010–0.044	0.002
CSA	*LINE1*	0.018	−0.025–0.061	0.41
	Age	0.025	0.008–0.042	0.004
CTA	*LINE1*	0.062	0.022–0.103	0.003
	Age	0.009	−0.007–0.025	0.28
CA	TL	−0.72	−1.4 to −0.002	0.049
	LINE1	0.042	−0.00021–0.084	0.051
	Age	0.025	0.008–0.042	0.003
CSA	TL	−1.0	−1.8 to −0.19	0.015
	LINE1	0.020	−0.023–0.063	0.37
	Age	0.024	0.007–0.041	0.007
CTA	TL	−0.37	−1.0–0.31	0.29
	LINE1	0.060	0.019–0.10	0.004
	Age	0.009	−0.007–0.025	0.29

compared with non-exposed ($\beta = -1.3$, $P = 0.017$). A weak effect of smoking was found on telomere length (Table 2), but only in former smokers ($\beta = -0.11$, $P = 0.034$) but there was no effect on telomere length of exposure status.

Ordinal regression showed that telomere length was associated with total chromosomal aberrations ($\beta = -0.76$, corresponding to an OR = 0.47, $P = 0.022$, Table 3): the shorter relative telomere length, the higher frequency of chromosomal aberrations. Telomere length was strongly associated with chromosome-type aberrations ($\beta = -1.00$, OR = 0.37, $P = 0.006$), but not with chromatid-type aberrations ($\beta = -0.49$, OR = 0.61,

TABLE 4. Associations Between Telomere Length, *LINE1* Methylation and Total Chromosomal Aberrations in Peripheral blood Lymphocytes and Cancer Risk in a Matched Nested Case–Control Population by Logistic Regression

		Case/control	Odds ratio	95%CI	*P*	Odds ratio	95% CI	*P* adj.
Model 1								
Telomere length	Long	19/33	1.0			1.0		
	Medium	11/32	0.60	0.25–1.5	0.25	0.55	0.22–1.4	0.19
	Short	19/33	1.0	0.45–2.2	1.0	0.87	0.38–2.0	0.73
	Age		–	–	–	1.03	1.0–1.1	0.10
Model 2								
Chromosomal aberrations	Low	9/25	1.0			1.0		
	Medium	12/28	1.2	0.43–3.3	0.74	1.2	0.42–3.3	0.75
	High	27/45	1.7	0.68–4.1	0.26	1.6	0.63–3.9	0.34
	Age		–	–	–	1.03	0.99–1.1	0.10
Model 3								
LINE1 methylation	Low	18/30	1.0			1.0		
	Medium	12/29	0.69	0.28–1.7	0.41	0.79	0.31–2.0	0.61
	High	16/29	0.92	0.39–2.1	0.85	1.04	0.43–2.5	0.93
	Age		–	–	–	1.02	0.99–1.1	0.24
Model 4								
Telomere length	Long	18/30	1.0			1.0		
	Medium	10/30	0.52	0.20–1.4	0.19	0.48	0.18–1.3	0.14
	Short	18/28	1.1	0.46–2.6	0.85	0.97	0.40–2.3	0.94
Chromosomal aberrations	Low	8/25	1.0			1.0		
	Medium	11/23	0.73	0.23–2.3	0.60	0.70	0.22–2.3	0.56
	High	26/40	1.4	0.45–4.1	0.58	1.2	0.40–3.8	0.71
LINE1 methylation	Low	18/30	1.0			1.0		
	Medium	12/29	0.58	0.22–1.5	0.26	0.69	0.26–1.9	0.47
	High	16/29	0.81	0.33–2.0	0.65	0.94	0.37–2.4	0.89
	Age		–	–	–	1.02	0.98–1.1	0.25

$P = 0.115$). *LINE1* methylation was also associated with total chromosomal aberrations ($\beta = 0.042$, OR = 1.04, $P = 0.048$): the higher degree of *LINE1* methylation the higher frequency of chromosomal aberrations. *LINE1* was strongly associates with chromatid-type aberrations ($\beta = 0.062$, OR = 1.06, $P = 0.003$), but not with chromosome-type aberrations ($\beta = 0.018$, OR = 1.02, $P = 0.41$). When telomere length and *LINE1* methylation were included in the same model, both were associated with total chromosomal aberrations: telomere length was associated with chromosome-type aberrations while *LINE1* methylation was associated with chromatid-type aberrations (Table 3). When including smoking or occupational exposure into the models, a significant interaction was found between *LINE1* methylation and occupational exposure for total chromosomal aberrations (Supporting Information Table). However, there was no clear pattern of effects of *LINE1* methylation on chromosomal aberrations in different exposure groups.

In the nested case–control study, the mean age of matched cases and controls were 51.4 and 48.3 years respectively ($P = 0.11$, *t*-test) and the median age (5/95th percentile) of case and control groups were 54 years (27–65) and 51 years (25–63). No significant associations between telomere length, *LINE1* methylation or chromosomal aberrations versus cancer risk were found (Table 4), although the risk increased non-significantly with having high frequency of chromosomal aberrations. The results were similar when including telomere length, *LINE1* methylation, chromosomal aberrations and age in the model simultaneously. The confidence intervals for the effect estimates were generally wide, indicating low statistical precision.

DISCUSSION

The results suggest that the telomere length and *LINE1* methylation in peripheral blood are predisposition factors for chromosomal aberrations in healthy individuals. The associations generally remained after adjustment for age and are probably fairly robust as the chromosomal aberrations scoring has been validated (Savage, 1976; Brøgger et al., 1990) and the telomere length and the *LINE1* methylation analyses demonstrated low coefficients of variation (Jang et al., 2008;

Mumford et al., 2008; Shen et al., 2009; Hossain et al., 2012).

The finding of more *LINE1* methylation with higher frequency of chromosomal aberrations was in contrast to expected. However, Pavanello et al. (2009) reported a weak positive association between *LINE1* methylation and frequency of micronuclei in peripheral blood, another cancer risk marker that arises from chromosomal aberrations. These results indicate that there is not a clear link between hypomethylation and chromosomal aberrations, as previously suggested. The mechanism for the finding may lie in the chromatin structure, which is important for controlling the compaction of chromosomes and regulating access to specific DNA sequences during gene transcription, DNA replication, and repair (Schneider and Grosschedl, 2007; Misteli and Soutoglou, 2009). Both global and localized changes in the chromatin structure are driven by DNA methylation and histone modifications, which directly affect the conformation of chromatin. While DNA lesions in euchromatin are detected and rejoined without any delay, DNA packaging in heterochromatin, which *LINE1* elements are part of, appears to retard DNA strand break processing, leading to slower repair kinetics (Rübe et al., 2011). Hypothetically, more methylation of *LINE1* results in less accessible chromatin that may slow down DNA repair and result in higher frequency of chromosomal aberrations (Baylin and Ohm, 2006).

Shorter telomere length was found to be more related with chromosome-type than chromatid-type aberrations, which is in line with chromosomal aberration patterns observed in tumor cells with short telomeres (Gisselsson et al., 2001; Swiggers et al., 2006), and further, stresses the notion that shorter telomere length may cause chromosomal instability that develop into neoplasia. Opposite to telomere length, higher degree of average *LINE1* methylation was more related with chromatid-type than chromosome-type aberrations. The different effects on type of chromosomal damage remained when both genetic biomarkers were included in the same model. The formation mechanisms for chromosome-type and chromatid-type aberrations involve different kinds of DNA lesions and different repair systems (Pfeiffer et al., 2000). Chromosome-type aberrations arise from double-strand breaks in resting lymphocytes *in vivo* in the G_0-phase, whereas chromatid-type from DNA double strand breaks formed from initial DNA lesions (base modifications or single strand breaks) *in vitro* in the S-phase. Our results suggest that telomere dynamics and epigenetic alterations are involved in different types of DNA lesions.

Still, telomere length and *LINE1* can only explain a fraction of the variation of chromosomal aberrations in peripheral blood. Earlier studies (Skjelbred et al., 2006, 2011) suggested that genetic polymorphisms of xenobiotic and folate metabolisms and DNA repair affect chromosomal aberrations levels, but that each gene contributes to a small part of the variation. Thus, apart from telomere length, *LINE1* methylation and genotype, probably other unidentified factors are influential for the occurrence of chromosomal aberrations as well.

Telomere length and degree of *LINE1* methylation were not better risk markers than chromosomal aberrations for cancer risk: the group with shortest telomeres did not have increased cancer risk, whereas there was a trend for higher fraction of chromosomal aberrations and increasing cancer risk. This may reflect that chromosomal aberrations per se are intermediate endpoints in carcinogenesis, whereas shorter telomere length rather represents the biological age of cells (Blasco, 2005) and DNA methylation is a process for keeping the *LINE1* elements non-expressed. However, these results need to be interpreted very cautiously due to large statistical uncertainty. The small number of cancer cases that had occurred up to year 2008 after baseline hampered the analysis.

Lower degree of *LINE1* methylation was found in relation to increasing age. This association was also shown in a study on twins, where hypomethylation of repetitive elements, including Alu, increased with age (Fraga et al., 2005). Age was, as expected, also a strong determinant of telomere length, as well as chromosomal aberrations. It is known from studies of ours and of others that with increasing age the average telomere length in blood is decreasing (Brouilette et al., 2007; Shen et al., 2009; Li et al., 2011). Also a larger number of chromosomal aberrations were found among elderly individuals compared with younger (Bolognesi et al., 1997). One can hypothesize that these events actually are linked—the age-induced telomere length shortening result in more chromosomal aberrations.

Exposure and smoking increased the degree of methylation of *LINE1*, but there was no clear pattern indicating an interaction between *LINE1* and genotoxic exposures on the frequency of

chromosomal aberrations. In a study on coke-oven workers, mainly exposed to polycyclic aromatic hydrocarbons, higher *LINE1* methylation was found in exposed workers compared to controls (Pavanello et al., 2009). There was no clear effect of genotoxic exposures from smoking or occupational exposure on telomere length, nor was there evidence for that the telomere length modified the effect of genotoxic exposure on the frequency of chromosomal aberrations. The unclear associations between exposure at work and genetic biomarkers may be due to crude exposure assessment, since this was done retrospectively. Alternatively, the effect of telomeres on chromosomal aberrations is rather exposure-specific and this phenomenon could not be captured in this cohort encompassing different type of exposures classified only by retrospective data. In a previous cross-sectional study from the rubber industry, we found an association between increasing exposure to N-nitrosamines in air and toluidine in urine and shorter telomeres, but there was no effect on telomeres by, for example, exposure to polycyclic aromatic hydrocarbons measured in urine (Li et al., 2011).

Smoking did not clearly influence the telomere length, which is similar to results from the above mentioned study on rubber workers' (Li et al., 2011). However, this is contrast to other studies that reported a negative association between smoking and telomere length (Valdes et al., 2005; Morla et al., 2006; McGrath et al., 2007; Babizhayev and Yegorov, 2011). Since no information about pack-year but only smoking status was available in this study, it may be a too crude classification to discern a possible effect if the effect of smoking on telomere length is small. Also, other factors such as nutritional ones may modify the effect of genotoxic exposures (Valdes et al., 2005). Marine omega-3 polyunsaturated fatty acids have been found to be a protective factor for telomere length shortening in a cohort of individuals with coronary artery disease (Farzaneh-Far et al., 2010). Since the cohort in this study is based on Norwegians, who in general has a much higher fish intake than many other European populations (Engeset et al., 2006; Laerum et al., 2007), marine fatty acids may have modified the effect of smoking or other types of genotoxic exposures on telomere length. Also there is increasing evidence that the diet influences the DNA methylation (Arasaradnam et al., 2008; Zeisel, 2011). Unfortunately, we did not have dietary information for the cohort.

In conclusion, results of this study contribute to our understanding of the mechanisms for chromosomal aberrations in healthy individuals. Shorter telomere length appears to cause chromosome-type, whereas higher *LINE1* methylation influences chromatide-type aberrations in peripheral lymphocytes.

ACKNOWLEDGMENTS

The authors thank Karin Paulsson for help with the telomere length determination. Some of the data in this article are from the Cancer Registry of Norway.

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