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http://dx.doi.org/10.1289/ehp.1103979

Online 17 October 2011

NIEHS
National Institute of Environmental Health Sciences

National Institutes of Health
U.S. Department of Health and Human Services

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Running Title: Low-Dose Benzene and Mitochondrial DNA Copy Number

Key Words: benzene, biomarkers, low exposures, methylation, mitochondrial DNA copy number.

Acknowledgements: Thanks to Anna Gillio-Tos for her critical comments, and Francesco Nordio for assistance with multiple imputation and spline models. The present work is part of Dr. Carugno's dissertation project for the Epidemiology Master Program, University of Turin, Italy. This work was supported by Italian Association for Research against Cancer (AIRC-6016); INAIL Delib-277-05/06/2008; CARIPLO Foundation (2007-5469); NIEHS (P30ES000002); MIUR-PRIN (2003065175/2003); European Commission BMH4-CT98-3785 and IC20-CT98-0201.

The authors declare they have no competing financial interests.

Abbreviations:

95%CI: 95% Confidence Interval

IQR: interquartile-range

mtDNAcn: mitochondrial DNA copy number

ppb: part per billion

ppm: part per million

SD: Standard Deviation

ABSTRACT

BACKGROUND: Benzene is an established leukemogen at high-exposure levels. While low-level benzene exposure is widespread and may induce oxidative damage, no mechanistic biomarkers are available to detect biological dysfunction at low doses.

OBJECTIVES: To determine in a large multi-center cross-sectional study whether low-level benzene is associated with increased blood mitochondrial DNA copy number (mtDNAcn), a biological oxidative response to mitochondrial DNA damage and dysfunction; explore potential links between mtDNAcn and leukemia-related epigenetic markers.

METHODS: We measured blood relative mtDNAcn by real-time PCR in 341 individuals selected from various occupational groups with low-level benzene exposures (>100 times lower than the OSHA/EU standards), and 178 referents from three Italian cities (Genoa, Milan, Cagliari).

RESULTS: In each city, benzene-exposed participants showed higher mtDNAcn than referents: mtDNAcn was 0.90 relative units in Genoa bus drivers, and 0.75 in referents (p=0.019); 0.90 in Milan gas-station attendants, 1.10 in policemen, and 0.75 in referents (p-trend=0.008); 1.63 in Cagliari petrochemical-plant workers, 1.25 in referents close to the plant, and 0.90 in farther referents (p-trend=0.046). Using covariate-adjusted regression models, we estimated that an interquartile-range increase in personal airborne benzene was associated with percent increases in mtDNAcn equal to 10.5% in Genoa (p=0.014); 8.2% (p=0.008) in Milan; 7.5% in Cagliari (p=0.22), and 10.3% in all cities combined (p<0.001). Using methylation data available in the Milan participants, we found that mtDNAcn was associated with *LINE-1* hypomethylation (-2.41%, p=0.007), and *p15* hypermethylation (+15.95%, p=0.008).

CONCLUSIONS: MtDNAcn was increased at low-benzene levels, potentially reflecting mitochondrial DNA damage and dysfunction.

INTRODUCTION

Benzene is a widespread environmental chemical that has been associated with increased risk of hematological malignancies, particularly with acute nonlymphocytic (myeloid) leukemia (Baan et al. 2009; IARC 1982, 1987). Benzene ranks among the top 20 chemicals for production volume in the United States (CDC 2006). Outdoor air contains low levels of benzene from several sources, including gas stations, motor vehicle exhaust, and industrial emissions (ATSDR 2011). Most of the current epidemiologic evidence for benzene-related leukemia risks stems from studies among workers exposed to very high levels of benzene (Smith 2010). Multiple investigations have suggested potential hematotoxicity at levels below the occupational exposure limit of 1 ppm (equivalent to 1000 ppb or 3250 $\mu g/m^3$, 8-h Time-Weighted Average) recommended by the U.S. Occupational Safety and Health Administration (OSHA) and European Union (EU) (EU 1997; Forastiere et al. 1994; Lan et al. 2004; OSHA 2003). Nonetheless, uncertainties remain about the effects of benzene at low levels. In particular, as remarked in a recent review of benzene health effects (Smith 2010), epidemiology and animal studies have not yet provided conclusive insights about "the shape of the exposure-response relationship, particularly at low doses at or below 1 ppm in air". In this context, the development and use of mechanism-based biological markers has been suggested to hold substantial value in the risk-assessment process (Albertini et al. 2003; Smith 2010).

An important limitation in current understanding of benzene carcinogenesis is that mechanisms that are activated at low doses are still largely undefined (Atkinson 2009). In-vitro models have shown that some of the reactive metabolites of benzene (such as phenol, catechol, hydroquinone) can bind to and damage macromolecules, including the DNA (Ross 2000). These reactive metabolites may also generate reactive oxygen species (ROS) that can

exacerbate DNA damage (Palackal et al. 2002). Recently, Bollati et al. (2007) have shown that low-dose exposure to airborne benzene is associated with alterations in DNA methylation in blood DNA of healthy individuals that resemble those found in hematological malignancies, including hypomethylation of *LINE-1* and *Alu* repetitive elements, hypermethylation of the *p15* tumor suppressor gene, and hypomethylation of *MAGEA1* (melanoma-associated antigen 1 gene). Consistently, global DNA hypomethylation has been recently shown in in-vitro experiments on hydroquinone-treated human lymphoblastoid cells (Ji et al. 2010). These effects of benzene on DNA methylation have been suggested to result from ROS-induced DNA damage (Baccarelli and Bollati 2009).

Mitochondria are both the major intracellular source and primary target of ROS, which are generated under normal conditions as by-products of aerobic metabolism in animal and human cells (Han et al. 2001). Each human and animal cell contains between several hundred and over a thousand mitochondria, each carrying 2-10 copies of mitochondrial DNA (mtDNA) (Cavelier et al. 2000). MtDNA copy number (mtDNAcn) is correlated with number and size of mitochondria (Lee and Wei 2000). Compared with nuclear DNA, mtDNA has diminished protective histones and DNA repair capacity, and is therefore particularly susceptible to ROS-induced damage. Cells challenged with ROS have been shown to synthesize more copies of their mtDNA, and increase their mitochondrial abundance to compensate for damage and meet the increased respiratory demand required for ROS clearance (Lee and Wei 2000). Conversely, ROS are also generated from the increased mitochondria and can in turn cause additional oxidative damage to mitochondria and other intracellular constituents, including DNA, RNA, proteins, and lipids.

In a recent study of 40 shoe and clothing Chinese manufacturing workers (Shen et al. 2008), individuals exposed to benzene levels >1 ppm exhibited higher mtDNAcn in peripheral blood leukocytes than subjects with lower exposure. MtDNAcn has never been studied in larger studies, particularly at the levels of exposure often found in populations in North America and Europe. In the present work, we conducted a multi-center cross-sectional study in Italian cities on individuals exposed to low-level benzene from a variety of sources to examine whether low doses of benzene exposure determine alterations in mtDNAcn.

METHODS

Study Population

We enrolled 519 participants from three Italian cities (Genoa, Milan, Cagliari). In each city, we included individuals with low-level benzene exposures and referents. Exposed subjects were selected from occupational categories that entail exposure to low levels of benzene (Fustinoni et al. 2005; Merlo et al. 2003), including 153 bus drivers in Genoa; 78 gas-station attendants and 77 police officers in Milan; and 33 workers in a modern petrochemical plant in Cagliari. In Genoa and Milan, referents were occupationally active people from the same area as the exposed participants. In Cagliari, we selected referents who were residents of two small towns located at 2 and 5 km from the petrochemical plant (close referents). An additional sample of referents (distant referents) was selected from an area further away (≥20 km) from the plant. Both exposed and referent individuals had been actively employed for ≥1 year. We used the same standardized procedures for the recruitment of all exposed and unexposed individuals in all cities. The smaller numbers of referents compared to the numbers of exposed workers was determined based on the balance between the efforts required to motivate and recruit referents vs. loss in statistical power. A standardized structured self-administered questionnaire was used to collect information on lifestyle and

risk factors. All participants provided written informed consent to the study, which was approved by the Local Institutional Review Boards.

Personal Exposure Assessment

Personal exposure to airborne benzene was determined using passive samplers worn by the study participants near the breathing zone during their work shifts for 5-6 hours (approximately 8:00-9:30 am to 1:00-2:30 pm). In Milan and Genoa, we used passive samplers (stainless-steel tube, 9 mm internal diameter, 90 mm length) containing Chromosorb 106 and equipped with a diffusion chamber (Brown 1999). At the end of the monitoring period, the passive sampler was closed with a brass cap and nut, equipped with a polyperfluoroethylene ferule, and kept at -20°C until analysis, performed by thermal desorption followed by gas chromatography/flame ionization detector analysis (Fustinoni et al. 2005). In Cagliari, we used Radiello® passive samplers, equipped with a 35-50 mesh charcoal cartridge (Supelco, Sigma-Aldrich, Milan, Italy). At the end of sampling, the cartridge was sealed in glass tubes and kept in a clean box at room temperature until gas chromatography/mass spectrometry analysis (Fustinoni et al. 2010a), which occurred within 30 days from collection, as per manufacturer's instructions. The two sampling methods have been previously shown to have similar recovery performances (Hayes 2009). All benzene analyses were performed at the Environmental Chemistry Unit of the National Cancer Research Institute, Genoa, Italy. The detection limit for airborne benzene was 6 $\mu g/m^3$ (1.85 ppb). 65 individuals (12.5%) had benzene levels below the detection limit (DL) and were assigned a value corresponding to $DL/\sqrt{2}$ (Hornung and Reed 1990).

Mitochondrial DNA Copy Number (mtDNAcn) Analysis

Total DNA was extracted using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI) from whole blood collected in EDTA tubes at the beginning of the work shift. Relative mtDNAcn was measured by quantitative real-time-PCR, as previously described (Hou et al. 2010). All samples were run in triplicates in 384-plates on a 7900HT Fast Real-Time PCR System (Applied Biosystem, Foster City, California, USA). The assay is based on the ratio of copy number estimates of a mitochondrial gene (mtND1) to those of a nuclear gene (human beta globin [hbg]). The mtND1/hbg ratio thus calculated in experimental samples is then scaled to a standard DNA sample to obtain relative mtDNAcn values controlled for plate effects. The standard DNA sample was obtained by pooling DNA from 20 participants, randomly selected from the Milan referents and used to generate a fresh five-point standard curve (range: 20-0.247 ng) in every mtND1 and hbg run. Primers and conditions for mtDNAcn analysis are reported in the Supplemental Material.

In addition to the mtDNAcn data, DNA methylation measures on blood DNA by PCR-Pyrosequencing were available for the subset of Milan participants, as part of previous work that evaluated the effects of benzene exposure on DNA methylation (Bollati et al. 2007).

Statistical Analysis

We used standard descriptive statistics (means, Standard Deviations [SDs], medians, Interquartile-Ranges [IQRs], and proportions) to summarize data. MtDNAcn showed asymmetric distributions within each city and exposure group and was log-transformed to approximate normality. Consistently, we report geometric means and corresponding 95% Confidence Intervals (95%CIs). Differences in mtDNAcn across exposure groups were evaluated using one-way ANOVA and tests for trend computed via linear regression analysis. Also, we evaluated the association between mtDNAcn and exposure groups by fitting

multivariate models adjusting for age (continuous), sex (male, female), smoking (never, former, current smoker), and number of cigarettes/day (continuous). We used linear regression models to examine the association of airborne benzene levels with mtDNAcn. Scatterplots of airborne benzene vs. mtDNAcn showed a non-linear relation that approximated linearity when both variables were log-transformed. All models were thus fitted by regressing log[mtDNAcn] over log[benzene]. To exclude confounding from factors associated to differences across cities, including potential differences in Cagliari from the different protocol used for air benzene sampling, we first fitted unadjusted and adjusted models for each of the cities separately and then models for all participants combined. In the models on all participants combined, we fitted an independent indicator variable for each of the cities (Genoa, Milan, Cagliari) in both unadjusted and adjusted models. In both city-specific and combined analyses, adjusted models included age, sex, smoking, and number of cigarettes/day as independent variables. To facilitate understanding of effect sizes, effects are expressed throughout the paper as percent variation in mtDNAcn for an IQR increase in benzene exposure. To confirm the results from multiple linear regression models evaluating the association between relative mtDNAcn and airborne benzene, we performed a set of sensitivity analyses that we report in the Supplemental Material.

In the subset of the study participants with DNA methylation data (n=212), we fitted multiple regression models (adjusted for age, sex, smoking and number of cigarettes/day) to evaluate the association of log[mtDNAcn] with *LINE-1*, *Alu*, *p15*, or *MAGEA1* DNA methylation. DNA methylation variables were also log-transformed to approximate normality; effects are expressed as percent variation in DNA methylation for an IQR change in mtDNAcn.

Outliers were excluded from all regression analyses by dropping observations with studentized residuals that exceeded +3 or -3. Using these criteria, a variable number of

observations (between one and seven) was dropped from each model. All tests of statistical significance were two sided.

Statistical analyses were performed using Stata/MP 11.1 (Stata Corporation, College Station, TX), R (R Foundation for Statistical Computing, Vienna, Austria), and SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Study Population

The characteristics of the study population, by city and exposure group, are summarized in Table 1. Median age was 39, considering all participants, with median values ranging from a minimum of 30 years (Milan police officers) to a maximum of 55 years (Cagliari distant referents). In all cities combined, males represented 81% of the study population. The proportion of overall current smokers (32%) was similar to the proportion of smokers in the Italian male adult population (ISTAT 2009). In all participants combined, the mean number of cigarettes/day was equal to 16. Most of the study participants (66%) resided in the suburbs at the time of enrollment.

Exposure Levels to Airborne Benzene

Across all cities, individuals in the exposed groups had higher airborne benzene exposure levels, as measured on personal samplers, than the referents (Table 2). Among the occupationally exposed participants, the highest exposure levels were observed in the Milan gas-station attendants (geometric mean=69.9 $\mu g/m^3$, 95%CI 57.4-85.2 [21.5 ppb, 95%CI 17.7-26.2]), followed by the Cagliari petrochemical workers (geometric mean=35.4 $\mu g/m^3$, 95%CI 20.5-61.0 [10.9 ppb 95%CI 6.31-18.8]). Geometric means across different referent

groups were all between 5.9 μg/m^3 (1.82 ppb, Cagliari distant referents) and 8.7 μg/m^3 (2.68 ppb, Genoa referents).

Relative mtDNAcn by Exposure Groups

In each city, exposed participants had consistently higher mtDNAcn levels than referents (Table 3). In Genoa, mean relative mtDNAcn was 0.75 (95%CI 0.66-0.85) in referents, and 0.90 (95%CI 0.84-0.97) in bus drivers (p=0.019) in analysis adjusted for age, sex, smoking, and number of cigarettes/day. In Milan, adjusted mean relative mtDNAcn was 0.75 (95%CI 0.69-0.82) in referents, 1.10 (95%CI 1.01-1.19) in police officers, and 0.90 (95%CI 0.83-0.98) in gas-station attendants (p-trend=0.008). In Cagliari, adjusted mean relative mtDNAcn was 0.90 (95%CI 0.60-1.41) in distant referents, 1.25 (95%CI 1.03-1.51) in close referents, and 1.63 (95%CI 1.22-2.18) in petrochemical workers (p-trend=0.046). Referents exhibited different mtDNAcn across cities, with higher mean levels in Cagliari compared to Milan and Genoa (Table 3).

Relative mtDNAcn and Airborne Benzene

Relative mtDNAcn showed a positive correlation with airborne benzene concentrations in each of the cities (Figure 1A-1C). In multivariate regression models adjusting for age, sex, smoking, and number of cigarettes/day, we estimated a significant 10.5% increase (95%CI 2.1 to 19.6, p=0.014) in relative mtDNAcn for an IQR benzene increase in Genoa (Figure 1A), and a significant 8.2% increase (95%CI 2.2 to 14.7, p=0.008) in Milan (Figure 1B). In Cagliari, the correlation between relative mtDNAcn and airborne benzene was also positive (7.5% increase, 95%CI -4.2 to 20.6, Figure 1C), but not statistically significant (p=0.22). City-specific unadjusted analyses showed similar results, except for Milan, where the percent increase was lower than in the multivariate analyses (10.7% increase, 95%CI 2.4 to 19.7,

p=0.011 in Genoa; 3.3% increase, 95%CI -2.7 to 9.7, p=0.29 in Milan; 9.0% increase, 95%CI -1.6 to 20.8, p=0.10 in Cagliari). Analyses on all participants combined (Figure 1D) showed a highly statistically significant increase in relative mtDNAcn associated with benzene levels for both unadjusted (7.8% increase, 95%CI 2.9 to 13.0, p=0.002) and adjusted regressions (10.3% increase, 95%CI 5.4 to 15.5, p<0.001).

DNA Methylation and Relative mtDNAcn

Taking advantage of extant epigenetic data in the Milan subset of the study population (n=212), we also explored whether mtDNAcn was associated with DNA methylation in *LINE-1*, *Alu*, *p15*, and *MAGEA1* by multivariate regression models (adjusted for age, sex, smoking, and number of cigarettes/day). Correlations of mtDNAcn with DNA methylation measures are shown in Figure 2A-D. *LINE-1* methylation showed a significant decrease associated with an IQR increase in relative mtDNAcn (-2.4% change, 95%CI -4.1 to -0.7, p=0.007, Figure 2A); *p15* methylation exhibited a significant percent increase (16.0% change, 95%CI 4.1 to 29.2, p=0.008, Figure 2C); *Alu* and *MAGEA1* methylation (Figure 2B and 2D) did not show significant variations correlated with increasing relative mtDNAcn (-0.4% change, 95%CI -2.5 to 1.7, p=0.69 for *Alu*; 0.2% change, 95%CI -0.1 to 0.6, p=0.14 for *MAGEA1*). Unadjusted analyses showed very similar results (-2.3% change, 95%CI -3.9 to -0.7, p=0.005 for *LINE-1*; 13.0% change, 95%CI 2.3 to 24.8, p=0.017 for *p15*; 0.4% change, 95%CI -1.5 to 2.4, p=0.69 for *Alu*; 0.3% change, 95%CI -0.1 to 0.6, p=0.06 for *MAGEA1*).

DISCUSSION

In this multi-city investigation of individuals with low-level exposure to benzene, we found that blood mtDNAcn was increased in association with airborne benzene exposure. We observed differences in mtDNA levels by comparing different exposure groups within each

city. We demonstrated a dose-response relationship between mtDNAcn and benzene exposure levels within each city and overall using personal sampler data as direct measures of individual benzene exposure. In the Milan study participants, we correlated increasing mtDNAcn with decreased *LINE-1* methylation and increased *p15* methylation.

Benzene exposure has been consistently linked with hematological malignancies (Baan et al. 2009; IARC 1982, 1987) in cohorts of workers occupationally exposed to levels substantially higher than those found in the environment or even in modern facilities where appropriate occupational safety procedures are implemented. In our study population, benzene exposure levels (median=19.2 $\mu g/m^3$, equivalent to 6 ppb) were on average more than 100 times lower than the occupational standard limit of 1 ppm (equivalent to 1000 ppb or 3250 $\mu g/m^3$) set by OSHA/EU (EU 1997; OSHA 2003). Even in the most exposed participant, benzene exposure level (1250 $\mu g/m^3$, 380 ppb) was still about one third of the occupational exposure limit.

MtDNAcn has been previously associated with benzene exposure in Chinese workers exposed to levels of benzene (reported mean=14.3 ppm, SD=20.4) comparable to those associated with hematological malignancies (Shen et al. 2008). Our study indicates that mtDNAcn alterations can be observed also at low exposure doses, potentially reflecting the activation of key cellular processes, such as oxidative stress, that are known to operate in early carcinogenesis. Increased mtDNAcn has been suggested to have a dual role in cells challenged by oxidative stress. On one hand, it stimulates mitochondrial proliferation to supply energy to meet the need for cell survival, including repair of damage and synthesis of new proteins (Lee and Wei 2000). On the other hand, the increasing abundance of dysfunctional mitochondria causes excess ROS production and further oxidative damage that may initiate cell senescence or death (Lee and Wei 2005). Extensive work has shown that alterations in DNA methylation can also result from oxidative insults (Baccarelli and Bollati

2009). Aberrant DNA methylation, including hypomethylation of repetitive elements and hypermethylation of tumor suppressor genes, is increasingly recognized as a critical step in malignant transformation (Rodriguez-Paredes and Esteller 2011). In particular, *LINE-1* hypomethylation and *p15* hypermethylation are commonly found in acute nonlymphocytic leukemia and other hematological malignancies (Deneberg et al. 2010). Using methylation data available to us from a previous investigations on the Milan study participants, we found a linear association of mtDNAcn with both *LINE-1* hypomethylation and *p15* hypermethylation, while no association was observed between mtDNAcn and *Alu* or *MAGEA1* methylation. Hypomethylation of *LINE-1*, which has often been used as a surrogate for global methylation, is believed to contribute to determine chromosomal instability and breakage. The tumor suppressor gene *p15* shows low or no methylation in normal cells, whereas it is hypermethylated in acute nonlymphocytic leukemia cells (Claus and Lubbert 2003), as well as in other hematological malignancies. *p15* encodes a cyclin-dependent kinase inhibitor, which functions as a cell growth regulator that controls cell cycle G1 progression (Hannon and Beach 1994). *p15* hypermethylation is widely considered to contribute to the loss of cell cycle arrest responses in malignant cells. Our findings on *LINE-1* methylation follow the same direction as in a recent investigation on the Normative Aging Study cohort (Baccarelli et al. 2009) that showed an association between exposure to air pollution from traffic particles and DNA methylation of *LINE-1*, but no association with *Alu* methylation. Even though *LINE-1* and *Alu* repetitive element methylation has been demonstrated to correlate with global DNA methylation in cancer tissues (Weisenberger et al. 2005), the two repetitive elements are controlled through different mechanisms and might respond differently to oxidative stress (Bollati et al. 2009). We did not observe any association between mtDNAcn and *MAGEA1* methylation. We surmise that *MAGEA1* may be part of a benzene-induced pathway that does not directly involve oxidative stress. Moreover, it is

worth noting that in our previous work on effects of benzene exposure on DNA methylation (Bollati et al. 2007) we found only a weak, borderline significant association between benzene exposure and *MAGEA1* methylation.

Whether mtDNAcn has a direct role in carcinogenesis is still under investigation. Recent longitudinal studies have shown that individuals with higher blood mtDNAcn at baseline have higher risk of developing non-Hodgkin lymphoma and lung cancer (Hosgood et al. 2010; Lan et al. 2008). Also, mtDNAcn alterations are associated with impaired apoptosis and subsequent increased cellular proliferation (Eliseev et al. 2003), as well as with nuclear DNA mutations following mtDNA insertion into the genome (Hazkani-Covo et al. 2010). While these results are suggestive of potential roles of mtDNAcn in carcinogenesis, whether mtDNAcn alterations contribute to determine increased risks of malignancies in benzene-exposed individuals remains to be determined.

We note that at least some of our findings might be explained by exposure to co-pollutants whose levels may track together with airborne benzene levels. In all the cities in the present study, exposed individuals had higher mtDNAcn levels than referents. Police officers in Milan however, who had exposure levels that were intermediate between gas-station attendants and referents, showed higher mtDNAcn levels than the gas-station attendants. Conversely, Milan gas-station attendants, who were the exposure group with the highest exposure across cities, were the group with the lowest mtDNAcn among exposed subjects. Although both exposed to environmental benzene, these occupational categories work in different exposure settings – gas-station attendants are exposed mainly to benzene vapors during filling operations, whereas police officers receive most of their exposure from vehicular combustion by-products (Cattaneo et al. 2010). Combustion byproducts from traffic

include not only benzene but also particulate matter and nitric oxide, which can all contribute to generate oxidative stress (Risom et al. 2005). Because particulate matter and nitric oxide exposures were not measured in our study, we cannot determine their possible contributions to the increase in mtDNAcn among the Milan police officers. Levels of mtDNAcn in the referent group in Cagliari were higher than those found in the Milan and Genoa referents. Cagliari is located in the Sardinia island, whose inhabitants mostly have a unique genetic background that goes back approximately 8,000 years to the island's original settlers (NIA 2009; Pilia et al. 2006). Consequently, Sardinians have genetic characteristics that are remarkably different from individuals living in other Italian regions (Calò et al. 2008). Twin studies have shown that mtDNA content is a trait with high genetic heritability (Xing et al. 2008). Based on these observations, the higher mtDNAcn values in the Cagliari participants might be determined by the different genetic background of the study individuals.

We used the same standardized study procedures across the three cities, including uniform questionnaires, data and blood collection, and mtDNAcn analysis. Air benzene sampling was performed using Chromosorb 106 stainless steel passive samplers in Milan and Genoa, and Radiello® passive samplers in Cagliari. A head-to-head comparison of the two different passive samplers showed no difference in recovery performances (Hayes 2009). The populations of the three cities in our study are likely to differ for multiple factors, including but not limited to lifestyle, diet, and climatic conditions. In order to take into account these differences and avoid potential confounding, we first analyzed each city separately. Then, analyses on all participants combined were conducted by fitting regression models that included an independent indicator variable for each of the cities.

Our study had the advantage of relying on personal measurements of benzene exposure obtained from portable passive samplers. Although limited to one day of airborne benzene sampling, airborne benzene measures showed significant differences between the exposure groups and were therefore taken in this cross-sectional study as a proxy of the usual exposure of the study participants. We have recently re-evaluated in a new recruitment campaign a subset of 53 Milan study participants (18 referents and 35 gas station attendants) from the original study, and found high correlations of benzene concentration between the original study and current follow-up (Fustinoni et al. 2010b). Exposure levels in the exposed groups were comparable to those reported in previous studies on individuals in similar occupations (Angelini et al. 2011; Cattaneo et al. 2010; Lovreglio et al. 2010; Ruchirawat et al. 2010). We used a relative measure of mtDNAcn, which has been shown to be highly precise and reproducible (Xing et al. 2008). This method, which has been widely used in large human studies (Shen et al. 2010; Xing et al. 2008), expresses mtDNAcn as a relative measure in relation to standard DNA. Whereas this method is well suited for comparisons between groups that, as in our study, are usually all scaled to the same standard DNA, the use of different standard DNA samples in different studies may limit external comparability.

CONCLUSIONS

Our investigation of individuals with low-level exposure to benzene in Italian cities showed increased mtDNAcn in association with airborne benzene exposure. Whether mitochondrial damage and dysfunction potentially related with increased mtDNAcn reflects the risk of haematological malignancies due to low-dose benzene remains to be determined in future prospective investigations.

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Table 1: Characteristics of the Study Population

City	Exposure Group	N	Age[a]	Sex[b]		Smoking[b]			Cig/Day[c]	Home Address[b, d]	
				Male	Female	Never	Former	Current		City	Suburbs
Genoa	Referents	49	42 (9)	47 (96)	2 (4)	25 (51)	11 (22)	13 (27)	16 +/- 7	23 (47)	26 (53)
	Bus Drivers	153	38 (23)	150 (98)	3 (2)	61 (40)	45 (29)	47 (31)	17 +/- 9	50 (33)	103 (67)
Milan	Referents	57	36 (18)	38 (67)	19 (33)	26 (46)	8 (14)	23 (40)	15 +/- 10	19 (33)	38 (67)
	Police Officers	77	30 (8)	47 (61)	30 (39)	40 (52)	9 (12)	28 (36)	15 +/- 7	10 (13)	65 (87)
	Gas-station Attendants	78	41 (18)	69 (88)	9 (12)	30 (38)	16 (21)	32 (41)	17 +/- 8	27 (35)	51 (65)
Cagliari	Distant Referents	16	55 (15)	10 (62)	6 (38)	8 (50)	6 (38)	2 (12)	13 +/- 4	9 (56)	7 (44)
	Close Referents	56	46 (17)	27 (48)	29 (52)	31 (55)	15 (27)	10 (18)	16 +/- 6	21 (38)	35 (62)
	Petrochemical Workers	33	36 (21)	32 (97)	1 (3)	10 (30)	10 (30)	13 (40)	15 +/- 9	16 (48)	17 (52)
All Subjects		519	39 (16)	420 (81)	99 (19)	231 (45)	120 (23)	168 (32)	16 +/- 8	175 (34)	342 (66)

[a]Median (IQR).
[b]N (%).
[c]Mean +/- SD; number of cigarettes/day calculated among current smokers only.
[d]The counts do not add up to the total number of participants because of two missing values.

Table 2: Airborne Benzene (µg/m^3) by City and Exposure Group

City	Exposure Group	Benzene Exposure Levels (µg/m^3)[a]							
		Minimum	25th p	Median	75th p	Maximum	Geometric Mean	(95%CI)	p-value
Genoa	Referents	4.2	4.2	8.6	13.8	45.8	8.7	(7.3-10.5)	
	Bus Drivers	4.2	14.8	20.5	30.9	92.1	20.5	(18.7-22.4)	<0.001[*]
Milan	Referents	4.2	4.2	6.3	12.8	57.1	8.1	(6.6-10.0)	
	Police Officers	9.03	19.0	21.8	31.1	315.7	25.0	(22.0-28.3)	
	Gas-station Attendants	11.5	37.9	60.9	130.9	477.9	69.9	(57.4-85.2)	<0.001[**]
Cagliari	Distant Referents	4.2	4.2	6.0	7.0	9.0	5.9	(4.9-7.1)	
	Close Referents	4.2	5.1	8.0	11.0	27.0	8.2	(7.1-9.4)	
	Petrochemical Workers	6.00	11.0	25.0	63.0	1250.0	35.4	(20.5-61.0)	<0.001[**]

[a]To convert µg/m^3 to ppb divide by 3.25.
[*]Mann-Whitney U non-parametric test for difference between referents and bus drivers.
[**]Nonparametric test (Cuzick) for trend across exposure categories.

Table 3: Relative mtDNAcn by City and Exposure Group

City	Exposure Group	N	MtDNAcn (Unadjusted)				MtDNAcn (Adjusted)			
			Mean[a]	(95%CI)[a]	p[*]	p-trend[**]	Mean[b]	(95%CI)[b]	p[*]	p-trend[**]
Genoa	Referents	48	0.75	(0.65-0.86)			0.75	(0.66-0.85)		
	Bus Drivers	151	0.90	(0.84-0.97)	0.013	-	0.90	(0.84-0.97)	0.019	-
Milan	Referents	56	0.76	(0.68-0.84)			0.75	(0.69-0.82)		
	Police Officers	77	1.14	(1.07-1.22)	<0.001		1.10	(1.01-1.19)	<0.001	
	Gas-station Attendants	76	0.86	(0.79-0.94)	0.037	0.180	0.90	(0.83-0.98)	0.005	0.008
Cagliari	Distant Referents	10	0.94	(0.59-1.48)			0.90	(0.60-1.41)		
	Close Referents	47	1.24	(1.01-1.52)	0.215		1.25	(1.03-1.51)	0.206	
	Petrochemical Workers	24	1.64	(1.30-2.07)	0.024	0.020	1.63	(1.22-2.18)	0.041	0.046

MtDNAcn: mitochondrial DNA copy number.
[a]Geometric mean and 95% confidence interval.
[b]Geometric mean and 95% confidence interval adjusted for age, sex, smoking (never, former, current), number of cigarettes/day.
[*]One-way ANOVA for difference vs. referents.
[**]Linear regression analysis for test for trend across exposure categories.

FIGURE LEGENDS

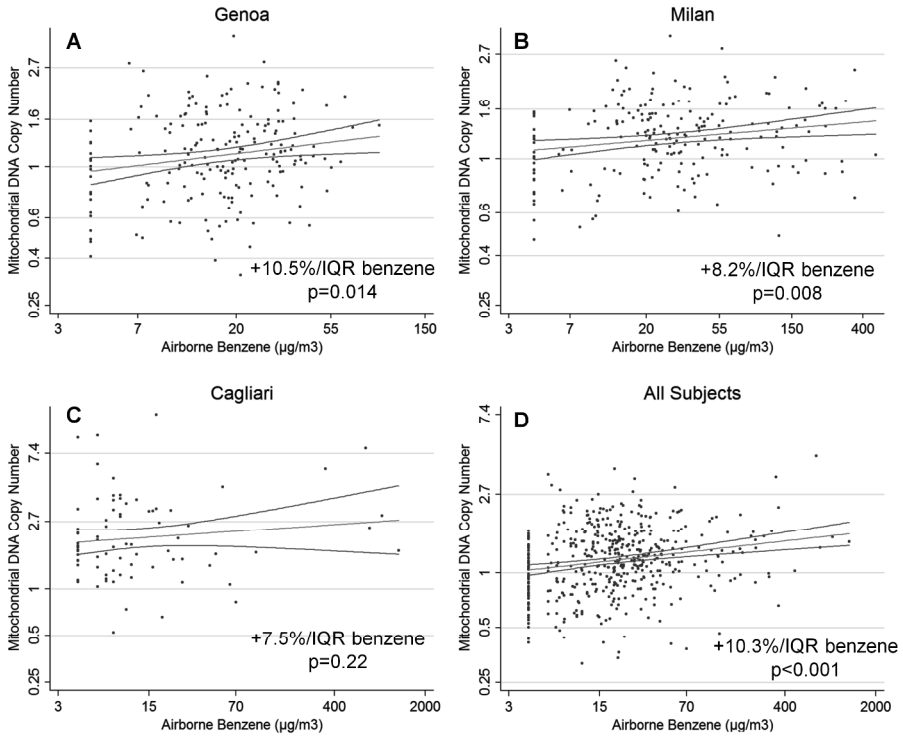
Figure 1: Association between Relative mtDNAcn and Airborne Benzene.

Scatterplots of mitochondrial DNA copy number (mtDNAcn) vs. airborne benzene levels for each of the cities (Figure 1A-1C) and for all participants combined (n=519) (Figure 1D). Covariate-adjusted percent changes in mtDNAcn estimated per an interquartile-range increase in personal airborne benzene are shown.

Figure 2: Association between DNA Methylation and Relative mtDNAcn.

Scatterplots of *LINE-1* (A), *Alu* (B), *p15* (C), or *MAGEA1* (D) methylation vs. mitochondrial DNA copy number (mtDNAcn) in the Milan subset of the study population (n=212). Covariate-adjusted percent changes in DNA methylation estimated per an interquartile-range increase in mtDNAcn are shown.

Figure 1



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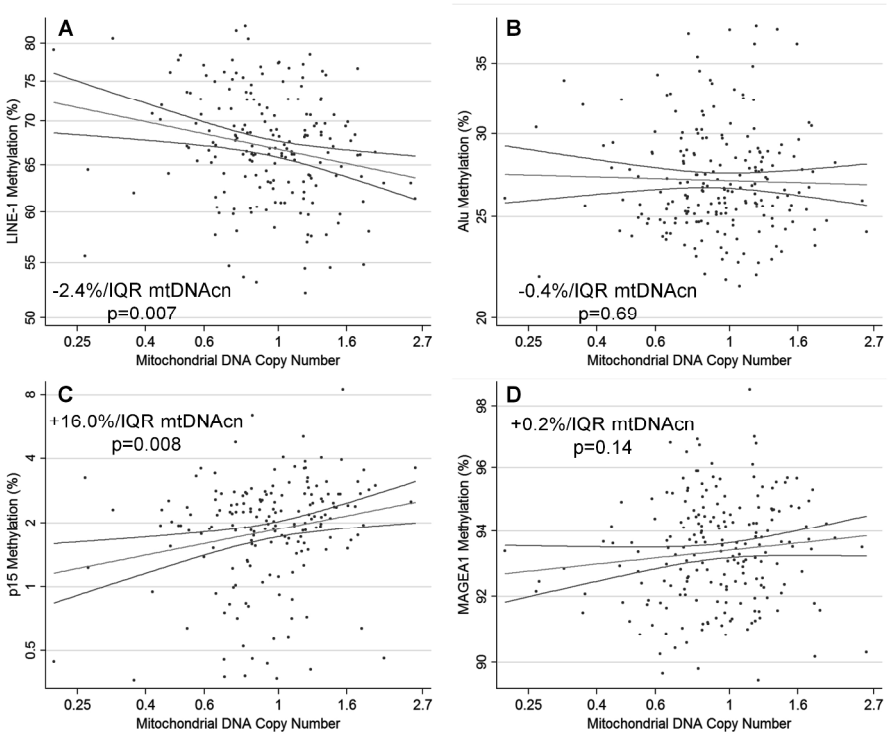
254x190mm (300 x 300 DPI)

Figure 2



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