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EPIGENETIC MARKERS
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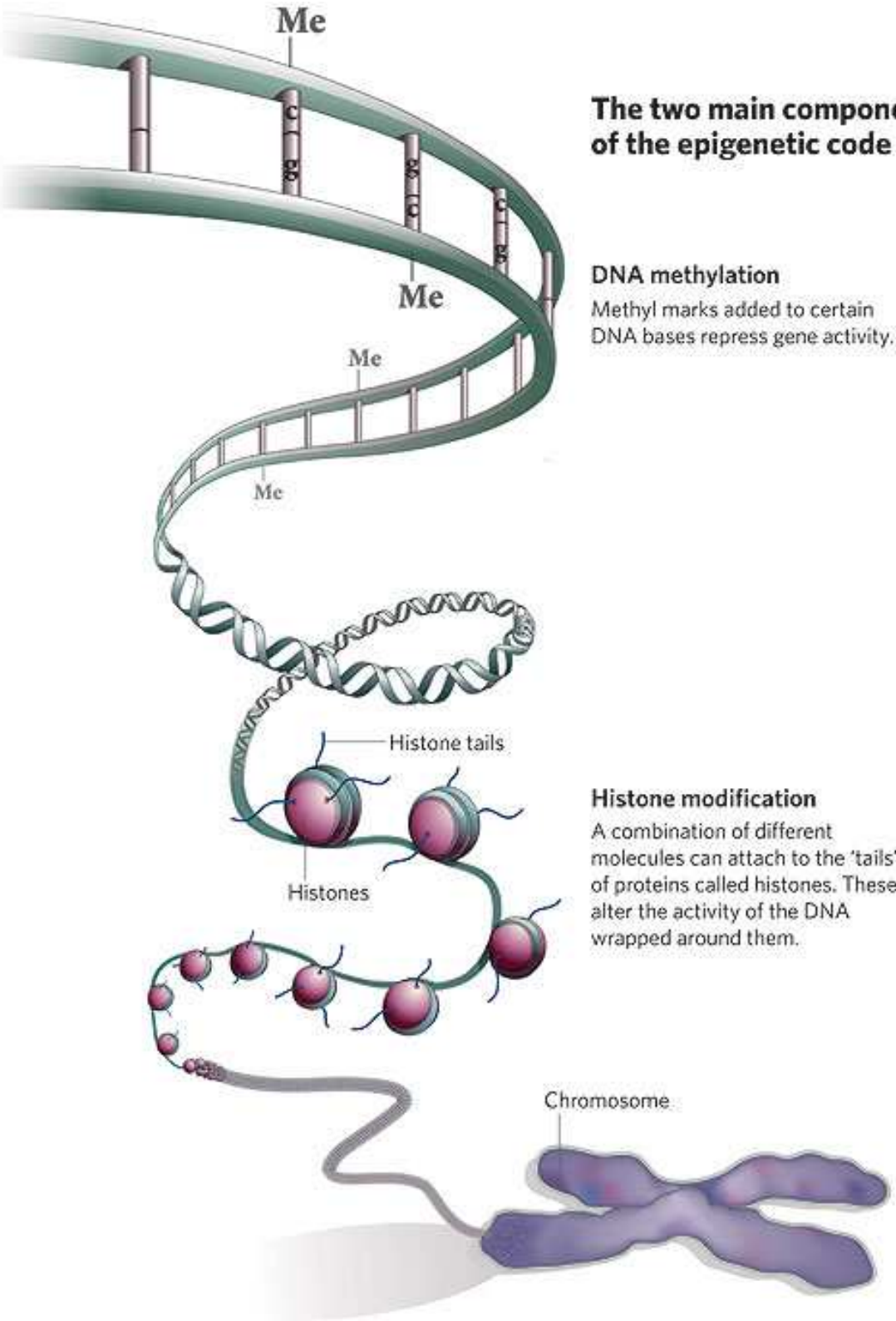
INTRODUCTION

The Epigenetics

The credit for coining in 1942, the term epigenetics as "the branch of biology that studies the causal interactions between genes and their products, which bring the phenotype into being", is attributed to Conrad Waddington (1905-1975) . By the mid-nineteenth century Epigenetics in literature, although the conceptual origins date back to Aristotle (384-322 BC) who believed in: the development of individual organic form from the unformed. This controversial view was the main argument against the notion that human beings developed from tiny fully-formed bodies. Even today, the opinions about what we are preprogrammed versus environmentally shaped are not unanimous. Epigenetics has emerged to explain the gap between nature and environmental effects. In the twenty-first century is largely defined as "the study of heritable changes in genome function that occur without changes in DNA sequence."

DNA methylation and histone modifications are the two most important epigenetic phenomena. These epigenetic phenomena alter physical accessibility to the genome by molecular complexes involved in gene expression and thus alter the levels of functioning of genes



The two main components of the epigenetic code

DNA methylation

Methyl marks added to certain DNA bases repress gene activity.

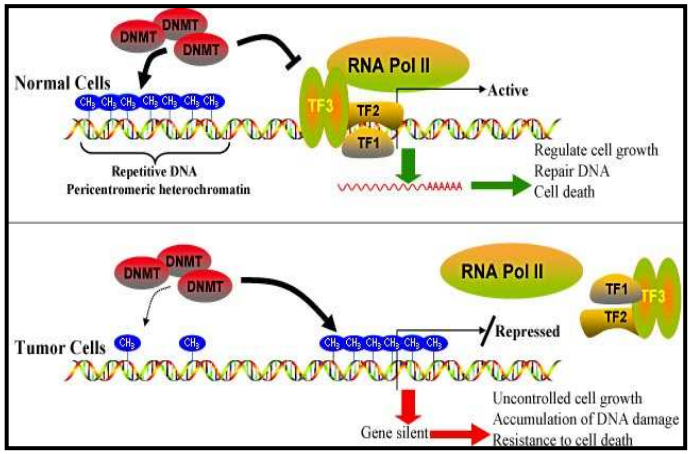
Histone modification

A combination of different molecules can attach to the 'tails' of proteins called histones. These alter the activity of the DNA wrapped around them.

DNA Methylation

As shown by numerous studies, the DNA methylation level of cytosine, which gives rise to 5-methyl-cytosine, is crucial in the development and progression of cancer (1, 2, 3).

The 5-methyl-cytosine represent 3-4% of all cytosine present in the mammalian genome (4, 5) and are located mainly in CpG dinucleotides. Clusters of CpG sequences, called CpG islands, are mainly located near the 5 'end of genes and are usually not methylated, although the methylation can occur in certain phases of life, or in some cell types, silencing the gene adjacent to the sequence. Approximately half of the promoters of human genes contain CpG islands (6).



The main consequence of DNA methylation is the alteration of the degree of compactness of chromatin by which DNA is organized. This more compact chromatin excludes transcriptional machinery of the cell and "takes off" gene expression (7).

Cancer cells may have an abnormal methylation pattern has been proposed that this disruption in the methylation of DNA, also observed in relation to environmental and occupational exposures, may be responsible for the processes of carcinogenesis..

In cancer, while CpG island become hypermethylated, the genome of cancer cells goes through a global hypomethylation and can have up to 20-60% 5mc less than normal cells (4, 5). In particular, hypomethylation or hypermethylation of CpG Islands may interfere with the constitutive expression of oncogenes or tumor suppressor gene silencing. A decrease in the global level of methylation observed in many types of cancer, could contribute directly to transformation through mobilization of transposable elements that are normally silent. (8). In addition, hypomethylation could lead to chromosomal instability and loss of imprinting. However, it is not yet clear whether the changes in methylation of DNA to cause cancer or are a consequence of the cancer itself (9).

In the human genome there are 1.4 million Alu repetitive elements and half a million long interspersed nuclear elements-1 (LINE-1) (10) that are normally heavily methylated, and it is estimated that more than one third of DNA methylation occurs in repetitive elements. Thus, the analysis of methylation (percent 5-mC) of repetitive elements may be a marker for global methylation of genomic DNA (10, 11)

Histone modifications

The DNA is wrapped by histones. Histones are positively charged proteins, almost always with a basic pH and consist of lysine, arginine, and a phosphate group that reacts with neighboring cells. There are five types of histones: H1, called joints, H2A, H2B, H3, H4. In each of these there is a polar region (positively charged, depending on the intensity) and a central non-polar, which can change their amino acid or remain constant.

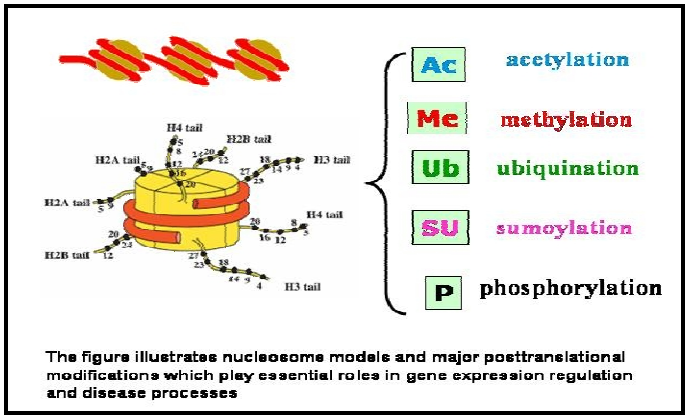
The histones H2 A and B, H3, H4, taken twice each form an octamer, which is surrounded by 1 ¾-turning of DNA, equal to 46 DNA bases. Many octamers are joined together by DNA link composed of 15 to 55 bp (base pairs). The octamer together with DNA link is called nucleosome, fundamental unit of chromatin, which looks like a necklace of pearls. There are different forms of histone H1 in different tissues of the same body and there is much variability among species (in yeast is absent). All of the histone H1 can be removed without altering the structure of the nucleosome, suggesting that is outside of the particle. The nucleosome is shaped like a cylinder 6 nm high with a diameter of 11 nm and a circumference of 34 nm. The DNA associated with nucleosome has a length of 67 nm (2 times the diameter): DNA makes two laps around the octamer. The DNA is bound to leave the nucleosome at two points close to each other: the binding site of histone H1.

In past years, post-translational modifications of histones have been intensively studied and has been proposed a model that attempts to summarize and shed light on the complex relations that bind them to the metabolism of DNA.

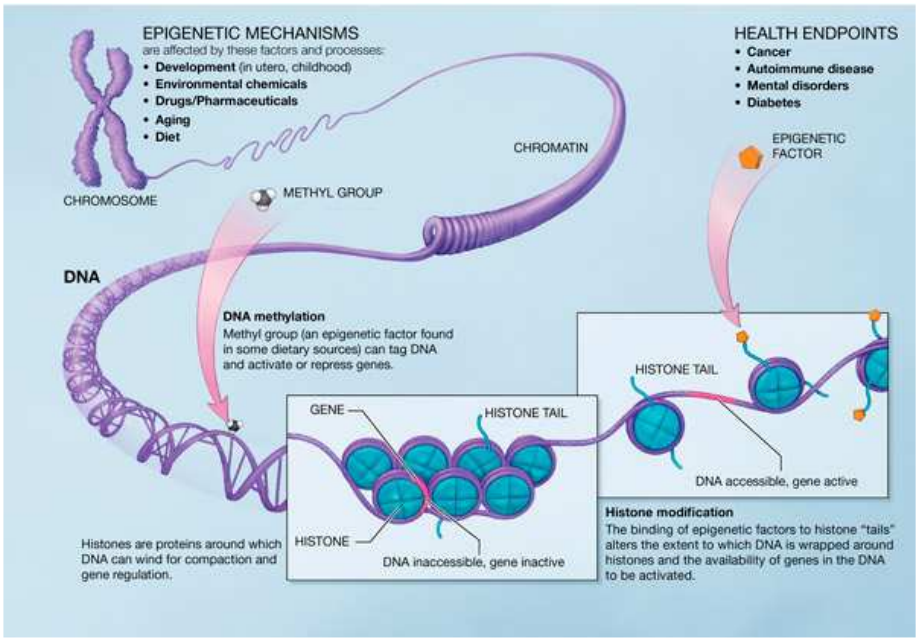
According to the model of the histone code, modifications of histone tails that affect chromatin structure, make it more or less accessible for transcription factors of the basal machinery, operating so as "receptors" for multiprotein complexes.

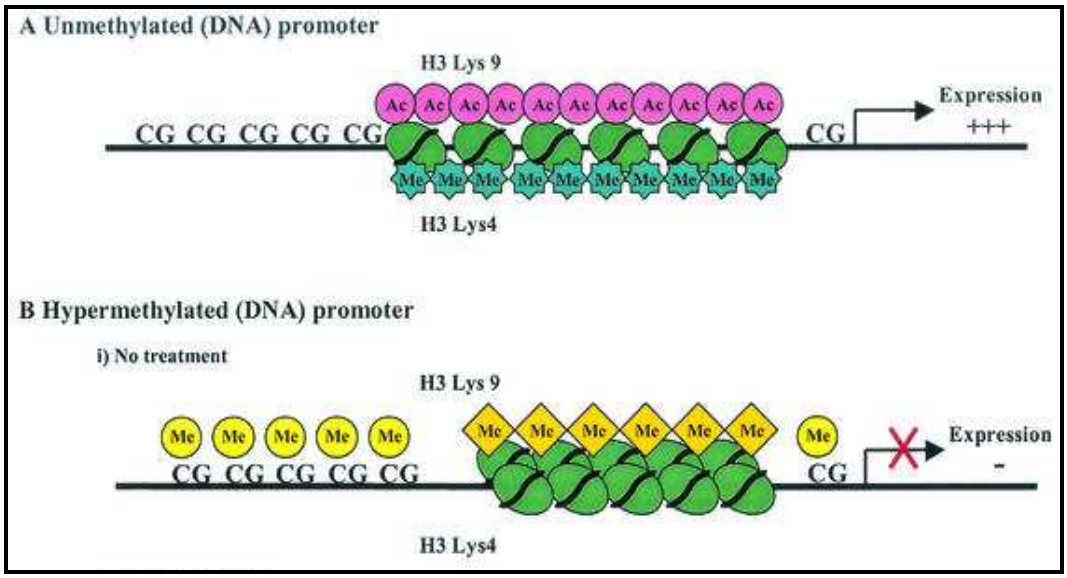
Modification of histone tails identified and characterized are methylation, acetylation, ADP-ribosylation, ubiquitination and phosphorylation. The

acetylation of histones has long been associated with regions in chromatin, transcriptionally active (12, 13), by contrast, the deacetylation with the induction of repression in many inducible genes. The link between transcriptional activity and histone acetylation status was determined through the intensive use of antibodies that specifically recognize acetylated histones



The figure illustrates nucleosome models and major posttranslational modifications which play essential roles in gene expression regulation and disease processes





A schematic representation of the proposed mechanism in the role of histone modifications in unmethylated and methylated DNA promoters

Overview of environmental and occupational carcinogenesis

Although environmental and occupational agents are implicated as significant contributors to the increased incidence of human carcinogenesis, interpretations as to the extent of such contributions remain largely unclear. One consideration suggests that environmental pollutants play only a minor role in cancer incidence, with lifestyle influences (tobacco, high fat diet and alcohol usage) acting as major contributing factor(s) (14-16). Conversely, other groups have suggested that environmental exposure to diverse physical, chemical and biological agents play a significant role in the occurrence of cancer, with lifestyle factor(s) contributing only a minor fraction (14,17). Nevertheless, data have clearly shown that many exogenous environmental factors can significantly contribute to the causation of a variety of human cancers (14,18). For example, natural and synthetic chemicals (i.e. fossil fuels, plastics, herbicides, pesticides, fungicides, tobacco smoke, food additives, etc.) found in the ambient environment, in drinking water and in food, have been shown to alter gene expression, as well as cellular metabolism (19,20).

Tobacco smoke, perhaps one of the most well established environmental carcinogenic factors, has been estimated to account for 30% of all cancer deaths and 85% of lung cancer deaths (19). In fact, the mixture of tobacco smoke and tar is defined as a “complete carcinogen” due to the presence of thousands of mutagenic compounds, including polycyclic aromatic hydrocarbons (PAH) and nitrosamines (14). On the other hand, one of the most well-established occupational carcinogenic agents known to cause mesothelioma and approximately 10% of lung cancers is asbestos. Other common agents involved in

the induction of occupational carcinogenesis include: wood-dust particulates (common to cabinet makers), solvents, paints, dye products and by-products of aromatic amines and/or aminophenol groups (common to painters), gasoline, petroleum-based mixtures, benzene, mineral oils and phthalates (used for their plasticizing and emulsifying properties in medical and cosmetic devices) (14). The combustion of a variety of these elements (i.e. vehicle exhaust, factory smoke, tobacco smoke, waste incinerators) yields a plethora of outdoor air pollutants known to adhere to fine carbon particles. Such particulates are able to remain suspended in the air and consequently penetrate into an organism and thus contributing to an increased cancer risk (14). A recent European study showed that the risk of lung cancer due to environmental (second hand) tobacco smoke is slightly higher at work than at home, and significantly higher for ex-smokers than for never smokers (21). Other outdoor air pollutants (such as biocides and pesticides) have also been linked to an increased risk for developing cancer. In fact, studies have revealed a causative relationship between parental and child pesticide exposure and an increased risk of developing leukemia, brain tumors, Wilm's tumors, Ewing's sarcoma and germ cell tumors (14). Pesticides are commonly referred to as persistent organic pollutants (POPs) due to their ability to withstand environmental degradation, thereby contaminating drinking water and food (22).

The IARC has identified a variety of metals and metalloids (as antimony, arsenic, chromium, cobalt, nickel, vanadium) as being carcinogenic with an ability to contribute to a wide spectrum of cancers (14,23). For example, whereas arsenic inhalation can cause lung cancer, its ingestion can cause bladder, kidney and lung

cancers (14,24). While the exact molecular mechanism(s) behind metal-induced carcinogenesis remain unclear, recent evidence has indicated that various metals act as catalysts in the oxidative deterioration of biological macromolecules with the ability to induce free radical generation (25). Metal ions (such as copper, cadmium, chromium, nickel, arsenic, cobalt, vanadium, and iron) are known to generate reactive oxygen species (ROS) and thus contribute to an imbalance observed between normal and pathologic conditions where free radicals are generated (25,26). Although ROS (i.e. superoxide radical, hydrogen peroxide, hydroxyl radical, etc.) are present in cells under normal physiological conditions, accumulative toxic effects contribute to an increased rate of ROS generation that ultimately supersedes the cellular antioxidant defense capacity thus generating a cellular toxic state known as “oxidative stress” (27). Strong evidence supports the involvement of oxidative stress in the process of carcinogenesis (28). For example, intracellular ROS accumulation has been shown to induce deleterious damage to a variety of biomolecules, leading to protein oxidation, lipid peroxidation, DNA damage, depletion of sulfhydryl groups and alteration(s) in signal transduction pathways (25). In addition, evidence has linked intracellular ROS formation to carcinogenesis through either direct genotoxic effects or indirect modification(s) of signaling pathways leading to altered expression of numerous genes (26,28). For example, recent data have suggested that certain carcinogenic metals (arsenic, cadmium, nickel, cobalt and lead) inhibit zinc fingercontaining DNA repair proteins and that such damage may be regarded as a novel mechanism involved in metal-induced carcinogenesis (14). Finally, free

radical-induced damage is suggested to be involved in aberrant epigenetic changes observed during the multistage carcinogenic process (27).

Effect of particulate matter (PM) and its metal carcinogenic components on epigenetics

In the last few years, several investigations have examined the relation between exposure to environmental/occupational and epigenetics and identified several toxicants that modify epigenetic marks (29-36).

Environmental and occupational exposure to inhalable particulate matter (PM) has been associated with increased risk of lung cancer (37-39). Epidemiologic and in-vivo studies suggest that the metal components of PM may be responsible for PM health effects, including lung cancer (40-48). In ranking the carcinogens, heavy metals have been classified by the International Agency for Research on Cancer (IARC) and Environmental Protection Agency (EPA) as the first group, except for selenium that has been listed within group 3 (not carcinogen to humans) of the IARC classification (49).

In spite of the well-recognized carcinogenic potentials of several toxic metals, the molecular mechanisms underlying their associations with cancer risk remain poorly understood. In particular, most carcinogenic metals are weak mutagens and do not induce DNA adduct formation, a key initiating event caused by other carcinogens (50).

Growing evidence indicates that epigenetic disregulation of gene expression plays a primary role in cancer etiology (29,51). Several toxic metals have been shown to bind more avidly to histone proteins than to other biopolymers such as DNA or

RNA (44,52,53). Recent in-vitro studies have shown that carcinogenic metals cause posttranslational epigenetic modifications of histone proteins, thus derailing the normal programming of gene expression (29-32). In lung epithelial cell lines and malignant transformation models, arsenic (32,33,35), nickel (35), and chromium (32,36) have been linked with specific activating histone modifications, such as H3K4 dimethylation, that contribute to the formation of a relaxed or 'open' chromatin structure permissive for gene transcription (31,54). Metal-related induction of activating histone modifications have been suggested to contribute to metal carcinogenesis by causing the expression of tumor suppressor or other cancer-promoting genes (34,55).

Inhaled particulate pollutants have been previously shown to produce systemic changes in gene expression, which can be detected in peripheral blood of exposed individuals (50).

Several metal components of PM have been also reported to modify DNA methylation patterns in vitro and animal studies (56). ROS, such as those produced following PM exposure (57) may interfere with DNA methylation processes (58) thus resulting in genomic hypomethylation.

Changes in DNA methylation have also been observed *in vivo* in nickel-induced tumors of wild type C57BL/6 mice and mice heterozygous for the tumor suppressor p53 gene injected with nickel sulfide. The promoter of the tumor suppressor gene p16 was found hypermethylated in all tumors of these mice (59,60).

The promoter region of the p53 tumor suppressor gene was also found hypermethylated as a result of arsenic exposure in a tissue culture model system and in arsenic-induced skin cancer patients (61,62).

However, whether metals in inhalable air particles induce alterations of histone modifications and DNA methylation in human subjects has never been evaluated.

OBJECTIVES

Although wide strata of the general population are exposed to low, background levels of toxic and carcinogenic inhalable compounds, industrial workers have been often exposed to significantly higher concentrations of potentially carcinogenic agents released from multiple sources (63). Foundry work is a specific condition of exposure to inhalable metal-rich particles that has been associated with increased risk of lung cancer in several early investigations (64). Even in modern foundry facilities, particles exhibit levels that are well above the concentrations found in ambient outdoor air, and also have a larger proportion of toxic metal components (65-68).

In the present work, we investigated both short- and long-term effects of particle exposure on histone modifications and DNA methylation in workers with well-characterized exposure to a wide range of PM level in a foundry steel plant.

We measured Histone 3 Lysine 4 (H3K4) dimethylation and Histone 3 Lysine 9 (H3K9) acetylation and DNA methylation in *Alu* and long interspersed nuclear element-1 (*LINE-1*) repetitive elements – as a surrogate of genome-wide methylation content – in peripheral blood leukocytes from foundry workers with well-characterized exposure.

METHODS

Study Subjects

We recruited 63 male healthy workers, free of cancer and cardiopulmonary disease, who had been working in a steel production plant in Brescia, Northern Italy for at least one year. Individual written informed consent and approval from the local Institutional Review Board were obtained before the study. All of the study subjects had a rotating weekly schedule based on four consecutive working days of eight hours each, followed by two days of rest. The study subjects worked in 11 different areas within the plant, which were selected to provide a wide contrast of exposures between the study subjects. The exposure of each of the study subjects in the plant was monitored for the first three working days of a work week. On the fourth day, each subject donated a 20 ml peripheral blood sample. We used EDTA tubes to collect 7 mL whole blood that was promptly (within 30 minutes from the blood drawing) centrifuged on site at 2,500 rpm for 15 min. The buffy coat (400 μL) was separated and transferred in a cryovial, immediately snap frozen in vapor phase of liquid nitrogen, shipped in nitrogen dry shippers to the laboratory and kept stored in vapor phase of liquid nitrogen until histone extraction. A self-administered questionnaire was used to collect detailed information on lifestyle, drug use, medical conditions, BMI, education and residential history. Records from the factory administrative files were used to extract information on occupational history.

In order to discriminate short term changes from effects occurring over a longer timeframe, we obtained blood samples for DNA methylation analysis in two

occasions: i) Sample 1 was collected in the morning of the first day of work – following two days off work – before the beginning of any work activity; ii) Sample 2 was collected at approximately the same time on the fourth day of work of the same week.

Exposure assessment

Particle metal components (aluminum, manganese, nickel, zinc, arsenic, lead, iron) and particle mass (PM with aerodynamic diameters <10 μm [PM_{10}]; <1 μm [PM_1]) were measured in each of the 11 work areas of the steel production plant. We measured air concentrations of individual metal particle components in PM_{10}, through multi-elemental analysis performed by means of inductively coupled-plasma mass spectrometer (ICP-MS, ELAN DRC II, Perkin Elmer, Waltham, USA) using the Total Quant method. External calibration was performed using calibration standard 3, stock multielement (10 μg/ml; Perkin Elmer, Waltham, USA). PM_{10} and PM_1 were measured using a GRIMM 1100 light-scattering dust analyzer (Grimm Technologies, Inc. Douglasville, GA, USA).

Each of the study subjects recorded in a personal log the time he spent in each of the work areas. Personal exposure was calculated as the average of work area levels weighted by the time spent in each area. All metal and particle levels were expressed in $\mu g/m^3$. In the plant, exposure levels have shown very little variability over time, as measures repeated over three years in a subset of the study population showed very high correlations (r^2>0.90). Therefore, the time-weighted levels of metals and particles represented, in addition to the exposure during the week of the study, also a measure of the usual exposure of the study

subjects (Tarantini et al. 2009). We estimated cumulative exposures as the product of the time-weighted levels of metals and particles during the study by the years of employment in the plant.

Total histone extraction and histone modification analysis

We obtained buffy coat from peripheral blood collected in EDTA tubes centrifuged at room temperature (22-25° C) for 15 minutes at 1,500 x g. Red blood cell lysis solution (Promega s.r.l. cod.A7933) was added to the buffy coat to wash out red blood cells. After 10 min at room temperature, the mixture was centrifuged at 2500 x g for 15 min, the supernatant was discarded. Remaining monolayer cells were processed according to protocol used by Chen et al. (69). Briefly, cells were lysed in 1ml of ice-cold radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with a protease inhibitor mixture (Roche Applied Sciences, Indianapolis, IN) for 10 min. The sample was then collected and centrifuged at 10000 x g for 10 min. After discarding supernatant, remaining pellet was resuspended in 0.4 N H2SO4. After incubation on ice for 90 min, the sample was centrifuged at 14000 x g for 15 min. The supernatant was mixed with cold acetone and kept at -20°C overnight. The histones were collected by centrifugation at 14000 x g for 15 min. After one wash with acetone, the histones were air dried and resuspended in 500 µl water. We measured total proteins in each samples by means of the Bradford assay according to manufacturer's instructions (Bio-Rad protein assay kit 500-0002. Bio-Rad laboratories, Milan, Italy). We used equal amounts of proteins (4µg) to normalize results of the subsequent analysis on histones.

We used a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA), using monoclonal antibodies, to detect endogenous levels of dimethylated H3K4 and acetylated H3K9 (PathScan® Sandwich ELISA Kit, Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocol. The assays we used in our study are the PathScan® Di-Methyl-Histone H3 (Lys4) Sandwich ELISA Kit #7124 and PathScan® Acetyl-Histone H3 (Lys9) Sandwich ELISA Kit #7121. The assays use the Di-Methyl-Histone H3 (Lys4) (C64G9) Rabbit mAb Antibody #9725 and Acetyl-Histone H3 (Lys9) Antibody #9671, respectively, which have been shown to be specific for the histone modifications of concern. Sample measurements were perfomed in duplicate. We used a Synergy HT-BioTek spectrophotometer to read 450nm absorbance. The absorbance values at 450nm directly reflected the concentration of modified histones (Deligezer et al. 2010). According to the Beer-Lambert law, optical density (O.D., absorbance) is used for colorimetric analysis so that readings relate directly to concentration. The coefficient of variation (CV) in replicate samples of the assays was 0.30% for H3K4 dimethylation and 0.42% for H3K9 acetylation.

Statistical analysis for histone modifications

We evaluated the association of PM metal components and PM mass levels with histone modifications using simple linear regression models, as well as multivariable models adjusting for age, body mass index, pack-years, education and percent granulocytes in the differential blood count. The independent variables used in multivariable models were selected a priori and included general characteristics potentially associated with cancer risks or other carcinogenic exposures. In addition, we adjusted for percent granulocytes to account for possible shifts in the proportion of leukocytes subtypes associated with the exposures. As sensitivity analyses, we fitted in the models as independent variables data from differential white blood counts (i.e., percent lymphocytes, monocytes, *eosinophils,* or basophils) or duration of sample storage and found no major changes in the results. Regression diagnostics were performed separately for each model. We examined whether the exposure-response relationships were linear through graphical inspection. Furthermore, we fitted a polynomial regression by including a quadratic term for exposure and compared these models with the linear model using the Likelihood Ratio Test. Neither graphical inspection, nor the Likelihood Ratio Tests suggested any departure from linearity. Outliers were excluded from regression analysis by dropping observations with studentized residuals that exceeded +3 or -3. The numbers of outliers removed ranges from a minimum of 0 to a maximum of 3.

Regression coefficients were computed with OLS estimators. In order to compare the magnitude of the associations of H3K4 dimethylation and H3K9 acetylation with different exposures, we calculated standardized regression

coefficients which express the effects on H3K4 dimethylation or H3K9 acetylation as the fraction of a standard deviation of H3K4 dimethylation or H3K9 acetylation associated with a standard deviation increase in exposure. We checked regression assumptions by performing diagnostic tests for each model, which included the Shapiro-Wilk test to verify normality of residuals and the White test to verify the homogeneity of variance of the residuals. Statistical analyses were performed in SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and R (The R Foundation for Statistical Computing, Vienna, Austria).

DNA extraction

We used EDTA tubes to collect 7 ml whole blood that was immediately centrifuged at 2500rpm for 15 minute. The buffy coat (400µl) was transferred in a cryovial and stored at -20°C until DNA extraction. DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI) following the manufacturer's instructions. Purified DNA was resuspended on the kit hydration solution, quantified and stored at -20° C until use.

Bisulfite DNA treatment

We performed DNA methylation analyses on bisulfite-treated DNA, using highly-quantitative, state-of-art analysis based on PCR-Pyrosequencing. 1µg DNA (concentration 50 ng/µl) was treated using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Final elution was performed with 30 µl M-Elution Buffer.

Pyrosequencing method for DNA methylation analysis

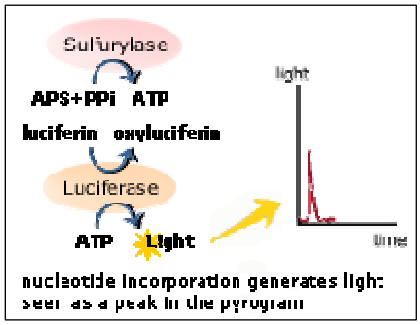
The biotinylated PCR products can be purified and denatured to single strand to be used as template in the pyrosequencing reaction. The Pyrosequencing is a direct sequencing, with synthesis method, developed to remove artifacts due to secondary structure that is observed in the more traditional electrophoresis.

With this method it is possible to analyze many sites of methylation in the same time without having the limit to locate the restriction sites within the site of methylation (the alternative method provides, in fact, digestion of DNA with restriction enzymes capable of recognizing between 5mc methylated and C non-methylated transformed into U by bisulfite); in this way you avoid the sequencing of multiple clones and you can accurately quantificate methylation sites because most sites are analyzed in the same reaction.

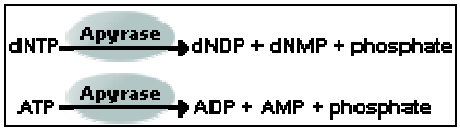
1. A primer is hybridized with a single strand DNA template and amplified by PCR and incubated with the enzymes (DNA pol, fosforilase ATP, and luciferase and apyrase) and substrates (adenosine-5- phosphosulfate and luciferin).
2. The first of the four dNTPs is added to the reaction. The DNA polymerase catalyzes the incorporation of dNTP only if it is complementary to the base of the template. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of nucleotide incorporated.

$(DNA)_n$ + dNTP -- **POLYMERASE**-->$(dna)_{n+1}$+PPi

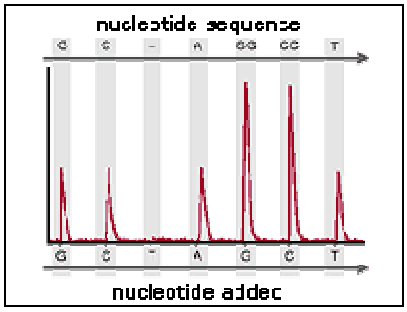
3. ATP sulfurylase quantitatively converts pyrophosphate into ATP in the presence of adenosine-5-phosphosulfate.



4. This allows the conversion of ATP to oxiluciferina luciferin by luciferase, and generates visible light in a quantity that is proportional to the amount of ATP. The light is detected by a CCD camera and is shown as a spike in a program. Each light signal is proportional to the number of nucleotides incorporated.
5. The apyrase, an enzyme that degrades nucleotides, continuously degrades unincorporated dNTPs and excess ATP. When degradation is complete nucleotide is added to another one.



6. Nucleotides are added one at a time. As the process continues, the complementary strand of DNA is synthesized and the nucleotide sequence is determined as peaks in the program.



We developed the assay for Alu and LINE-1 methylation by locating the *Alu and LINE-1* promoters using the Genomatix Software (Genomatix Software Inc, Ann Arbor, MI). A 50 µL PCR was carried out in 25 µL GoTaq Green Master mix (Promega, Madison, WI, USA), 10 pmol forward primer, 10 pmol reverse primer, 50 ng bisulfite-treated genomic DNA, and water. PCR cycling conditions were 95°C for 30 s, 50°C for 30 s and 72°C for 30 s for 40 cycles. PCR products were purified and sequenced by pyrosequencing as previously described (70) using 0.3 µM sequencing primer.

We performed DNA methylation analyses of *Alu* and *LINE-1* repeated sequences, which allow for the amplification of a representative pool of repetitive elements, as previously described (70). Measures of *Alu* and *LINE-1* methylation have been shown to be highly correlated with 5-methylcytosine content measured through high performance liquid chromatography, and are commonly used as a surrogate of global methylation (71,72). Primers for *Alu* and *LINE-1* assay are shown in table 1. For all assays we used built-in controls to verify bisulfite conversion efficiency. Compared to other common methods of DNA methylation analysis, Pyrosequencing-based assays have the advantage to produce individual measures of methylation at more than one CpG dinucleotide.

In the *Alu or LINE-1* assays, we measured the percentage of methylated cytosines (%mC) in three individual CpG dinucleotides within the sequence of interest. In addition, every sample was tested two times for each assay to confirm reproducibility. The resulting data were analyzed using mixed models, as described in the statistical analysis section below.

Table 1: Primers for DNA methylation analysis

Sequence ID	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Sequencing Primer (5' to 3')	Sequence to analyze*
Alu	Biotin-TTTTTATTAAAAATATAAAAATT	CCCAAACTAAAATACAATAA	AATAACTAAAATTACAAAC	G/AC/TG/AC/TG/ACCACCA
LINE-1	TTTTGAGTTAGGTGTGGGATATA	Biotin-AAAATCAAAAAATTCCCTTTC	AGTTAGGTGTGGGATATAGT	TTC/TGTGGTGC/TGTC/TG

* Nucleotides at which DNA methylation was measured are underlined

Statistical analysis for DNA methylation

In each blood sample, the Pyrosequencing-based analysis of DNA methylation produced six measurements (three individual CpG dinucleotides replicated in two measurements) for *LINE-1* and *Alu*. Each subject was tested twice (at the beginning of the work-week [sample 1] and after three days of work [sample 2]). To account for the data structure, we used mixed effects models.

Analysis of short-term effects

We first evaluated short-term changes in DNA methylation by contrasting measures on sample 1 vs. measures on sample 2 in two-way crossed random effects models:

$$y_{i(j_1,j_2)} = \beta_0 + \beta_1(sample) + \delta_{j_1} + \delta_{j_2} + e_{i(j_1,j_2)} \quad [1]$$

where β_0 represents mean DNA methylation; β_1 is the regression coefficient for the sample contrast; j_1 represents the subject, j_2 represents the CpG dinucleotide; δ_{j_1} is the random effect for subject j_1, δ_{j_2} is the random effect for CpG dinucleotide; and $e_{i(j_1,j_2)}$ is the residual error term.

Likelihood ratio tests were used to test for the difference between sample 1 and 2.

We then evaluated whether DNA methylation measured after three days of work (sample 2) was associated with the PM_{10} exposure level estimated during the previous three days, using two-way crossed effects models:

$$y_{i(j_1,j_2)} = \beta_0 + \beta_1(\mathrm{PM}_{10}) + \beta_2 X_2 + ... + \beta_n X_n + \delta_{j_1} + \delta_{j_2} + e_{i(j_1,j_2)} \quad [2]$$

where β_0 represents the mean of DNA methylation; β_1 is the regression coefficient for PM_{10} exposure; $\beta_2 \ldots \beta_n$ are the regression coefficients for the covariates included in multivariate models; j_1 represents the subject, j_2 represents the CpG dinucleotide; δ_{j_1} is the random effect for subject j_1, δ_{j_2} is the random effect for CpG dinucleotides, and $e_{i(j_1,j_2)}$ is the residual error term.

Analysis of long-term effects

As noted in the exposure assessment section, PM_{10} exposure levels estimated during the study also represented a measure of the usual exposure of the study subjects. To estimate long-term effects of PM_{10} on DNA methylation, we evaluated the level of individual exposure to PM_{10} in relation to all the measures of DNA methylation performed in the study, regardless of whether they were measured on samples taken on the first day of work (i.e., following two days off), or after three consecutive days of exposure to PM_{10} in the foundry, thus assuming that PM_{10} effects operating over an extended timeframe produced similar modifications at the two time points.

For DNA methylation measures that did not show changes in the analysis of short-term effects, we fit two-way error-components models, as described in the formula [2] above. If a significant difference between sample 1 and 2 was found in the analysis of short term effects, we fit a three-way error-components model, as described in the following notation:

$$y_{i(j_1,j_2,j_3)} = \beta_0 + \beta_1(PM_{10}) + \beta_2 X_2 + ... + \beta_n X_n + \delta_{j_1} + \delta_{j_2} + \delta_{j_3} + e_{i(j_1,j_2,j_3)} \quad [3]$$

where β_0 represents the mean of DNA methylation , β_1 represents the mean PM_{10} effect; $\beta_2 \ldots \beta_n$ are the regression coefficients for the covariates included in multivariate models; j_1 represents the subject, j_2 represents the CpG dinucleotide, and j_3 represent the blood sample (Sample 1 or 2); δ_{j_1} is the random effect for subject j_1, δ_{j_2} is the random effect for CpG dinucleotide j_2, and δ_{j_3} is the random effect for blood sample j_3; $e_{i(j_1, j_2, j_3)}$ is the residual error term.

All analyses were performed in Stata 10.0 (Stata Corp., College Station, TX). For all models including PM10 levels, we report beta coefficients estimating the changes in DNA methylation for an increment equal to the difference between the 90^{th} and 10^{th} percentile of PM_{10}. Covariates for multivariate models included the following potential confounders that were chosen *a priori* and included in the analysis: age, body mass index, smoking, and number of cigarettes/day.
A p-value<0.05 was considered statistically significant.

RESULTS

Subjects' characteristics and exposure level

The mean age of the study subjects was 44 years (range between 27 and 55 years). Twenty-five subjects (40%) were current smokers, who reported a mean number of 13.0 (SD=7.2) cigarettes smoked every day. The average body mass index of the study participants was 26.5 Kg/m^2 (SD=2.7) (Table 2). The average levels of inhalable air metal concentrations and PM mass estimated are shown in Table 3. For both metal levels and PM mass, the study subjects showed wide ranges of exposures. The subject with the maximum individual exposure level was at least 17-fold more exposed than the subject with the minimum exposure level (i.e., for PM_{10}, the maximum level was 1220.17 $\mu g/m^3$ vs. minimum level of 73.72 $\mu g/m^3$). For some of the exposures (i.e., aluminum, manganese, zinc, lead) the maximum individual exposure was more than 200 times higher than the minimum individual exposure (Table 3).

The correlation matrix between exposure levels showed correlations of different strengths between the exposures (Table 4). We found high correlations (Pearson's correlation coefficient [r] between 0.7–1) for aluminum with manganese, lead, and PM_{10}; for manganese with lead and PM_{10}; for nickel with arsenic and iron; and between arsenic and iron; PM_{10} and lead; and PM_1 and PM_{10}. Correlations were moderate (r=0.4–0.7) for PM_1 with aluminum, manganese, and lead; and between nickel and aluminum; and iron and zinc. Low positive correlations (r=0-0.4) were found for nickel with manganese, zinc, lead, PM_{10} and PM_1; for zinc with aluminum, manganese, arsenic, lead, PM_{10} and PM_1; for arsenic with aluminum,

manganese, lead, and PM_{10}; and for iron with aluminum, manganese, lead, PM_{10} and PM_1.

Table 2: Characteristics of the study subjects

Variable	
Age, mean (SD) [years]	44 (7.6)
Body Mass Index, mean (SD) [Kg/m^2]	26.5 (2.7)
Current smokers, n (%)	
No	38 (60%)
Yes	25 (40%)
Number of cigarettes, mean (SD) [n/day]	13 (7.2)
Education, n (%)	
Primary School (completed grade 5)	12 (19%)
Middle School (completed grade 8)	37 (59%)
High School, completed	14 (22%)
Use of non-steroidal anti-inflammatory drugs, n (%)	
No	51 (81%)
Yes	12 (19%)
Liver diseases, n (%)	
No	59 (94%)
Yes	4 (6%)
PM_{10} exposure,* n (%)	
74-162 µg/m^3	23 (36.5%)
165-210 µg/m^3	24 (38.1%)
223-1220 µg/m^3	16 (25.4%)

* Average level of particulate matter < 10 µm in aerodynamic diameter (PM_{10}) estimated for each subject, categorized based on tertiles

Table 3. Levels of personal exposure to metal components of particulate matter (PM) and total PM mass

Exposure* ($\mu g/m^3$)	N	Mean	(SD)	Min	Percentile			Max
					25^{th}	50^{th}	75^{th}	
Aluminum	63	8.50	(18.07)	0.40	1.48	2.05	7.41	84.07
Manganese	63	11.26	(30.41)	0.11	1.20	4.63	10.77	174.79
Nickel	63	0.30	(0.18)	0.02	0.23	0.25	0.46	0.72
Zinc	63	18.85	(26.37)	0.26	1.47	8.45	32.28	129.06
Arsenic	63	0.10	(0.1)	0.01	0.02	0.07	0.17	0.31
Lead	63	7.53	(17.46)	0.13	0.63	2.87	9.52	99.90
Iron	63	32.02	(22.08)	0.96	18.00	25.64	48.69	88.43
PM_{10}	63	233.42	(214.56)	73.72	152.23	179.45	222.86	1220.17
PM_1	63	8.48	(6.18)	1.71	3.51	9.01	11.35	30.49

Abbreviations: SD, Standard Deviation ; Min, minimum; Max, maximum; PM_1 , particulate matter with aerodynamic diameter <1μm; PM_{10}, particulate matter with aerodynamic diameter <10μm

*Metal components were measured on the PM_{10} fraction of PM mass. Coarse particle levels were calculated as the difference between PM_{10} and PM_1

Table 4. Matrix of correlations (r*) among individual exposures (PM metal components and PM mass measures)

	Aluminum	**Manganese**	**Nickel**	**Zinc**	**Arsenic**	**Lead**	**Iron**	**PM_{10}**	**PM_1**
Aluminum	1								
Manganese	0.75 *<0.0001*	1							
Nickel	0.46 *0.0001*	0.39 *0.0017*	1						
Zinc	0.18 *0.1625*	0.21 *0.1034*	0.32 *0.0105*	1					
Arsenic	0.15 *0.2378*	0.31 *0.0143*	0.84 *0.0000*	0.25 *0.0483*	1				
Lead	0.75 *<0.0001*	0.99 *<0.0001*	0.38 *0.0018*	0.35 *0.0047*	0.28 *0.0265*	1			
Iron	0.15 *0.2451*	0.28 *0.0261*	0.76 *<0.0001*	0.48 *0.0001*	0.70 *<0.0001*	0.32 *0.0113*	1		
PM_{10}	0.81 *<0.0001*	0.82 *<0.0001*	0.34 *0.0056*	0.34 *0.0070*	0.04 *0.7446*	0.85 *<0.0001*	0.27 *0.0294*	1	
PM_1	0.63 *<0.0001*	0.60 *<0.0001*	0.22 *0.0897*	0.28 *0.0277*	-0.20 *0.1195*	0.64 *<0.0001*	0.26 *0.0408*	0.90 *<0.0001*	1

* Pearson's product-moment correlation coefficient.

Associations of histone modifications with subjects' characteristics and years of employment

H3K4 dimethylation and H3K9 acetylation after three consecutive days of work (sample 2) were similar to those measured at the beginning of the workweek (sample 1) (p=0.59, paired t-test for the difference between sample 1 and 2 for H3K4 dimethylation ; p=0.57, paired t-test for the difference between sample 1 and 2 for H3K9 acetylation).

The range of values of the histone modifications was between 0.26 to 1.20 optical density (O.D.) for H3K4 dimethylation, with a mean of 0.94 O.D. (SD=0.20); and between 0.50 to 1.06 O.D. for H3K9 acetylation, with a mean of 0.42 O.D. (SD=0.35).

H3K4 dimethylation was moderately, but significantly correlated with H3K9 acetylation (Pearson's r =0.45, p<0.001). H3K4 dimethylation and H3K9 acetylation were not associated with age, body mass index, smoking, number of cigarettes/days, smoking duration, pack-years, area of residence (city center, suburbs, rural), self-reported traffic intensity near home and percent granulocytes (Table 5). H3K9 acetylation was positively associated with education (p=0.04, Table 5). Both H3K4 dimethylation and H3K9 acetylation increased in association with the years of employment of the study subjects in the steel plant (Table 5). The association between years of employment and histone modifications was confirmed in age-adjusted regression models: we estimated that age-adjusted H3K4 dimethylation was 0.87 (95% CI 0.77-0.97) in subjects with less than 9

years of employment, 0.96 (95% CI 0.88-1.05) in subjects with 9-21 years of employment, and 1.03 (95% CI 0.90-1.16) in those with more than 21 years of employment (p-trend=0.02); age-adjusted H3K9 acetylation was 0.23 (95% CI 0.043-0.42) in subjects with less than 9 years of employment, 0.41 (95% CI 0.25-0.58) in subjects with 9-21 years of employment, and 0.47 (95% CI 0.22-0.71) in those with more than 21 years of employment (p-trend=0.004).

Table 5. Association of the subjects' characteristics with H3K4 dimethylation and H3K9 acetylation (in Optical Density [O.D.] units) measured on the fourth day of a workweek.

		H3K4 dimethylation			H3K9 acetylation		
Variable	**N**	**Mean (O.D.)**	**(95% CI)**	**P value***	**Mean (O.D.)**	**(95% CI)**	**P value***
Age, years							
< 39	22	0.90	(0.79; 1.02)		0.41	(0.22; 0.59)	
39 - 47	20	1.00	(0.96; 1.05)		0.43	(0.26; 0.59)	
> 47	21	0.94	(0.88; 1.00)	0.47	0.43	(0.27; 0.58)	0.88
Body Mass Index, Kg/m^2							
< 25	21	0.96	(0.9; 1.02)		0.48	(0.33; 0.62)	
25 - 27.5	21	0.89	(0.78; 1.01)		0.20	(0.04; 0.35)	
> 27.5	21	0.99	(0.95; 1.03)	0.57	0.59	(0.45; 0.74)	0.36
Smoking							
Non smoker	24	0,96	(0.88; 1.04)		0,44	(0.28; 0.61)	
Former smoker	14	0,87	(0.76; 0.97)		0,35	(0.12; 0.59)	
Current smoker	25	0,98	(0.92; 1.05)	0.14$^{\#}$	0,43	(0.30; 0.57)	0.76$^{\#}$
Number of cigarettes/day							
0	38	0.93	(0.87; 0.99)		0.41	(0.28; 0.54)	
1 - 10	10	0.98	(0.92; 1.05)		0.39	(0.23; 0.55)	
> 10	15	0.98	(0.87; 1.09)	0.29	0.46	(0.24; 0.68)	0.69
Smoking duration, years							
0	23	0.97	(0.88; 1.05)		0.45	(0.28; 0.62)	
0 - 19	19	0.90	(0.8; 1.00)		0.35	(0.15; 0.54)	
> 19	21	0.97	(0.91; 1.03)	0.94	0.45	(0.32; 0.58)	0.98
Pack-years							
0	24	0,96	(0.88; 1.04)		0,44	(0.28; 0.61)	
0-4	17	0,92	(0.84; 0.99)		0,39	(0.22; 0.57)	
>4	20	0,95	(0.86; 1.04)	0.79	0,43	(0.25; 0.61)	0.90
Education							
Primary school	12	0.96	(0.83; 1.09)		0.25	(0.05; 0.45)	
Middle school	37	0.94	(0.89; 0.99)		0.43	(0.31; 0.56)	
High school	14	0.96	(0.81; 1.10)	0.98	0.55	(0.36; 0.74)	0.04
Area of residence							
City centre	8	0.89	(0.54; 1.23)		0.30	(0.05; 0.64)	
Suburbs	41	0.97	(0.92; 1.01)		0.49	(0.38; 0.60)	
Rural	12	0.91	(0.79; 1.03)	0.94	0.26	(0.02; 0.50)	0.43
Granulocyte, %							
45 - 55	21	0.95	(0.88; 1.01)		0.43	(0.3; 0.57)	
55 - 61	21	0.94	(0.82; 1.06)		0.42	(0.19; 0.65)	
61 - 76	21	0.96	(0.91; 1.02)	0.76	0.41	(0.28; 0.54)	0.86
Years of Employment							
< 9	20	0.88	(0.75; 1.00)		0.22	(0.04; 0.39)	
9 - 21	17	0.96	(0.91; 1.02)		0.43	(0.28; 0.58)	
> 21	22	0.99	(0.95; 1.04)	0.04	0.51	(0.37; 0.66)	0.006

** p - value test for trend; $^{\#}$ p one-way ANOVA*

Association of levels of exposure to inhalable metals with histone modifications

We evaluated whether levels of H3K4 dimethylation and H3K9 acetylation were associated with the levels of personal exposure to metals in inhalable particles, as well as to PM mass, in both simple regression models and multivariable models adjusted for age, BMI, education, pack-years, and percent granulocytes. Results from unadjusted and adjusted models showed similar results (Table 6). In adjusted models, H3K4 dimethylation increased in association with nickel, arsenic, and iron. H3K4 dimethylation was not associated with the levels of exposure to aluminum, manganese, zinc, lead, PM_{10}, and PM_1 levels in both unadjusted and adjusted regression models (Table 6). H3K9 acetylation showed borderline significant associations with nickel and iron levels. H3K9 acetylation was not associated with the levels of the remaining metals, PM_{10} and PM_1 (Table 6).

Table 6: Association of personal level of exposure to PM metal components and total PM mass with H3K4 dimethylation and H3K9 acetylation

	Association with H3K4 dimethylation								Association with H3K9 acetylation							
	Unadjusted				Adjusted*				Unadjusted				Adjusted*			
Exposure	**β[#]**	**(95% CI)[#]**		***P* value**	**β[#]**	**(95% CI)[#]**		***P* value**	**β[#]**	**(95% CI)[#]**		***P* value**	**β[#]**	**(95% CI)[#]**		***P* value**
Aluminium	0.01	(-0.02;	0.04)	0.36	0.02	(-0.01;	0.05)	0.24	0.04	(-0.11;	0.02)	0.15	0.03	(-0.1;	0.03)	0.33
Manganese	0.02	(-0.01;	0.04)	0.21	0.02	(-0.01;	0.05)	0.17	0.03	(-0.08;	0.02)	0.25	0.01	(-0.06;	0.04)	0.73
Nickel	0.15	(0.03;	0.28)	0.02	0.16	(0.03;	0.3)	0.02	0.22	(-0.04;	0.48)	0.10	0.24	(-0.02;	0.51)	0.07
Zinc	0.05	(-0.01;	0.11)	0.08	0.05	(-0.01;	0.12)	0.12	0.05	(-0.18;	0.07)	0.41	0.06	(-0.19;	0.07)	0.38
Arsenic	0.16	(0.03;	0.28)	0.02	0.16	(0.02;	0.3)	0.02	0.18	(-0.08;	0.45)	0.17	0.21	(-0.06;	0.48)	0.13
Lead	0.02	(-0.01;	0.04)	0.19	0.02	(-0.01;	0.05)	0.16	0.04	(-0.09;	0.02)	0.16	0.02	(-0.08;	0.04)	0.52
Iron	0.12	(0.01;	0.24)	0.04	0.14	(0.01;	0.26)	0.03	0.21	(-0.02;	0.45)	0.08	0.22	(-0.03;	0.47)	0.08
PM_{10}	0.03	(-0.03;	0.09)	0.34	0.04	(-0.03;	0.11)	0.23	0.08	(-0.2;	0.04)	0.17	0.04	(-0.17;	0.09)	0.51
PM_1	0.02	(-0.06;	0.1)	0.64	0.03	(-0.06;	0.13)	0.45	0.04	(-0.2;	0.13)	0.65	0.00	(-0.18;	0.18)	0.97

[*] Multivariable regression models adjusted for age, body mass index, education, pack-years, %granulocyte

[#] Regression coefficient (β) and 95% Confidence interval expressing the change in histone modifications (Optical Density [O.D.] units) associated with an increase in exposure equal to the difference between the 90th and 10th percentile of the exposure distribution.

Association of cumulative exposure to inhalable metals with histone modifications

Results on the associations of cumulative exposure, estimated as the product of personal levels of exposure to PM metal components or total PM mass by the years of employment, with H3K4 dimethylation and H3K9 acetylation are shown in Table 7. Again, unadjusted and adjusted models showed similar results. In adjusted models, H3K4 dimethylation increased in association with cumulative exposure to nickel and arsenic. Cumulative exposure to iron was not significantly associated with H3K4 dimethylation. H3K9 acetylation showed significant associations with cumulative exposure to nickel and arsenic.

Table 7: Association of cumulative level of exposure* to PM metal components and total PM mass with H3K4 dimethylation and H3K9 acetylation

	Association with H3K4 dimethylation						Association with H3K9 acetylation					
	Unadjusted			Adjusted[#]			Unadjusted			Adjusted[#]		
Exposure	**β**[†]	**(95% CI)**[†]	***P* value**	**β**[†]	**(95% CI)**[†]	***P* value**	**β**[†]	**(95% CI)**[†]	***P* value**	**β**[†]	**(95% CI)**[†]	***P* value**
Aluminium	0.01	(-0.03; 0.04)	0.61	0.02	(-0.02; 0.06)	0.44	0.02	(-0.09; 0.04)	0.48	0.03	(-0.1; 0.05)	0.48
Manganese	0.01	(-0.02; 0.04)	0.33	0.02	(-0.02; 0.05)	0.35	0.01	(-0.07; 0.04)	0.63	0.00	(-0.06; 0.05)	0.86
Nickel	0.11	(-0.01; 0.23)	0.07	0.16	(0.01; 0.3)	0.03	0.26	(0.04; 0.49)	0.02	0.27	(0.01; 0.54)	0.04
Zinc	0.06	(-0.03; 0.15)	0.21	0.06	(-0.05; 0.17)	0.30	0.02	(-0.21; 0.16)	0.80	0.03	(-0.24; 0.18)	0.78
Arsenic	0.14	(0.03; 0.26)	0.01	0.16	(0.03; 0.29)	0.02	0.30	(0.09; 0.51)	0.01	0.28	(0.04; 0.51)	0.02
Lead	0.02	(-0.02; 0.06)	0.35	0.02	(-0.03; 0.06)	0.38	0.03	(-0.11; 0.05)	0.42	0.02	(-0.1; 0.06)	0.65
Iron	0.09	(-0.03; 0.21)	0.14	0.12	(-0.02; 0.27)	0.09	0.25	(0.03; 0.48)	0.03	0.24	(-0.03; 0.5)	0.08
PM_{10}	0.03	(-0.04; 0.11)	0.41	0.05	(-0.04; 0.14)	0.26	0.00	(-0.14; 0.15)	0.99	0.00	(-0.17; 0.17)	0.97
PM_1	0.03	(-0.07; 0.13)	0.53	0.07	(-0.06; 0.21)	0.29	0.08	(-0.11; 0.28)	0.40	0.08	(-0.17; 0.32)	0.54

*Cumulative levels of exposure were estimated as the product of personal level of exposure to PM metal components or total PM mass by the years of employment in the job

#Multivariable regression models adjusted for age, body mass index, education, pack-years, %granulocyte

†Regression coefficient (β) and 95% Confidence interval expressing the change in histone modifications (Optical Density [O.D.] units) associated with an increase in exposure equal to the difference between the 90th and 10th percentile of the exposure distribution.

Short-term effects of PM_{10} exposure on DNA methylation

As shown in Table 8, DNA methylation of *Alu* and *LINE-1* repeated elements did not change after three days of work. The level of individual exposure to PM_{10} showed weak negative correlations with DNA methylation of *Alu and LINE-1* measured after three days of exposure, with associations that were not statistically significant in unadjusted analysis, as well as in models adjusted for age, body mass index, smoking, and number of cigarettes/day (Table 9).

Table 8. Change in methylation of *LINE-1* and *Alu* in foundry workers measured after three days of work (sample 2), compared to measures on the first day of work (sample 1)*

	Number of subjects	Sample 1 (Mean)	Sample 2 (Mean)	Difference between sample 2 vs. 1 (Beta)	Standard error	P-value
Alu	61	25.8	25.8	0.001	0.083	0.99
LINE-1	61	78.8	78.8	0.02	0.11	0.89

*Sample 1 was collected at the beginning of the first day of work, following two days off; Sample 2 was collected in the same week at the beginning of the fourth day of work, following three consecutive days of work.

Table 9: Association of PM_{10} average exposure with DNA methylation measured in foundry workers after three consecutive work days of exposure

	Unadjusted Regression			Adjusted Regression*		
	β	Standard error	P-value	$\beta^{\dagger}$	Standard error	P-value
Alu	-0.18	0.10	0.08	-0.18	0.10	0.071
LINE-1	-0.25	0.25	0.31	-0.28	0.25	0.26

* Multivariable regression models adjusted for age, body mass index, smoking, number of cigarettes/day
† Beta for an increment equal to the difference between the 90^{th} and 10^{th} percentile of PM_{10}.

Long-term effects of PM10 exposure on DNA methylation

To identify long-term effects of PM_{10} exposure, we evaluated the level of individual exposure to PM_{10}, taken as a measure of usual exposure to particles, in relation to all the measures of DNA methylation performed in the study, regardless of whether they were measured on samples taken on the first day of work (i.e., following two days off), or after three consecutive days of exposure to PM_{10} in the foundry (Table 10).

Table 10: Association of PM_{10} average level with all measures of DNA methylation in blood samples taken from exposed foundry workers*

	Unadjusted Regression			**Adjusted Regression***		
	β	**Standard error**	**P-value**	**β†**	**Standard error**	**P-value**
Alu	-0.18	0.09	0.04	-0.19	0.09	0.04
LINE-1	-0.30	0.17	0.07	-0.34	0.17	0.04

* To estimate long-term effects of PM_{10}, the level of individual exposure to PM_{10}, taken as a measure of usual exposure to particles, was examined in relation to all the measures of DNA methylation performed in the study, regardless of whether they were measured on samples taken on the first day of work (i.e., following two days off), or after three consecutive days of exposure to PM_{10} in the foundry. In the models, the two samples collected at different times are exchangeable, thus assuming that PM_{10} effects operating over an extended timeframe produced similar modifications at the two time points.
† Multivariable mixed-models adjusted for age, body mass index, smoking, number of cigarettes/day

In unadjusted models, the average PM_{10} levels were significantly associated with decreased *Alu* methylation (β=-0.18; P=0.04). A negative, non significant association was also observed for *LINE-1* methylation (β=-0.30; P=0.07). In multivariable regression analysis adjusting for age, body mass index, smoking and number of

cigarettes – the average PM_{10} levels were significantly associated with both *Alu* (β = -0.19; P = 0.04) and *LINE-1* (β = -0.34; P = 0.04) methylation.

Association of levels of exposure to inhalable metals with DNA methylation

We evaluated whether levels of DNA methylation in Alu and LINE-1 elements were associated with the levels of personal exposure to metals in inhalable particles, as well as to PM mass, in both simple regression models, not adjusted, and multivariable models adjusted age, BMI, smoking, number of cigarettes/day and percent of granulocytes . Results from unadjusted and adjusted models showed similar results (Table 11). In unadjusted models, DNA methylation of Alu decreased in association with zinc and PM_{10}, while DNA methylation of LINE-1 decreased in association with the levels of exposure to aluminium. LINE-1 also decreased in a borderline significant association with $PM_{10.}$

For LINE-1 methylation, the association with levels of exposures was confirmed in the adjusted model. For Alu, methylation, instead, only the association with PM_{10} was confirmed, while the association with zinc was not confirmed. In the adjusted model, Alu methylation showed a borderline significant association with lead exposure (Table 11).

Table 11. Association of effects of PM mass and PM metal component exposure with DNA methylation of Alu and LINE 1 in blood samples measured in sample 2.

	Alu			LINE-1		
Exposures	**β†**	**SE**	**p-Value**	**β†**	**SE**	**p-Value**
Unadjusted regression						
Aluminium	-0.05	0.05	0.26	-0.18	0.09	0.05
Manganese	-0.05	0.04	0.23	-0.08	0.07	0.28
Nickel	-0.10	0.2	0.62	-0.06	0.38	0.87
Zinc	-0.19	0.09	0.04	-0.11	0.18	0.56
Arsenic	-0.08	0.2	0.71	0.16	0.38	0.68
Lead	-0.06	0.04	0.13	-0.09	0.08	0.24
Iron	-0.12	0.19	0.52	0.18	0.35	0.61
PM_{10}	-0.18	0.09	0.05	-0.31	0.17	0.07
PM_1	-0.17	0.12	0.19	-0.3	0.23	0.19
Multivariable regression*						
Aluminium	-0.05	0.05	0.25	-0.19	0.09	0.03
Manganese	-0.06	0.04	0.12	-0.1	0.07	0.19
Nickel	-0.07	0.19	0.71	-0.11	0.38	0.78
Zinc	-0.16	0.09	0.08	-0.11	0.18	0.54
Arsenic	-0.04	0.19	0.85	0.13	0.38	0.73
Lead	-0.07	0.04	0.07	-0.11	0.08	0.16
Iron	-0.08	0.18	0.65	0.15	0.35	0.67
PM_{10}	-0.19	0.09	0.03	-0.35	0.17	0.04
PM_1	-0.19	0.12	0.11	-0.36	0.24	0.13

*Multivariable mixed effects models (adjusted for age, BMI, smoking, number of cigarettes/day, % of granulocytes) ; †β for an increment equal to the difference between the 90th and 10th percentile of pollutant concentration**.**

DISCUSSION

Discussion for histone modifications:

The present study, based on a healthy worker population from a steel plant near Brescia, Italy, showed that exposure to some metal components of PM was associated with increased activating histone modifications measured in blood leukocyte samples. In particular, we found that both H3K4 dimethylation and H3K9 acetylation were higher in individuals with more years of employment in the plant and higher estimated cumulative exposures to arsenic and nickel.

To the best of our knowledge this is the first study showing that metal exposures, such as nickel, arsenic and iron, affect histone modifications in human subjects. Our study was based on measures of histone modifications in blood leukocyte samples from healthy subjects, thus suggesting that exposure-related alterations of histone modifications may occur in normal tissues and possibly anticipate the onset of disease. The effects of metal exposures on the gene-activating mark H3K4 dimethylation that we observed in blood leukocytes is consistent with previous in-vitro toxicology studies that showed that carcinogenic metals increased H3K4 dimethylation in A549 human lung carcinoma cells (32,36).

In particular, nickel and arsenic, which were most consistently associated with increased H3K4 dimethylation in our analyses, were also previously found to increase H3K4 dimethylation in vitro (32). In our study, we found that H3K4 dimethylation was correlated with the levels of exposure to nickel, arsenic, and iron, but only nickel and arsenic showed significant associations with H3K4

dimethylation when we evaluated cumulative exposures. Also, we found significant associations of cumulative exposures to nickel and arsenic with increased H3K9 acetylation.

The changes observed in previous in-vitro studies on lung carcinoma cells (32,36) and in our study in blood leukocyte samples suggest that induction of activating histone modifications such as H3K4 dimethylation and H3K9 acetylation might be a systemic process detectable across different tissues. However, whether H3K4 dimethylation and H3K9 acetylation are induced in human lung tissues exposed in vivo to carcinogenic metals needs to be confirmed in future investigations. Metal components of inhaled particles have been shown to induce oxidative stress and inflammatory processes, which are known to affect histone modifications (73,74) and might specifically impact on blood leukocyte measures. A series of experimental studies using ambient PM_{10} collected in the Utah valley near a local steel plant demonstrated that the lung dose of bioavailable transition metals, and not just instilled PM mass, determined acute inflammatory responses, as well as that PM oxidant generation ability was enhanced in PM with higher metal content (75-77). Previous studies have shown that exposures to PM or to related airborne pollutants produce in blood leukocytes changes in gene expression (45,46,50,51), as well as in mechanisms of gene expression control such as DNA methylation (78-81).

We investigated a population with well-characterized exposure that allowed for contrasting subjects over a wide range of different exposure levels. We controlled several potential confounders by fitting multivariable models that included several individual characteristics as independent variables. However, we cannot exclude that other unmeasured exposures that are present in foundry facilities, such as heat, carbon monoxide, and non-ionizing radiations (82, 83), might have influenced H3K4 dimethylation and H3K9 acetylation. In univariate analyses, we found that H3K9 acetylation was positively associated with the education level of the study subjects. Whereas our study did not provide information to evaluate the biological basis for this association, our results showed that adjusted and unadjusted estimates for metal effects on histone modifications were remarkably similar, thus excluding confounding from education, as well as from the other variables that we included in the models.

Discussion for DNA methylation

In the present study on foundry workers with well-characterized measures of exposure to a wide range of particulate matter concentrations, we observed no short-term changes for methylation in *Alu* and *LINE-1* repeated elements but they were negatively associated with individual PM_{10} exposure, potentially reflecting effects operating over a longer timeframe.

Several epidemiologic investigations have demonstrated that exposure to ambient PM is associated with increased risk of cardiovascular disease in the general population (84).

Lower levels of genome-wide methylation measured in blood DNA have been found in subjects with cardiovascular disease (85). Recent indirect evidence suggested that decreases in genome-wide DNA methylation may mediate the effects of PM (86), possibly through the effects of their metal components.(56) In the present work, we demonstrated for the first time a direct correlation between PM exposure and lower genome-wide blood DNA methylation. Our findings indicate that decreases in global DNA methylation may be a common mechanism of PM effects, regardless of the source of exposure. Metal PM components have not only been shown to cause genetic damage via oxidative mechanisms, but can also induce silencing or reactivation of gene expression through DNA methylation (50). In our study, the association between PM_{10} level and methylation in *Alu* and *LINE-1* was significant only when the two measurements of methylation taken before and after three consecutive work days were both included in repeated-measure models. In such models, we assumed that PM_{10} exposure levels reflected the usual exposure levels of the study subjects, as suggested by both the high correlation between PM_{10} measures taken over the year before the study and the standard routine of the foundry workers in this plant. The use in repeated-measure models of both methylation measures taken at the beginning and at the end of the work week provided our statistical analysis with added power to detect the PM_{10} effects. At the opposite, we found no evidence of PM-induced short-term changes in *Alu* and *LINE-1* methylation. These results suggest that PM_{10} operates on genome-wide methylation over a longer timeframe, possibly causing a persistent suppression of methylation levels which were not reset to baseline over the two

days off between consecutive work weeks in the work schedule of our study subjects.

Our study was based on quantitative analysis of DNA methylation using pyrosequencing which is highly reproducible and accurate at measuring small changes in DNA methylation. DNA methylation analysis measured multiple individual CpG dinucleotides for each marker, and was repeated twice on each sample to minimize the assay variability. We used mixed-models to fully represent the structure of the data and take advantage of the multiple measurements, while also adjusting for potential confounders.

OBSERVATIONS

Because of the limited number of study subjects, it is possible that the associations observed were due to chance. However, the occupational exposure and relatively controlled environment of a foundry provide a good setting for evaluating these mechanistic questions and limit bias and chance findings. Our study was based on subjects working in several work areas of the same factory and did not include a different population of subjects without a specific condition of exposure to inhaled pollutants. Limiting our investigation to individuals who have all been working in the same work facility avoided potential concerns related to the selection of external referents who might have differed from the exposed population in terms of socioeconomic factors and other characteristics determining hiring into the plant (87). Nonetheless, the differences in the personal levels of exposure in our study group were large, providing sufficient contrast for identifying exposure-related changes in histone modifications. For example, the lowest level of PM_{10} observed in our study population (73.72µg/m^3) was only marginally higher than ambient PM_{10} levels measured in the geographic area in which the plant is located [average annual ambient PM_{10} levels between 41 and 57 µg/m^3 were recorded in the year of the study by different ambient monitoring stations in Brescia area] (88), whereas the highest level was 1220.17 µg/m^3. The subjects with the highest exposures to arsenic and nickel had exposure levels that were 36 and 31 times respectively higher than those for the lowest-exposed subjects. It is worth noting that, although our study was based on a group of steel workers with higher average exposures than the general

population, the levels of exposure to metals in our study were all lower than the commonly accepted threshold limits for industrial settings (89).

Because differences in exposures within our study population were determined by the different tasks routinely performed by each of the study subjects, the personal exposure levels we measured in the week of the study reflected the usual exposure of the study subjects. This was confirmed by the high correlation (r^2>0.90) between exposure measures repeated over three years in a subset of the study population. Employment records showed that all the subjects included in the present study performed the same tasks for all the years they had been employed in the plant, suggesting that contrasts of exposure within the study population might have remained stable over time. Therefore, we estimated long-term cumulative exposure as the product of the levels of exposure by years of employment in the plant. However, because most of the study subjects had worked for more than a decade in the plant, it is possible that changes in production or exposure protection regulations might have occurred over such an extended period of time. Therefore, we recognize that the estimated cumulative exposures we used in the present study is prone to exposure misclassification and the results based on these metrics of exposure should be interpreted with caution.

We investigated a well-characterized population with a wide range of PM_{10} exposure that allowed for contrasting subjects over a wide range of different exposure levels. In addition, to PM, foundry workers have additional exposures, including heat, , polycyclic aromatic hydrocarbons, (90,91) carbon monoxide

(83,92) non-ionizing radiations (82). Thus, we cannot exclude that co-exposures might have contributed to the observed effects.

CONCLUSIONS

Our results indicate that exposure to some metal components of PM, including nickel and arsenic , increased H3K4 dimethylation and H3K9 acetylation in blood leukocytes from a population of healthy steel workers. These changes in the genomic levels of histone modifications may produce aberrant activation of gene expression which may help explain the carcinogenic properties of inhalable nickel and arsenic.

We showed also, for the first time, that PM exposure is associated with altered blood DNA methylation patterns that reproduce epigenetic changes found in cardiovascular disease, including decreased methylation in *Alu* and *LINE-1* repetitive elements.

Further studies are required to directly link these changes with exposure-related increases in the risk of cancer, as well as to identify specific genes and pathways that are affected by the exposure-related changes in histone modifications and DNA methylation.

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