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Oxidative damage, genetic and epigenetic alterations in hexavalent chromium exposed workers - A cross-sectional study within the SafeChrom project

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ABSTRACT

Background: Hexavalent chromium (Cr(VI)) is a lung cancer carcinogen. However, the genotoxic and mutagenic effects of Cr(VI) in humans at low-to-moderate occupational exposure levels are unknown. This study aims to investigate the relationship between occupational exposure to Cr(VI) and the presence of oxidative damage, genetic and epigenetic alterations.

Methods: We included 113 Cr(VI) exposed workers in 14 companies and 72 controls recruited within the SafeChrom project. Cr(VI) was measured in inhalable dust and total chromium in urine (U-Cr) and red blood cells (RBC-Cr). Analysed effect biomarkers included urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG), micronuclei in peripheral blood reticulocytes (MNRET), blood relative mitochondrial DNA copy number (mtDNA-cn), relative telomere length (TL), and blood DNA methylation of four lung cancer-related genes (*F2RL3*, *LINE-1*, *MGMT* promoter and *SEMA4B*).

Results: The median inhalable Cr(VI) concentration among the exposed workers was 0.11 μg/m^3 (5th-95th percentile: 0.02–8.44). Exposed workers showed higher 8-OHdG, TL, and *MGMT* promoter methylation levels and lower mtDNA-cn and MNRET compared with controls. Company-based differences in biomarkers were observed. Univariate analysis showed that TL was positively correlated with U-Cr, and 8-OHdG and *MGMT* promoter methylation were positively correlated with RBC-Cr. Multivariate analyses with adjustment for possible confounders showed higher 8-OHdG, TL, and *MGMT* promoter methylation in exposed workers compared with controls.

Conclusions: Low-to-moderate Cr(VI) exposure was associated with higher oxidative stress, longer telomeres and epigenetic alterations, changes that previously have been linked to lung cancer risk. This study highlights the molecular impacts of Cr(VI) exposure, underscoring the importance of reducing the exposure to Cr(VI).

Abbreviations

8-OHdG	8-hydroxy-2′-deoxyguanosine
8-oxodG	8-oxo-7,8-dihydro-2′-deoxyguanosine

(*continued on next column*)

(*continued*)

BMI	Body mass index
Cd	Cadmium
COPD	Chronic obstructive pulmonary disease

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(*continued*)

CpG	Cytosine-phosphate-guanine
Cr(III)	Trivalent chromium
Cr(VI)	Hexavalent chromium
Cys	Cysteine
F2RL3	F2R like thrombin or trypsin receptor 3
GSH	Glutathione
HBB	Hemoglobin beta
IARC	International Agency for Research on Cancer
ICP-MS	Inductively coupled plasma mass spectrometry
LINE-1	Long interspersed nuclear element-1
LOD	Limit of detection
MGMT	O-6-methylguanine-DNA methyltransferase promoter
MN	Micronuclei
MNRET	Micronuclei in peripheral blood reticulocytes
mtDNA	Mitochondrial DNA
mtDNA-cn	Mitochondrial DNA copy number
P5	5th percentiles
P95	95th percentiles
RBC	Red blood cells
RBC-Cr	Chromium concentration in red blood cells
ROS	Reactive oxygen species
r_S	Spearman's rank correlation coefficients
SEMA4B	Semaphorin 4B
TL	Telomere length
U-Cr	Chromium concentration in urine adjusted by density

1. Introduction

Hexavalent chromium (Cr(VI)) and its compounds are widely used in industrial applications, including agricultural fertilizers, ammunition, cement, chromates, electroplating, leather, metallurgy, pigments, and weapons because they provide superior hardenability, durability, and corrosion resistance compared with other metals (Alvarez et al., 2021). Environmental exposure to Cr(VI) can occur through tobacco smoke and inhalation of polluted air or ingestion of contaminated water, particularly for individuals living in industrial-contaminated areas (Tumolo et al., 2020; Williams et al., 2017). Occupational exposure to Cr(VI) may occur from several occupational activities, including chromate production, Cr(VI) electroplating, steel passivation, welding and grinding in stainless steel (Tavares et al., 2022b). The main route of occupational exposure to Cr-containing compounds is through the inhalation of dust, mists or fumes (Tavares et al., 2022a). Respirable particles can penetrate the non-ciliated regions of the lungs, and depending on their water solubility, Cr(VI) may be released and absorbed into the bloodstream up to 72 h after exposure, with maximum uptake occurring after 6 h (Kozłowska et al., 2022). Dermal contact and ingestion due to hand-to-mouth activity are also relevant when the skin is not adequately protected and exposed to liquid forms of Cr(VI) (NIOSH, 2013). Strong evidence linking lung and other respiratory tract cancers with occupational exposure to Cr(VI) has led to the classification of Cr(VI) compounds as group 1 carcinogens by the International Agency for Research on Cancer (IARC) (IARC, 2012). However, as a non-threshold carcinogen, Cr(VI) poses uncertainties regarding the risks of developing mutagenic and genotoxic effects at low-to-moderate exposure levels.

The precise mechanisms behind Cr(VI) carcinogenicity are not fully understood, but several mechanisms have been suggested including oxidative stress, DNA damage, genomic instability, inflammation, and epigenetic modulation (Zhang et al., 2021). Soluble Cr(VI) primarily exists as the chromate oxyanion (CrO_4^{2-}), which structurally resembles sulfate oxyanions, allowing it to enter cells via sulfate transporters on the cell surface (Salnikow and Zhitkovich, 2008). Once inside the cell, Cr(VI) is metabolically reduced to trivalent chromium (Cr(III)) through the action of enzymatic and non-enzymatic antioxidants, primarily ascorbate and biological thiols such as glutathione (GSH) and cysteine (Cys) (O'Brien et al., 2003). Ascorbate transfers two electrons to Cr(VI) yielding Cr(IV) as the sole intermediate, while GSH and Cys reduce Cr(VI) through sequential one-electron transfers, producing Cr(V) and Cr(IV) intermediates (Krawic and Zhitkovich, 2023). The intermediates can deplete cellular antioxidants and generate reactive oxygen species (ROS) during their re-oxidation processes (Yao et al., 2008). Excessive production of ROS can lead to lipid peroxidation, DNA damage, and subsequent cellular injury causing cell death through apoptosis or necrosis (Sun et al., 2015). Moreover, intermediate Cr species can form DNA-protein adducts, such as DNA-amino acid cross-links, which have the potential to induce DNA single- and double-strand breaks (Stearns et al., 1995). Intracellular Cr(III) can form DNA adducts as well, including binary adducts (formed by DNA binding of Cr(III)) and ternary adducts (Cr(III)-ligand complexes) (Krawic and Zhitkovich, 2023). These DNA adducts may cause DNA mutations and chromosome damage (Zhitkovich, 2005).

8-hydroxy-2′-deoxyguanosine (8-OHdG) or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) is the product of cellular repair of oxidatively damaged DNA, and urinary 8-OHdG has been suggested as a biomarker of ROS (Roszkowski et al., 2011). Associations have been observed between 8-OHdG and lung cancer risk (Loft et al., 2012). Micronuclei (MN) are fragments of extranuclear chromatin that form when chromosome segments or lagging chromosomes fail to integrate into daughter nuclei during cell division (Dertinger et al., 2007). They are associated with chromosome instability, genomic rearrangements, and mutagenesis (Krupina et al., 2021). MN in peripheral blood reticulocytes (MNRET) is used to study chemicals with potential genotoxic damage (Tavares et al., 2022a). Mitochondria are essential for energy production and play a crucial role in regulating redox-dependent pathways, influencing cell fate and homeostasis by controlling apoptosis, differentiation and proliferation (Alur et al., 2024). Mitochondrial DNA (mtDNA) copy number (mtDNA-cn) reflects mitochondrial function and overall health (Cai et al., 2024). Studies showed that mtDNA-cn is inversely associated with lung cancer risk (Kim et al., 2014). Telomeres are highly specialized chromatin structures consisting of tandem repeats of the TTAGGG sequence located at the ends of linear chromosomes (Palm and de Lange, 2008). These functional non-coding sequences, aided by shelterin proteins, help maintain chromosome stability and safeguard them against degradation and damage (Shay and Wright, 2019). Telomere length (TL) decreases with age, and progressive shortening of telomeres triggers senescence and apoptosis in somatic cells, impacting an individual's health and lifespan (Shammas, 2011). While shorter telomeres are recognized as markers of poor health and advanced biological age, longer telomeres, associated with increased cell growth potential, may promote the accumulation and expression of cancer-initiating somatic mutations (Tsatsakis et al., 2023).

Epigenetic DNA methylation is the process of adding a covalent methyl group to the 5th carbon of cytosine, forming 5-methylcytosine in cytosine-phosphate-guanine (CpG) sequences (Moore et al., 2013). The cancer genome is often marked by a global reduction in 5-methylcytosine level, accompanied by the hypermethylation of specific genes, including the promoters of tumor suppressor and homeobox genes (Ehrlich, 2009; Watanabe and Maekawa, 2010). Abnormalities in the DNA methylation process have emerged as promising biomarkers for the early detection and prognosis of cancer (Ibrahim et al., 2023). Hypomethylation of F2R like thrombin or trypsin receptor 3 (*F2RL3*) has been identified as a biomarker of smoking exposure and is a powerful predictor of lung cancer risk and mortality (Zhang et al., 2015). Long interspersed nuclear element-1 (*LINE-1*) hypomethylation was found to be associated with cadmium (Cd) and benzene exposure (Bollati et al., 2007; Virani et al., 2016). O-6-methylguanine-DNA methyltransferase is a DNA repair gene and hypermethylation of its gene promoter (*MGMT*) is associated with lung cancer (Yang and Li, 2016). DNA methylation levels of Semaphorin 4B (*SEMA4B*) have been found to be associated with Cr(VI) exposure in electroplating workers (Feng et al., 2020).

As a non-threshold carcinogen, Cr(VI) raises concerns about the potential for oxidative damage, DNA damage, and epigenetic modifications even at low-to-moderate exposure levels. To this end, we analysed effect biomarkers reflecting oxidative stress, genetic and

epigenetic alterations (urinary 8-OHdG concentrations, blood MNRET, mtDNA-cn, TL, and DNA methylation of *F2RL3*, *LINE-1*, *MGMT* and *SEMA4B)* in relation to low to moderate occupational Cr(VI) exposure.

2. Material and methods

2.1. Study population and sample collection

This study included 113 Cr(VI) exposed workers and 72 non-occupationally exposed controls from the SafeChrom project. The details of participant recruitment and sample collection were published elsewhere (Jiang et al., 2024b). In short, the exposed workers were recruited from 14 companies that were categorised into four groups: manufacture/processing of metal products, steel production, bath plating and non-categorised. In addition, work tasks were categorised into four groups: welding, process operation, machining, and others. The controls were recruited from construction and storage companies, an agricultural operator, a nursing home, and a restaurant without any known occupational exposure to Cr(VI). Participants should be non-smokers for more than 6 months. However, during the recruitment phase, a significant proportion of potentially exposed workers were current smokers. To ensure sufficient sample size while maintaining scientific rigor, we included current smokers in both the exposed and control groups.

Three venous blood samples were collected in vacutainer tubes (BD, Plymouth, UK) for all participants: two sodium-heparin tubes of which one was used for measuring Cr concentration in red blood cells (RBC-Cr), and one for measuring MNRET; and one EDTA tube for measuring TL, mtDNA-cn and DNA methylation. Post-shift urine samples (after at least 4 h of work) were collected to measure Cr and 8-OHdG concentrations. Blood and urine samples were kept at 4 °C and transported to the laboratory at the Div. of Occupational and Environmental Medicine. After arrival, blood and urine samples were stored at −20 °C until analysis. Sodium-heparin tubes for MNRET were transported to the Finnish Institute of Occupational Health and stored refrigerated until processing and analysis. All participants filled in a questionnaire including questions about birth year, sex, height, weight, diet (mix/vegetarian/vegan), current tobacco smoking (yes/no), consumption of alcoholic beverages (yes/no), cancer history, and infection history during the last two weeks (cancer and infection may impact on level of effect biomarkers (Barturen et al., 2022; García-Guede et al., 2020)). Each participant gave informed written consent to participate in the study. The study was approved by the Swedish Ethical Review Authority (Dnr, 2021-00641).

2.2. Assessment of Cr(VI) exposure

Personal air sampling was performed in exposed workers and filter samples of the inhalable Cr(VI) fraction were analysed by ion chromatography with conductivity detection, or by inductively coupled plasma mass spectrometry (ICP-MS). Biomonitoring for Cr(VI) exposure was carried out by measuring Cr concentration with ICP-MS in post-shift urine (U-Cr) and red blood cells (RBC) for all participants. In addition, manganese, cobalt, nickel, copper, zinc, selenium, Cd, antimony, mercury, and lead were measured in RBC along with Cr. The details of the analysis of inhalable Cr(VI), U-Cr and RBC-Cr were published elsewhere (Jiang et al., 2024b).

The measurement of urinary creatinine and density was published elsewhere (Jiang et al., 2024b). Briefly, density and creatinine were measured in all urine samples for correction of dilution. The density was measured with a hand-held refractometer (30PX; Mettler Toledo, USA). Creatinine was measured with Atellica (Siemens Healthcare Diagnostics, Munich, Germany). Density-adjusted U-Cr was present in this study.

2.3. 8-OHdG concentration in urine

The urine samples were analysed for 8-OHdG using liquid chromatography-tandem MS (LC-MS/MS; QTRAP 5500, AB Sciex, Framingham, MA, USA). A full description of the method is provided in the supplementary text. In brief, 0.2 mL of urine sample was prepared in 96-well plates, ammonium acetate (pH 6.5) and β-glucuronidase (*Escherichia coli*) were added and incubated for 30 min at 37 °C for enzymatic deconjugation. Isotopically labelled internal standard ($^{15}N_5$-8-OHdG) was added and the sample plates were centrifuged for 10 min at 3000 g prior to analysis. Each analysed sample batch included a matrix-matched standard curve, six blanks, and nine quality control samples at three concentrations. The limit of detection (LOD) was calculated as the mean value of all blanks plus 3 × the standard deviation of the blanks and is reported as 0.02 ng/mL. No sample was below the LOD. The between-run precision determined by analyses of QC samples ranged from 9 to 12 %. The between-batch precision (or reproducibility) was determined by comparing duplicate analyses of 39 % of the samples and was 11 %.

2.4. MNRET

The measurement of MNRET was described previously (Andersen et al., 2021; Tavares et al., 2022a). Briefly, the whole blood samples were stored at 4 °C and processed within seven days after collection. Transferrin-positive (+CD71) reticulocytes were isolated by immunomagnetic separation according to the instructions of the CELLection Pan Mouse IgG Kit (Thermo Fisher Scientific, Waltham, USA) using a FITC Mouse Anti-human CD71 antibody (BD Biosciences, San Jose, USA). Thereafter, samples were fixed in 2 % paraformaldehyde in PBS with 10 μg/mL of sodium dodecyl sulfate (Sigma-Aldrich, Darmstadt, Germany) and kept at 4 °C until analysis. Before the analysis, DNA was stained with Hoechst 33342 (Thermo Fisher Scientific). The samples were analysed using blue (488 nm) laser for the identification of +CD71 reticulocytes and near UV (375 nm) laser for the detection of DNA-containing MN. A CytoFlex S flow cytometer and CytExpert software version 2.3 (Beckman Coulter, Brea, USA) were used for data acquisition and analysis. The MN frequency was quantified as permille of micronucleated + CD71 reticulocytes from all analysed + CD71 reticulocytes.

Due to 6 missing blood samples, a total of 179 samples were available for MNRET analysis. Given the constraints of the sampling schedule and the maximum number of samples that could be processed in a single analytical run, the analysis was conducted in three separate batches. The first batch included 70 exposed workers and 15 controls, the second batch comprised 37 exposed workers and 32 controls, and the final batch consisted of 25 controls. Additionally, four samples contained an insufficient number of cells for analysis, resulting in a final dataset of 105 exposed workers and 70 controls.

2.5. DNA extraction, mtDNA-cn and TL

Genomic DNA was isolated from 200 μL whole blood sample using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) was used to determine DNA quantity and purity (median (25th, 75th percentiles) 260/280 ratio: 1.81 (1.74, 1.88)). DNA samples were diluted to a concentration of 25 ng/μL.

The methods of mtDNA-cn and TL measurement were described previously (Xu et al., 2018). Briefly, the mtDNA-cn and TL were measured by SYBR Green-based quantitative PCR in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, USA). DNA Samples were diluted to a concentration of 5 ng/μL. The master mix for mtDNA-cn was prepared with PerfeCTa SYBR Green FastMix with ROX (Quantabio, Beverly, U.S.A.) and 0.20 μM mtDNA primers. Master mix for TL was prepared with 0.45 μM telomere primers, Platinum Taq DNA Polymerase, dNTPs mix, SybrGreen, and ROX Reference Dye (reagents

were all purchased from Thermo Fisher Scientific). A single-copy gene (hemoglobin beta (*HBB*)) was used as a reference to determine the copies per cell of the mtDNA and telomere. The master mix for *HBB* was prepared with Fast SYBR Green Master Mix (Thermo Fisher Scientific) and 0.20 μM *HBB* primers. The corresponding primers and qPCR protocol for mtDNA, telomere and *HBB* are listed in Supplementary Tables 2 and 3

One reference DNA sample was diluted serially twofold per dilution to produce six concentrations of 0.5–16 ng/μL for the standard curve. A control sample was included in each run to monitor the variance between runs. The standard curve, samples (with 2.5 μL DNA diluted to 5 ng/μL), control sample and one blank were run in triplicates. SDS 2.4.1 software (Thermo Fisher Scientific) calculated the relative quantity of mtDNA, TL and *HBB* for each reaction based on the standard curve. Then, the relative quantity of mtDNA was divided by the quantity of *HBB* to calculate the mtDNA/*HBB* ratio (mtDNA-cn). Likewise, the TL was the quotient of the quantity of telomere and *HBB*. Both mtDNA-cn and TL are therefore arbitrary values. The coefficient of variation (CV) of the control sample based on two runs was 0.62 % for mtDNA, 1.45 % for telomere and 1.72 % for *HBB*.

2.6. DNA methylation

Bisulfite conversion of 20 μL DNA (25 ng/μL) was performed with EZ DNA Methylation-Gold kit (Zymo Research, Irvine, USA) according to the manufacturer's protocol, and the bisulfite-converted DNA was eluted in 20 μL elution buffer for each sample. The PCR amplifications were performed on a T100 Thermal Cycler (Bio-Rad, Hercules, USA) with PyroMark PCR Kit (Qiagen) and PCR primers. The corresponding primers are listed in Supplementary Table 4. Gradient PCR and agarose gel electrophoresis were performed to find the appropriate annealing temperature for each gene (for *F2RL3* was 57 °C; *LINE-1* 53 °C; *MGMT* 57 °C; and *SEMA4B* 58 °C). The PCR protocol is provided in Supplementary Table 5. Pyrosequencing was analysed on the PyroMark Q48 Autoprep Instrument (Qiagen) with PyroMark Q48 Advanced CpG reagents (Qiagen) and sequencing primers, according to the manufacturer's protocol. Sequencing primers used for pyrosequencing are provided in Supplementary Table 4. Four controls were included in each run, one bisulfite-converted methylated DNA, one bisulfite-converted unmethylated DNA (EpiTect PCR Control DNA Set, Qiagen), one pooled DNA sample and one blank control consisting of RNase-free water. All PCR products and controls were prepared in duplicate. The results of pyrosequencing were recorded in PyroMark Q48 Autoprep software for further analysis.

2.7. Statistical analysis

Age was calculated based on recruitment date and birth. Body mass index (BMI) was obtained using the formula BMI = weight in kilograms/(height in meters)^2 according to self-reported data. Descriptive statistics including median, 5th and 95th percentiles (P5, P95) were calculated. Mann-Whitney *U* test was used to compare differences between continuous variables and Pearson Chi-square test was used to compare differences in the distribution of categorical variables. Spearman's correlation was used to examine correlations between the effect biomarkers and markers of Cr(VI) exposure. Multivariate linear regression models were constructed to assess differences in effect biomarkers between exposed workers and controls, as well as to examine associations between effect biomarkers and RBC-Cr concentrations. The models were adjusted for potential covariates and confounders, including age, sex, BMI, diet, smoking, alcohol consumption, leisure activity with risk of Cr exposure, and metals with significantly different concentrations in RBC between the two groups (antimony, cobalt, copper, and zinc). To deal with skewed data, natural logarithm transformation was used for all effect biomarkers. Due to the presence of samples with 0 % methylation, a log transformation was applied using formula Y = ln(X+1) for the methylation level of *MGMT* CpG1 and CpG2 (Booeshaghi and Pachter, 2021).

The statistical analyses above were conducted with SPSS 28.0 (IBM Corp., Armonk, NY, USA) and statistical significance (two-tailed) was denoted at P value < 0.05.

3. Results

3.1. Characteristics of the study participants

The demographic and lifestyle characteristics of the exposed workers and controls are, along with the inhalable Cr(VI) in exposed workers, U-Cr and RBC-Cr in all participants, summarised in Table 1. Exposed workers were significantly younger, more likely to be male and more likely to drink alcohol than controls ($P < 0.05$). No significant differences were found between exposed workers and controls for BMI, current smoking, leisure activity with Cr exposure, self-reported cancer history, or infection during the last two weeks. The U-Cr and RBC-Cr concentrations were significantly higher in the exposed workers than in controls. The median (P5, P95) inhalable Cr(VI) concentration among the exposed workers was 0.11 (0.02, 8.44) μg/m^3. Since the inhalable Cr (VI) levels observed in this study were generally (93 %) below the Swedish OEL of 5 μg/m^3, and the median urinary and RBC Cr concentrations were considerably lower than those reported in the HBM4EU study (0.77 μg/g creatinine for urine and 4.34 μg/L for RBC, among chrome platers) and Danish SAM-Krom study (2.42 μg/L for urine and 0.89 μg/L for RBCs), we considered the workers in the present study to be exposed to low-to-moderate levels of Cr(VI).

3.2. The results of 8-OHdG, genetic biomarkers and DNA methylation levels

The levels of the effect biomarkers 8-OHdG, mtDNA-cn, TL, MNRET and DNA methylation of *F2RL3*, *LINE-1*, *MGMT* and *SEMA4B* were compared between Cr(VI) exposed workers and controls (Table 2). Since creatinine excretion, due to gender differences in muscle mass, is often higher in men (Thomas et al., 2012), and slightly skewed gender distribution between exposed workers and controls, density adjustment

Table 1
Basic characteristics and exposure markers of hexavalent chromium in exposed workers and controls.

	Exposed workers (n = 113)	Controls (n = 72)	*P*
Age, median (P5, P95)	39 (22, 60)	44 (28, 60)	0.02[a]
Male, n (%)	98 (86.7)	50 (69.4)	0.004[b]
BMI, median (P5, P95)	27.8 (20.0, 37.6)	26.8 (20.6, 35.1)	0.37[a]
Diet (mix, vegetarian, vegan), n (%)	113/0/0 (100/0/0)	70/1/1 (97.2/ 1.4/1.4)	0.205[c]
Current smokers, n (%)	10 (8.8)	4 (5.6)	0.41[b]
Alcohol drinkers, n (%)	103 (91.2)	56 (77.8)	0.01[b]
Leisure activity with Cr, n (%)	10 (8.8)	4 (5.6)	0.41[b]
Cancer[d], n (%)	0 (0)	1 (1.4)	0.39[c]
Infection in two weeks, n (%)	13 (11.5)	6 (8.3)	0.49[b]
Inhalable Cr(VI)[e], median (P5, P95)	0.11 (0.02, 8.44)		
U-Cr[f], median (P5, P95)	0.60 (0.10, 3.20)	0.10 (0.06, 0.56)	<0.001[a]
RBC-Cr[g], median (P5, P95)	0.73 (0.51 2.33)	0.53 (0.42, 0.72)	<0.001[a]

[a] Mann-Whitney *U* test.
[b] Pearson Chi-Square test.
[c] Fisher's Exact Test.
[d] Self-reported cancer by questionnaire.
[e] Inhalable Cr(VI), concentrations of inhalable hexavalent chromium in air samples (only performed in exposed workers) (μg/m^3).
[f] U-Cr, post-work urinary chromium concentration (μg/L) adjusted by density.
[g] RBC-Cr, chromium concentration in red blood cells (μg/L).

Table 2
Median levels of biomarkers of effect in Cr(VI) exposed workers and controls; data presented as median (P5, P95).

	Exposed workers (n = 113)	Controls (n = 72)	Mann-Whitney *U* test
8-OHdG[a]	9.42 (3.55, 20.09)	7.77 (3.02, 19.35)	0.02
mtDNA-cn[b]	1.56 (0.97, 2.30)	1.68 (0.97, 2.48)	0.03
TL[c]	0.80 (0.51, 1.43)	0.70 (0.41, 1.09)	<0.001
MNRET[d]	1.98 (0.62, 6.04)	2.49 (0.95, 8.26)	0.01
% *F2RL3* (average)	84.42 (75.03, 87.80)	84.33 (70.29, 88.01)	0.91
% *F2RL3* (CpG1)	83.86 (73.56, 87.42)	83.54 (68.25, 87.96)	0.80
% *F2RL3* (CpG2)	84.69 (75.74, 88.93)	84.60 (72.61, 88.54)	0.83
% *LINE-1* (average)	63.08 (60.91, 65.11)	62.97 (60.40, 65.10)	0.86
% *LINE-1* (CpG1)	77.12 (74.94, 79.65)	77.31 (74.06, 79.61)	0.42
% *LINE-1* (CpG2)	54.54 (52.37, 57.02)	54.43 (52.48, 57.03)	0.43
% *LINE-1* (CpG3)	57.66 (54.18, 59.94)	57.39 (53.97, 60.37)	0.59
% *MGMT* (average)	1.91 (1.48, 3.57)	1.78 (1.34, 2.67)	0.04
% *MGMT* (CpG1)	1.21 (0.47, 3.05)	1.14 (0.00, 2.41)	0.08
% *MGMT* (CpG2)	1.03 (0.00, 2.38)	1.00 (0.00, 1.75)	0.40
% *MGMT* (CpG3)	2.32 (1.71, 4.14)	2.21 (1.56, 3.36)	0.11
% *MGMT* (CpG4)	3.13 (2.33, 4.64)	2.91 (2.37, 4.18)	0.10
% *SEMA4B*	73.83 (36.03, 78.31)	72.79 (35.81, 77.40)	0.08

[a] 8-hydroxy-2′-deoxyguanosine concentration in urine, density adjusted.
[b] Relative mitochondrial DNA copy number.
[c] Relative telomere length.
[d] Micronuclei in peripheral blood reticulocytes (‰). Due to 6 missing samples and 4 samples having too few cells, the result was for 105 exposed workers and 70 controls.

was considered more appropriate for the correction of urinary dilution, and accordingly, density-adjusted urinary 8-OHdG was presented in this study. Nevertheless, we also provide the un-adjusted urinary 8-OHdG and creatinine-adjusted urinary 8-OHdG in Supplementary Table 6 for comparison with other studies. Exposed workers had significantly higher 8-OHdG, longer TL and higher average methylation level of *MGMT* compared with controls. MNRET and mtDNA-cn were significantly lower in the exposed group compared with controls. Sensitivity analyses were conducted by excluding females, non-alcohol drinkers, and individuals with infection within the last two weeks. Excluding non-alcohol drinkers did not change effect estimates or *P*-values for associations with 8-OHdG, MNRET, mtDNA-cn, TL or *MGMT* methylation (Supplementary Table 7). However, excluding females and individuals with infection within the last two weeks, resulted in changes in effect estimates that became non-significant for the associations with mtDNA-cn. After excluding females, 8-OHdG became non-significant as well. In addition, sensitivity analyses were also conducted by matching the propensity score with age, sex, BMI, smoking, alcohol drinking, diet, and leisure activity with Cr (exposed worker vs. control = 1 vs. 1, match tolerance=0.2). After matching, 70 controls and 70 matched exposed workers were retained (Supplementary Table 8). Consistent with our primary findings, exposed workers had significantly higher levels of urinary 8-OHdG, longer TL, and lower MNRET compared to controls. However, differences in mtDNA-cn ($P = 0.06$) and MGMT promoter methylation ($P = 0.22$) were no longer statistically significant in the matched sample.

To investigate the dose-dependent effects of Cr(VI) exposure, we analysed multiple biomarkers across quartiles (Q) of inhalable Cr(VI), U-Cr, and RBC-Cr levels (Fig. 1). A significant increase in 8-OHdG levels was found in Q2 of RBC-Cr compared to Q1. MtDNA-cn was significantly higher in the highest quartile of U-Cr compared to Q1. TL exhibited an inconsistent pattern across exposure quartiles: a significant elevation was observed between Q3 and Q4 for inhalable Cr(VI), whereas decreasing trends were noted for both U-Cr and RBC-Cr. No significant associations were found between exposure quartiles and either MNRET or *MGMT* promoter methylation.

Analysing the biomarkers in relation to the type of company did not identify specific company types as being clearly associated with an altered level of effect markers. Significant differences were found for mtDNA-cn, MNRET, and average and CpG3 methylation of *MGMT* within different companies (Supplementary Table 9). Non-categorised companies had the highest mtDNA-cn and MNRET with the median (P5, P95) of 2.1 (1.5, 2.6) and 4.8 (1.9, 6.1), respectively. Manufacture/processing of metal products companies had the lowest mtDNA-cn (1.5 (0.8, 2.2)) and MNRET (1.5 (0.6, 4.9)). Steel production companies had the highest methylation level of *MGMT* (average) (2.1 (1.5, 3.9)) and bath plating companies had the highest methylation level of *MGMT* (CpG3) (2.5 (1.9, 4.7)). Non-categorised companies had the lowest methylation level of both *MGMT* average and CpG3 (1.6 (1.3, 2.2) and 2.0 (1.2, 2.3), respectively). In relation to work tasks, no significant difference was found for any effect biomarker (Supplementary Table 9).

3.3. Univariate analysis for markers of Cr(VI) exposure and effect biomarkers

Table 3 shows Spearman's rank correlation coefficients (r_S) between the effect biomarkers and age, inhalable Cr(VI), U-Cr, and RBC-Cr. Age was significantly negatively correlated with TL and *F2RL3* methylation in both the all participant group and the subgroup of exposed workers ($r_S = -0.16$ to -0.30). No significant correlation was found between inhalable Cr(VI) and any effect biomarker. In all participants, TL was significantly positively correlated with U-Cr; 8-OHdG was positively and MNRET was negatively correlated with RBC-Cr. However, those correlations became non-significant if only exposed workers were considered. *MGMT* methylation was significantly positively correlated with U-Cr and RBC-Cr in all participants and exposed workers only ($r_S = 0.17$ to 0.29). Furthermore, in exposed workers, the methylation of *LINE-1* (CpG2) was significantly negatively correlated with U-Cr and RBC-Cr.

The correlations between effect biomarkers and other metals measured in RBC were relatively weak and are shown in Supplementary Table 10. In summary, 8-OHdG showed significant negative correlations with cobalt ($r_S = -0.22$) and Cd (-0.22) and positive correlations with copper (0.24) and zinc (0.15). MtDNA-cn was negatively correlated with antimony (-0.22). TL had significant negative correlations with nickel, zinc, selenium, and antimony (-0.25 to -0.31). MNRET exhibited no significant correlations with metals in RBC. *F2RL3* methylation showed inverse associations with Cd and lead (-0.16 to -0.31). *LINE-1*, *MGMT* and *SEMA4B* methylation displayed a limited number and relatively weak (-0.19 to 0.18) but significant associations with metals in RBC.

3.4. Multivariate analysis for effect biomarkers

In a general linear regression model (Table 4), exposed workers had significantly higher 8-OHdG and TL, and lower MNRET (unadjusted model). When taking possible confounders and covariates into consideration (partly adjusted model adjusting for age, sex and alcohol drinking; and fully adjusted model with further adjustment for BMI, smoking, diet, and leisure activity with Cr), the effect estimates of TL MNRET did not differ much and remained statistically significant. However, effect estimates of 8-OHdG became non-significant. Furthermore, in partly and fully adjusted models, exposed workers showed significantly higher methylation level of *MGMT* compared with controls. Other metals were adjusted for in the linear regression models, as well (Supplementary Table 11). The exposed workers still had significantly

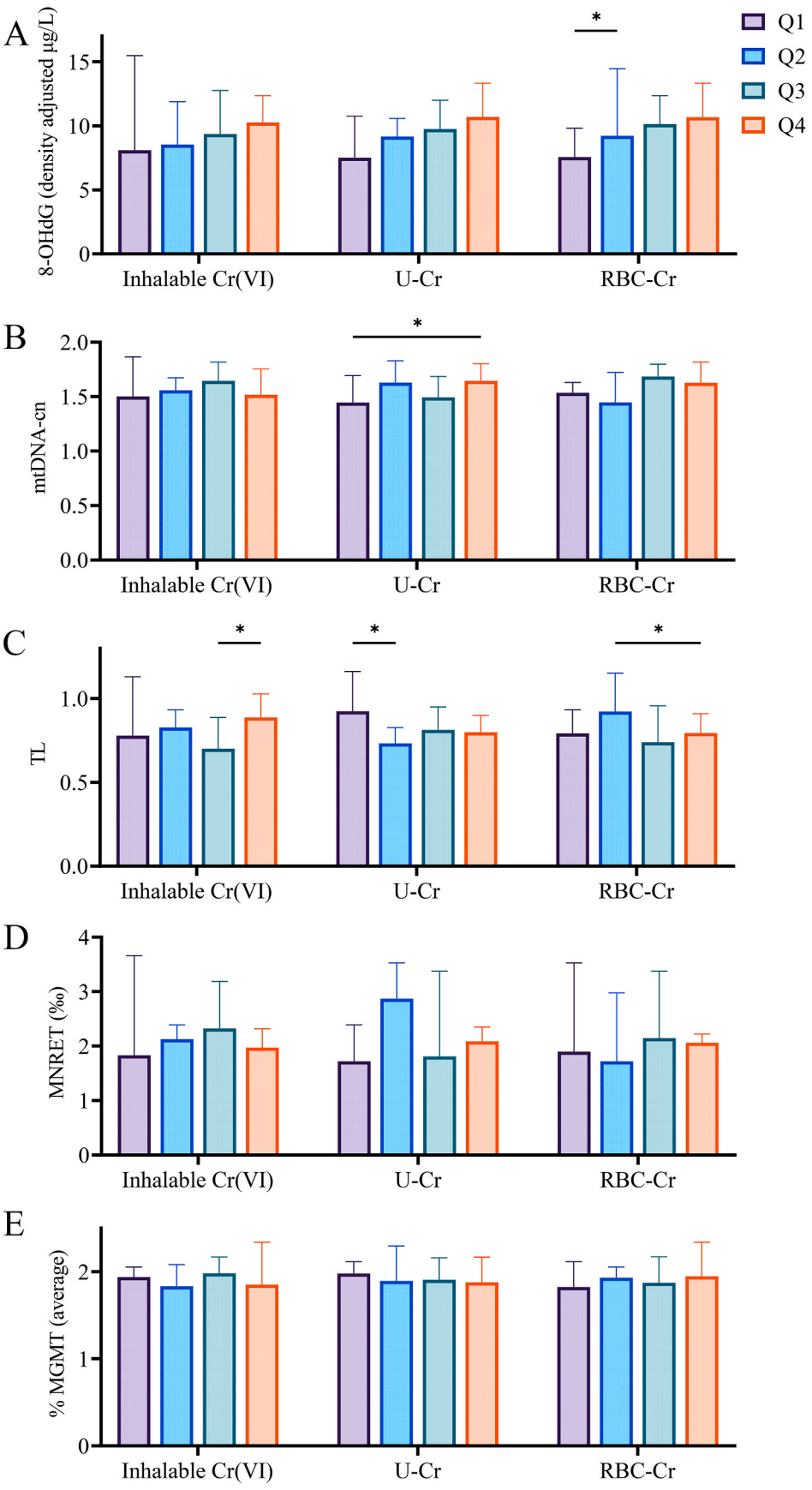

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Fig. 1. Associations between Cr(VI) exposure biomarkers quartiles and effect biomarkers in exposed workers. Bar plots showing the distribution of five biomarkers across quartiles (Q1–Q4) of inhalable Cr(VI), urinary Cr(U-Cr), and Cr in red blood cells (RBC-Cr). The data are presented as median with 95 % confidence interval. A. 8-hydroxy-2′-deoxyguanosine concentration in urine. B. Relative mitochondrial DNA copy number. C. Relative telomere length. D. Micronuclei in peripheral blood reticulocytes. E. Average methylation level of MGMT promoter. *Mann-Whitney *U* test, P < 0.05. Sample sizes: Q1: n = 17 (inhalable Cr(VI)), n = 29 (U-Cr), n = 28 (RBC-Cr). Q2: n = 38 (inhalable Cr(VI)), n = 28 (U-Cr), n = 28 (RBC-Cr). Q3: n = 29 (inhalable Cr(VI)), n = 28 (U-Cr), n = 27 (RBC-Cr). Q4: n = 28 (inhalable Cr(VI)), n = 28 (U-Cr), n = 27 (RBC-Cr). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Spearman rank correlation coefficients (β) between effect biomarkers and markers of hexavalent chromium (Cr(VI)) exposure.

	All participants			Exposed workers			
	Age	U-Cr[e]	RBC-Cr[f]	Age	Inhalable Cr(VI)	U-Cr	RBC-Cr
8-OHdG[a]	−0.14	0.14	0.20*	−0.15	0.09	0.17	0.16
mtDNA-cn[b]	0.11	−0.01	−0.02	0.12	0.10	0.14	0.16
TL[c]	−0.30*	0.15*	0.09	−0.26*	0.03	−0.18	−0.13
MNRET[d]	0.06	−0.10	−0.21*	0.06	0.03	0.08	−0.03
% *F2RL3* (average)	−0.17*	−0.04	0.03	−0.26*	0.04	0.00	0.01
% *F2RL3* (CpG1)	−0.16*	−0.03	0.06	−0.22*	0.05	0.05	0.02
% *F2RL3* (CpG2)	−0.18*	−0.03	0.03	−0.30*	0.05	0.00	0.01
% *LINE-1* (average)	0.04	−0.03	−0.06	0.10	−0.05	−0.12	−0.11
% *LINE-1* (CpG1)	−0.06	0.04	−0.05	−0.05	0.11	0.09	0.05
% *LINE-1* (CpG2)	0.06	−0.04	−0.11	0.10	−0.12	−0.21*	−0.27*
% *LINE-1* (CpG3)	0.06	−0.02	0.00	0.12	−0.08	−0.10	−0.04
% *MGMT* (average)	0.09	0.10	0.17*	0.16	0.05	0.04	0.13
% *MGMT* (CpG1)	0.08	0.17*	0.21*	0.10	0.02	0.21*	0.29*
% *MGMT* (CpG2)	0.07	−0.04	0.01	0.04	−0.03	−0.14	−0.02
% *MGMT* (CpG3)	0.10	0.09	0.10	0.20	−0.01	0.00	0.03
% *MGMT* (CpG4)	0.05	0.07	0.17*	0.11	0.07	0.03	0.15
% *SEMA4B*	0.02	0.01	0.05	0.02	0.09	−0.15	−0.09

* Spearman rank correlation, $P < 0.05$.
[a] 8-hydroxy-2′-deoxyguanosine concentration in urine, density adjusted.
[b] Relative mitochondrial DNA copy number.
[c] Relative telomere length.
[d] Micronuclei in peripheral blood reticulocytes (‰). Due to 6 missing samples and 4 samples having too few cells, the result was for 105 exposed workers and 70 controls.
[e] Post-work urinary chromium concentration adjusted by density (μg/L).
[f] Chromium concentration in red blood cells (μg/L).

Table 4
Multiple linear regression models for natural logarithm-transformed effect biomarkers between exposed workers and controls. Data are presented as beta coefficient (β), 95 % confidence interval (95 % CI) and p-value (*P*). Significant associations are shown in bold.

	Unadjusted			Partly adjusted[f]			Fully adjusted[g]		
	β	95 % CI	*P*	β	95 % CI	*P*	β	95 % CI	*P*
8-OHdG[a]	**0.21**	**0.04, 0.37**	**0.01**	0.09	−0.07, 0.25	0.29	0.10	−0.07, 0.26	0.26
mtDNA-cn[b]	−0.08	−0.15, 0.00	0.06	−0.04	−0.12, 0.05	0.39	−0.05	−0.13, 0.04	0.28
TL[c]	**0.19**	**0.10, 0.29**	**<0.001**	**0.19**	**0.10, 0.29**	**<0.001**	**0.18**	**0.08, 0.27**	**<0.001**
MNRET[d]	**−0.27**	**−0.48, -0.07**	**0.01**	**−0.26**	**−0.47, -0.04**	**0.02**	**−0.24**	**−0.46, -0.02**	**0.04**
% *F2RL3* (average)	0.01	−0.01, 0.03	0.38	0.01	−0.01, 0.03	0.54	0.01	−0.01, 0.02	0.39
% *F2RL3* (CpG1)	0.01	−0.01, 0.03	0.37	0.01	−0.01, 0.03	0.49	0.01	−0.01, 0.03	0.38
% *F2RL3* (CpG2)	0.01	−0.01, 0.03	0.40	0.01	−0.01, 0.02	0.61	0.01	−0.01, 0.02	0.42
% *LINE-1* (average)	0.00	−0.01, 0.01	0.76	0.00	−0.01, 0.01	0.96	0.00	−0.01, 0.01	0.82
% *LINE-1* (CpG1)	0.00	−0.01, 0.01	0.90	0.00	−0.01, 0.00	0.55	0.00	−0.01, 0.01	0.60
% *LINE-1* (CpG2)	0.00	−0.01, 0.01	0.64	0.00	−0.01, 0.01	0.72	0.00	−0.01, 0.01	0.90
% *LINE-1* (CpG3)	0.00	−0.01, 0.01	0.66	0.00	−0.01, 0.01	0.91	0.00	−0.01, 0.01	0.89
% *MGMT* (average)	0.07	−0.01, 0.15	0.07	**0.10**	**0.01, 0.18**	**0.02**	**0.09**	**0.00, 0.17**	**0.05**
% *MGMT* (CpG1)[e]	0.09	−0.01, 0.18	0.07	**0.11**	**0.01, 0.21**	**0.04**	0.09	−0.02, 0.19	0.10
% *MGMT* (CpG2)[e]	0.04	−0.05, 0.13	0.39	0.03	−0.07, 0.13	0.57	0.02	−0.09, 0.11	0.77
% *MGMT* (CpG3)	0.05	−0.03, 0.14	0.21	0.07	−0.02, 0.16	0.11	0.06	−0.03, 0.15	0.17
% *MGMT* (CpG4)	0.05	−0.02, 0.12	0.19	0.07	0.00, 0.15	0.05	0.07	0.00, 0.15	0.06
% *SEMA4B*	0.05	−0.17, 0.28	0.63	−0.03	−0.26, 0.20	0.79	−0.03	−0.27, 0.21	0.79

[a] 8-hydroxy-2′-deoxyguanosine concentration in urine.
[b] Relative mitochondrial DNA copy number.
[c] Relative telomere length.
[d] Micronuclei in peripheral blood reticulocytes.
[e] Values were transformed using Y = ln(x+1) to handle zeros in the dataset.
[f] The partly adjusted model was adjusted for age, sex and alcohol drinking. The reference is controls.
[g] The fully adjusted model was adjusted for age, sex, BMI, smoking, alcohol drinking, diet, leisure activity with chromium, infection in two weeks. The reference is controls.

higher 8-OHdG and TL, and lower MNRET and methylation level of *MGMT* after adjustment for the metals (antimony, cobalt, copper and zinc) that had significantly different concentrations in the two groups.

Multiple linear regression models were applied to assess the associations between effect biomarkers and RBC-Cr concentrations (Table 5). *MGMT* methylation was positively associated with RBC-Cr in the unadjusted model, and this association remained statistically significant after adjusting for potential confounders and covariates in both the partly and fully adjusted models. Furthermore, additional adjustments were made for metals that significantly differed between exposed workers and controls (Supplementary Table 12), and the association with *MGMT* methylation remained robust. No other biomarkers showed statistically significant associations with RBC-Cr across the models.

3.5. Correlations between effect biomarkers

Supplementary Fig. 1 shows heatmaps on the Spearman's correlations between effect biomarkers. The average methylation and the methylation of CpG sites within each gene showed high correlation coefficients. Considering all participants, mtDNA-cn was significantly positively correlated with MNRET (r_S = 0.21) and TL (0.19), and inversely correlated with *MGMT* (CpG2) methylation (−0.17). In addition, the methylation of *F2RL3* (CpG2) was significantly positively correlated with *SEMA4B* methylation, but the correlation coefficient was relatively weak (0.16). When exposed workers were analysed separately, the coefficients of the correlations mentioned above became non-significant except for the correlation between *F2RL3* (CpG2) and *SEMA4B*. Furthermore, in exposed workers, 8-OHdG showed a positive correlation with *F2RL3* (CpG2) and a negative correlation with *MGMT*; mtDNA-cn showed a negative correlation with *LINE-1* (CpG1) and *MGMT* (CpG2); TL showed a positive correlation with *SEMA4B* and negative correlation with *LINE-1* (CpG3).

4. Discussion

In this study examining low-to-moderate Cr(VI) exposure and different oxidative stress, genetic and epigenetic effect biomarkers, exposed workers showed significantly higher 8-OHdG, TL, and *MGMT* methylation, alongside lower mtDNA-cn and MNRET compared to controls. Further, some effect biomarkers correlated with exposure biomarkers: U-Cr was positively correlated with TL, while RBC-Cr correlated positively with 8-OHdG and *MGMT* methylation. Multivariate analysis showed significant differences in 8-OHdG, MNRET, TL, and *MGMT* methylation between exposed workers and controls. Interrelationships were also found within biomarkers, suggesting that Cr (VI) exposure may modulate multiple pathways associated with DNA damage and repair.

4.1. Cr(VI) and oxidative stress

Cr(VI)-induced oxidative stress is recognized as a key molecular mechanism underlying Cr(VI) toxicity and carcinogenesis (DesMarais and Costa, 2019). ROS could be generated during the intracellular reduction of Cr(VI) to other valence chromate compounds, which can contribute to oxidative stress, genetic instability, inflammation, and ultimately tumor development (O'Brien et al., 2003; Valko et al., 2005). ROS are able to react with deoxyguanosine at the C-8 position, leading to the formation of 8-OHdG (Kasai et al., 1986). Measuring urinary 8-OHdG as an index of oxidative DNA damage in Cr(VI) exposure has been commonly used in epidemiological research because it is non-invasive and easy to perform (Su et al., 2024). Our study is in agreement with previous studies that showed that urinary 8-OHdG concentrations among Cr(VI) exposed workers were higher than controls (Hu et al., 2022; Kuo et al., 2003; Li et al., 2014; Pan et al., 2018; Su et al., 2024; Zhang et al., 2024). Of these studies, one reported urinary 8-OHdG positively associated with airborne Cr, two with urinary Cr and four with blood Cr. In the present study, a significant positive correlation was observed between urinary 8-OHdG and RBC-Cr, but no correlation was found with U-Cr. The lack of correlation between urinary 8-OHdG and U-Cr could be attributed to several factors. One possibility is that U-Cr levels may not accurately reflect long-term Cr(VI) exposure, as it is more influenced by recent exposure or renal clearance (Jiang et al., 2024b). However, some of the absorbed Cr remains in the body, where it is slowly excreted and may continuously generate oxidative stress (Tavares et al., 2022a). Moreover, U-Cr reflects both Cr(III) and Cr(VI) exposure (Santonen et al., 2022), thus, variation in Cr(III) exposure in

Table 5
Multiple linear regression models for associations between logarithm-transformed effect biomarkers and chromium concentration in red blood cells, data are presented as beta coefficient (β), 95 % confidence interval (95 % CI) and P-value (P). Significant associations are shown in bold.

	Unadjusted			Partly adjusted[f]			Fully adjusted[g]		
	β	95 % CI	*P*	β	95 % CI	*P*	β	95 % CI	*P*
8-OHdG[a]	0.16	−0.04, 0.35	0.11	0.08	−0.10, 0.26	0.39	0.09	−0.10, 0.27	0.37
mtDNA-cn[b]	0.02	−0.07, 0.12	0.61	0.04	−0.05, 0.13	0.35	0.04	−0.06, 0.13	0.45
TL[c]	−0.04	−0.15, 0.07	0.48	−0.02	−0.13, 0.09	0.70	−0.03	−0.14, 0.08	0.59
MNRET[d]	−0.07	−0.31, 0.17	0.57	−0.05	−0.29, 0.20	0.71	−0.03	−0.28, 0.21	0.79
% *F2RL3* (average)	0.00	−0.02, 0.02	0.79	0.00	−0.02, 0.03	0.70	0.01	−0.01, 0.02	0.57
% *F2RL3* (CpG1)	0.01	−0.02, 0.03	0.67	0.01	−0.02, 0.03	0.58	0.01	−0.01, 0.03	0.46
% *F2RL3* (CpG2)	0.00	−0.02, 0.02	0.93	0.00	−0.02, 0.02	0.84	0.00	−0.02, 0.02	0.71
% *LINE-1* (average)	0.00	−0.01, 0.01	0.67	0.00	−0.01, 0.01	0.85	0.00	−0.01, 0.01	0.86
% *LINE-1* (CpG1)	0.01	0.00, 0.01	0.15	0.01	0.00, 0.01	0.19	0.01	0.00, 0.01	0.19
% *LINE-1* (CpG2)	0.00	−0.01, 0.01	0.38	−0.01	−0.01, 0.00	0.29	0.01	−0.01, 0.00	0.29
% *LINE-1* (CpG3)	0.00	−0.01, 0.01	0.70	0.00	−0.01, 0.01	0.93	0.00	−0.01, 0.01	0.94
% *MGMT* (average)	0.09	0.00, 0.18	0.06	**0.09**	**0.01, 0.19**	**0.05**	0.09	0.00, 0.19	0.05
% *MGMT* (CpG1)[e]	**0.15**	**0.04, 0.26**	**0.01**	**0.15**	**0.04, 0.26**	**0.01**	**0.15**	**0.04, 0.26**	**0.01**
% *MGMT* (CpG2)[e]	0.01	−0.10, 0.11	0.91	0.00	−0.11, 0.10	0.94	0.00	−0.11, 0.10	0.94
% *MGMT* (CpG3)	0.02	−0.08, 0.12	0.69	0.02	−0.08, 0.12	0.70	0.02	−0.08, 0.12	0.70
% *MGMT* (CpG4)	**0.09**	**0.01, 0.17**	**0.03**	**0.10**	**0.02, 0.18**	**0.02**	**0.10**	**0.02, 0.18**	**0.02**
% *SEMA4B*	0.04	−0.21, 0.30	0.74	0.01	−0.25, 0.27	0.95	0.00	−0.26, 0.26	0.99

[a] 8-hydroxy-2′-deoxyguanosine concentration in urine.
[b] Relative mitochondrial DNA copy number.
[c] Relative telomere length.
[d] Micronuclei in peripheral blood reticulocytes.
[e] Values were transformed using Y = ln(x+1) to handle zeros in the dataset.
[f] The partly adjusted model was adjusted for age, sex and alcohol drinking.
[g] The fully adjusted model was adjusted for age, sex, BMI, smoking, alcohol drinking, diet, leisure activity with chromium and infection.

the studied occupations may weaken a possible correlation between urinary excreted Cr(VI) and 8-OHdG. Finally, the use of 8-OHdG as a ROS biomarker should be carefully evaluated since urinary 8-OGdG levels may be attributed to DNA (and nucleotide) related protective processes, and not only to oxidative stress (Møller et al., 2012).

4.2. Cr(VI) and MN

Studies have shown that Cr(VI)-induced ROS can impact genetic stability, causing oxidative DNA lesions, DNA crosslinks, and both single- and double-strand breaks (Zhitkovich, 2005). Additionally, Cr(VI) and its reduction products can directly cause genetic damage through interactions with proteins, amino acids, or DNA itself (Chen et al., 2019). MN serves as a biomarker for genetic damage. They arise from lagging chromosomes or acentric chromosome fragments that fail to integrate into daughter nuclei and are instead encapsulated in a separate nuclear envelope (Krupina et al., 2021). The MN test is increasingly recognized as a reliable biomarker of genotoxicity in individuals occupationally exposed to Cr(VI). Studies have shown that Cr(VI) exposure was positively associated with MN frequency in buccal cells (Sudha et al., 2011) and in peripheral lymphocytes (Li et al., 2014).

Flow cytometric analysis of MNRET is regarded as a sensitive and high-throughput method for detecting genotoxicity in biomonitoring studies (Tavares et al., 2022a). Recently, the European Human Biomonitoring Initiative (HBM4EU) (Tavares et al., 2022a) and the Danish SAM-Krom project (Saber et al., 2024) found significantly higher levels of MNRET in Cr(VI) exposed workers compared with controls. However, the results were the opposite in our study. A detailed comparison of the data revealed that exposed workers in all three studies had similar mean levels of MNRET, in which exposed workers in HBM4EU had 2.75 ‰, SAM-Krom had 2.27 ‰ and our study had 2.48 ‰. However, the MNRET levels for the control group varied across the three studies. The mean level of MNRET in SAM-Krom was 1.14 ‰. HBM4EU categorised controls into two groups, within company controls and outwith company controls, and within company controls were exposed to low levels of Cr (VI). The average level of MNRET in within company controls was 3.13 ‰ and in outwith company controls was 1.92 ‰. Our controls had the highest mean value of 3.25 ‰. The higher MNRET levels in our control group could be attributed to several factors, including differences in population characteristics. However, even after adjusting for demographic and lifestyle factors (e.g., gender, alcohol drinking, diet) or exposures to other metals, the exposed workers still exhibited lower MNRET levels compared to the controls. All three studies employed the same method for sample collection, and MNRET analysis was conducted in the same laboratory, making it unlikely that the differences were due to methodological variations. We also evaluated potential differences between analytical batches. Among exposed workers, the median MNRET was slightly lower in the first batch (1.97 ‰) compared to the second batch (2.01 ‰). Among controls, the highest MNRET was observed in the first batch (4.71 ‰), compared to the second (2.46 ‰) and third (2.33 ‰) batches. These findings suggest that the observed differences between exposed workers and controls are unlikely to be attributed to batch effects in the analysis. In our study, controls can be classified into four groups: one agricultural operator (n = 15), one care home (n = 20), one storage company (n = 25) and other (n = 10). The mean level of MNRET according to this categorization was 4.95 ‰, 3.41 ‰, 2.23 ‰ and 2.94 ‰, respectively. The HBM4EU study reported a negative correlation between RBC-Cr and MNRET ($P = 0.12$), which was possibly attributed to eryptosis triggered by the toxic effects of Cr(VI), leading to the removal of the most damaged erythrocytes. However, this did not result in a lower MNRET frequency compared to the external company controls in that study. In our study, the control group from the agricultural sector exhibited the highest MNRET frequency (Kruskal--Wallis H Test, $P < 0.01$). Previous research has shown significantly elevated MNRET levels among agricultural workers, likely due to pesticide exposure. We infer that the employees at this company may be exposed to factors that contribute to an increased frequency of MN. More studies are needed to further investigate the factors, instead of metals, contributing to the higher MNRET levels. Identifying these factors and understanding their impact on genetic damage is essential for accurately assessing genotoxicity risks in various work environments. Another potential explanation could be adaptive responses induced by Cr(VI), whereby prior exposure to low doses of Cr(VI) may activate cellular defense mechanisms, such as enhanced DNA repair or antioxidant responses, resulting in reduced biological damage during subsequent higher-dose exposures. Previous studies have demonstrated that micronucleus formation can exhibit adaptive responses to certain mutagens, including methyl methanesulfonate, mitomycin C, furylfuramide, and colchicine (Sutou et al., 2024). However, the mechanisms underlying such responses in the context of Cr(VI) exposure remain unclear, and further research is needed to determine whether adaptive responses truly contribute to the observed MNRET patterns and to better understand the complexity of interpreting MNRET results in chronically exposed populations. In addition, MNRET represent genotoxic damage that has occurred during a rather narrow time window approximately three days prior to the sampling and therefore the blood sampling schedule in relation to the potential exposure is crucial and could affect the results.

4.3. Cr(VI), mtDNA-cn and TL

Mitochondria are essential organelles for energy production and play a vital role in regulating redox-dependent pathways (Alur et al., 2024). *In vitro* studies showed that Cr(VI) can induce mitochondrial bioenergetics perturbation (Fernandes et al., 2002), biogenesis imbalance (Zhong et al., 2017) and mitophagy (Dlamini et al., 2021). Furthermore, it has been found that exposure to Cr(VI) initiates cell apoptosis through a mitochondria-dependent process (Chiu et al., 2010). This suggests that mitochondria are among the most sensitive targets of Cr(VI) toxicity. The mitochondrial genome is a circular, double-stranded, supercoiled molecule, present in varying quantities ranging from one to several thousand copies per cell (Takamatsu et al., 2002). Unlike nuclear DNA, mtDNA is much smaller in size, lacks histone packaging, and has a less efficient DNA repair system (Yang et al., 2016). As a result, mtDNA is particularly susceptible to oxidative stress, being over five times more sensitive to such damage compared to nuclear DNA (Byun and Baccarelli, 2014). Zhong et al. observed decreased mtDNA-cn, mitochondrial mass and function in HepG2 human hepatoma cells exposed to 20 and 40 μM of Cr(VI), indicating that high Cr(VI) exposure leads to the downregulation of mitochondrial biogenesis (Zhong et al., 2017). Li et al. reported reduced blood mtDNA-cn in lung tissue of rats exposed to both low (0.05 mg/kg w) and high (0.25 mg/kg w) doses of Cr(VI) (Li et al., 2024). Similarly, our results showed that workers exposed to Cr (VI) had lower mtDNA-cn compared to controls. Alteration of mtDNA-cn may contribute to the development of lung cancer (Liu et al., 2017). It has been reported that mtDNA-cn is reduced in the plasma (Chen et al., 2018) and cancer tissue (Dai et al., 2013) of lung cancer patients. The lower mtDNA-cn may be a consequence of exposure to excessive ROS and may result in decreased mitochondrial function and energy metabolism, leading to alterations in mitochondrial biosynthetic and bioenergetic (Chen et al., 2018). Further studies are required to elucidate the mechanisms and consequences of decreased mtDNA content in Cr (VI)-induced carcinogenesis.

Telomeres help maintain genome stability by protecting against the gradual loss of terminal DNA during cell division and shielding the genome from other forms of cellular damage (Blackburn, 2001). Telomeres naturally shorten as part of the aging process, and shorter TL have been linked to non-cancer age-related diseases (Blackburn et al., 2015). Our study also found a significant negative correlation between TL and age in both all participants and the exposed workers. Longer telomeres, by extending cellular growth potential, have been associated with cancer-initiating somatic mutations, as they allow cells to accumulate

genetic alterations that would otherwise be limited by normal telomere shortening (Nakao & Natarajan, 2023; Tsatsakis et al., 2023). Several studies suggest that longer TL increases lung cancer risk, especially lung adenocarcinoma risk (Rode et al., 2016; Samavat et al., 2021; Sanchez-Espiridion et al., 2014; C. Zhang et al., 2015). In addition, Córdoba-Lanús et al. reported that chronic obstructive pulmonary disease (COPD) patients who developed lung cancer had longer TL three years before their lung cancer diagnosis, compared with the controls (COPD patients who did not develop lung cancer) (Córdoba-Lanús et al., 2024). Therefore, they suggested that longer leukocyte TL could serve as an early biomarker for the risk of future lung cancer development in COPD patients. An animal study has demonstrated a significant increase in the TL of peripheral blood mononuclear cells in rats following exposure to welding fumes containing Cr (Shoeb et al., 2017). Consistent with our findings, Ahlers et al. observed a rapid and significant increase in TL among steel workers exposed to metal-rich particulates (Dioni et al., 2011). However, a cross-sectional study found a negative association between blood Cr concentrations and TL in chromate production workers (Zhang et al., 2024). In our dataset, we also observed a negative association between RBC-Cr and TL among exposed workers, though not statistically significant. In addition, a significant positive correlation was found between TL and U-Cr in the overall study population ($r_S = 0.15$), however, this relationship appears to become negative (though nonsignificant, $r_S = -0.18$) in the exposed workers. This could be due to several potential factors: it is possible that the relationship between TL and U-Cr is not linear, particularly at higher levels of Cr(VI) exposure. At lower exposure levels, Cr(VI) may have a mild oxidative effect that could potentially influence TL positively. Study shows that 8-OHdG frequently mispairs with adenine during replication; the mutated telomeric DNA sequence will interfere with binding of shelterin components and consequently increases TL (Ahmed and Lingner, 2018). However, at higher exposures, more pronounced oxidative stress might overwhelm the body's repair mechanisms, leading to telomere shortening (Houben et al., 2008), as observed in our exposed workers. Alternatively, it may resemble the effect of arsenic on telomeres, where low exposure levels activate telomerase and lead to telomere elongation, whereas high exposure levels result in telomere shortening due to excessive oxidative stress (Wai et al., 2022). Another plausible explanation involves competing mechanisms that might affect TL in exposed workers. Exposure to low level of Cr(VI) may trigger DNA repair mechanisms or activate cellular stress responses (Ferreira et al., 2019; Zhang et al., 2023). Those may act as compensatory mechanisms and mask or alter the relationship between TL and U-Cr. Finally, the relationship between TL and biomarkers like U-Cr might also be influenced by additional confounding variables not captured in this analysis, such as other occupational exposures, individual health conditions, or lifestyle factors. Future studies with more detailed data on these factors could help clarify the mechanisms at play.

4.4. Cr(VI) and DNA methylation

Although genomic instability and intracellular metabolism mediated by ROS are thought to be the primary mechanisms behind Cr(VI)-induced carcinogenesis (Zhao et al., 2022), Cr(VI) has been shown to alter DNA methylation and histone modifications, as well as non-coding RNA expression (Iyer et al., 2023). These epigenetic changes can regulate the expression of genes that affect cell death, cell migration, cell proliferation, DNA repair, genomic stability and inflammation without changes to the DNA sequence (Chen et al., 2019). Our previous results found that circulating non-coding RNA dysregulation in populations with occupational Cr(VI) exposure (Jiang et al., 2025; Jiang et al., 2024a). Given the wide-ranging cellular effects of Cr(VI), it is likely that multiple mechanisms work together to promote the neoplastic transformation of normal cells and tumor progression. *In vitro* (Lou et al., 2013), *in vivo* (Wang et al., 2016) and population (Wang et al., 2012) studies have shown that global DNA hypomethylation is linked to Cr(VI) exposure.

Consistent with our findings, Yang et al. reported no changes in *LINE-1* gene methylation levels among chrome plating workers (Yang et al., 2016). Feng et al. utilized a combination of Infinium Methylation450K Chip and targeted-bisulfite sequencing to measure DNA methylation, identifying 131 differentially methylated CpG sites, including *SEMA4B* (Feng et al., 2020). They also proposed that *SEMA4B* methylation could serve as a potential biomarker for Cr(VI) exposure. In contrast, our study found that Cr(VI) exposed workers had similar *SEMA4B* methylation levels to those of the controls. Previous studies showed that *F2RL3* hypomethylation was associated with smoking and welding fumes exposure (Bhardwaj et al., 2023; Hossain et al., 2015). In addition, the hypomethylation of *F2RL3* may mediate the effect of tobacco on lung cancer risk (Fasanelli et al., 2015). Researchers believe that Cd exposure caused by smoking triggers differential methylation of *F2RL3* (Domingo-Relloso et al., 2020; Lee et al., 2020). In our study, the methylation level of *F2RL3* showed no significant correlation with RBC-Cr but had significantly negative correlations with Cd in RBC. Confirmed with previous studies, our findings suggest that Cd exposure, rather than Cr, may play a more significant role in influencing *F2RL3* methylation levels. However, after excluding smokers, *F2RL3* methylation is still significantly correlated with RBC-Cd but with a smaller r_S, suggesting that the association is not solely driven by smoking-related Cd exposure but may also reflect other environmental or occupational sources of Cd exposure. More studies are needed to determine whether this epigenetic alteration occurs with Cd exposure alone, independent of smoking, and to further explore the mechanistic pathways linking Cd exposure to *F2RL3* methylation and its potential contribution to adverse health outcomes, particularly non-smokers.

A recent study in human bronchial epithelial BEAS-2B cells revealed that chronic Cr(VI) exposure lowered MGMT protein concentrations by increasing methylation of *MGMT* (Wang et al., 2024). Epigenetic downregulation of DNA damage repair genes plays a critical role in cancer development, with *MGMT* being one of the most frequently suppressed DNA repair genes in cancer cells due to increased promoter region methylation (Christmann and Kaina, 2019). The *MGMT* gene encodes DNA repair protein essential for cellular defense against mutagenesis and toxicity caused by alkylating agents (Ali-Osman et al., 2001). A meta-analysis revealed a strong association between *MGMT* methylation and non-small cell lung cancer (Gu et al., 2013). In the present study, *MGMT* methylation levels were significantly higher in exposed workers, indicating a potential association between Cr(VI) exposure and MGMT hypermethylation, which may play a role in Cr (VI)-related carcinogenic processes. Future research should explore the mechanisms of *MGMT* in Cr(VI) carcinogenesis using *MGMT* knockout or other transgenic mouse models.

4.5. Strengths and limitations

This study simultaneously investigated multiple classes of effect biomarkers — including markers of oxidative damage, chromosomal damage, mitochondrial DNA content, telomere length, and epigenetic alterations in lung cancer–related genes — in the same population, offering an integrative perspective on the molecular impacts of Cr(VI) exposure. By focusing on low-to-moderate occupational exposure levels, the study fills a critical gap in the current literature, which has primarily emphasized high-exposure groups. The use of both external (airborne Cr (VI)) and internal (U-Cr and RBC-Cr) exposure measurements enabled robust exposure–response analyses. The study's comprehensive adjustment for confounders strengthens the validity and generalizability of our findings.

This study has several limitations. First, its cross-sectional design limits the ability to infer causal relationships between Cr(VI) exposure and the observed biomarker changes. Second, although we adjusted for major confounders and conducted propensity score matching, residual confounding from unmeasured variables cannot be excluded. Third,

lifestyle and health-related factors were self-reported and may be affected by recall bias. Fourth, the group size imbalance may have influenced statistical power and model stability. Fifth, the observed lower MNRET levels in exposed workers contrast with findings from some previous studies; further studies with larger samples and longitudinal designs are needed to clarify this discrepancy and explore underlying mechanisms.

5. Conclusions

This study highlights that occupational Cr(VI) exposure, even at low-to-moderate exposure level, is associated with significant alterations in key biomarkers of oxidative stress, DNA changes and damage, and epigenetic changes. Exposed workers had a higher level of 8-OHdG, longer TL, a higher level of *MGMT* methylation, and lower levels of mtDNA-cn compared to controls, suggesting that Cr(VI) may cause oxidative damage, activate telomere lengthening and disrupt mitochondria biogenesis, and DNA repair pathways. Positive correlations between U-Cr, RBC-Cr, and TL and *MGMT* methylation further support the role of Cr(VI) in these genetic and epigenetic alterations. Together, these findings indicate that Cr(VI) exposure at low-to-moderate levels is associated with markers of genomic instability and may be linked to increased cancer risk on a group level, highlighting the importance of stringent exposure monitoring, protective measures in workplaces, and rigorous enforcement of regulatory standards to minimize worker exposure.

CRediT authorship contribution statement

Zheshun Jiang: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Agneta Runkel:** Writing – review & editing, Methodology, Formal analysis. **Christian Lindh:** Writing – review & editing, Methodology, Formal analysis. **Aimonen Kukka:** Writing – review & editing, Methodology, Formal analysis. **Julia Catalán:** Writing – review & editing, Methodology. **Daniela Pineda:** Writing – review & editing, Project administration, Methodology, Formal analysis. **Thomas Lundh:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Ulla Vogel:** Writing – review & editing. **Anne T. Saber:** Writing – review & editing. **Martin Tondel:** Writing – review & editing. **Malin Engfeldt:** Writing – review & editing, Resources, Project administration, Investigation, Formal analysis. **Annette M. Krais:** Writing – review & editing, Investigation. **Karin Broberg:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Karin Broberg reports financial support was provided by Forskningsrådet för hälsa, arbetsliv och välfärd (Forte, Swedish Research Council for Health Working Life and Welfare). Karin Broberg reports financial support was provided by AFA Försäkring (Afa Insurance). No a If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2025.122123.

Data availability

Data will be made available on request.

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