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Occupational Exposure to Hexavalent Chromium in Sweden and its Toxicity

Evidence from the SafeChrom project

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--Evidence from the SafeChrom project

Occupational Exposure to Hexavalent Chromium in Sweden and its Toxicity

Evidence from the SafeChrom project

Zheshun Jiang
蒋哲舜

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Abstract: Hexavalent chromium (Cr(VI)) is classified as a Group 1 human carcinogen and increases the risk of lung cancer because it can generate reactive oxygen species, induce DNA damage, and trigger epigenetic alterations Occupational Cr(VI) exposure can occur during different work processes, but when this PhD study was initiated, the exposure level to Cr(VI) at Swedish workplaces and its health risk were unknown. Within the SafeChrom project, 113 Cr(VI) exposed workers and 72 controls were recruited. Inhalable Cr(VI) was measured by personal air sampling. Cr was measured in urine (U-Cr) and red blood cells (RBC-Cr). Effect biomarkers were measured, including 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine, relative mitochondrial DNA copy number (mtDNA-cn), relative telomere length (TL), micronuclei in peripheral blood reticulocytes (MNRET), DNA methylation level of four candidate genes, expression levels of three lncRNAs and four miRNAs. After excluding females and current smokers, miRNA sequencing, differentially expressed miRNAs (DEMs) identification, and bioinformatics analysis were conducted. Finally, the expression levels of potential target genes of the DEMs were validated. We found that workers were exposed to low-to-moderate levels of Cr(VI) with a median inhalable Cr(VI) concentration of 0.13 µg/m^3. Eight exposed workers (7%) exceeded the Swedish occupational exposure limit of 5 µg/m^3. Median U-Cr (0.60 µg/L) and RBC-Cr (0.73 µg/L) were significantly higher in the exposed workers compared with the controls (0.10 and 0.53 µg/L, respectively). Inhalable Cr(VI) correlated with urinary Cr (Spearman's rank correlation coefficient (r_S)=0.64) and RBC-Cr (r_S=0.53). Exposed workers had higher levels of 8-OHdG, longer TL, and *MGMT* promoter methylation, and lower levels of mtDNA-cn and MNRET, compared to controls. TL was positively correlated with U-Cr, while 8-OHdG and *MGMT* were positively associated with RBC-Cr. Expression levels of lncRNAs *MALAT1* and *NORAD*, and all four miRNAs, were significantly lower in exposed workers compared with controls, and significantly negatively correlated with RBC-Cr concentrations. *H19* was significantly correlated with *miR-142-3p* (r_S=-0.33) and *miR-15b-5p* (r_S=-0.30), and *NORAD* was significantly positively correlated with all four miRNAs (r_S=0.17 to 0.46). In the exposed workers, 21 up-regulated and 38 down-regulated DEMs were identified. Target genes of DEMs were significantly enriched in three KEGG terms: miRNAs in cancer, small cell lung cancer and non-small cell lung cancer. The expression of target genes *CCNE2*, *CDK4* and *E2F1* was significantly higher in the exposed workers compared with controls. Overall, this thesis reveals that while most workers in Sweden were exposed to low-to-moderate levels of Cr(VI), certain individuals were subjected to high levels of this non-threshold carcinogen. Cr(VI) exposure was associated with notable molecular changes, including oxidative stress, genomic instability, and epigenetic alteration, which stresses that exposure to Cr(VI) should be minimised as much as possible. Multiple mechanisms may interact to drive the toxicity and carcinogenesis associated with Cr(VI) exposure.

Key words: Hexavalent chromium; Occupational Exposure; Biomonitoring; Toxicity; Effect biomarkers

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Evidence from the SafeChrom project

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炉火照天地，红星乱紫烟。

赧郎明月夜，歌曲动寒川。

--李白

The furnace fire illuminates heaven and earth,

Crimson sparks scatter through the purple smoke.

Under the moonlit sky, the steelworker toils,

Songs echo through the chilly river valley.

--Li Bai

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Abstract

Background Hexavalent chromium (Cr(VI)) is classified as a Group 1 human carcinogen and increases the risk of lung cancer because it can generate reactive oxygen species, induce DNA damage, and trigger epigenetic alterations Occupational Cr(VI) exposure can occur during different work processes, but when this Ph.D. study was initiated, the exposure level to Cr(VI) at Swedish workplaces and its health risk were unknown.

Method Within the SafeChrom project, 113 Cr(VI) exposed workers and 72 controls were recruited. Inhalable Cr(VI) was measured by personal air sampling. Cr was measured in urine (U-Cr) and red blood cells (RBC-Cr). Effect biomarkers were measured, including 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine, relative mitochondrial DNA copy number (mtDNA-cn), relative telomere length (TL), micronuclei in peripheral blood reticulocytes (MNRET), DNA methylation level of four candidate genes, expression levels of three lncRNAs and four miRNAs. After excluding females and current smokers, miRNA sequencing, differentially expressed miRNAs (DEMs) identification, and bioinformatics analysis were conducted. Finally, the expression levels of potential target genes of the DEMs were validated.

Results We found that workers were exposed to low-to-moderate levels of Cr(VI) with a median inhalable Cr(VI) concentration of 0.13 $\mu g/m^3$. Eight exposed workers (7%) exceeded the Swedish occupational exposure limit of 5 µg/m3. Median U-Cr (0.60 µg/L) and RBC-Cr (0.73 µg/L) were significantly higher in the exposed workers compared with the controls (0.10 and 0.53 µg/L, respectively). Inhalable Cr(VI) correlated with urinary Cr (Spearman's rank correlation coefficient (r_S)=0.64) and RBC-Cr (r_S=0.53). Exposed workers had higher levels of 8-OHdG, longer TL, and *MGMT* promoter methylation, and lower levels of mtDNA-cn and MNRET, compared to controls. TL was positively correlated with U-Cr, while 8-OHdG and *MGMT* were positively associated with RBC-Cr. Expression levels of lncRNAs *MALAT1* and *NORAD*, and all four miRNAs, were significantly lower in exposed workers compared with controls, and significantly negatively correlated with RBC-Cr concentrations. *H19* was significantly correlated with *miR-142-3p* (r_S=-0.33) and *miR-15b-5p* (r_S=-0.30), and *NORAD* was significantly positively correlated with all four miRNAs (r_S=0.17 to 0.46). In the exposed workers, 21 up-regulated and 38 down-regulated DEMs were identified. Target genes of DEMs were significantly enriched in three KEGG terms: miRNAs in cancer, small cell lung cancer and non-small cell lung cancer. The expression of target genes *CCNE2*, *CDK4* and *E2F1* was significantly higher in the exposed workers compared with controls.

Conclusion Overall, this thesis reveals that while most workers in Sweden were exposed to low-to-moderate levels of Cr(VI), certain individuals were subjected to high levels of this non-threshold carcinogen. Cr(VI) exposure was associated with

notable molecular changes, including oxidative stress, genomic instability, and epigenetic alteration, which stresses that exposure to Cr(VI) should be minimised as much as possible. Multiple mechanisms may interact to drive the toxicity and carcinogenesis associated with Cr(VI) exposure.

Popular Scientific Summary

We are trailing behind our neighbours. At the time the SafeChrom project was initiated, Sweden's occupational exposure limit (OEL) for hexavalent chromium (Cr(VI)) was 5 µg/m³. In contrast, Denmark had already implemented a more restrictive OEL of 1 µg/m³, with a further reduction to 0.25 µg/m³ anticipated.

Moreover, it was recognized that an exposure limit of 5 µg/m³ corresponds to an estimated 40 lung cancer cases per 1000 exposed workers—around 500 times higher than the risk accepted for other carcinogens in the general environment. However, the actual exposure levels in Swedish workplaces were not known. These observations motivated the launch of the SafeChrom project, a collaboration involving all seven Occupational and Environmental Medicine clinics in Sweden and their affiliated university departments, together with the Danish National Research Centre for the Working Environment, and the Finnish Institute of Occupational Health. The project aims to characterize and reduce occupational exposure to Cr(VI) and its associated health risks in Sweden.

Cr(VI) and its compounds are valued for their properties, including corrosion resistance, hardness, and durability. Workers can be occupationally exposed to Cr(VI) when Cr(VI) compounds are used as a start-product (e.g. bath plating, steel passivation, and manufacture of Cr pigment), when Cr(VI) compounds are formed as a by-product (e.g. during welding, polishing, sanding, and grinding), and when Cr(VI) compounds are manufactured as end-product (e.g. chromate production). The main route of occupational exposure to Cr-containing products is through the inhalation of dust, mists, or fumes. Dermal contact and ingestion due to hand-to-mouth activity are also relevant when the skin is not adequately protected and is exposed to liquid forms of Cr(VI). Cr(VI) has been classified as a Group 1 human carcinogen and increases the risk of lung cancer. In the 1990s, it was estimated that around 21000 workers in Sweden were occupationally exposed to Cr(VI). However, at the start of the project, it was unknown how many workers are exposed today and at what levels, and what health risks can arise at this exposure level.

In SafeChrom, we recruited 113 Cr(VI) exposed workers and 72 controls (unexposed to Cr(VI)). Cr(VI) concentration in the air was measured by personal air sampling, and Cr was measured in urine and red blood cells. To evaluate the toxicity of Cr(VI) exposure, we analysed several effect biomarkers. Our results showed that workers were exposed to low-to-moderate levels of Cr(VI) with a median inhalable Cr(VI) concentration of 0.13 µg/m³. However, there were eight exposed workers (7%) who exceeded the Swedish OEL. Exposed workers had higher concentrations of Cr in urine and blood compared with controls. Exposed workers showed several significant cancer-related biological changes compared to controls, indicating that although the exposure level was relatively low, it may still cause potential health risks, and those risks may relate to lung cancer.

We reported these findings to the Swedish Work Environment Authority (Arbetsmiljöverket), which drew significant attention from the relevant bodies. After discussions with expert groups, the Arbetsmiljöverket decided to lower the Swedish OEL from the current 5 $\mu g/m^3$ to 1 $\mu g/m^3$ by the 9th of April 2026, in order to reduce the cancer risk for workers exposed to Cr(VI).

List of Papers

Paper I

Jiang Z, Schenk L, Assarsson E, Albin M, Bertilsson H, Dock E, Hagberg J, Karlsson LE, Kines P, Krais AM, Ljunggren S, Lundh T, Modig L, Möller R, Pineda D, Ricklund N, Saber AT, Storsjö T, Amir ET, Tinnerberg H, Tondel M, Vogel U, Wiebert P, Broberg K, Engfeldt M. Hexavalent chromium still a concern in Sweden - Evidence from a cross-sectional study within the SafeChrom project. *International Journal of Hygiene and Environmental Health*, 2024 Mar;256:114298.

Paper II

Jiang Z, Runkel A, Lindh C, Kukka A, Catalán J, Pineda D, Lundh T, Vogel U, Saber AT, Tondel M, Engfeldt M, Krais AM, Broberg K; SafeChrom Project Team. Oxidative damage, genetic and epigenetic alterations in hexavalent chromium exposed workers - A cross-sectional study within the SafeChrom project. *Environmental Research*. 2025 Jun;283:122123.

Paper III

Jiang Z, Person R, Lundh T, Pineda D, Engfeldt M, Krais AM, Hagberg J, Ricklund N, Vogel U, Saber AT, Tondel M, Albin M, Broberg K; SafeChrom Project Team. Circulating lung-cancer-related non-coding RNAs are associated with occupational exposure to hexavalent chromium - A cross-sectional study within the SafeChrom project. *Environment International*. 2024 Aug;190:108874.

Paper IV

Jiang Z, Pan M, Liu Y, Lundh T, Pineda D, Schenk L, Saber AT, Vogel U, Ljunggren S, Ricklund N, Engfeldt M, Krais AM, Broberg K; SafeChrom Project Team. Integrative analyses of circulating microRNA expression profile in hexavalent chromium exposed workers - A cross-sectional study within the SafeChrom project. *Journal of Hazardous Materials*. 2025 Jan;488:137367

Author’s contribution to the papers

Paper I

I am the first author. I contributed to data curation, formal analysis, investigation, visualization, writing – original draft, and writing – review & editing.

Paper II

I am the first author. I contributed to data curation, formal analysis, investigation, methodology, visualization, writing – original draft, and writing – review & editing.

Paper III

I am the first author. I contributed to writing – review & editing, writing – original draft, visualization, methodology, investigation, formal analysis, and data curation.

Paper IV

I am the first author. I contributed to writing – review & editing, writing – original draft, visualization, methodology, investigation, formal analysis, and data curation.

List of Papers not included in the thesis

Paper I
Saber AT, Levin M, Kines P, Aimonen K, Givelet L, Andersen C, Huusom AJ, Carøe T, Ebbehøj NE, Christensen FM, **Jiang Z**, Lundh T, Tinnerberg H, Albin M, Engfeldt M, Broberg K, Catalan J, Loeschner K, Fuglsang K, Vogel U. The SAM-Krom biomonitoring study shows occupational exposure to hexavalent chromium and increased genotoxicity in Denmark. *International Journal of Hygiene and Environmental Health*. 2024 Sep; 16:114444.

Paper II
Liu Y, **Jiang Z**, Sundquist J, Sundquist K, Ji J. Long-Term Pattern of Psychotropic Medication Uses Among Swedish Parents of Children Diagnosed With Cancer. *Journal of the National Comprehensive Cancer Network: JNCCN*. 2024 Nov; 22(9):e247048.

Paper III
Jiang Z, Liu Y, Lindh C, Pineda D, Carøe TK, Catalán J, Ebbehøj NE, Givelet L, Huusom AJ, Kines P, Krais AM, Aimonen K, Lundh T, Loeschner K, Rastkhani H, Tondel M, Saber AT, Vogel U, Broberg K; SafeChrom project team and SAM-Krom project team. Per- and polyfluoroalkyl substances exposure in hexavalent chromium exposed workers and the effects of exposure mixtures on oxidative stress and genomic instability. *Environmental Pollution*. 2025 Oct; 12:127255

Paper IV
Broberg K, Saber AT, Engfeldt M, Kines P, **Jiang Z**, Tondel M, Albin M, Schenk L, Vogel U. From biomonitoring studies to lowered occupational exposure limits for hexavalent chromium. *Societal Impacts*. 2026 Jan; 7: p. 100165.

Abbreviations

8-OHdG	8-hydroxy-2'-deoxyguanosine
cDNA	Complementary DNA
CI	Confidence interval
Cr	Chromium
Cr(VI)	Hexavalent chromium
DEMs	Differential expressed miRNAs
H19	H19 imprinted maternally expressed transcript
HBM4EU	The European Human Biomonitoring Initiative
ICP-MS	Inductively coupled plasma mass spectrometry
KEGG	Kyoto Encyclopedia of Genes and Genomes
lncRNA	Long non-coding RNA
MALAT1	Metastasis associated lung adenocarcinoma transcript 1
MGMT	O-6-methylguanine-DNA methyltransferase
miRNA	MicroRNA
MNRET	Micronuclei in peripheral blood reticulocytes
mtDNA-cn	Mitochondrial DNA copy number
ncRNA	Non-coding RNA
NORAD	Non-coding RNA activated by DNA damage
OEL	Occupational exposure limit
PPI	Protein-protein interactions
qPCR	Quantitative real-time polymerase chain reaction
RBC-Cr	Chromium concentration in red blood cells
ROS	Reactive oxygen species
r_S	Spearman's rank correlation coefficient
TL	Telomere length
U-Cr	Post-work urinary chromium concentration adjusted by density

Introduction

Chromium (Cr) is a transition metal with the atomic number 24[1]. It's the 21st most abundant element in the earth's crust[2]. Cr exists in seven oxidation states, ranging from -2 to +6, and the common stable states in the environment are the trivalent (Cr(III)) and the hexavalent (Cr(VI)) forms[3]. Cr(III) is the most naturally occurring form, and it is a trace element that is involved in regulating carbohydrate and lipid metabolism by stimulating insulin kinase activity in the human body[4]. Cr(VI), on the other hand, is mainly released from industrial processes and is considered mutagenic and carcinogenic[4]. The focus of this thesis is Cr(VI).

Cr(VI)

Cr(VI) rarely occurs in nature and is produced mainly from commercial and industrial processes[5]. Cr(VI) and its compounds provide superior hardenability, durability, and corrosion resistance compared to other metals, thus, they are widely used in several industrial applications, including agricultural fertilizers, ammunition, cement, chromates, electroplating, leather, metallurgy, pigments, and weapons[6].

Occupational exposure to Cr(VI)

Figure 1. Welding. Picture by Zheshun Jiang.

Occupational exposure to Cr(VI) occurs when Cr(VI) compounds are used as a starting product (e.g. bath plating, steel passivation, and manufacture of Cr pigment), when Cr(VI) compounds are formed as a by-product (e.g. during stainless steel welding (Figure 1), polishing, sanding and grinding of Cr-containing alloys), and when Cr(VI) compounds are manufactured as end-product (e.g. chromate production)[7,8]. The main route of occupational exposure to Cr(VI)-containing products is through the inhalation of dust, mists, or fumes[9]. Dermal contact and ingestion due to hand-to-mouth activity are

also relevant when the skin is not adequately protected and particularly when exposed to liquid forms of Cr(VI)[10].

Respirable particles can penetrate the non-ciliated regions of the lungs, and Cr(VI) may be released and absorbed into the bloodstream up to 72 h after exposure, with maximum uptake occurring after 6 h[11]. The extent to which Cr(VI) is absorbed after inhalation depends on particle size and the solubility of the Cr compound[6]. Soluble Cr(VI) primarily exists as the chromate oxyanion ($CrO4^{2-}$), which structurally resembles sulfate oxyanions[12]. Once it has entered the blood, Cr(VI) can pass through the cell membrane of red blood cells (RBC) via non-specific sulfate/phosphate anionic transporters on the cell surface, intracellularly be reduced to Cr(III)[13]. It is then eliminated mainly via urine[13,14].

Air and biological monitoring of Cr(VI)

Exposure to Cr(VI) is often assessed by measurements in air, urine, or blood[15]. The previous binding occupational exposure limit (OEL) set under the European Union (EU) Directive 2004/37/EC was 10 µg/m^3 (8-hour time-weighted average, 8-h TWA) and transitional arrangements allowed a higher limit value of 25 µg/m^3 for welding, plasma-cutting, and similar processes until 17 January 2025, after which, all limit value was reduced to 5 µg/m^3 through subsequent amendments[16,17]. In France and the Netherlands, OELs of 1 µg/m^3 have already been set for Cr(VI)[18]. In Denmark, an OEL of 1 µg/m^3 was implemented on July 1, 2020, which was further lowered to 0.25 µg/m^3 on January 1, 2025[19]. The current OEL for Cr(VI) in Sweden is 5 µg/m^3, and it has been estimated that exposure to air concentrations of 5 µg/m^3 corresponds to 20 extra lung cancer cases per 1000 exposed workers after 40 years of occupational exposure (*i.e.*, lifetime risk)[20,21]. In Germany and the Netherlands, acceptable risk is considered to be an additional risk of < 4 cases per 100,000 after 40 years, and tolerable risk (during a transitional period) is considered to be < 4/1000[22]. Recently, this approach has also been adopted at the EU-level by the Advisory Committee on Safety and Health at Work as a low-to-high risk level range, within which future EU OELs should be set[23].

Urinary Cr is a common internal exposure biomarker for total Cr, accounting for both Cr(VI) and Cr(III) exposure[24]. It has an approximate half-life of 7 h, reflects short-term exposure, and shows variation in half-lives depending on the route of exposure[25]. Post-shift urine is often compared with pre-shift urine to identify possible work-related Cr exposure[26]. A weakness with urinary Cr as a biomarker for Cr(VI) exposure is that it represents total Cr and is not specific for Cr(VI)[8]. However, since Cr(VI) is the only oxidation state of Cr that can cross the cell membrane, Cr in RBC is a specific indicator of Cr(VI) exposure[27]. As Cr is bound to haemoglobin within the RBC, it is assumed that the half-life of RBC-Cr corresponds to the half-life of RBC[16,28]. Hence, it is suggested that RBC-Cr values reflect the exposure to Cr(VI) over the past four months[16].

There are no EU-wide biological limit values (BLV) for Cr(VI); however, certain Member States, but not Sweden, have established BLVs for occupational exposure to Cr(VI), using urinary Cr as a measurement indicator[18]. For instance, Finland has established a BLV of 10 µg/L urinary Cr, corresponding to its OEL of 5 µg/m³ for Cr(VI) in the air; France and the Netherlands have implemented the most stringent BLV for European workers, setting it at 2.5 µg/L urinary Cr, derived from an OEL of 1 µg/m³ for Cr(VI)[8].

In addition to OELs and BLVs, changes in permitted uses and industrial applications have influenced occupational exposure to Cr(VI) in Europe. Since the introduction of authorisation requirements for most Cr(VI) substances under the EU chemicals regulation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), the number of workplaces and processes involving Cr(VI) has decreased, as reflected by a substantial decline in registered use volumes over the past decade[29]. Available EU-wide data indicate that registered volumes of Cr(VI) substances in 2022 were approximately 60% of their 2010 levels, with an even larger reduction observed for chromium trioxide[29]. This decline suggests that substitution, process closure, and reduced use intensity have contributed to lower opportunities for occupational exposure, particularly in sectors where Cr(VI) applications were technically replaceable.

Nevertheless, remaining uses of Cr(VI) are largely confined to specific industrial activities, including welding, electroplating, and surface treatment[30]. These uses are characterised by task-based exposure scenarios, where worker exposure depends strongly on operational conditions and risk management measures. Under this background, the SafeChrom project provides exposure measurements that are representative of contemporary, task-based Cr(VI) exposure scenarios in Sweden. This contextualisation is essential when comparing current exposure levels with earlier data or when assessing trends in occupational exposure over time.

The toxicity of Cr(VI)

Carcinogenicity

Cr(VI) is listed as 17th on the Agency for Toxic Substances and Disease Registry's Hazards Priority List[31]. It has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC) since 1990, based on sufficient evidence from occupational epidemiological studies showing strong and consistent excess risks of lung cancer among workers in chromate production, chromate pigment manufacturing, and chromium plating[32]. These epidemiological findings were later corroborated by mechanistic evidence demonstrating that Cr(VI) induces DNA damage, chromosomal aberrations, and oxidative stress, ultimately

leading to lung cancer, with animal studies further confirming the predominance of squamous cell carcinomas[33]. Epidemiological evidence also suggests that workers exposed to Cr(VI) have an increased risk of nose and nasal sinus cancer, as well as non-cancer effects, particularly affecting the respiratory system (nasal septum perforation, respiratory allergies, and other chronic pulmonary diseases) and the skin (skin irritation and allergic contact dermatitis), with additional adverse effects reported for the gastrointestinal tract, liver and kidney function, immune responses, reproductive and developmental outcomes, the cardiovascular system, and the nervous system[34,35]. Cr(VI) is considered a non-threshold carcinogen, meaning that even minimal exposure entails some cancer risk[36]. Accordingly, the guiding principle is that the exposure should be 'as low as reasonably achievable'[37].

The precise mechanisms behind Cr(VI) carcinogenicity are not fully understood, but several potential mechanisms have been suggested, including oxidative stress, genetic damage, genomic instability, and epigenetic modulation[38].

Oxidative stress

During intracellular reduction, Cr(VI) is primarily converted to Cr(III), a more thermodynamically stable form of Cr, by various reducing agents such as ascorbate, glutathione, and cysteine[39]. Throughout this process, multiple reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and hydroxyl radicals (HO•), can be generated[40]. ROS act as key secondary messengers and activators in various pathways and mediate Cr(VI)-induced alterations in apoptosis, cell signaling, and homeostasis[41]. Intracellular ROS induce damage to DNA, RNA, proteins, and mitochondria and are considered the primary contributors to Cr(VI)-induced cellular damage (Figure 2)[42]. The accumulation of ROS induces oxidative stress, promoting chronic inflammation, genetic instability, and metabolic reprogramming, which ultimately contribute to tumor development[43].

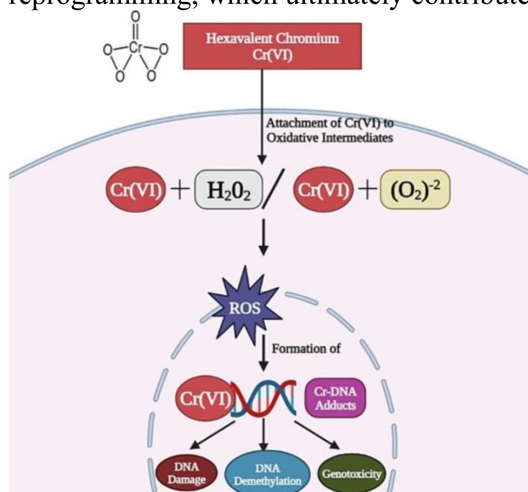

Figure 2. Cr(VI) and reactive oxygen species (ROS). Reprinted from Science of The Total Environment, Vol /882, Mahalaxmi Iyer, Uttpal Anand, Saranya Thiruvenkataswamy, Harysh Winster Suresh Babu, Arul Narayanasamy, Vijay Kumar Prajapati, Chandan Kumar Tiwari, Abilash Valsala Gopalakrishnan, Elza Bontempi, Christian Sonne, Damià Barceló, Balachandar Vellingiri, A review of chromium (Cr) epigenetic toxicity and health hazards, Page 4, Copyright (2023), with permission from Elsevier.

8-hydroxy-2'-deoxyguanosine (8-OHdG)

ROS can oxidize purine and pyrimidine bases, with guanine being particularly susceptible due to its higher oxidation potential compared to cytosine, thymine, and adenine[44]. Hydroxyl radicals readily interact with guanine residues, targeting the C-8 position of the nucleotide base guanine and forming radical adducts such as 8-OHdG (Figure 3), which is a widely recognized biomarker of ROS-induced toxicity and carcinogenicity[45]. Measuring urinary 8-OHdG as an index of oxidative DNA damage after Cr(VI) exposure has been commonly used in epidemiological research, as it provides a non-invasive and stable biomarker[46].

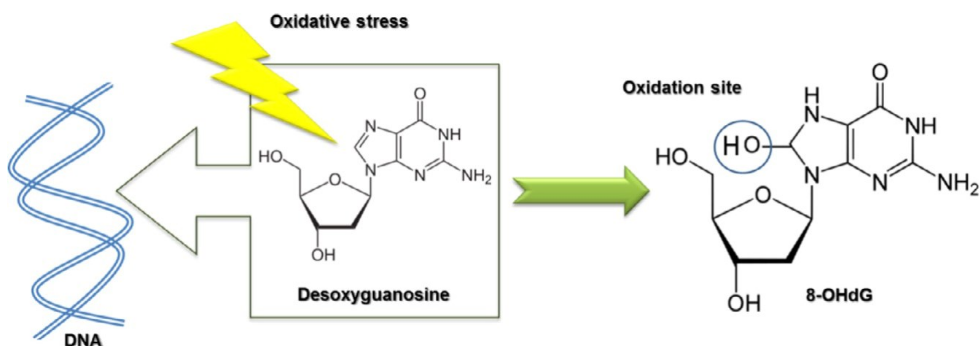

Figure 3. Formation process of 8-OHdG adduct. Reprinted from Advances in Molecular Toxicology, Vol 8, Ahmed Nabile Emam, Emad Girgis, Wagdy K.B. Khalil, Mona Bakr Mohamed, Chapter Five - Toxicity of Plasmonic Nanomaterials and Their Hybrid Nanocomposites, Page 185, Copyright (2014), with permission from Elsevier.

Genotoxicity

Genotoxicity refers to the ability to interact with DNA in cells, altering its structure and function, as well as disrupting DNA repair mechanisms[47]. Cr(VI) has been shown to cause DNA damage, gene mutations, and chromosome aberrations in both *in vitro* and *in vivo* studies[48]. Cr(VI)-induced DNA damage can indirectly be attributed to the production of ROS. The interaction of DNA with ROS causes oxidative damage to its structure[49]. This damage can result in mutations, strand breaks, or modifications to the DNA bases, ultimately impairing the integrity of the genetic material[50]. Additionally, ROS can disrupt the function of DNA repair mechanisms by direct oxidative damage to repair proteins or by overloading repair pathways, preventing the proper repair of DNA damage[51-53]. Therefore, 8-OHdG, which results from oxidative damage to DNA, is widely used as a biomarker of oxidative stress and DNA damage[54]. On the other hand, during the reduction of Cr(VI) to Cr(III), reactive Cr intermediates, including tetravalent Cr (Cr(IV)) and pentavalent Cr (Cr(V)), are generated and accumulated[55]. These intermediates play a significant role in the genotoxicity of Cr(VI). The intermediate Cr species and Cr(III) can form DNA adducts and DNA-protein adducts (*e.g.*, DNA-amino acid cross-links) that may give rise to DNA single- and double-strand breaks (Figure 4)[12].

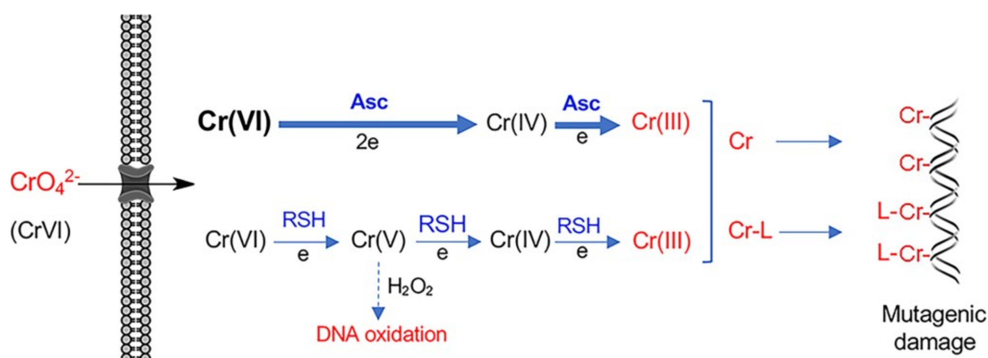

Figure 4. Metabolism and formation of DNA damage by Cr(VI). Reprinted from Advances in Pharmacology, Vol 96, Casey Krawic, Anatoly Zhitkovich, Chapter Two - Chemical mechanisms of DNA damage by carcinogenic chromium(VI), Page 40, Copyright (2023), with permission from Elsevier. Asc, ascorbate; RSH, nonprotein thiols (e.g., glutathione and cysteine).

Micronuclei in peripheral blood reticulocytes (MNRET)

Micronuclei (MN) are extranuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that were not incorporated into the nucleus after cell division[56]. These structures persist into interphase if they fail to be reincorporated into the primary nucleus following the completion of mitosis or meiosis[57]. MN can be induced by DNA damage, chromosomal aberrations and defects in the cell repair machinery[58]. MN in RBC originates from chromosome missegregation during mitosis in nucleated erythroblasts within the bone marrow (Figure 5)[59]. As the mature erythroblast expels its nucleus, the resulting reticulocyte retains the MN and eventually exits the bone marrow, entering the bloodstream[59]. The measurement of MNRET by flow cytometric analysis is regarded as a sensitive, high-throughput method for assessing the genotoxic potential of chemicals in biomonitoring studies[9].

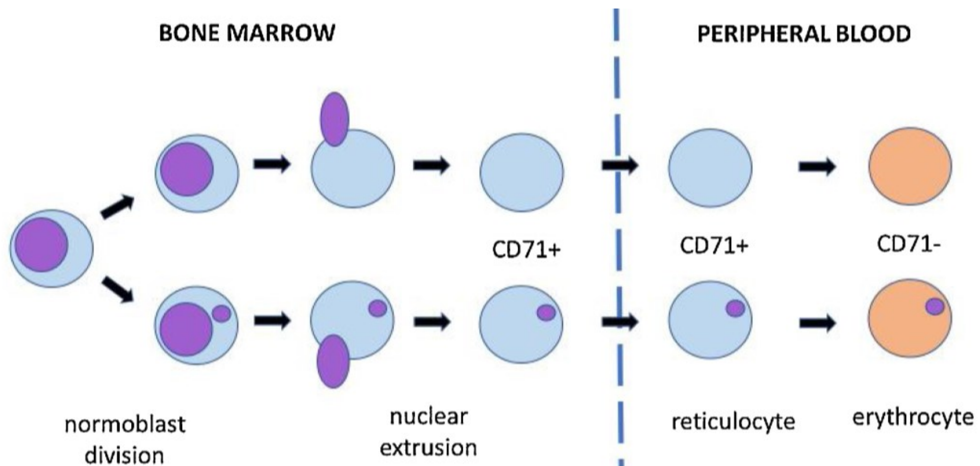

Figure 5. The formation of micronuclei in peripheral blood reticulocytes. Reprinted from Mutation Research/Reviews in Mutation Research, Vol 786, Michael Fenech, Siegfried Knasmueller, Claudia Bolognesi, Nina Holland, Stefano Bonassi, Micheline Kirsch-Volders, Micronuclei as biomarkers of DNA damage, aneuploidy, inducers of chromosomal hypermutation and as sources of pro-inflammatory DNA in humans, Pages 4, Copyright (2020), with permission from Elsevier. CD71, cluster of differentiation 71.

Mitochondrial DNA copy number (mtDNA-cn)

Mitochondria are cytoplasmic organelles known as the powerhouse of the cell, essential for metabolism and involved in critical processes such as adenosine triphosphate production, apoptosis, and the biosynthesis of macromolecules (such as lipids and heme)[60]. Mitochondrial DNA (mtDNA) is a circular, double-stranded, haploid DNA molecule[61]. Each mitochondrion contains multiple copies of mtDNA within the mitochondrial matrix[62]. MtDNA-cn reflects the amount of mtDNA per cell and is present in varying quantities, ranging from one to several thousand copies[63]. While mtDNA-cn is not a direct indicator of mitochondrial function, its

levels are closely associated with changes in mitochondrial membrane potential, energy reserves, and oxidative stress (Figure 6)[64]. In epidemiological studies, mtDNA-cn is recognized as a biomarker that reflects mitochondrial damage and dysfunction[65]. In general, lower mtDNA-cn has been associated with impaired mitochondrial function[66], whereas higher mtDNA-cn is often interpreted as reflecting enhanced bioenergetic capacity[67].

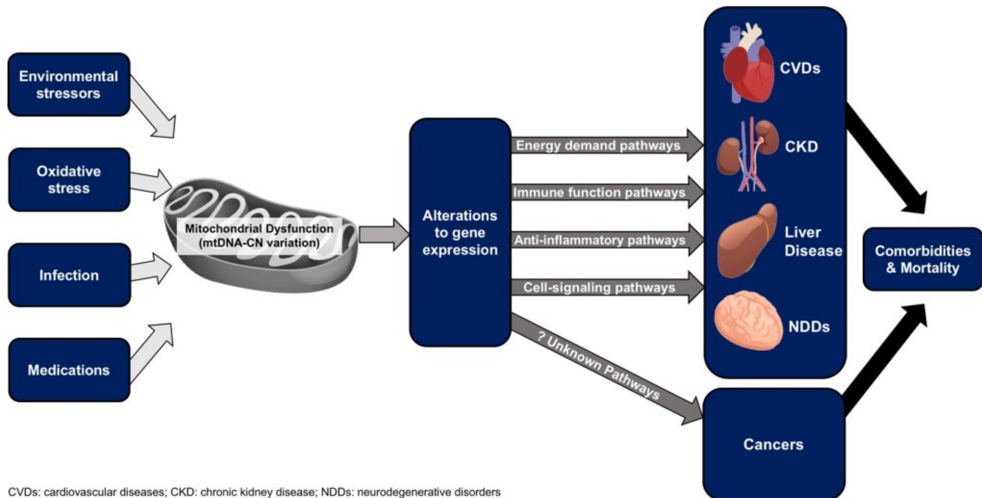

Figure 6. Summary of the proposed relationship between mtDNA-cn and disease. Permission to reuse via license: CC BY-NC-ND 4.0. Christina A. Castellani, Ryan J. Longchamps, Jing Sun, Eliseo Guallar, Dan E. Arking, Thinking outside the nucleus: Mitochondrial DNA copy number in health and disease. Mitochondrion vol. 53 (2020): 214-223. doi:10.1016/j.mito.2020.06.004. mtDNA-CN, mitochondrial DNA copy number; CVDs, cardiovascular diseases; CKD, chronic kidney disease; NDDs, neurodegenerative diseases

Telomere length (TL)

Telomeres are highly specialized chromatin structures consisting of tandem repeats of the TTAGGG sequence located at the ends of linear chromosomes[68]. These functional non-coding sequences, aided by shelterin proteins, help maintain chromosome stability and safeguard them against degradation and damage[69]. Telomeres shorten with each cell division due to the inherent limitations of DNA polymerase in fully replicating chromosome ends[70]. Telomere length decreases with age, and progressive shortening of telomeres triggers senescence and apoptosis in somatic cells, impacting an individual's health and lifespan[71]. Various stressors, including psychological stress, inflammation, and oxidative stress, have been shown to accelerate telomere shortening[72,73]. While shorter telomeres are recognized as markers of poor health and advanced biological age, longer telomeres can allow additional cell divisions, support cellular immortality, accumulate genetic alterations, and contribute to abnormal cell proliferation and tumor development[74,75].

Epigenetic modulation

Epigenetic modulations, including DNA methylation, non-coding RNAs (ncRNAs), and histone modifications, play a crucial role in regulating gene expression without changing the DNA sequence[76]. DNA methylation (changing the activity of a DNA segment) and histone modification (affecting the ease of DNA being transcribed) govern gene transcription, and post-transcriptional regulation is mediated by mechanisms such as ncRNAs[77,78]. These layers of epigenetic control form an intricate feedback network that shapes gene expression[79]. Unlike genomic alterations, epigenetic changes are typically reversible and can be rapidly adjusted[80].

DNA methylation

DNA methylation, catalysed by DNA methyltransferases, is the covalent addition of a methyl group to the carbon-5 position of cytosine, forming 5-methylcytosine, often found within cytosine-guanine (CpG) island regions (Figure 7)[81]. DNA methylation and demethylation are dynamic processes that play a crucial role in regulating gene expression[82]. DNA methylation mainly induces gene silencing by recruiting gene-repressive proteins or preventing transcription factors from binding to DNA[82]. Conversely, DNA demethylation typically leads to the reactivation of genes that were silenced by DNA methylation[83]. DNA methylation and demethylation are involved in various cellular processes, including apoptosis, cell cycle, cell proliferation, invasion and migration, due to their crucial role in gene regulation[84]. Abnormal DNA methylation and demethylation have been implicated in various human diseases, including cancer[85]. For instance, hypermethylation of tumor suppressor promoter region results in the loss of expression of tumor suppressive transcript, leading to an increased risk of tumor development[86]. Global DNA hypomethylation can, on the other hand, play a significant role in cancer development by promoting genomic instability and increasing susceptibility to genetic mutations[87].

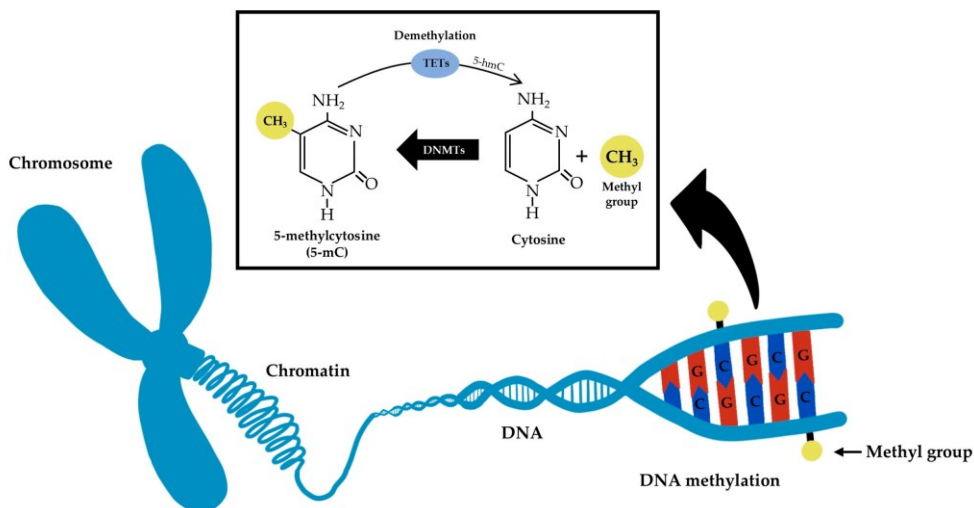

Figure 7. Schematic representation of DNA methylation and demethylation molecular mechanisms. Permission to reuse via license: CC BY 4.0. Ana Valente, Luís Vieira, Maria João Silva, Célia Ventura, The Effect of Nanomaterials on DNA Methylation: A Review. Nanomaterials (Basel, Switzerland) vol. 13,12 1880. 17 Jun. 2023, doi:10.3390/nano13121880. TETs, ten-eleven translocation enzymes; DNMTs, DNA methyltransferases.

NcRNAs

NcRNAs are RNAs that do not translate into proteins, and they are primarily classified based on their length into long ncRNAs (lncRNAs, greater than 200 nucleotides (nt)) and small ncRNAs (less than 200 nt)[88]. LncRNAs are a class of linear ncRNAs that mainly function through regulating chromatin remodeling, transcription, pre-messenger RNAs splicing, mRNA translation, micro RNAs (miRNAs) processing and miRNA functions[89]. LncRNAs are involved in various cellular functions and biological processes, including cell differentiation, proliferation, apoptosis, cell cycle regulation, metabolism, migration, and invasion[90]. MiRNAs are short, single-stranded ncRNAs ranging from 18 to 25 nt in length[91]. MiRNAs regulate gene expression by binding to seed sequences in the 3′-untranslated region of target mRNAs, leading to their degradation or translational inhibition (Figure 8A)[92]. MiRNAs are estimated to regulate approximately 30% of human protein-coding genes[93]. A single miRNA can regulate multiple transcripts, while a single mRNA can be targeted by multiple miRNAs, creating a complex regulatory network[94]. MiRNAs are involved in nearly all critical biological processes, including cell apoptosis, differentiation, metabolism and proliferation[95]. Dysregulation of miRNAs plays a key role in metal carcinogen-induced malignant cell transformation, tumor growth, and angiogenesis[96]. Carcinogens can increase the expression of tumor-promoting genes by inhibiting miRNAs, leading to reduced translation repression[97].

The lncRNA-miRNA-mRNA regulatory network is a molecular hypothesis that explains the functional relationship between lncRNA and miRNA: lncRNAs could act as competing endogenous RNAs to sponge miRNAs by binding and sequestering miRNAs away from their target mRNAs (Figure 8B). This subsequently weakens the impact of miRNAs on downstream mRNAs[98].

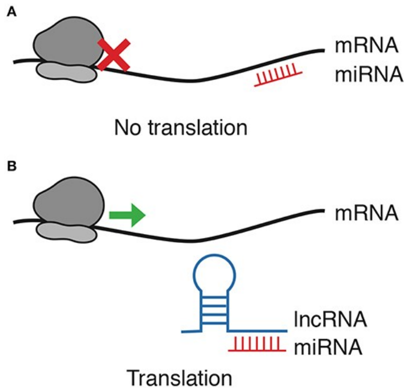

Figure 8. lncRNA/miRNA/mRNA axis regulation. A. miRNAs block translation by binding mRNA. B. As lncRNAs function as decoys for miRNAs, mRNA translation is allowed. Permission to reuse via license: CC BY 4.0. Eduardo López-Urrutia, Lilia P Bustamante Montes, Diego Ladrón de Guevara Cervantes, Carlos Pérez-Plasencia, Alma D Campos-Parra, Crosstalk Between Long Non-coding RNAs, Micro-RNAs and mRNAs: Deciphering Molecular Mechanisms of Master Regulators in Cancer. Frontiers in oncology vol. 9 669. 25 Jul. 2019, doi:10.3389/fonc.2019.00669.

Aim

The overall aim of this thesis was to characterize the current level of occupational exposure to Cr(VI) in Sweden and evaluate the toxicity and health risks.

Specific aims

Paper I

To characterize Cr(VI) exposure at different workplaces by air monitoring of inhalable Cr(VI) and biomonitoring of Cr in urine and RBC, and to identify adequate monitoring methods for Cr(VI) exposure.

Paper II

To investigate the effect of Cr(VI) on oxidative DNA damage, genetic and epigenetic alterations in occupationally exposed workers.

Paper III

To measure the expression of lung cancer-related circulating ncRNAs and to assess associations between ncRNAs expression and Cr exposure and evaluate correlations between the ncRNAs.

Paper IV

To explore the association of miRNAs with occupational exposure to Cr(VI), characterize circulating miRNA expression profile and identify candidate protein-coding target genes of the Cr-associated miRNA changes.

Materials and methods

Study design

The SafeChrom project of Cr(VI) exposure in the Swedish work environment was carried out by all seven Occupational and Environmental Medicine clinics in Sweden (Lund, Gothenburg, Linköping, Örebro, Stockholm, Uppsala, and Umeå) in collaboration with their corresponding university divisions. The occupational exposure assessment, along with the air and biological sampling, was performed by standard operating procedures (SOPs) used by all partners. SafeChrom was designed to be as similar as possible to the European Human Biomonitoring Initiative (HBM4EU) chromates study protocol[99]. The recruitment of exposed workers was performed between June 2021 and May 2022. Controls were recruited between March 2022 and October 2022.

Study participants and ethics

The inclusion criteria for both exposed workers and controls were the following: being 20–68 years of age, and non-smoker >6 months (as tobacco smoke may contain Cr(VI)[100]) prior to participation. The exposed workers should have potential exposure to Cr(VI), while controls should not have had potential exposure to Cr(VI) or other genotoxic agents via work.

Occupational hygienists approached 44 companies where workers might be exposed to Cr(VI). Of these, 14 companies located throughout Sweden, from the northern to the southern regions, consented to take part in the study, corresponding to a participation rate of 31.8% (Figure 9). The companies were categorised into four groups: (i) manufacture/processing of metal products, (ii) steel production, (iii)bath plating and (iv) non-categorised (one of the companies had two different divisions and was thus categorised into both steel production and manufacture/processing of metal products). In total, one hundred and sixteen exposed workers were included in the project and subsequently categorised into four groups: welding, process operation, machining, and others. Additionally, seventy-two controls participated in the project.

For exposed workers, the air sampling and biological sampling were performed on the same day, and this was when the study participants had worked for at least three previous consecutive days (for those who worked Monday to Friday, sampling was carried out on Wednesday at the earliest). For controls, biological sampling was possible on each working day.

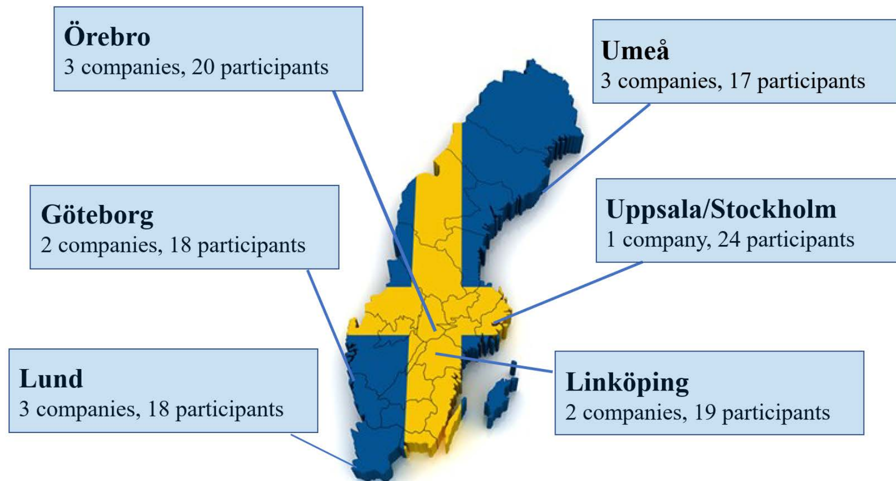

Figure 9. Recruitment of exposed workers. Adapted from Geografi åk 4 – Sverige (partially reproduced), by Skolbanken/Unikum, 2024. Retrieved from https://skolbanken.unikum.net/plans/plan/5430678696.

All study participants answered the same questionnaire to collect basic information, lifestyle factors, disease history, and work details, except for questions relating to work tasks that differed between exposed workers and controls. All study participants gave informed written consent to participate in this project. This project was approved by the Swedish Ethical Review Authority (Dnr 2021-00641).

Air exposure monitoring

Air sampling

Figure 10. Air sampling. Picture by Zheshun Jiang.

The inhalable Cr(VI) fraction was collected using a conical inhalable sampler (CIS) (Casella, Rutland, United States) mounted with a 37 mm polyvinyl chloride filter, pore size 5 µm (Merck Millipore, Cork, Ireland). Battery-powered sampling pumps were used to provide a flow rate of 3.5 L/min (Figure 10). Due to a temporary shortage of CIS samplers, another type of sampler for inhalable dust was used in ten individuals, who were sampled with a Gesamtstaubprobenahme sampler (GSP) mounted with the same filter and run at the same flow rate. The sampler was placed within the breathing zone. For workers who were wearing respiratory protection equipment (RPE) (*e.g.*, powered air-purifying respirators, full- or half-mask, or filtering half-mask) during sampling, the air outside the RPE was sampled. At least one field blank was collected per sampling day. Field blanks were analysed in parallel with the samples. One pump did not work during sampling; thus, 115 valid inhalable Cr(VI) filter samples were collected.

Chemical analysis of air samples

Filter samples of inhalable Cr(VI) were sent for analysis to the Occupational and Environmental Medicine Laboratory, University Hospital, Örebro. The samples were analysed by ion chromatography with conductivity detection (Thermo Fisher Scientific, GmbH, Bremen, Germany, Dionex ICS-2100) using a method modified from the National Institute for Occupational Safety and Health (NIOSH)[101]. The limit of detection (LOD) was 0.08 µg/sample. Due to a delayed delivery of solid phase extraction columns, four samples were analysed by ALS Scandinavia AB in Luleå by a method based upon SS-EN ISO 17294-2:2016[102] and EPA Method 200.8:1994[103] using inductively coupled plasma mass spectrometry (ICP-MS). The ALS laboratory used the limit of quantification (LOQ) as the reporting limit of the applied method (0.3 µg/sample).

Biological exposure monitoring

Sampling of blood and urine

The biological sampling was the same for the exposed workers and controls. A urine sampling kit (including instructions, two acid-washed tubes, and one acid-washed cup) for the morning urine (pre-shift urine) was sent out to every participant before the sampling day (Figure 11). On the day of the visit, trained nurses collected the pre-shift urine sample and, after the participants had worked for at least 4 h, they collected blood and after-shift urine. Blood samples were collected in four vacutainer tubes (Becton, Dickinson and Company, Plymouth, UK): two sodium-heparin tubes for analysis of metals and MNRET, one vacutainer EDTA tube for analysis of biomarkers of genotoxicity and DNA methylation, and one PAXgene blood RNA tube for analysis of gene expression level. Blood samples were also collected in a VACUETTE Serum Separator Clot Activator tube (Greiner Bio-One GmbH, Kremsmünster, Austria) containing an inert gel, and in another vacutainer EDTA tube, which, following centrifugation to separate serum and plasma, respectively. Urine and blood samples were kept at 4 °C and transported to the laboratory at the Division of Occupational and Environmental Medicine, Lund University, and then stored at -20 °C until analysis. Serum and plasma samples were kept at -20 °C and transported to the same lab, then stored at -80 °C until analysis. Sodium-heparin tubes for MNRET were transported to the Finnish Institute of Occupational Health and stored refrigerated until processing and analysis.

Figure 11. Collecting urine samples. Picture by Eva Assarsson, used with permission.

Of the exposed workers, three participants only provided air samples. Six participants abstained from providing blood samples shipping to Finland; in those six, three participants also abstained from providing the other three tubes of blood samples, two abstained from providing plasma and serum, and one abstained from providing a morning urine sample.

Chemical analysis of blood and urine samples

To avoid haemolysis, plasma and RBC separation was conducted following the method described by Devoy *et al*[27]. Since occupational exposure to Cr(VI) generally occurs together with other metals in industrial settings, and such co-exposures may influence health risks, confound exposure–response analyses, and support exposure characterization, Cr, and a panel of metals—manganese, cobalt, nickel, copper (Cu), zinc, selenium, cadmium (Cd), antimony, mercury, and lead—were quantified in RBC and urine using ICP-MS (Thermo Fisher Scientific, iCAP Q) equipped with collision cell with kinetic energy discrimination and helium as collision gas. A sample volume of 100 µL (RBC) and 250 µL (urine) was diluted 20 times with an alkaline solution according to Barany *et al*.[104] and analysed in peak-jumping mode, with scandium, rhodium, terbium, and iridium used as internal standards. The detection limits were calculated as three times the standard deviation (SD) of the blank and were 0.20 µg/L for Cr in both blood and urine. All analysed samples were prepared and measured in duplicate, and the mean value was used in subsequent statistical analyses.

Measurement of creatinine and density in urine

Density and creatinine were measured in all urine samples for correction of dilution. Creatinine was measured with Atellica (Siemens Healthcare Diagnostics, Munich, Germany; accredited analysis) at the Clinical Chemistry University Hospital, Lund. The density was measured with a hand-held refractometer (30PX; Mettler Toledo, USA). The density adjustment was calculated using the following formula: $C_{(density\text{-}adjusted)} = C \times (1-\rho_{mean})/(1-\rho_{sample\ density})$, where C = the determined Cr concentration in the sample, ρ_{mean} = the mean of the urine density of all participants, and $\rho_{sample\ density}$ = the density of the urine sample. Since creatinine excretion is often higher in men (due to gender differences in muscle mass[105]), density adjustment was more appropriate for the correction of urine dilution. Post-work urinary Cr concentration, adjusted for density (U-Cr), was selected as the biomarker for occupational Cr(VI) exposure in urine in studies II, III, and IV.

Toxicity biomarkers

8-OHdG

The urine samples were analysed for 8-OHdG using liquid chromatography tandem mass spectrometry (LC-MS/MS; QTRAP 5500, AB Sciex, Framingham, MA, USA). 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 before analysis. Each analysed sample batch included a matrix-matched standard curve, six blanks, and nine quality control samples at three concentrations. The 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.

TL and mtDNA-cn

A QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used for extracting genomic DNA from whole blood samples. NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used to determine DNA quantity and purity. DNA Samples above 25 ng/µl were diluted to a concentration of 25 ng/µl.

SYBR green-based real-time quantitative polymerase chain reaction (qPCR) was used to determine TL and mtDNA-cn, as described previously[106]. Briefly, the mtDNA and telomere were measured 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 was prepared with PerfeCTa SYBR Green FastMix (Quantabio, Beverly, U.S.A.) and 0.20 µM mtDNA primers. Master mix for telomere 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.

One reference DNA sample was diluted serially twofold per dilution to produce 6 concentrations of 16 - 0.5 ng/µL for the standard curve. The standard curve, samples (with 2.5 µl DNA (5 ng/µL)) and one blank were run in triplicates. SDS 2.4.1 software (Thermo Fisher Scientific) calculated the relative quantity of mtDNA, telomere 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.

MNRET

The micronuclei frequency in peripheral blood reticulocytes was assessed by the micronucleus assay using flow cytometry, as described elsewhere[107], and the analyses were conducted at the Finnish Institute of Occupational Health. Briefly, the whole blood samples were stored at 4 °C and processed within 7 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) using a FITC Mouse Anti-human CD71 antibody (BD Biosciences, San Jose, USA). Thereafter, the samples were fixed in 2% paraformaldehyde in PBS with 10 µg/mL of sodium dodecyl sulfate (Sigma-Aldrich, Darmstadt, Germany) and kept refrigerated (4 °C) until analysis. Prior to the analysis, DNA was stained with Hoechst 33342 (Invitrogen, 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 micronuclei. A CytoFlex S flow cytometer and CytExpert software version 2.3 (Beckman Coulter, Brea, CA, USA) were used for data acquisition and analysis. The micronuclei frequency was quantified as per-mille of micronucleated + CD71 reticulocytes from all analysed + CD71 reticulocytes. A minimum of 20000 + CD71 reticulocytes per sample was required to ensure reliable data, resulting in the exclusion of four samples.

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). Each PCR reaction contained 12.5 µL of PyroMark PCR Master Mix (2x), 2.5 µL of CoralLoad Concentrate (10x), 0.5 µL of forward primer (10 µM), 0.5 µL of biotinylated reverse primer (10 µM), 8 µL of RNase-free water, and 1 µL of bisulfite-converted DNA. The methylation levels of four candidate genes were analysed in this thesis, including F2R like thrombin or trypsin receptor 3 (*F2RL3*), long interspersed nuclear element-1 (*LINE-1*), O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter and semaphorin 4B (*SEMA4B*). A gradient PCR was initially completed 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) and agarose gel electrophoresis was performed to verify the PCR fragment length.

Pyrosequencing was analysed on the PyroMark Q48 Autoprep Instrument (Qiagen) with PyroMark Q48 Advanced CpG reagents (Qiagen) according to the manufacturer´s protocol. Briefly, 10 µL of biotinylated PCR product was loaded into the PyroMark Q48 disk (Qiagen) together with 3 µL of PyroMark Q48 Magnetic Beads (Qiagen). 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. Sequencing primers, together with the PyroMark Q48 Advanced CpG reagents, were added to the cartridges in the instrument. The results of pyrosequencing were recorded in PyroMark Q48 Autoprep software for further analysis.

Target lncRNA and miRNA expression

Total RNA was isolated from 200 µL of plasma sample using the miRNeasy Serum/Plasma Advanced Kit (Qiagen) according to the manufacturer's protocol. An additional step of DNAse treatment with the RNase-Free DNase Set (Qiagen) was added according to the supplier's instructions to digest DNA during RNA purification. Total RNA from 200 µL plasma was eluted in a final volume of 20µL of RNAse free water.

Expression levels of three lncRNAs (H19 imprinted maternally expressed transcript (*H19*), metastasis associated lung adenocarcinoma transcript 1 (*MALAT1)* and non-coding RNA activated by DNA damage (*NORAD*)) and four miRNAs (*miR-142-3p*, *miR-15b-5p*, *miR-3940-5p* and *miR-451a*) were detected by qPCR. For lncRNAs, total RNA was reverse transcribed using the high-capacity cDNA reverse transcription Kit (Thermo Fisher Scientific). TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assay (Thermo Fisher Scientific) were used to measure expression levels of target lncRNAs in 7900HT Fast Real-Time PCR System according to the manufacturer's protocol. The assays used were as follows: *GAPDH* (Assay ID Hs99999905_m1), *H19* (Hs00399294_g1), *MALAT1* (Hs00273907_s1) and *NORAD* (Hs05023182_g1). For miRNAs, total RNA was reverse transcribed using the miRCURY LNA RT Kit (Qiagen) according to the manufacturer's protocol. UniSp6 RNA (spike-in RNA) was added as an exogenous control. The miRCURY LNA SYBR Green PCR Kit and miRCURY LNA miRNA PCR Assay (Qiagen) were used to measure the expression levels of target miRNAs in the 7900HT Fast Real-Time PCR System according to the manufacturer's protocol. The assays used were as follows: *UniSp6* (GeneGlobe ID - YP00203954), *hsa-miR-142-3p* (YP00204291), *hsa-miR-15b-5p* (YP00204243), *hsa-miR-3940-5p* (YP02114897), *hsa-miR-451a* (YP02119305) and *hsa-miR-23a-3p* (YP00204772).

MiRNA sequencing and bioinformatic analysis

To evaluate the expression profile of circulating miRNAs, RNA samples were sent to SciLifeLab National Genomics Infrastructure, Stockholm for library preparation and miRNA Sequencing. The libraries were constructed following the manufacturer's instruction of QIAseq miRNA Library Kit (Qiagen). Libraries that passed quality control (QC) were then pooled and sequenced on two lanes of an SP-100 (v1.5) flowcell through the Illumina NextSeq 6000 sequencing platform (Illumina, San Diego, USA).

Raw reads of sequencing were preprocessed by the nf-core/smrnaseq pipeline (https://nf-co.re/smrnaseq)[108] and converted to a count matrix. For comparison between exposed workers and controls, the identification of differentially expressed miRNAs (DEMs) was based on adjusting the linear model with empirical Bayes moderated t-statistic, conducted by the limma package[109,110]. MiRNAs with $|\log_2$ fold change$| \geq 2$ and $P < 0.05$ were considered to be differentially expressed.

Target gene prediction was generated using miRTarBase[111] and TargetScanHuman[112] databases. Putative target genes listed in miRTarBase, as validated by either reporter assay, Western blot analysis, or qPCR, were selected. During the TargetScanHuman screening process, target genes with a Cumulative weighted context++ score higher than -0.8 were eliminated[113]. Ultimately, the final set of target genes for DEMs was determined by assembling two databases and were selected for enrichment analysis and protein-protein interactions (PPIs) network.

The Gene Ontology (GO) category analysis provides a comprehensive annotation for functional attributes of genes, encompassing their biological process, cellular component, and molecular function[114]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) employs a pathway-centric approach to gene classification, whereby genes with similar functions are organized into distinct pathways[115]. The GO and KEGG enrichment analyses were performed using SRplot (https://www.bioinformatics.com.cn/SRplot, an online platform for data analysis and visualization) to enrich the target genes of DEMs[116]. The thresholds for enrichment analyses were set as *P*-value less than 0.05. Histograms, bubble maps and chord diagrams were generated by Bioinformatics. Construction and visualization of the miRNA-target gene interaction network were carried out using Cytoscape software v.3.10.2[117]. PPIs were evaluated using the STRING 12.0 database[118]. Interaction scores for PPIs above 0.9 were considered statistically significant[119]. Unconnected nodes were removed and Cytoscape was used to establish and visualize the PPIs network.

mRNA expression

PAXgene tubes were incubated for at least 2 h and total RNA was isolated using the PAXgene Blood RNA Kit (Qiagen), according to the manufacturer's protocol. RNA

samples were diluted to 40 ng/µL and 13 µL of the extracted RNA was reverse transcribed using the QuantiNova Reverse Transcription Kit (Qiagen). Briefly, template RNA was mixed with gDNA removal mix (provided with the reverse transcription kit) and incubated for 2 min at 45 °C and then mixed with reverse transcriptase and reverse transcription mix. The reverse-transcription reaction was diluted 10 times before qPCR. The expressions of genes were measured using QuantiNova Probe PCR Kit (Qiagen) in a QuantStudio 5 Real-Time PCR Systems (Applied Biosystems). The qPCR amplification mixtures contained 5 µL QuantiNova Probe PCR Master Mix, 0.05 µL ROX Reference Dye, 3.95 µL template cDNA and 1 µL QuantiNova LNA Probe PCR Assay (Qiagen). Samples were run in triplicate for each assay.

The use of generative AI

In this thesis, generative AI tools were used only for language editing, including grammar and textual flow. All academic and scientific content was independently produced by the author.

Statistical analysis

For inhalable Cr(VI), concentrations below the LOD (0.08 µg/sample) or below the LOQ (0.3 µg/sample) were substituted by values equal to half of the LOD (0.04 µg/sample) or LOQ (0.15 µg/sample)[120]. Age was calculated based on birth and recruitment dates. Body mass index (BMI) was obtained using the formula BMI = weight in kilograms/ $(\text{height in meters})^2$. Exposure to Cr(VI) among workers in Sweden was estimated using a job-exposure matrix (JEM) linked to occupational register data from Statistics Sweden, with adaptations to Swedish working conditions made by Wiebert and Tinnerberg *et al.*[121]

Normalized quantification cycle values (referred to as ΔCt, where Ct is the cycle threshold) were calculated as the difference between the mean Ct of the candidate miRNAs and the Ct of *Unisp6*, the difference between the mean Ct of the candidate lncRNAs and *GAPDH,* and the difference between the mean Ct of the candidate mRNAs and *GAPDH*, for miRNAs, lncRNAs and gene expression levels, respectively[122,123]. The relative expression levels were then calculated using the $2^{-\Delta\Delta Ct}$ method[123-125].

Descriptive statistics, including mean, standard deviation (SD), median, geometric mean (GM), 95% confidence interval (95% CI), 5th, 25th, 75th and 95th percentiles (P5, P25, P75 and P95) were calculated. Student's t-test, Mann-Whitney U test,

Kruskal-Wallis test and Wilcoxon signed ranks test were used to compare differences between continuous variables. The Pearson Chi-square test and Fisher's exact test were used to compare differences in distribution of categorical variables between groups. Spearman's correlation was used to examine correlations between variables. 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 biomarkers for Cr(VI) exposure. The models were adjusted for potential covariates and confounders. 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 the formula $Y=\ln(X+1)$ for the methylation level of *MGMT* CpG1 and CpG2[126].

The statistical analyses above were conducted with SPSS 28.0 (IBM SPSS Statistics, NY) and statistical significance (two-tailed) was denoted at *P* value <0.05. Limma was implemented with the R package "limma" (version 3.62.2).

Main results

Paper I

One hundred and fifteen valid inhalable Cr(VI) results were obtained and 113 workers completed the questionnaire and donated biological samples. The exposed workers and controls were similar in BMI, smoking history, and leisure activities with Cr. Exposed workers were younger, more likely to be male and less likely to drink tea than the controls (P<0.05). There were 74 air samples (64.3%) below 0.25 µg/m^3, 20 samples (17.4%) between 0.25 and 1 µg/m^3, 13 samples (11.3%) between 1 and 5 µg/m^3 and eight samples (7%) higher than 5 µg/m^3. Both pre-shift (median, 0.51 density-adjusted µg/L) and post-shift (0.60) urinary Cr in the exposed workers were significantly higher compared with controls (0.10 for both pre-shift and post-shift urine) (P<0.001). The median RBC-Cr in the exposed workers (0.73 µg/L) was significantly higher than in the controls (0.53 µg/L). Notably, among exposed workers with inhalable Cr(VI) concentrations ≤0.25 µg/m^3 (n=70), 15 (21.4%) exhibited urinary and RBC Cr concentrations above the P95 of the controls. There were strong correlations between pre-shift and post-shift urinary Cr concentrations in exposed workers (Spearman's rank correlation coefficients (r_S) =0.89). Inhalable Cr(VI) correlated with post-shift urinary Cr (r_S=0.64) and RBC-Cr (r_S=0.53). Urinary Cr correlated with RBC-Cr (r_S=0.72). In multivariate regression analysis, post-shift urinary and RBC Cr concentrations were significantly higher in the exposed workers compared with the controls before and after adjustment for potential covariates and confounders. In addition, workers with inferred non-acceptable local exhaust ventilation (LEV) showed significantly higher exposure levels compared with those with inferred acceptable LEV, including higher inhalable Cr(VI), post-shift urinary, and RBC Cr concentrations. However, workers with inferred correct use of RPE were exposed to higher levels of Cr(VI) compared with those without RPE. Finally, based on the Swedish job-exposure matrix, approximately 17,900 workers are estimated to be currently occupationally exposed to Cr(VI).

Paper II

In this study, we analysed effect biomarkers in exposed workers and controls, including 8-OHdG in urine, mtDNA-cn, TL, MNRET and DNA methylation of *F2RL3*, *LINE-1*, *MGMT* and *SEMA4B*. Exposed workers had significantly higher 8-OHdG (median, 9.42 density adjusted µg/L), longer TL (0.80) and higher average methylation level of *MGMT* (1.91%) compared with controls (7.77 µg/L, 0.70 and 1.78%, respectively). MtDNA-cn (1.56) and MNRET (1.98‰) were significantly lower in the exposed workers compared with controls (1.68 and 2.49‰, respectively). Age was significantly negatively correlated with TL and *F2RL3* methylation. TL was significantly positively correlated with U-Cr; 8-OHdG was positively and MNRET was negatively correlated with RBC-Cr; *MGMT* was significantly positively correlated with U-Cr and RBC-Cr. In the general linear regression models, exposed workers had significantly higher 8-OHdG, TL, methylation level of *MGMT*, and lower MNRET. Multiple linear regression models were also used to examine associations between effect biomarkers and RBC-Cr. Only *MGMT* methylation showed a significant association with RBC-Cr (positive).

Paper III

In this study, we analysed the expression levels of three lncRNAs (*H19*, *MALAT1*, *NORAD*) and four miRNAs (*miR-142-3p*, *miR-15b-5p*, *miR-3940-5p*, *miR-451a*) in plasma from the exposed workers and controls. Exposed workers had a non-significantly higher expression of *H19* compared with controls. The expressions of *MALAT1* and *NORAD* were found to be significantly lower in exposed workers than in controls. For miRNAs, the expression of all four miRNAs was found to be significantly lower in Cr(VI) exposed workers compared with controls. Significantly negative correlations were found between U-Cr with *miR-142-3p* (r_S=-0.20), *miR-3940-5p* (r_S=-0.25) and *miR-451a* (r_S=-0.33). Furthermore, *H19* was positively correlated (r_S=0.16) with RBC-Cr, and *MALAT1* (r_S=-0.16), *NORAD* (r_S=-0.26), *miR-142-3p* (r_S=-0.38), *miR-15b-5p* (r_S=-0.28), *miR-3940-5p* (r_S=-0.26) and *miR-451a* (r_S=-0.36) were negatively correlated with RBC-Cr. In a general linear regression model, the exposed workers had significantly lower expressions of *MALAT1*, *NORAD* and all four miRNAs. In addition, *H19* was significantly correlated with *miR-142-3p* (r_S=-0.33) and *miR-15b-5p* (r_S=-0.30). *NORAD* was significantly positively correlated with all four miRNAs (r_S=0.46 for *miR-142-3p*; r_S=0.42 for *miR-15b-5p*; r_S=0.17 for *miR-3940-5p*, and r_S=0.33 for *miR-451a*).

Paper IV

In this study, we characterized the miRNA expression profile in Cr(VI) exposed workers and identified dysregulated miRNAs linked to carcinogenesis. A total of 2100 miRNAs were detected in plasma samples, including 1964 in exposed workers and 1810 in controls; 1674 miRNAs were expressed in both groups. A total of 59 DEMs were identified in the exposed workers, in which 21 miRNAs were up-regulated, and 38 miRNAs were down-regulated. Of all DEMs, five were significantly correlated with inhalable Cr(VI), 23 with U-Cr, and 22 with RBC-Cr, and the r_S were low to moderate (range -0.37 to 0.18). A total of 169 target genes associated with up-regulated DEMs and 609 target genes associated with down-regulated DEMs were identified. The KEGG pathway enrichment analysis indicated that target genes for both up- and down-regulated DEMs were significantly enriched in three terms: miRNAs in cancer, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). PPIs encoded by the target genes identified from the STRING database showed a high number of interactions and confirmed the results obtained by enrichment analysis, that the majority of central proteins are involved in these three KEGG pathways. The hub genes were identified as *BAX*, *CCND1*, *CCNE2*, *CDK4*, *E2F1* and *MYC*. Blood mRNA expression levels of hub genes were analysed for their associations with Cr(VI) by comparing their relative expression between Cr(VI) exposed workers and controls. The expressions of *CCNE2*, *CDK4* and *E2F1* were found to be significantly higher in exposed workers as compared with controls.

Discussion

Occupational exposure assessment of Cr(VI)

In occupational settings, external exposure is traditionally assessed through occupational hygiene measurements like air monitoring, which is considered the gold standard. However, internal exposure assessment through biomonitoring—typically analysing workers' urine or blood—has also been a longstanding practice in occupational health[127]. Biomonitoring provides valuable additional insights, as it comprehensively reflects an individual's actual exposure when conducted properly. Unlike external monitoring alone, it accounts for all relevant exposure pathways, including inhalation, skin absorption, and unintentional ingestion via hand-to-mouth contact[128]. Biomonitoring can also be a useful tool for assessing the effectiveness of RPE. In addition, urine sampling and analysis offer a practical and feasible approach for monitoring exposure in larger groups of workers.

Inhalable Cr(VI)

In literature, airborne Cr exposure has been monitored as inhalable total Cr, inhalable Cr(VI), respirable total Cr, or respirable Cr(VI). Additionally, several studies have measured airborne Cr(VI) without specifying which particle fraction was measured. A study conducted in Iran reported a mean value of 2 µg/m^3 for welding and higher mean value of 5 µg/m^3 when welding inside confined spaces[129]. Studies in India and Egypt measured airborne Cr(VI) in the leather tanning industry with a mean value of 21 µg/m^3 and 10.4 µg/m^3, respectively[130,131]. The median concentration of inhalable Cr(VI) in HBM4EU chromates study (0.43 µg/m^3) was higher compared with our study (0.1 µg/m^3), but their P95 value was lower (5.13 µg/m^3 *vs.* 8.03 µg/m^3)[132]. The same trend was also observed if only welders were considered. The median and P95 of inhalable Cr(VI) for welders in HBM4EU chromates study were 0.5 and 4.06 µg/m^3, while in our study, they were 0.1 and 14.73 µg/m^3. The lower median value in our study suggests that, on average, the inhalable Cr(VI) concentrations among exposed workers were relatively low in Sweden. However, the higher P95 value indicates a higher upper range of exposure. When comparing with the SAM-Krom project[133], a sister project conducted in Denmark, similar overall exposure levels were observed in the SAM-Krom and SafeChrom studies. In SAM-Krom, the geometric mean inhalable Cr(VI)

concentration among exposed workers was 0.26 µg/m³ (95% CI: 0.12–0.57), whereas the corresponding geometric mean in the present SafeChrom study was 0.15 µg/m³ (95% CI: 0.11–0.21). In both studies, the majority of measured inhalable Cr(VI) concentrations were below the respective national occupational exposure limits, although higher task-specific exposures were reported in SAM-Krom, particularly among apprentices performing manual metal arc welding, with geometric mean levels reaching 3.69 µg/m³.

Biomonitoring of Cr(VI)

Urinary Cr

Both France and the Netherlands have a biological limit value (BLV) of 2.5 µg/L of Cr in urine based on their OEL of 1 µg/m³ for Cr(VI) in air, and Finland has a BLV of 0.2 µmol/L (ca. 10 µg/L) in urine corresponding to its OEL of 5 µg/m³ in air[8]. The UK has set a biological monitoring guidance value of 4.6 µg/g creatinine (ca. 6.3 µg/L)[134]. In comparison, two exposed workers (1.8%) in our study exceeded 10 µg/L of urinary Cr and 13 (11.5%) exceeded 2.5 µg/L. In a systematic review of biomonitoring data on occupational exposure to Cr(VI)[8], the median or mean urinary Cr levels were lower in European countries (ranging from 0.96 µg/L to 5.81 µg/L) compared with non-European countries (ranging from 1.66 µg/L to 48.4 µg/L). In HBM4EU chromates study, the median and P95 concentrations of post-shift urinary Cr in exposed workers were 1.7 and 5.1 µg/g creatinine. In our study, the median urinary Cr was 0.55 µg/L, and the creatinine-adjusted median and P95 were 0.41 and 2.12 µg/g creatinine, respectively, showing a lower result than all studies above. In HBM4EU chromates study, reference values were obtained by recruiting controls from both the same companies as the exposed workers (within company controls) and from other companies where no known Cr(VI) exposure occurred (outwith company controls). The urinary Cr in our control group (median and P95, 0.08 and 0.54 µg/g creatinine) is similar to the outwith company controls in HBM4EU chromates study (0.1 and 0.4 µg/g creatinine)[135]. In addition, U-Cr concentrations among exposed workers were lower in the present study (median 0.60 density-adjusted µg/L; P5–P95: 0.10–3.20) compared with those reported in the SAM-Krom study (median 2.42 µg/L; P5–P95: 0.28–58.39)[133]. A similar pattern was observed among controls, with lower median U-Cr concentrations in the present study (0.10 µg/L) than in SAM-Krom (0.40 µg/L).

The primary limitation of the traditional biomonitoring method for assessing Cr(IV) exposure—urinary Cr levels—is its tendency to overestimate Cr(VI) exposure, as it accounts for both Cr(III) and Cr(VI)[8]. Consequently, there is a need for more specific biomarkers, such as Cr levels in RBC, to accurately quantify Cr(VI) exposure.

RBC-Cr

RBC-Cr has been suggested as a more specific biomarker for occupational Cr(VI) exposure, as its concentration may reflect the fraction of Cr(VI) that enters the bloodstream in its non-reduced form[136]. Additionally, RBC-Cr levels may directly correlate with the inhaled dose through the airways[137]. Despite Cr in RBC being considered a specific biomarker of Cr(VI), there is no established BLV for RBC-Cr. RBC-Cr was lower in our study (median, 0.73 µg/L and mean, 0.89 µg/L) compared to welders in a German study (median, 1.95 µg/L)[138], electroplaters in Italy (median, 3.4 µg/L)[136] and China (median, 4.41 µg/L)[139], and chromate production workers in China (mean, 12.45 µg/L). However, the median value of RBC-Cr in our study was in concordance with HBM4EU chromates study (0.73 µg/L) and SAM-Krom study (0.89 µg/L), but HBM4EU chromates study had the highest and SAM-Krom study had the second highest P95 (5.83 µg/L *vs.* 4.92 µg/L *vs.* 2.33 µg/L)[133,140]. With respect to controls, one study in China measured RBC-Cr in 93 controls (median, 1.54 µg/L)[139], HBM4EU chromates study measured 175 controls (median, 0.63 µg/L)[140] and SAM-Krom study had a median RBC-Cr concentration of 0.60 µg/L. The median concentration of RBC-Cr in our controls was 0.53 µg/L, similar to HBM4EU chromates study and SAM-Krom study.

Correlations of exposure markers

Correlations between airborne Cr(VI) levels, urinary Cr levels and RBC-Cr levels provide insight into the efficiency of Cr(VI) absorption, its reduction to Cr(III) within the body, and subsequent metabolic processing and excretion[16]. The positive correlation between inhalable Cr(VI) and urinary Cr (r_S=0.64) in our study indicates that exposures to Cr(VI) occurred mainly via inhalation[141]. Moderate correlations were also found between inhalable Cr(VI) and urinary Cr in welders in Poland (r_S=0.58)[142], electroplaters in Great Britain (r_S=0.62)[25] and in the HBM4EU chromates study (r_S=0.46)[135]. The specific Cr species differ between plating and welding processes. In plating, exposure primarily involves highly soluble Cr(VI) compounds, such as Cr trioxide (chromic acid), in aerosol form[143]. These soluble Cr(VI) particles are readily absorbed in the lungs, reduced to Cr(III) within the body, and rapidly excreted in the urine[16]. However, welding generates ultra-fine and nano-sized Cr oxides, which are encapsulated in welding fumes and exhibit significantly lower water solubility compared to highly soluble chromates[144]. This reduced solubility leads to prolonged lung retention, a delayed and gradual release into the systemic circulation, and consequently, a much slower rate of urinary excretion compared to more soluble Cr compounds[145]. Furthermore, it is important to highlight that a considerable portion of total Cr exposure in chrome plating activities may result from dermal contamination[146]. This complicates the interpretation of U–Cr levels and complicates effective hazard and risk assessment in occupational environments.

To date, only a few studies have investigated the correlation between inhalable Cr(VI) and RBC-Cr concentrations. No correlation (r_S=-0.06; *P*=0.73) was reported for Polish welders[142]. Similarly, in HBM4EU chromates study, no correlation was reported when all groups of workers were combined, but among chrome-platers, the correlation between inhalable Cr(VI) and RBC-Cr was stronger (r_S=0.54)[140]. A similar correlation was found in our study for all exposed workers (r_S=0.53). There is a limited number of studies that have investigated the correlation between urinary and RBC Cr. An Italian study found a significant positive correlation between urinary and RBC Cr (r_S=0.74) in chrome-platers, while the correlation was weak (r_S=0.21) in chromate production workers in China[136,147]. Likewise, a poor correlation between urinary and RBC Cr was found in HBM4EU chromates study, but when only considering chrome-platers, the r_S coefficient became higher[132]. The correlation between urinary and RBC Cr in our study is relatively strong (r_S=0.72). Since air concentrations can vary between days, the short-term nature of total urinary Cr may mislead exposure to Cr(VI). A better strategy when assessing long-term Cr(VI) exposure would be repeated air measurement combined with biomonitoring of RBC-Cr.

The toxicity of Cr(VI)

Oxidative stress

Cr(VI)-induced oxidative stress is recognized as a key molecular mechanism underlying Cr(VI) toxicity and carcinogenesis[3]. 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[148,149]. 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[46]. Our study agrees with previous studies that showed that urinary 8-OHdG concentrations among Cr(VI) exposed workers were higher than controls[46,150-154]. Of these studies, one reported urinary 8-OHdG to be positively associated with airborne Cr, two with urinary Cr, and four with blood Cr. The present study observed a significant positive correlation 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[155]. However, some of the absorbed Cr remains in the body, where it is slowly excreted and may continuously generate oxidative stress[9]. Moreover, U-Cr reflects both Cr(III) and Cr(VI) exposure[16], thus, variation in Cr(III) exposure in the studied occupations may weaken a possible correlation between urinary excreted Cr(VI) and 8-OHdG[156].

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 such as nucleotide excision repair, and not only to oxidative stress[157,158].

Genetic Damage

Cr(VI) and TL

Telomeres help maintain genome stability by protecting against the gradual loss of chromosomal end sequences during cell division and shielding the genome from other forms of cellular damage[159]. Telomeres naturally shorten as part of the aging process, and shorter TL have been linked to non-cancer age-related diseases[160]. Our study also found a significant negative correlation between TL and age. Longer TL has been associated with increased risks for several cancer types. Several studies suggest that longer TL increases lung cancer risk, especially lung adenocarcinoma risk[161-164]. 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)[165]. 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[166]. Consistent with our findings, Ahlers *et al.* observed a significant increase in TL among steel workers short-term exposed to metal-rich particulates[167]. However, a cross-sectional study found a negative association between blood Cr concentrations and TL in chromate production workers[153]. In our dataset, we also observed a negative association between RBC-Cr and TL among exposed workers, though it was not statistically significant. This could be explained by several potential factors, including: (1) a non-linear relationship between TL and Cr(VI) exposure, where low levels of Cr(VI) exposure may exert mild oxidative effects that potentially lengthen telomeres, while higher exposures could induce stronger oxidative stress that overwhelms repair mechanisms and leads to telomere shortening; (2) the presence of competing biological mechanisms, such as enhanced DNA repair pathways and antioxidant responses caused by increased ROS, that influence TL in exposed workers; and (3) the influence of additional confounding variables, such as other occupational exposures, individual health conditions, or lifestyle factors, which may affect the observed relationship between TL and Cr(VI) exposure.

Cr(VI) and mtDNA-cn

Mitochondria are essential organelles for energy production and play a vital role in regulating redox-dependent pathways[168]. *In vitro* studies showed that Cr(VI) can induce mitochondrial bioenergetics perturbation[169], biogenesis imbalance[170] and

mitophagy[171]. Furthermore, it has been found that exposure to Cr(VI) initiates cell apoptosis through a mitochondria-dependent process[172]. This suggests that mitochondria are among the most sensitive organelles to Cr(VI) toxicity. Unlike nuclear DNA, mtDNA is much smaller in size, lacks histone packaging, and has a less efficient DNA repair system[173]. As a result, mtDNA is particularly susceptible to oxidative stress, being more sensitive to such damage compared to nuclear DNA[174]. 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[170]. 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)[175]. 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[176]. It has been reported that mtDNA-cn is reduced in the plasma[177] and cancer tissue[178] 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[177].

Cr(VI) and MN

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[57]. The MN test is 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[179] and in peripheral lymphocytes[150]. The HBM4EU[9] and the Danish SAM-Krom project[180] 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 control group was 1.14‰. In the HBM4EU chromate study, 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 controls could be attributed to several factors, including differences in population characteristics. However, even after adjusting 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. Workers in the agricultural operator had the highest MNRET within the four groups (Kruskal-Wallis H Test, $P<0.01$). Previous research has shown significantly elevated MNRET levels among agricultural workers, likely due to pesticide exposure[181]. 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, other than metals, contributing to the higher MNRET levels.

Epigenetic Alterations

Although intracellular metabolism mediated by ROS and genomic instability are thought to be the primary mechanisms behind Cr(VI)-induced carcinogenesis[182], Cr(VI) has been shown to alter DNA methylation and histone modifications, as well as non-coding RNA expression[183]. 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[41].

Cr(VI) and DNA methylation

Studies have shown that global DNA hypomethylation is linked to Cr(VI) exposure[184,185]. Consistent with our findings, Yang *et al.* reported no changes in *LINE-1* gene methylation levels among chrome plating workers[173]. 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*[186]. 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 fume exposure[187,188]. In addition, the hypomethylation of *F2RL3* may mediate the effect of tobacco on lung cancer risk[189]. Researchers found that Cd exposure caused by smoking triggers differential methylation of *F2RL3*[190,191]. In our study, the methylation level of *F2RL3* showed no significant correlation with RBC-Cr but had a significantly negative correlation with Cd in RBC. As 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 was 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. 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*[192]. 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[193]. The *MGMT* gene encodes DNA repair protein essential for cellular defense against mutagenesis and toxicity caused by alkylating agents[194]. A meta-analysis revealed a strong association between *MGMT* methylation and non-small cell lung cancer[195]. In the present study, *MGMT* methylation levels were significantly higher in exposed workers and demonstrated a dose-response relationship with U-Cr and RBC-Cr, suggesting that *MGMT* hypermethylation plays a role in Cr(VI)-induced carcinogenesis.

Cr(VI) and lncRNAs

LncRNAs have been linked to almost every characteristic of cancer, including cell proliferation and survival, metabolism, and tumor microenvironment[196]. The number of lncRNAs associated with cancer initiation and progression is continuously growing, but data is still scarce on the role of lncRNAs in Cr(VI)-related toxicity. One *in vitro* study on Cr(VI) exposure in human bronchial epithelial cells reported 1484 differentially expressed lncRNAs associated with chromatin assembly, DNA repair and DNA replication[197], and another *in vitro* study in the same type of cells identified 4098 differentially expressed lncRNAs associated with DNA damage, DNA repair, and immune response[198]. *H19* acts as an oncogene in most tumors and tumor cells increase *H19* expression to promote their progression[199]. The expression level of *H19* was found to be significantly higher in the plasma[200] and sputum[201] of lung cancer patients compared with benign lung disease patients. In our study, we found that *H19* was significantly positively correlated with RBC-Cr but not significantly higher expressed in exposed workers. *MALAT1* has been found to regulate alternate splicing, apoptosis, autophagy, and epithelial-mesenchymal transition, thus promoting cancer development and metastasis[202]. *MALAT1* in whole blood[203] and serum[204] of lung cancer patients has been found to be lower compared with healthy controls. Our study showed that *MALAT1* was significantly lower in the plasma of Cr(VI) exposed workers compared with controls. This result is consistent with trends found in lung cancer patients' biofluid. *NORAD* is activated by DNA damage and modulates genome stability[205]. *NORAD* has been found to be overexpressed in lung cancer cell lines and tissues, enhancing migration and proliferation of cancer cells[206]. However, in our study, *NORAD* was downregulated in the plasma of Cr(VI) exposed workers, which is contradictory to the previous studies of lung cancer. This might be due to

sample differences. Most of the studies analysed tumor tissues or cell lines, whereas we studied lncRNA in plasma from a study group without cancer diagnosis. The expression of lncRNA in biofluids may be contrary to that in tumor tissues[203], as reported for *MALAT1*.

Cr(VI) and miRNAs

MiRNAs regulate multiple pathways, including apoptosis, cell differentiation, developmental timing, embryogenesis, intercellular communication, metabolism, and organogenesis[41,207]. Exposure to metals (such as lead, Cd, mercury, selenium, and Cr) may alter miRNA expression[208,209]. In study III, we used qPCR to analyse four miRNAs and they were all down-expressed in exposed workers. In study IV, we ran miRNA sequencing and identified 21 up-regulated and 38 down-regulated miRNAs in exposed workers. MiRNAs exert their regulatory function by binding to their target mRNA and leading to mRNA destabilization and degradation, translational inhibition, or direct cleavage[210]. The mRNA destabilization and degradation are common mechanisms for regulating protein-coding gene expression, thereby reducing the overall rate of protein synthesis[211]. This complex regulation of miRNAs plays a key role in cancer development[212], and dysregulated miRNAs can function as either oncogenes or tumor suppressors[213]. In human cancer tissues, the majority of miRNAs are downregulated relative to normal tissues, where they typically act as tumor suppressor-like miRNAs by targeting oncogenes. Conversely, only a small fraction of miRNAs is upregulated in cancer tissues, behaving like oncogenes by inhibiting tumor suppressor genes[214]. Speer *et al.* found exposure to particulate Cr(VI) caused concentration- and time-dependent downregulation of numerous miRNAs in a non-cancerous human lung cell line[215]. Similarly, we found that more miRNAs were down-regulated in the Cr(VI) exposed workers than in controls. This finding indicated that the potential carcinogenic effects caused by Cr(VI) exposure may mainly be caused by down-regulating tumor suppressor-like miRNAs, which could promote oncogene activity and increase cancer risk.

LncRNA-miRNA-mRNA regulatory network

The lncRNA-miRNA-mRNA regulatory network is a molecular hypothesis that explains the functional relationship between lncRNA and miRNA: lncRNAs could act as competing endogenous RNAs to sponge miRNAs by binding and sequestering miRNAs away from their target mRNAs. This, in turn, weakens the impact of miRNAs on downstream mRNAs[98]. One study showed that *H19* can regulate $A\beta_{1-42}$ deposition through the *H19/miR-15b/BACE1* axis in diabetic encephalopathy[216]. *H19* can also positively regulate *CDC42* via sponging *miR-15b* (*H19/miR-15b/CDC42/PAK1* axis) to promote cell proliferation, migration, and invasion in hepatocellular carcinoma[217]. Sweef *et al*. identified in Cr(VI)-transformed cells three oncogenic and three tumor-suppressive lncRNA-miRNA-mRNA regulatory axes by using miRNA interaction and target prediction algorithms[218]. The oncogenic

lncRNA-miRNA-mRNA network is defined as up-regulated oncogenic lncRNAs, down-regulated tumor suppressive miRNAs, and up-regulated oncogenic mRNAs[218], which is consistent with our finding that *H19* was up-regulated (although not significant) and *miR-142-3p* and *miR-15b-5p* were down-regulated. Moreover, our study found *H19* to be inversely correlated with *miR-142-3p* and *miR-15b-5p*, which all suggested that they may participate in the same oncogenic lncRNA-miRNA-mRNA module.

In addition, several Cr(VI)/miRNA/gene signaling pathways, such as *miR-3940-5p*/*XRCC2*, *miR-143*/*IGF-IR* & *IRS1*, *miR-21*/*PDCD4*, and *miR-494*/*c-Myc*, have been shown to play a crucial role in Cr(VI)-induced toxicity and carcinogenicity *in vitro*[208,219-221]. In study IV, only one significant correlation was found that was relevant for those miRNA/gene signaling pathways, namely between *hsa-miR-106a-5p* and *CCND1*. The lack of significant correlations between the hub genes and DEMs could be attributed to the complexity of miRNA-gene regulatory networks. MiRNAs often exhibit combinatorial regulation, where multiple miRNAs target the same gene, or a single miRNA regulates multiple genes[222]. In addition, since this is a cross-sectional study, the regulatory effects of miRNAs on their target genes may depend on the timing between exposure and sampling. If the effects of miRNA activity are transient or delayed, the correlations may not be detectable at a single time point[223].

Conclusion

This thesis investigated occupational exposure to Cr(VI) in Sweden and some related health effects. The overall conclusion is that the workers were exposed to a low-to-moderate level of Cr(VI), which may cause health risks due to its association with increased oxidative stress, elevated DNA damage, cancer-related DNA methylation, and dysregulation of non-coding RNAs. From the results of this thesis, I can also draw the following conclusions:

- Some workers experienced high levels of Cr(VI) exposure, exceeding existing OEL, despite overall air measurements being relatively low. Biological monitoring revealed elevated levels of Cr in urine and RBC compared to controls in individuals with low levels in air, suggesting that air measurements alone may have underestimated actual exposure levels and that additional routes of exposure may have contributed.
- Cr(VI) exposure was linked to significant alterations in biomarkers of oxidative stress, telomere length, mitochondrial dysfunction, and DNA methylation, all of which may contribute to genomic instability and increased cancer risk.
- Exposure to Cr(VI) was associated with the dysregulation of non-coding RNAs, which might serve as early effect biomarkers and indicated a potential epigenetic link between Cr(VI) toxicity and carcinogenesis.
- National policies should be strengthened to raise awareness of the risks associated with non-threshold carcinogens like Cr(VI) and to ensure implementation of mitigation measures and continuous surveillance of exposure levels in the workforce.
- Further research is needed to elucidate the molecular mechanisms underlying Cr(VI)-induced toxicity and its long-term health consequences, including sinonasal cancers and other non-cancer health outcomes.

Perspectives on Future Development

Implications for Occupational Health Practice and Regulation

Risk assessment

In study I, by using the Swedish Job Exposure Matrix, we estimated that there were approximately 16000 men and 1900 women distributed within 14 different occupations exposed to Cr(VI) in 2021. Assuming that the study population is representative of the Swedish labour market, we can speculate that around 1250 workers are at risk of exceeding the Swedish OEL. In addition, we used the Bayesian tool Expostats to estimate the overall risk that the investigated workers would exceed the Swedish OEL at any other time, assuming similar conditions as during the performed measurements. The analysis indicated that there was a non-negligible, and often high, probability that the Swedish OEL was exceeded for at least 5% of the investigated occupational groups. Since most of the companies involved in this project were large corporations, smaller companies with limited resources for preventive measures were underrepresented. Consequently, the exceedance of the OEL might be underestimated. We also examined the effectiveness of using LEV and RPE. In study I, the use of LEV corresponded to about 50% lower airborne Cr concentrations. Moreover, workers who correctly used RPE were exposed to around four times higher inhalable Cr(VI) compared with those who did not, and higher concentrations of urinary and RBC Cr were found in workers who used RPE correctly compared to those who used it incorrectly or not at all. Hence, a conclusion is that many workplaces are aware that there is a Cr(VI) exposure and have taken the measure of using RPE, but that this measure is not sufficient. The functional inadequacy of RPE might have several explanations: RPE is often used during certain work tasks, and it might be difficult for the individual to know when RPE is needed or not; additionally, RPE is often taken off too early (e.g., by welders who need to examine the goods). Furthermore, the exposed workers might have been subject to secondary exposure, or they might have been exposed to Cr(VI) via the skin. Yet another aspect is that RPE only guarantees protection if it fits properly on the wearer's face and doesn't leak[135]. Before January 1, 2025, the fit test was not formally required in Sweden, as opposed to many other countries. However, starting

January 1, 2025, the Swedish Work Environment Authority's more precise rules on fit testing of tight-fitting respirators came into force[224]. This means that employees' respirators must be individually tested and that those who use tight-fitting respirators must undergo a fit test.

In summary, to lower Cr(VI) exposure, more effective risk management measures and stronger incentives for workplaces to implement them are required. In this context, it is particularly important that the Swedish OEL for Cr(VI) will be lowered to 1 µg/m³, effective from 9 April 2026, a change that is expected to drive improved compliance and exposure reduction. Effective enforcement of the revised OEL, combined with targeted information campaigns, will be essential to support workplaces in adopting appropriate risk management measures. More information on risk assessment related to Cr(VI) exposure was published elsewhere[225].

In addition, Cr(VI) is not the only non-threshold carcinogen encountered in occupational settings. Other examples include benzene, benzopyrene, mineral oils, and soot, for which no safe level of exposure can be defined[36]. The findings of this thesis may therefore be relevant beyond Cr(VI), as they highlight challenges in exposure assessment and risk management that are common to non-threshold carcinogens. In particular, the observed discrepancies between airborne concentrations and internal exposure emphasize the importance of combining air monitoring with biological monitoring when assessing occupational cancer risks. Such an approach may also support improved risk management strategies for other non-threshold carcinogens, especially in settings where airborne levels are low but exposure may still occur via multiple routes.

Exposure mixture

Workers exposed to Cr(VI) might be exposed to other hazardous environmental substances in their working environment. In study I, we found Cr(VI) exposed workers had significantly higher urinary concentration of Cu, along with higher RBC concentration of Cu and zinc, compared with controls. In addition, Cr(VI)-exposed workers had higher urinary and RBC concentrations of lead than controls, but not statistically significant ($0.05<P<0.10$). This indicated that workers were exposed to multiple toxic elements. HBM4EU chromates study reported high perfluorooctane sulfonic acid (PFOS) exposure in platers and welders[226], and this may be explained by the application of PFOS as the mist suppressant in electroplating baths. Exposure to a mixture of environmental contaminants may lead to more serious health consequences than exposure only to one carcinogen, and thus, a more complex risk assessment is needed, including monitoring of multiple carcinogens. We measured per- and polyfluoroalkyl substance (PFAS) concentrations in serum and polycyclic aromatic hydrocarbons (PAH) concentrations in urine in the SafeChrom project. These results and the effect of mixture exposure were published elsewhere[227]. In brief, this study included participants from the SafeChrom and Danish SAM-Krom studies. After combining the datasets, exposed workers had higher concentrations of Cr, manganese, Cu, zinc, lead, and perfluoroheptanoic acid (PFHpA), lower mtDNA-cn and longer TL compared to controls. In the SAM-Krom study, PFOS level was significantly elevated among exposed workers, with the P95 reaching 2044 ng/mL. The exposure mixtures were associated with increased 8-OHdG and *MGMT* hypermethylation. Together, these findings highlight the complexity of multiple occupational exposures in Cr(VI)-related work environments and suggest that combined exposure may contribute to early biological alterations related to oxidative stress and DNA methylation.

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