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All authors have read and agreed to the published version of the manuscript.

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Abstract

Occupational exposures to hexavalent chromium (Cr(VI)) can occur in welding, hot working stainless steel processing, chrome plating, spray painting and coating activities. Recently, within the human biomonitoring for Europe initiative (HBM4EU), a study was performed to assess the suitability of different biomarkers to assess the exposure to Cr(VI) in various job tasks. Blood-based biomarkers may prove useful when more specific information on systemic and intracellular bioavailability is necessary. To this aim, concentrations of Cr in red blood cells (RBC-Cr) and in plasma (P-Cr) were analyzed in 345 Cr(VI) exposed workers and 175 controls to understand how these biomarkers may be affected by variable levels of exposure and job procedures.

Compared to controls, significantly higher RBC-Cr levels were observed in bath plating and paint application workers, but not in welders, while all the 3 groups had significantly greater P-Cr concentrations. RBC-Cr and P-Cr in chrome platers showed a high correlation with Cr(VI) in inhalable dust, outside respiratory protective equipment (RPE), while such correlation could not be determined in welders. In platers, the use of RPE had a significant

impact on the relationship between blood biomarkers and Cr(VI) in inhalable and respirable dust. Low correlations between P-Cr and RBC-Cr may reflect a difference in kinetics.
This study showed that Cr-blood-based biomarkers can provide information on how workplace exposure translates into systemic availability of Cr(III) (extracellular, P-Cr) and Cr(VI) (intracellular, RBC-Cr). Further studies are needed to fully appreciate their use in an occupational health and safety context.

Key words: Red Blood Cells Chromium, Plasma Chromium, Biological Monitoring, workplace, electroplating, Welding,

1. Introduction

Chromium (Cr) is a transition element belonging to the heavy metal group. It exists in oxidation states ranging from -2 to +6. Of these Cr species, metallic (Cr(0)), trivalent (Cr(III)) and hexavalent Chromium (Cr(VI)) species occur in the working environment. Particularly, Cr(VI) and its compounds have found wide industrial applications due to their capacity to confer hardenability, durability and corrosion resistance compared to other metals (Alvarez et al., 2021). Cr(VI) compounds may also be used as pigments in dyes, paints, inks, and plastics. Occupational exposures can occur mainly in welding and hot working stainless steel, high Cr alloys and Cr-coated metal; electrolytic Cr(VI) plating in baths; Cr-containing pigments, spray paints and coatings and removal of old paints and coatings (SCOEL, 2017).
Chronic occupational exposure to Cr(VI) can induce several adverse health effects, including lung impairments, pneumonia, bronchitis and asthma, as well as skin, sinonasal, kidney, liver, and hematological alterations (Khadem et al., 2017). The International Agency for Research on Cancer (IARC) classified Cr(VI) compounds as group I human carcinogenic agents (IARC, 2012), as these compounds can cause cancer of the lung. Recently, the Rijksinstituut voor Volksgezondheid en Milieu (RIVM) proposed sufficient evidence between exposure to Cr(VI) compounds and sinonasal cancer (den Braver-Sewradj et al., 2021). Additionally, positive associations have been observed with stomach and laryngeal cancer (den Braver-Sewradj et al., 2021). However, despite its carcinogenic properties, the use of Cr(VI) compounds, i.e. chromates, Cr trioxide and dichromium tris(chromate), are still authorized for specific purposes, under the EU Regulation on Registration, Evaluation, Authorisation and restriction

of CHemicals (REACH) (ECHA, 2021). Therefore, monitoring the exposure to these species is of particular importance in the context of occupational health protection.

Exposure assessment to Cr(VI) compounds can combine airborne environmental measurements with human biomonitoring (HBM) besides hand wipe samples to evaluate dermal contamination. The common biomarker used for the biomonitoring of Cr exposure at workplace is urinary Cr (U-Cr). Although U-Cr allows to achieve a suitable evaluation of the exposure experienced by workers, some improvements are still required (Devoy et al., 2016). In fact, U-Cr is not specific for Cr(VI) since it measures exposure to both Cr(III) and Cr(VI). The possibility for workers to be simultaneously exposed to a mixture of Cr(III) and Cr(VI) compounds make it difficult to interpret U-Cr results and to inform suitable hazard and risk assessment in occupational settings (SCOEL, 2017).

RBC-Cr has been proposed as a more specific biomarker for occupational Cr(VI) exposure. The concentration of Cr in red blood cells (RBC-Cr) may represent the portion of Cr(VI) able to reach the bloodstream in its non-reduced form and may be directly dependent on the airway inhaled dose (Goldoni et al., 2010). Cr(III) cannot pass the cell membranes, whereas Cr(VI) can be transported into the RBCs via active anion channels (Kortenkamp et al., 1987). Once in the RBCs, Cr(VI) is completely reduced to Cr(III) by the Fe(II) of haemoglobin, then trapped in the RBCs until the end of their life-span. Blood half-life of RBC-Cr is estimated to be approximately 63 days (half of the average RBC life span in humans) and follows zero order kinetics when eliminated from the blood compartment (Törnqvist et al., 2002). Thus RBC-Cr values reflect the average exposure to systemic Cr(VI) over the past four months. To date, few studies have investigated the RBC-Cr levels in occupationally exposed subjects.

Zhang et al. (2011) found significantly higher RBC-Cr concentrations in male chrome-plating workers than in controls. Comparably, a significantly higher mean RBC-Cr was found in chromate exposed workers compared to controls, with a positive correlation between RBC-Cr and U-Cr (Wang et al., 2011a; 2011b; 2012). Goldoni et al. (2010) found lower median RBC-Cr levels at the beginning of the workweek compared to the levels detected at the end of the working shift on Tuesday. RBC-Cr was significantly correlated to the U-Cr, at the beginning of the work-shift, and to the Cr levels in the exhaled breath condensate at the end of the work-shift. In welders, a low number of samples with detectable levels (Weiss et al., 2013) and low RBC-Cr concentrations (Scheepers et al., 2008; Stanislawska et al., 2020) were reported. The disparities reported in these studies probably relate to the differences in the occupational settings explored (Goldoni et al., 2010; Zhang et al., 2011; Wang et al., 2012; Weiss et al., 2013; Stanislawska et al., 2020), in line with the different kinetics and bioavailability of the Cr species present in the different contexts (Pesch et al., 2018). In addition, factors influencing RBC-Cr were not investigated.

Recently, within the HBM for Europe initiative (HBM4EU), a harmonized multicenter study was performed to gather data on the current occupational exposure to Cr(VI) in Europe and to assess the suitability and added value of different biomarkers of Cr(VI) exposure. This HBM4EU study has a unique set-up, including multiple countries collecting biomonitoring and industrial hygiene information using harmonized protocols. It allowed to overcome the typical challenge in occupational studies related to the low number of workers that can be recruited in national studies. From the HBM4EU results, the complementary role of different types of biomarkers clearly emerged, with the suggested possibility to employ RBC-Cr when more specific information on the contribution to intracellular bioavailability of Cr(VI) is necessary, in addition to the excretion of total Cr in urine (Santonen et al., 2022).

In this paper, we report earlier unpublished results from the HBM4EU chromates study on Cr levels in RBC-Cr and plasma (P-Cr) to demonstrate their added value compared to other biomarkers of exposure. The main goal of the present study is to better understand the role of RBC-Cr and P-Cr as CrVI biomarkers in workers engaged in different job tasks with exposure to Cr(VI) compounds and in controls. Moreover, this research includes the careful investigation of the relationship between external exposure, RBC-Cr and P-Cr levels to understand the correlations between blood biomarkers and factors influencing their levels. In addition, we aim to improve our understanding how confounding factors and variables that affect the exposure levels may contribute to the interpretation of blood biomarkers.

2. Materials and methods

The study was performed using the design previously reported by Santonen et al. (2019) in order to collect data in a harmonized way in different industrial sectors across Europe. Study methods have been described in Standard Operating Procedures (SOPs) and can be found in the HBM4EU Library (https://www.hbm4eu.eu/online-library/).

2.1. Study population

The study was carried out in nine European countries, i.e., Belgium, Finland, France, Italy, Luxembourg, Poland, Portugal, UK and the Netherlands. In the UK no blood was collected. Thus, only eight countries participated in this part of the study. Workers engaged in job tasks resulting in exposure to Cr(VI), e.g., chrome plating, surface treatment by sanding, spraying or painting, and stainless-steel welding, were considered suitable to be included in the study, as detailed by Santonen et al. (2019). However, as some activities performed were not classifiable under the three categories previously used (Santonen et al., 2022), machining, thermal spraying, steel production, maintenance and laboratory work were also considered, leading to seven categories in total. Workers with no documented occupational exposure to Cr(VI) compounds (e.g., administrative workers), from the same or other companies, were enrolled as controls (respectively, “within company controls” or “outwith company controls”). Both companies and workers were invited to participate in the study and information was provided on the aims and study procedures. Written informed consent was given before enrollment in the survey (Santonen et al. (2019). Participation was completely voluntary, and participants could withdraw from the study at any time. The study protocol was approved by the Ethics Committees of the involved institutions in each of the participating country (Santonen et al., 2019; 2022).

2.2. Collection of contextual information

General information on the workplace, work practices and risk management measures (RMMs) were collected from a company representative prior to the sampling campaign. Enrolled workers provided (as close as possible to the end of work shift) a detailed description of their job activities, specific work tasks, use of personal protective equipment (PPE) and work organization during the week of sampling. Additionally, they were also asked to provide information on possible extra-occupational sources of exposure due to e.g., general living environments and habits, smoking, orthopedic and dental implants, food supplements and recreative activities.

2.3. Blood sample collection

One blood sample was collected from each (exposed or non-exposed) participant, preferentially on the 3rd - 5th day of the working week, at the end of the work shift. A venous blood sample was collected from the fore arm using a single-use syringe or winged needle and transferred to a tube appropriate for trace element analyses containing potassium ethylenediamine tetra acetic acid (K-EDTA) as anticoagulant.

The sample was kept at +4°C until transfer to the laboratory. To avoid hemolysis, separation of plasma and RBC was conducted, preferably within 8 h (and maximum 24 h) from the specimen collection, following the method described by Devoy et al. (2016). Samples were centrifuged (10 min at 1000–2000 x g or 5 min at 2700 x g) and the supernatants containing the plasma and white blood cells were stored at +4 °C up to 7 days or at -20°C until analysis. The pellet underwent three washing steps with 0.9% NaCl solution (with a volume corresponding to the initial volume of blood collected), in order to eliminate interfering plasma/Cr residues. The haematocrit (HT) values (measured before (HT1) and after the washing steps (HT2)) were determined to adjust for RBC loss during washing steps. After the last washing step, the tube containing RBCs was filled up with 1% Triton X-100 in deionized water/0.2% HNO_3 (GFAAS analysis) or 1% Triton X-100 in deionized water/0.2% NH_4OH (ICP-MS analysis) up to the initial volume. Washed RBCs were then stored under the required conditions (i.e., room temperature up to 3 days or at -20°C for longer time).

2.4. Blood sample analysis

Blood samples were analyzed by each participating country according to the analytical method used by the national laboratory involved in the chromate study. Inductively coupled plasma – mass spectrometry (ICP-MS) was used to quantify RBC-Cr and P-Cr, except for Portugal who performed analyses by graphite furnace atomic absorption spectrometry (GFAAS). Supplementary Table S1 gives an overview of the methods used to measure RBC-Cr and P-Cr by country. RBC-Cr concentration was divided by HT2 as an approximation of RBC volume and expressed in µg/L. Total P-Cr content was divided by the volume of the collected plasma aliquot and expressed in µg/L.

Successful participation in the HBM4EU Inter-Laboratory Comparison Investigations (ICI) was mandatory to perform analyses for the occupational Cr study. The aim of ICI was to provide laboratories with an assessment of their analytical performance and reliability of their data in comparison with other laboratories (Esteban López et al., 2021). Four rounds were organized from August 2018 to December 2019 by the Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine (IPASUM) at the Friedrich-Alexander University of Erlangen-Nuremberg, with the requirement to pass at least 2 rounds to perform analysis under HBM4EU. Two different test samples consisting of ~ 3 mL serum or blood spiked with Cr (Cr ICP standard, ammonium dichromate in H_2O, 1000 mg/L, J.T.Baker) at two different concentrations (low and high) were dispatched at each round (apart from the first round where 6 samples were distributed). Laboratory results were rated using Z-scores in accordance with ISO 13528 and ISO 17043. The standard deviation for proficiency assessment was set at 25%. Detailed results of Cr ICI studies are described by Nübler et al. (2021).

2.5. Urine and air sample collection and Cr analysis

Sampling protocols and sample analysis have been described in detail in Santonen et al., (2019).

In brief, two urines samples were collected from the exposed workers, the first before the start of the shift at the beginning of the working week, and the second at the end of the shift in the end of the working week. One spot urine sample was collected from the controls at any time of the working week. All the laboratory analyzing the urine samples had successfully passed ICI rounds within HBM4EU. The limits of quantification were between 0.05 and 0.31 µg/L, depending on the laboratory.

Simultaneous sampling of the inhalable dust and respirable dust fractions of Cr(VI) and total Cr was performed in the breathing zone following the standard CEN-EN481:1993 Workplace atmospheres – size fraction definitions for measurement of air particles. Inhalable dust, corresponding to mass fraction of total airborne particles which is inhaled through the nose and the mouth, was collected using an IOM sampling head. Respirable dust, corresponding to mass fraction of total airborne particles penetrating to the lower gas exchange regions (nonciliated airways), was collected using the Higgins Dewell type or similar cyclone sampling heads. Both inhalable and respirable dusts fractions were collected by personal air sampling for a representative period of the work shift (>75%). The air samples were first analysed gravimetrically and subsequently for total Cr and Cr(VI) by OSHA Method ID-125G (OSHA 2002) and ISO 16740 Method (ISO 2005) , respectively, with some minor adaptations performed by some laboratories.

2.6. Statistical analysis

Descriptive statistics

A descriptive statistics analysis reporting minimum (min), maximum (max), geometric mean (GM) and 95% confidence interval (CI), median and percentiles (P10, P25, P75, P90, P95) was performed on RBC-Cr and P-Cr concentrations, for all participants, and by exposure group. Data below the limit of quantification (LOQ) were substituted by LOQ/2.

Inferential statistics

A logarithmic transformation was applied to both RBC-Cr and P-Cr concentrations to reduce the skewness and variability of the data to meet assumptions of statistical parametric tests (ANOVA, linear regressions). In order to compare RBC-Cr and P-Cr levels between exposure groups and between countries, an ANOVA was performed on the log transformed data, with an "exposure" effect (controls vs exposed), a "country" effect, and the interaction between the two effects. When the "country" effect was significant, a multiple comparison *post hoc* test (with Bonferroni correction) was used to test the differences of levels between countries. To consider the variability of background levels by country, a mixed linear regression model was applied on the log transformed RBC-Cr and P-Cr data to test the "exposure group" fixed effect, including a "country" random effect. When the "exposure group" effect was significant, a multiple comparison *post hoc* test (with Bonferroni correction) was used to test the differences of levels between groups.

The correlations between P-Cr, RBC-Cr and other Cr measurements (e.g., U-Cr, inhalable/respirable Cr (VI) outside RPE) (Santonen et al., 2022) were estimated using the Spearman correlation coefficients ρ. For each correlation coefficient, the number of couples of measurements and the p-value of the correlation coefficient were reported. To assess the impact of wearing RPE on the P-Cr and RBC-Cr concentrations, correlations were calculated specifically for workers who did not wear RPE (bath plating and welding workers) and compared to workers who did. When high correlation was observed between P-Cr or RBC-Cr and other Cr measurements, a multiple linear regression model was applied on the log transformed [RBC_Cr] (or [P_Cr]), with the "log transformed Cr parameter" effect, the "RPE_wearing" effect, and their interaction. This regression allowed to estimate the slope of the relation (on a log scale) between [RBC-Cr] (or [P-Cr]) and [respirable Cr(VI) outside RPE] (or [inhalable Cr(VI) outside RPE]), and the slope difference between "with RPE" or "without RPE", due to the estimation of the interaction effect.

Finally, the effects of different influencing parameters were tested. A mixed linear regression model was applied to the log transformed RBC-Cr and P-Cr data, with an "exposure group" as fixed effect (exposed vs control), an "influencing parameter" effect and their interaction, including a "country" random effect. Influencing parameters were divided into three groups. The first group of individual parameters was related to the participant personal habits: alcohol consumption (yes/no) and the number of glasses by month (<20 or >20); smoking status (non-smoker/current smoker/former smoker) and the amount of cigarettes/day for smokers (<10 or

>10); hobbies (yes/no), presence of orthopedic or dental implants (yes/no), presence of dental fillings (yes/no). The second group of parameters were related to the living environment of participants: home location (rural/urban), road traffic (related vehicle emissions) close to home (low/medium/high intensity), and presence of industrial plants close to home (yes/no). The last group of parameters were related to some specific occupational activities: exposure tasks frequencies (hours/week), and for welders, the welded material (iron, stainless, other), and the welding method when stainless steel was welded (MAG/MIG/MMA/TIG/Other).

Statistical analyses were performed using Stata Statistical Software (Version 16.1, StatCorp, College Station, TX, USA). The statistical significance threshold was set at 5%.

3. Results

3.1. Study Population

A total of 345 exposed workers and 175 controls volunteered to provide a blood sample. The distribution of the study population across Europe and the distribution of workers according to the main tasks performed are summarized in Table 1. As discussed in Santonen et al. (2022), the tasks covered in each country were dependent on the participating companies and workers willing to participate to the study (see Table 1). The control group encompassed 134 “within company controls” and 41 “outwith company controls” (Table 1). The mean age (±SD) was 42 years (±11) and 44 years (±10) in the exposed group and control group, respectively. Most of the exposed workers were male (n=336, 97%), while in the control group, the proportion of males was lower (n=127, 72 %). A total of 164 workers (31%) were smokers, i.e. 143 (41%) among exposed workers and 21 (12%) among controls.

Table 1. Distribution of the study population and number of blood samples collected in each country (i.e., BE: Belgium, FI: Finland, FR: France, IT: Italy, LU: Luxembourg, NL: the Netherlands, PL: Poland and PT: Portugal).

Group	**BE**	**FI**	**FR**	**IT**	**LU**	**NL**	**PL**	**PT**	**Total**
Chrome plating	7	15	18	6	0	19	0	5	70
Paint applications	11	0	0	4	0	0	0	32	47
Machining	9	2	19	0	0	0	0	5	35
Welding	28	18	18	37	16	0	51	3	171

Thermal spraying	0	5	0	0	0	0	1	0	6
Steel production	9	0	0	0	0	0	0	0	9
Maintenance and laboratory	0	0	1	0	0	1	0	5	7
All exposed workers	**64**	**40**	**56**	**47**	**16**	**20**	**52**	**50**	**345**
Within company controls	31	9	22	31	8	12	19	2	134
Outwith company controls	0	16	0	0	0	0	0	25	41
All controls	**31**	**25**	**22**	**31**	**8**	**12**	**19**	**27**	**175**

3.2. RBC-Cr and P-Cr levels in the control group

RBC-Cr and P-Cr levels in the control group are detailed in Table 2. "Outwith company controls" showed significantly higher GM and median RBC-Cr compared to "within company controls" (1.11 and 1.02 vs 0.59 and 0.38 µg/L, respectively). However, P95 levels were somewhat higher among "within company controls" (5.06 µg/L, see Table 2). Higher P-Cr levels were observed in "within company controls" (GM 0.55, median 0.55 and P95 2.61 µg/L) as opposed to "outwith company controls" levels (GM 0.31, median 0.34 and P95 0.70 µg/L). However, no significant difference was found between the two control sub-groups (Figure S1 in supplementary materials) when the variability due to the diverse involved countries was taken into account. Thus, both sub-groups were mixed and only an "all controls" group was considered for the following statistical analyses.

Table 2. RBC-Cr and P-Cr levels (µg/L) in "within company controls" and "outwith company controls"

	Control group	n	Median	GM (CI)	P95
RBC-Cr	Within company controls	134	0.38	0.59 (0.46-0.76)	5.06
	Outwith company controls	41	1.02	1.11 (0.93-1.32)	3.0
P-Cr	Within company controls	134	0.55	0.55 (0.48-0.63)	2.61
	Outwith company controls	41	0.34	0.31 (0.25-0.38)	0.70

n: number of workers, GM: geometric mean, CI: 95% confidence interval, P95: 95th percentile.

3.3. RBC-Cr and P-Cr levels across countries

Differences in RBC-Cr and P-Cr levels were observed between countries, but also between controls and exposed workers (Table 3). The box plots of the distribution of RBC-Cr and P-Cr across participating countries are presented in supplementary material (Figure S2). A significant effect of the “exposure” and “country” was observed on the log transformed RBC-Cr and P-Cr data, while no interaction between these variables was observed.

Regarding RBC-Cr, the concentrations in samples of exposed workers from France and The Netherlands were significantly higher than the concentrations measured in the other countries with median levels above 4 µg/L (Note that in previous paper (Santonen et al., 2022) we erroneously stated that RBC-Cr results were lower in these two countries when compared to other countries). Italy and Poland exhibited the lowest RBC-Cr concentrations with median values below 0.5 µg/L. As for Belgium, more than 92% of samples had RBC-Cr concentration below the LOQ. However, Belgium reported the highest LOQ among the participating countries.

For P-Cr in exposed workers, the concentrations in The Netherlands were significantly higher than in all other countries, while Poland and Portugal had the lowest concentrations.

Table 3. Distribution of RBC-Cr and P-Cr concentrations (µg/L) in exposed workers and controls groups across participating countries (i.e., BE: Belgium, FI: Finland, FR: France, IT: Italy, LU: Luxembourg, NL: The Netherlands, PL: Poland and PT: Portugal).

	Group		**BE**	**FI**	**FR**	**IT**	**LU**	**NL**	**PL**	**PT**
RBC-Cr	Controls	n	31	25	22	31	8	12	19	27
		Min	<LOQ	0.92	3.58	0.04	<LOQ	4.17	0.05	0.56
		median	<LOQ	**1.14**	**4.45**	**0.09**	**0.74**	**4.67**	**0.36**	**0.66**
		max	<LOQ	1.57	5.56	4.14	3.22	5.34	0.74	3.40
	Exposed workers	n	64	40	56	47	16	20	52	50
		min	<LOQ	0.30	2.97	0.04	0.18	4.67	0.14	0.61
		median	<LOQ	**1.55**	**4.49**	**0.11**	**0.78**	**5.69**	**0.46**	**1.89**
		max	2.50	7.61	10.00	0.42	2.20	21.2	1.31	4.56
P-Cr	Controls	n	31	25	22	31	8	12	19	27
		min	<LOQ	0.40	0.60	0.24	0.79	2.46	0.09	<LOQ
		median	<LOQ	**0.54**	**0.74**	**0.75**	**0.95**	**2.63**	**0.16**	**<LOQ**
		max	2.05	0.71	1.09	1.16	1.09	3.36	0.45	0.86
	Exposed workers	n	64	40	56	47	16	20	52	50
		min	<LOQ	0.39	0.61	0.44	0.77	2.62	0.13	< LOQ

median	**1.08**	**0.70**	**0.83**	**1.10**	**0.96**	**3.71**	**0.37**	**< LOQ**
max	4.68	4.49	1.94	2.35	2.21	8.94	1.49	2.51

n: number of workers, min: minimum, max: maximum, LOQ: limit of quantification.

3.4. RBC-Cr and P-Cr levels in exposed workers

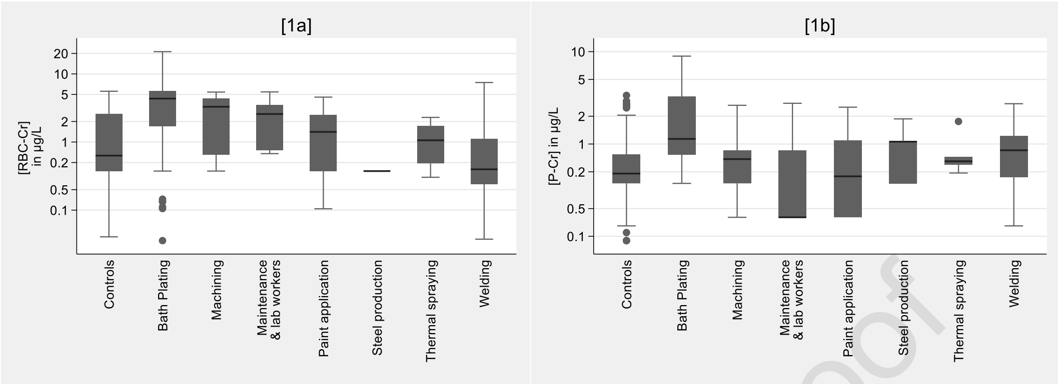
The highest RBC-Cr levels (median 4.34 and P95 8.37 µg/L) were observed in chrome plating workers, followed by machining (median 3.30 µg/L), maintenance and laboratory work (median 2.58 µg/L) and paint application (median 1.41 µg/L) (Table 4). Welders and steel production workers had the lowest RBC-Cr levels (median 0.40 µg/L and 0.38 µg/L, respectively). The box plots of the distribution of RBC-Cr and P-Cr according to the work activity are presented in Figure 1. RBC-Cr concentrations were significantly higher in the bath plating and paint application groups, when compared to controls and the welding group. Compared to the controls, P-Cr concentrations were significantly higher in bath plating workers (median 1.14 and P95 6.14 µg/L), paint application and welding groups.

Table 4. RBC-Cr and P-Cr concentrations (µg/L) in exposed workers and controls.

	Worker groups	n	GM	CI-	CI+	min	Median	P95	max
RBC-Cr	Chrome plating	70	2.44	1.79	3.34	<LOQ	4.34	8.37	21.22
	Steel production	9	0.38	0.38	0.38	<LOQ	0.38	0.38	0.38
	Maintenance and laboratory	7	2.05	1.15	3.64	0.68	2.58	5.44	5.44
	Machining	35	1.88	1.34	2.65	<LOQ	3.30	5.35	5.41
	Paint applications	47	1.07	0.80	1.43	<LOQ	1.41	3.57	4.56
	Thermal spraying	6	0.93	0.51	1.72	0.30	1.06	2.30	2.30
	Welding	171	0.49	0.41	0.58	<LOQ	0.40	5.12	7.48
	All workers	345	0.89	0.77	1.03	<LOQ	0.73	5.83	21.22
	All controls	175	0.68	0.56	0.83	<LOQ	0.63	5.00	5.56
P-Cr	Chrome plating	70	1.52	1.24	1.86	<LOQ	1.14	6.14	8.9
	Steel production	9	0.74	0.48	1.15	<LOQ	1.06	1.87	1.87
	Maintenance and laboratory	7	0.31	0.13	0.73	<LOQ	<LOQ	2.76	2.76
	Machining	35	0.59	0.46	0.75	<LOQ	0.69	2.61	2.63
	Paint applications	47	0.50	0.39	0.64	<LOQ	0.45	1.71	2.51
	Thermal spraying	6	0.73	0.51	1.05	0.49	0.65	1.76	1.76
	Welding	171	0.75	0.67	0.83	<LOQ	0.86	2.11	2.73
	All workers	345	0.78	0.71	0.85	<LOQ	0.82	3.27	8.94
	All controls	175	0.48	0.43	0.54	<LOQ	0.48	2.56	3.36

n: number of workers, GM : geometric mean, CI : 95% confidence interval of the geometric mean min: minimum, P95 : 95th percentile, max: maximum, LOQ : limit of quantification.

Figure 1. Distribution of RBC-Cr [1a] (µg/L) and P-Cr [1b] (µg/L) according to the work activity. Box plots: The bottom and top of the box are, respectively, the P25 and P75, and the horizontal line inside the box is the median (P50).



3.5. Correlation between RBC-Cr and P-Cr

The Spearman correlation coefficient ρ between RBC-Cr and P-Cr was 0.21 when all the involved participants were considered (n=520, sum of exposed workers and controls, $p<10^{-3}$). This ρ value was 0.24 in the control group (n=175, p=0.0012). In exposed workers, the highest correlation coefficients were found in chrome platers (ρ =0.66, n=70, $p<10^{-3}$), followed by machining workers (ρ =0.64, n=35, $p<10^{-3}$) and thermal sprayers (ρ =0.37, n=6, p=0.47), whereas very low correlation coefficients were obtained for the other groups (e.g., ρ =0.066 in welders, n=171, p=0.39).

3.6. Correlation between other Cr measurements and RBC/P-Cr

Taking into account exposed workers, RBC-Cr correlated only with respirable Cr(VI) outside RPE (ρ=0.49, n=86, $p<10^{-3}$) while P-Cr correlated with respirable Cr(VI) outside RPE (ρ=0.65, n=86, $p<10^{-3}$), total respirable Cr outside RPE (ρ=0.44, n=75, $p<10^{-3}$) and with post-shift U-Cr (ρ=0.52, n=318, $p<10^{-3}$). Supplementary Figure S3 shows a heatmap on the Spearman correlations between different markers of exposure regardless of the worker groups (i.e., all worker groups combined).

When worker groups were analyzed separately, somewhat higher correlations were found in chrome platers between both RBC-Cr and P-Cr and respirable Cr(VI) outside RPE (ρ=0.64, n=52, $p<10^{-3}$ and ρ=0.83, n=52, $p<10^{-3}$), inhalable Cr(VI) outside RPE (ρ=0.54, n=55, $p<10^{-3}$ and ρ=0.77, n=55, $p<10^{-3}$), total respirable Cr outside RPE (ρ=0.76, n=19, $p<10^{-3}$ and ρ=0.86, n=19, $p<10^{-3}$, respectively), total inhalable Cr outside RPE (ρ=0.49, n=16, p=0.05 and ρ=0.54, n=16, p=0.03, respectively), post-shift U-Cr (ρ=0.46, n=67, $p<10^{-3}$ and ρ=0.78, n=52, $p<10^{-3}$, respectively) and hand contamination (ρ=0.53, n=60, $p<10^{-3}$ and ρ=0.73, n=60, $p<10^{-3}$, respectively).

Likewise, in machining workers, RBC-Cr and P-Cr were moderately correlated with total respirable Cr outside RPE (ρ=0.59, n=6, p=0.22 and ρ=0.47, n=6, p=0.34 respectively) and highly correlated with total inhalable Cr outside RPE (ρ=1, n=5, $p<10^{-3}$ and ρ=0.89, n=5, p=0.04). The correlation with hand contamination was observed only with P-Cr (ρ=0.59, n=22, p=0.0035).

In welders, RBC-Cr and P-Cr were well correlated with respirable Cr(VI) outside RPE (ρ=0.59, n=17, p=0.013 and ρ=0.70, n=17, p=0.0015). A correlation with post-shift U-Cr was observed only with P-Cr (ρ=0.54, n=156, $p<10^{-3}$).

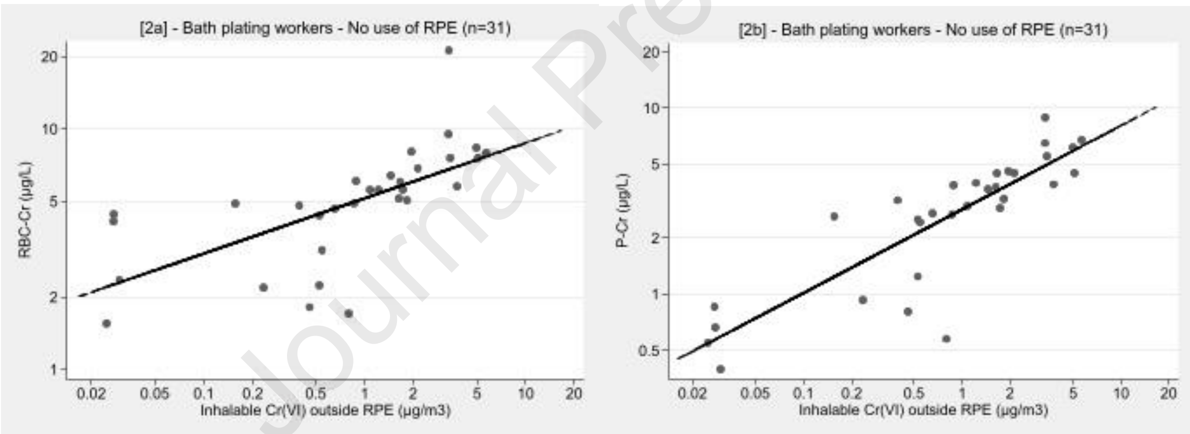
Stratifying by the use or not use of RPE, higher correlations were obtained between RBC-Cr [or P-Cr] and respirable/inhalable Cr(VI) outside RPE, in the chrome plating group (Table 5).

Table 5. Spearman correlations ρ between RBC-Cr or P-Cr and respirable Cr(VI) outside RPE or inhalable Cr(VI) outside RPE, in the chrome plating group stratified by the use or not use of RPE (n is the number of couples of measurements and p is the p-value of the correlation coefficient).

	RBC-Cr			P-Cr		
	All workers	Use of RPE	No use of RPE	All workers	Use of RPE	No use of RPE
Respirable Cr(VI) outside RPE	ρ =0.64, n=52, $p<10^{-3}$	ρ =0.34, n=12, p=0.27	ρ =0.81, n=31, $p<10^{-3}$	ρ =0.83, n=52, $p<10^{-3}$	ρ =0.65, n=12, p=0.02	ρ =0.87, n=31, $p<10^{-3}$
Inhalable Cr(VI) outside RPE	ρ =0.54, n=55, $p<10^{-3}$	ρ =-0.31, n=15, p=0.25	ρ =0.86, n=31, $p<10^{-3}$	ρ =0.77, n=55, $p<10^{-3}$	ρ =0.21, n=15, p=0.44	r ρ =0.89, n=31, $p<10^{-3}$

For the relations between [RBC-Cr] and [respirable Cr(VI) outside RPE], between [RBC-Cr] and [inhalable Cr(VI) outside RPE] and between [P-Cr] and [inhalable Cr(VI) outside RPE] in platers, the slope was significantly higher for workers not wearing RPE than for workers wearing RPE. These findings suggest that the use of RPE has an impact on the relation between [RBC-Cr] and [respirable Cr(VI) outside RPE]. In the same group of workers, for the relation between [P-Cr] and [respirable Cr(VI) outside RPE], the slope was not statistically different for workers not wearing RPE compared to those wearing RPE. Indeed, wearing RPE has no impact on the relation between [P-Cr] and [respirable Cr(VI) outside RPE]. Regression equation for RBC-Cr and inhalable Cr(VI) in platers was ln(RBC-Cr) = 1.64+0.23*ln(CrVI µg/m^3) (R^2=0.42) and for P-Cr and inhalable Cr(VI) levels ln(P-Cr) = 1.05+0.45*ln(CrVI µg/m^3) (R^2=0.72) suggesting that OEL of 5 µg/m^3 corresponds to RBC-Cr levels of 7.5 µg/l and P-Cr levels of 6 µg/l. This regression analysis is presented in Figure 2. Additional graphical representation of the linear regression models between RBC-Cr/P-Cr and respirable Cr(VI) outside RPE, for platers not wearing RPE, are provided in supplementary materials (Figure S4).

Figure 2: [2a] Linear regression model between RBC-Cr and inhalable Cr(VI) outside RPE. Regression equation: ln(RBC-Cr)=1.64+0.23*ln(InhCrVI_outside); R^2=0.42; n=31 (left panel). [2b] Linear regression model between P-Cr and inhalable Cr(VI) outside RPE. Regression equation: ln(P-Cr)= 1.05+0.45*ln(InhCrVI_outside); R^2=0.72; n=31 (right panel),



In the welding group, the coupled data count [RBC-Cr/respirable Cr(VI) outside RPE] was limited (n=17, see above). Stratifying by the use or not use of RPE gave a too low number of coupled data in each group to conclude.

3.7. Other influencing parameters

The statistical tests of the potential influencing parameters (personal habits, living environment, and occupational activities) showed no effect on P-Cr and RBC-Cr. Only a small effect of the road traffic (related to vehicle emissions) was found on both P-Cr and RBC-Cr. The difference in P-Cr and RBC-Cr between controls and exposed workers disappeared when controls were exposed to high intensity traffic.

4. Discussion

This paper is part of the HBM4EU chromates study, performed to collect EU-wide data on current occupational exposure to Cr(VI) and to assess the suitability and added value of different biological indicators in Cr biomonitoring (Santonen et al. 2022). The aim of this research was to assess the RBC-Cr and P-Cr levels in Cr(VI) exposed workers compared to controls in order to understand, also, which factors, related to the exposure levels or job procedures, may affect the concentrations of these biomarkers and the interpretation of the obtained results.

4.1. Exposure of chrome platers, welders and paint application workers

The highest RBC-Cr levels were observed in chrome plating workers. Exposures in surface treatment operations (both chrome plating and painting) were significantly higher compared to those experienced by welders and controls. These findings are in agreement with results earlier reported for Cr urinary excretion by Santonen et al., (2022) and Viegas al., (2022). Chrome platers showed higher exposure for U-Cr. The highest P95 value for inhalable Cr(VI) were also observed for chrome plating and painting. Differences in terms of processes, types of activities performed and Cr(VI) emissions, e.g. aerosols formed when hydrogen gas escapes from the warm baths or emissions produced in spray painting, paint removal, coating activities, water-solubility and size of particles, could be responsible for the diverse levels determined in such groups of workers (Viegas al., 2022).

Moreover, although the pulmonary epithelial lining fluid and lung macrophages have Cr(VI) reducing capacity, a fraction of the non-reduced Cr(VI) may reach the pulmonary tissue and enter the bloodstream. This kinetic process is more rapid in the case of more water-soluble compounds, while those with a low water solubility show less rapid toxicokinetics and may have much longer airway retention (Petrilli et al. 1986; De Flora et al. 1997; OSHA, 2006). Therefore, as the differences between occupational settings suggest, RBC-Cr may reflect primarily exposures with water soluble Cr, such as Cr acid and water-soluble chromates in paint, and much less clearly reflect exposure to welding fumes that more likely contain scarcely water-soluble chromates depending on the welding technique (Antonini et al., 2004). This could be demonstrated by the higher correlation between the inhalable and respirable Cr(VI) exposure and RBC-Cr and P-Cr in chrome platers compared to welders. A relation supported also by Viegas et al., (2022) who observed that similar air inhalable Cr(VI) levels resulted in almost two-times higher urinary Cr levels in platers compared to welders.

4.2. Factors affecting RBC-Cr and P-Cr levels

The acquired physicochemical features of the airborne Cr(VI) compounds, related to the different source processes, may affect their absorption, cell membrane penetration and cellular bioavailability. This may also explain the variable correlations found between RBC-Cr, P-Cr and environmental measurements in different job tasks. As an example, in chrome plating, the two biomarkers were highly correlated with the respirable Cr(VI) outside RPE, while in machining workers, such correlation failed to emerge, although this data may be affected by the low number of available observations. Additionally, we did not observe any significant impact of the type of welding process, the base metal and filler metals used, and exposure task frequencies on RBC-Cr and P-Cr results. These findings confirm those previously obtained by Scheepers et al. (2008), who did not observe differences in RBC-Cr median concentrations due to the mild, high alloy and stainless steel worked. However, not all occupational aspects could be analyzed in our study due to the considerable heterogeneity of the work situations reported in the questionnaire.

Once Cr(VI) compounds enter into the bloodstream most of them will readily be reduced to Cr(III) (Devoy et al. 2016). However, it remains to be clarified how the levels of exposure may affect the Cr(VI) reduction capacity. In fact, it cannot be excluded that once in the bloodstream, low levels of Cr(VI) could be metabolized, for the most part, into Cr(III), thus preventing Cr internalization in RBCs and favoring its persistence in plasma. Conversely, when the levels of exposure increase, at some point Cr(VI) scavenging capacity by glutathione and ascorbate may be exceeded resulting in accumulation of Cr(VI) in RBCs. Also, genetic polymorphisms may influence the Cr(VI) cellular uptake, intracellular reduction and trapping of reduced Cr and should be considered as important determinants of the accumulation of Cr in cells (Qu et al. 2008). Further research should elucidate these issues in order to achieve a suitable understanding of the results particularly concerning the representativeness of the P-Cr levels with respect to the Cr(VI) or Cr(III) exposure and find possible conditions of susceptibility.

Concerning other factors potentially responsible for the variability in the observed HBM results, our findings did not indicate a contribution of environmental, extra-professional co-exposures to RBC-Cr and P-Cr levels, including age and the smoking habit. This is in line with Scheepers et al. (2008) who did not find an influence of age on RBC-Cr and P-Cr and with Qu et al. (2008), Lukanova et al. (1996), and Zhang et al. (2011) who did not report a significant difference in RBC-Cr between smoker and non-smoker subjects.

The use of PPE should be deeply considered. With respect to the inhalable Cr(VI) exposure data, only 27% of chrome platers (15 out 55 workers) reported using RPE. A clear impact of the use of RPE on biomarkers concentrations was observed as better correlations between RBC-Cr and P-Cr and inhalable or respirable Cr(VI) were determined in the group without RPE with respect to those workers using RPE (Table 5). In the same line, previous publications (Santonen et al., 2022; Viegas at al., 2022) reported that the higher urinary Cr levels among platers when compared with other groups as welders or painters, maybe the results of ineffective protection by RPE.

Dermal and gastro-intestinal uptake, e.g. due to hand to mouth contact, in addition to uptake by inhalation may contribute to the observed exposure in chrome bath platers (Cherrie et al., 2006). Significant hand contamination was previously reported for chrome plating, welding and machining (Viegas et al., 2022). These observations suggest that the effect of PPE on Cr uptake needs further evaluation where HBM such as P-Cr and RBM-Cr could be useful.

4.3. Comparison with previous studies

Our results were in line with the median 1.95 μg/L RBC-Cr determined in German welders (Weiss et al. 2013); 1.5 and 3.4 μg/L in pre- and post-shift samples, respectively, in Italian Cr plating workers (Goldoni et al. 2010), 4.41 μg/L in Chinese platers (Zhang et al. 2011). Conversely, much higher levels, 19.02 μg/L, were detected in Bulgarian platers compared to our data (Lukanova et al. 1996). Our findings resulted in the same order of magnitude, or lower, than those determined in unexposed populations, such as the farmers recruited from an area about 90 miles away from a chromate facility in Shandong, China (median 2.64 μg/L) (Qu et al. 2008), the controls enrolled by Zhang et al. (2011) (median 1.54 μg/L) , and the levels determined in non-occupationally exposed subjects from heavily polluted Bulgarian areas and from a resort town on the Black Sea cost (median 2.02 μg/L) (Lukanova et al., 1996). Concerning available data on the P-Cr, our median result is lower than the median values reported by an Italian study (Goldoni et al. 2010) in chrome plating workers (3.0 μg/L at the end of the Tuesday working shift). However, suitable comparisons with previous studies are prevented by the different experimental settings explored, in terms of investigated populations, periods of investigation, diverse occupational practices and Cr exposure levels, as well as different HBM sampling and analytical strategies used. This strongly supports the need for harmonized and joined protocols for data gathering, sampling and chemical analyses, in order to achieve an acceptable comparison of the obtained results while reducing the influence of methodological differences (Santonen et al., 2019).

4.4. Correlation between RBC-Cr and P-Cr

P-Cr showed median levels significantly greater compared to controls (0.48 μg/L), not only in the employees performing chrome plating (1.14 μg/L), but also in welders (0.86 μg/L), while this latter group showed no significant differences in RBC-Cr compared to controls. This may suggest that Cr-compounds in fumes and gases produced during the welding activities may follow different kinetics, due to their physico-chemical characteristics, compared to the emissions generated from the other explored activities, including painting and spraying. This is supported by the high observed correlation between both RBC-Cr and P-Cr with respect to the inhalable and respirable Cr(VI) outside the RPE, and the lack of a correlation between the two biomarkers in welders. These findings may also reflect differences in composition with respect to Cr(III)and Cr(VI) in welding fumes and other sources of exposure. In fact, the Cr(VI) content in total Cr ranged from 4 to 82% in welding fumes (Pesch et al., 2018; Mei et al., 2018) and concentrations of inhalable Cr(III) (total or soluble) may exceed those of Cr(VI) (Stanislawska et al., 2020). Additionally, this latter study reported a positive correlation between the Cr(III) in the inhalable fraction and P-Cr in welders, supporting the role of Cr(III) exposure to explain the P-Cr levels in these workers.

Our results may also indicate that each specific biomarker may reflect different time windows of exposure. P-Cr follows 1st-order kinetics thus reflecting recent exposure, RBC-Cr, that follows a zero-order kinetics, may be more related to chronic exposure as it better reflects the moving average exposure over the past 4 months depending on the lifespan of RBCs (Goldoni et al. 2010). Once trapped in RBCs, Cr(VI) is maintained until the RBCs are sequestered by the spleen or until the Cr is eluted into the plasma. Given the long lifespan of RBCs in the body (up to 120 days), it can be estimated that RBC-Cr may be representative of the amount of Cr(VI) accumulated within the cells over the preceding eight to ten weeks (Miksche and Lewalter, 1997). This explains also the greater correlation found between P-Cr and U-Cr (both following 1st order kinetics), compared to the RBC-Cr. Conversely to the whole Cr content in blood, RBC-Cr can be specific for Cr(VI) exposures. Therefore, this study did not focus on the Cr concentrations in the whole blood, that may represent a mix between the Cr in plasma and in red blood cells, thus losing the possibility to understand the source of exposure. Overall, this underlines the importance to monitor the Cr concentrations in different biological matrices, including the RBCs and plasma, in order to better define the toxicokinetic profile of Cr with respect to both the quantitative and qualitative aspects of the external exposure, e.g. environmental concentrations, type of Cr compounds, and identify the most suitable biomarker to employ.

4.5. Background exposure and observed RBC-Cr and P-Cr in controls

Our findings highlighted a large variability in the background levels in controls between countries and depending on whether the controls were recruited "within" or "outwith" company. The higher levels observed in some control subjects of our study suggest the possibility that controls enrolled "within" companies may be unknowingly and indirectly exposed to Cr compounds. This "bystander exposure" may be explained by cross-contamination throughout workplace and common areas. Lack of industrial hygiene measurements and very limited contextual data related to activities of workers in the control group does not allow a definite interpretation. To this aim, further investigation is necessary to collect new data from adults of different countries, not occupationally exposed to Cr, in order to derive reference values for the general population for a suitable comparison. These may be important to better discriminate occupational exposure from the background one (Santonen et al., 2022) and detect individuals that are exposed at levels higher than expected, and that might need increased attention in risk assessment (Vogel et al. 2019). Reference values, together with limit values, should be viewed as a part of integrated guidance value systems that may assure an accurate interpretation of the HBM data and a suitable assessment of the exposure and possible adverse health effects (Iavicoli et al., 2019).

4.6. Strenghts and limitations

Our study has relevant strengths that deserve attention. These include the investigation of a number of workers, greater than the sample size achievable in national studies, coming from various European countries, and sharing harmonized HBM protocols. Enrolled workers were engaged in a series of different activities involving Cr(VI), and, although the subdivision of the different job tasks can limit the representativeness of the various group samples, this study represents a comprehensive attempt to define the contribution of different job practices in affecting the exposure levels and HBM results. The rich dataset obtained, in fact, allows the analysis of RBC-Cr and P-Cr in relation to the air concentration data, U-Cr, extraprofessional information, job procedures as well as information on the use of RPE. This allows to report regression equations between blood biomarkers and inhalable or respirable Cr(VI) in air samples for chrome platers. Such equations can be used to derive biological limit values corresponding to the occupational exposure level for Cr(VI). General limitations of HBM4EU chromates study have been discussed in previous paper (Galea et al., 2021; Santonen et al., 2022). Additionally, controls enrolled "within" the same companies limit the possibility to discriminate the contribution of the exposures exclusively related to the job tasks and prevent us to extrapolate definite considerations on the added values of both the blood biomarkers explored.

6. Conclusions

This study showed that Cr-blood based biomarkers can provide useful information on how workplace exposure translates into systemic availability of Cr(III) (extracellular, P-Cr) and Cr(VI) (intracellular, RBC-Cr). It shows that among the different job tasks, the highest exposure to Cr(VI) is related to chrome plating. RBC-Cr and P-Cr in chrome platers both demonstrated a high correlation with inhalable and respirable Cr(VI) outside RPE, while, in welders, such correlation was evident only of respirable Cr(VI). Different kinetics between RBC-Cr and P-Cr may explain the low correlation found between these two biomarkers.

Work is still necessary to fully appreciate RBC-Cr and P-Cr use in an occupational health and safety context. Future research should address potential intra- and inter-individual variations in RBC-Cr and P-Cr and background levels in non-occupationally exposed populations. Future work should also clarify how Cr-blood based biomarkers inform health risk and if these markers have added value in the existing framework of exposure and health risk surveillance.

Author contributions

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All authors have read and agreed to the published version of the manuscript.

Ethics:

The study involves human subjects. Consent from subjects participating in the study was received prior to conducting the study. Study protocols have been approved by ethical review boards in each of the participating countries with the approvals granted before recruiting the study participants. The ethical boards reviewing and approving the study are as follows:

•Belgium: Ethische Commissie Onderzoek UZ/KU Leuven, Belgium

•Finland: Coordinating ethics committee, HUS Joint Authority, Helsinki, Finland

•France: Comité de Protection des Personnes (CPP) Sud-Ouest

•Italy: Ethical committee at the Istituto Superiore di Sanità (ISS)

•The Netherlands: Medisch Ethische Toetsingscommissie (METC) Oost Nederland

•Poland: Bioethical Committee at the Nofer Institute of Occupational Medicine

•Portugal: Ethical Committees of Lisbon School of Health Technology and National Institute of Health Dr. Ricardo Jorge

•Luxembourg: CNER Comité National d'Ethique de Recherche (National Ethics Committee for research)

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Disclaimer

The contents, including any opinions and/or conclusions expressed in the manuscript, are those of the authors alone and do not necessarily reflect the opinions or policy of the organization to which they are employed.

Declaration of competing interest

The authors declare that they have no know competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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HBM4EU chromates study - Usefulness of measurement of blood Chromium levels in the assessment of occupational Cr(VI) exposure.

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Highlights

Suitability of blood biomarkers was evaluated to assess occupational Cr(VI)exposure

Bath platers and painters had higher Red Blood Cells-Cr levels compared to controls

Bath platers, painters and welders had higher plasma-Cr levels compared to controls

Red Blood Cells- and Plasma-Cr were highly correlated with inhalable Cr(VI) in platers

Blood-Cr biomarkers can provide information on systemic and intracellular availability

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: