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Setting up a collaborative European human biological monitoring study on occupational exposure to hexavalent chromium

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ABSTRACT

The EU human biomonitoring initiative, HBM4EU, aims to co-ordinate and advance human biomonitoring (HBM) across Europe. Within its remit, the project is gathering new, policy relevant, EU-wide data on occupational exposure to relevant priority chemicals and developing new approaches for occupational biomonitoring. In this manuscript, the hexavalent chromium [Cr(VI)] study design is presented as the first example of this HBM4EU approach. This study involves eight European countries and plans to recruit 400 workers performing Cr(VI) surface treatment e.g. electroplating or stainless steel welding activities. The aim is to collect new data on current occupational exposure to Cr(VI) in Europe and to test new methods for Cr biomonitoring, specifically the analysis of Cr(VI) in exhaled breath condensate (EBC) and Cr in red blood cells (RBC) in addition to traditional urinary total Cr analyses. Furthermore, exposure data will be complemented with early biological effects data, including genetic and epigenetic effects. Personal air samples and wipe samples are collected in parallel to help informing the biomonitoring results. We present standard operational procedures (SOPs) to support the harmonized methodologies for the collection of occupational hygiene and HBM samples in different countries.

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1. Introduction

The EU human biomonitoring initiative (HBM4EU, www.hbm4eu.eu/about-hbm4eu/) is a European Joint Programme, which aims to harmonize and use biomonitoring to understand human exposure to chemicals in the environment, in occupational settings or through the use of consumer products and the related health risks, in order to improve chemical risk management and to support policy making (Ganzleben et al., 2017). It is funded by the European Commission and national governments and includes experts from 28 countries and European Environment Agency (EEA). It runs from 2017 to 2021.

Human biomonitoring (HBM) provides important data on the combined exposure via all routes of exposure. It can complement measurements in food and environmental matrices and provide information on the effectiveness of preventive and protective measures. Thus, HBM has been considered as a beneficial approach for the health risk management e.g. under EU REACH regulation (Boogaard et al., 2011). Occupational exposures to specific chemicals may, in many instances, be several times higher than environmental exposures experienced by the general population. However, a typical challenge in occupational studies is the low number of workers that can be recruited in national studies. Furthermore, the studies performed by different researchers in individual countries are usually not aligned with respect to sampling, analytical methodologies or data collection, which complicates the comparison of the findings and use of the data in regulatory risk assessment at the European level. Combining national surveys using harmonized study designs and methodologies can potentially greatly improve the information collected and bring EU-added value for the data collected in different European countries as demonstrated in the earlier DEMOCOPHES project (www.eu-hbm.info/democophes). One of the most important aims of the whole HBM4EU project is the harmonization of methodologies and standardized collection of the data useful for EU decision making. In line with these overall goals, herein we present a multicenter study that intends to characterize occupational exposure to hexavalent chromium (Cr (VI)) in industrial settings across Europe.

Hexavalent chromium is an important occupational carcinogen and has been shown to cause lung cancer in humans. Positive associations have been also observed between Cr(VI) exposure and cancer of the nose and nasal sinuses (IARC, 2012). Exposure to Cr(VI) may occur e.g. in welding activities (Scheepers et al., 2008), in Cr(VI) electroplating and other surface treatment activities such as paint application and removal of old paint containing Cr(VI) (SCOEL, 2017). International Agency for Research on Cancer (IARC) has recently classified Welding as carcinogenic to humans (IARC Group 1, IARC, 2018).

Cr(VI) compounds (chromates, chromium trioxide and dichromium tris(chromate)) are authorized under the European regulation (EC, 1907/2006) concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). According to REACH, all companies using Cr(VI) compounds have to apply for authorisation for their uses. More than 100 authorizations for different uses of chromates have already been requested, some of these covering hundreds of workers, which means that potentially thousands of workers are exposed to Cr (VI) in these activities (ECHA, 2019).

REACH concerns only the use of Cr(VI) for specific purposes and does not cover process-generated fumes like welding fumes. Cr(VI) is formed, together with the less toxic trivalent chromium (Cr(III)), when welding metals containing Cr, such as stainless steel (Carre et al., 2005). Management of Cr(VI) formed during the welding process is achieved by compliance with occupational exposure limit values (OELs). The recent binding occupational limit value (BOELV) set under EU Directive 2004/37/EC on the protection of workers from the risks related to exposure to carcinogens or mutagens at work (EU, 2004) is 0.010 mg Cr (VI)/m^3 for a period of 5 years after the date of transposition of the directive; after that period a limit of 0.005 mg Cr(VI)/m^3 will apply. For welding or plasma-cutting processes or similar work processes that generate fumes, there is a derogation, with an OEL value of 0.025 mg Cr (VI)/m^3 until 5 years after the transposition date and after that period the limit will be 0.005 mg Cr(VI)/m^3. In France and the Netherlands, an OEL of 1 μg/m^3 has been set for Cr(VI) (MinSZW, 2016; ANSES, 2017). These are the most stringent OELs currently set for workplaces in the EU.

1.1. Biomonitoring of Cr(VI)

The common biomarker used for the biomonitoring of Cr exposure at the workplace is total urinary Cr (U-Cr). Different biological limit values (BLV) have been set on a national basis in Europe. For example, the Spanish authorities set a BLV of 10 μg/L for U-Cr measured during a shift and 25 μg/L at the end of the workweek (INSHT, 2019). In the UK, a biological monitoring guidance value (BMGV) of 10 μmol/mol creatinine (ca. 6.3 μg/L) in post shift urine has been established (HSE, 2018). France and Finland have derived BLVs of 2.5 μg/L and 10 μg/L corresponding to their OELs for Cr(VI) (ANSES, 2017; STM, 2018). The German DFG (Deutsche Forschungsgemeinschaft) has established EKA values (biological exposure equivalents for carcinogenic substances) ranging from 12 to 40 μg/L for total Cr at the end of shift in urine and from 9 to 35 μg/L in the erythrocyte fraction of whole blood at the end of the shift in the end of the work week. These values correspond to exposures to 0.03–0.1 mg/m^3 soluble alkaline chromate and/or hexavalent welding fumes (only for urine) over an 8 h work shift (DFG, 2018).

The main limitation of U-Cr is that it is not specific for Cr(VI) since it measures exposure to both Cr(III) and Cr(VI). Also, the lowest BLV given for Cr(VI) (2.5 μg/L in France) is close to background urinary Cr levels in populations with no known occupational exposure to Cr (e.g. in France 95th percentile in general population is 0.65 μg/L (ANSES, 2017). These circumstances illustrate the need to develop biomarkers specifically indicating Cr(VI) exposure. Even if U-Cr remains the most used approach for routine biomonitoring of Cr(VI) exposure, it is important to demonstrate how well it correlates with potentially more specific Cr(VI) exposure biomarkers in different work tasks. Such potential, new exposure biomarkers to Cr(VI) are Cr in red blood cells (RBC) and Cr(VI) in exhaled breath condensate (EBC).

Cr in RBC (Cr-RBC) reflects mainly the exposure to Cr(VI) since chromates that contain Cr(VI) can easily permeate through the membrane of the RBC as a tetrahedral divalent anion CrO_4^{2-}, by using the same anion transporter as SO_4^{2-} and PO_4^{2-} (Ray, 2016). In contrast, Cr (III) is poorly taken up by erythrocytes (Ray, 2016) at a rate three orders of magnitude lower than Cr(VI). Once Cr(VI) has entered the RBC it is rapidly reduced to unstable intermediates Cr(V) and Cr(IV) that bind to the beta chain of human hemoglobin and other ligands forming a stable Cr-hemoglobin complex. This Cr-hemoglobin complex remains stable within in the RBC for the cell's lifetime (~120 days) (Paustenbach et al., 2003). On this basis, Cr can be detected in RBC up to 120 days following exposure. In addition, Cr(VI) exposure can be differentiated from Cr (III) exposure by selecting this matrix instead of measuring Cr from whole blood which includes also Cr derived from the exposure to Cr(III) (Lewalter et al., 1985).

Cr(VI) in EBC (Cr-EBC) samples has been proposed as a new biomarker-matrix combination which can give specific information on the Cr(VI) levels in the main target tissue i.e. in the lungs (Leese et al., 2017). It is a less invasive biomarker than blood and Cr(VI) and Cr(III) can be analysed separately in the EBC samples. However, the possibility of some reduction of Cr(VI) to Cr(III) has been suggested for this matrix too, with kinetics not fully explored, yet.

In addition to exposure biomarkers, the characterization of effect biomarkers is of utmost importance to associate the exposure to Cr(VI) with its potential impact on human health, given that they comprise sensitive endpoints that reflect early biochemical changes (subclinical changes) before the onset of disease (Annangi et al., 2016). In addition, they may indicate combined effects of different substances, like Cr(VI)

and nickel (Ni) in welders. Most references have been related to genotoxicity biomarkers. Increased frequency of micronuclei in human peripheral blood lymphocytes have been shown to be predictive for cancer risk (Bonassi et al., 2007) and they have consistently shown correlations with the level of exposure to Cr(VI) (Annangi et al., 2016). Recently, a flow cytometry-based method has been developed for the analysis of micronuclei in peripheral blood reticulocytes, allowing a rapid evaluation of a large number of cells. It is considered very sensitive for the monitoring of genetic damage in humans (Abramsson-Zetterberg et al., 2000; Abramsson-Zetterberg, 2018). The alkaline comet assay, on the other hand, allows the identification of a broad spectrum of primary DNA lesions, such as single- and double-strand breaks (Azqueta and Collins, 2013) and there are studies reporting increased level of DNA damage measured by the comet assay in workers exposed to Cr(VI) (e.g. Balachandar et al., 2010; Zhang et al., 2011).

Oxidative stress, inflammation, oxidative DNA lesions, and telomere damage have been recognized as crucial events in the carcinogenicity process of many substances, including Cr(VI) (Arita and Costa, 2009; Annangi et al., 2016). The most used oxidative stress biomarkers in urine are markers of lipid peroxidation such as malondialdehyde (MDA) and isoprostanes or markers of DNA damage repair, such as 8-hydroxydeoxyguanosine (8-OHdG). Recently, a study showed that urinary 8-OHdG, and MDA levels in the Cr(VI) exposed electroplating workers exceeded those in the control subjects (Pan et al., 2017). Cr(VI) exposure has also been suggested to cause telomere damage and guanine residues of the telomeric repeats seem to be particularly susceptible to oxidative stress caused by Cr(VI) (Ko et al., 2017). Furthermore, epigenetic modifications (e.g. DNA methylation, histone modification) may contribute to the carcinogenicity of Cr(VI) compounds (Salnikow and Zhitkovich, 2008; Arita and Costa, 2009; Ray et al., 2014). In vitro, Cr has been associated with histone modifications in a dose-dependent manner (Sun et al., 2009; Ray et al., 2014). Also, metabolomic profiling, defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999) has been suggested to detect overall effects of welding fume exposure (Kuo et al., 2012; Wei et al., 2013).

1.2. Other hazardous agents in stainless steel welding and surface treatment activities

In addition to Cr(VI), stainless steel welding produces nickel (Ni) and manganese (Mn) fumes. Companies performing Cr(VI) electroplating, may also apply Ni electroplating. Like Cr(VI), inorganic Ni compounds are lung carcinogens, for which a health based limit value of 0.005 mg/m^3 (respirable fraction) has been recently proposed by ECHA Risk Assessment Committee (ECHA, 2018). Mn is a neurotoxic substance for which an indicative occupational exposure limit value (IOELV) has been also recently updated in the EU (EU, 2017). These metals are typically measured from the urine and blood samples but EBC has been suggested as a potential new matrix also for biomonitoring of Ni and Mn (Hulo et al., 2014).

In Cr(VI) electroplating, per- and polyfluoroalkyl substances (PFAS) may be used as mist suppressants to prevent aerosol formation and Cr (VI) exposure. Several long-chain PFAS have shown bioaccumulative properties; for example, for perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) as the best-known representatives, with serum half-lives of 5 and 2–4 years, respectively (EFSA, 2018). Cumulative exposure causes an increase in cholesterol levels and immunotoxic effects in humans and also association to reduced birth weight has been proposed (EFSA, 2018). There is, however, limited information on the exposure to PFAS in electroplating activities.

2. Aim of the HBM4EU occupational Cr(VI) study

The main aim of this study is to provide EU relevant data on Cr(VI) internal exposure and early biological effects in occupational settings, to be used as scientific evidence for regulatory risk assessment and decision-making under EU chemical legislation and under occupational safety and health legislation. The second important aim is to evaluate the capability and validity of different HBM parameters for the specific assessment of Cr(VI) exposure. This includes specific biomarkers for Cr (VI) exposure, Cr-RBC and Cr-EBC, as well as biomarkers of early biological effects, ranging from the classic micronucleus assay to epigenetics markers.

In addition, the study aims to provide information on welders' and platers' exposure to other relevant metals, especially to Ni and Mn, and of chrome platers exposure to mist suppressants containing PFAS.

This manuscript describes the study protocol, which provides an integrated model that can be used in future collaborative, multi-national occupational studies to support decision making in EU or in other regulatory regimes. Furthermore, it illustrates how the challenges related to the limitations of national studies regarding smaller sample sizes, comparability and representability of the results can be resolved.

3. Methodology

3.1. Company and workers recruitment, ethical approvals

Fig. 1 shows the project workflow. The target population includes workers undertaking activities resulting in occupational exposure to Cr (VI), e.g. chrome plating, surface treatment by sanding, spraying or painting, and stainless steel welding. Stainless steel welders were selected since they are expected to be exposed to higher Cr(VI) levels than mild steel welders. In addition, a control population of workers not involved in these activities will also be recruited. Samples will be collected from eight countries, namely, Belgium, Finland, France, Italy, Poland, Portugal, the Netherlands and the United Kingdom (UK).

Companies undertaking the above activities will be invited to participate in the study. Control subjects will be selected from companies in the same geographical area. Worker inclusion and exclusion criteria for the study are given in Table 1.

Recruitment of the companies and workers follows the standard operating procedure (SOP) developed under HBM4EU for the selection of participants, recruitment, informing participants and obtaining informed consent. Interested companies receive an information leaflet, explaining the aims, objectives of the study and what would be expected from them and their workers through their participation. If the company decides to participate an authorized representative completes an employer certificate of informed consent. Workers involved in the activities of interest will be approached to express their interest in participation. An information leaflet for the workers will be distributed and discussed during the first contact with the workers. Workers will complete a worker certificate of informed consent if they decide to participate. The same approach will be followed for controls.

Common information leaflets and informed consent forms, developed under HBM4EU, have been translated and provided in the national languages (English, Finnish, French, Polish, Italian, Portuguese and Dutch). National information, including information on specific national legislation and the contact details of the relevant national research group have been added. This approach ensures that all the participating companies and workers receive the same information on the study.

Study protocols were submitted for approval by medical ethics boards in each participating country with the necessary approvals being granted. The informed consent forms will be archived for the entire study duration and not less than 5 years.

3.2. Standard operating procedures for samplings

In order to collect comparable data in a harmonized way, great effort has been made in the development of SOPs for the collection,

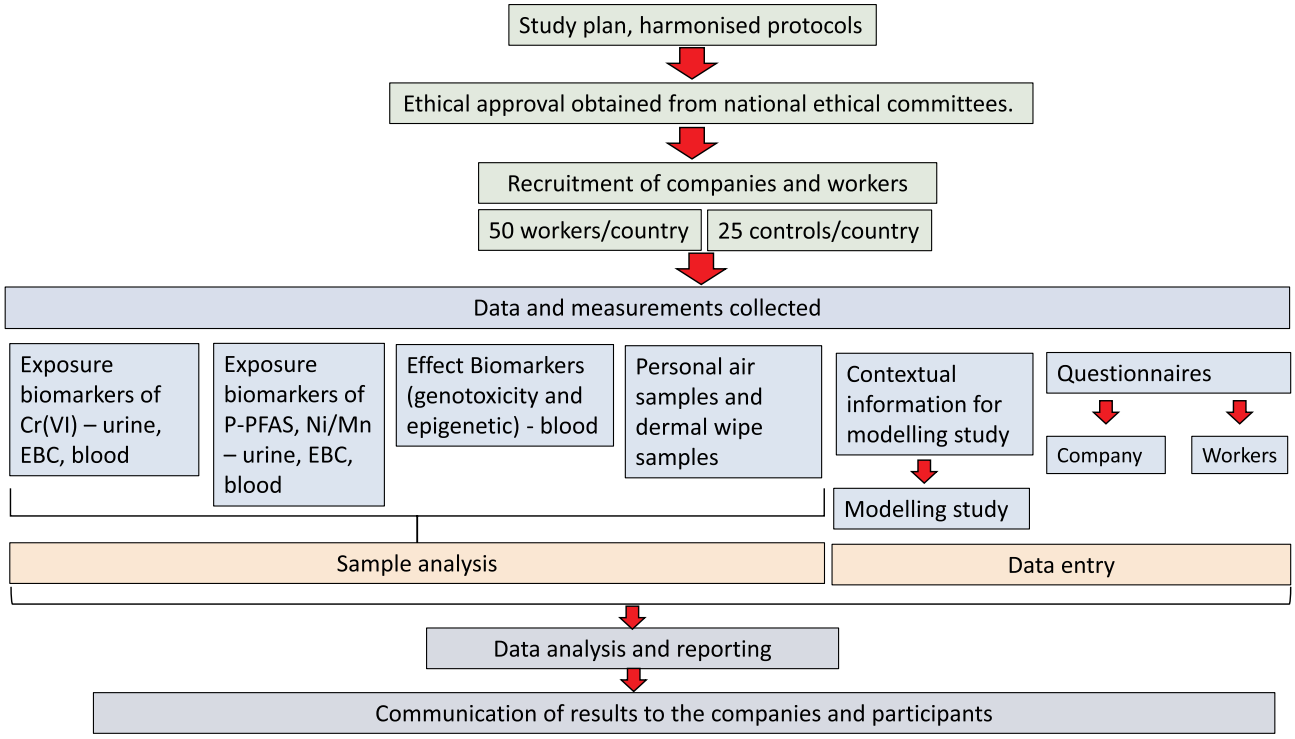

Fig. 1. Schematic illustration of the workflow.

Table 1
Worker inclusion and exclusion criteria for the chromate study.

Workers have occupational exposure to Cr(VI) and undertake either surface treatment (chrome-plating in baths, sanding, spraying or painting) or stainless steel welding activities.
All genders will be eligible, with ages ranging from 18 to 70 years.
Subjects should be in good health and present at work during the planned period of the study.
For genotoxicity biomarkers subjects should also fulfill the following inclusion criteria:
i) are under the age of 50
ii) are non-smokers or ex-smokers for more than six months
iii) have not been subjected to a medical exam such as a medical X-ray or Computerised Axial Tomography (CAT) scan in the last 3- months
iv) do not suffer or have suffered from cancer.

handling, sample storage and transfer of the biological and occupational hygiene samples covered within the Cr(VI) occupational study (Table 2 and supplementary material 1). These procedures were developed through active participation of the study team and agreed upon prior to commencing the measurement campaign, although some were refined following feedback from initial measurement campaigns undertaken. In addition to the SOPs specific for the chromate study, the participating countries follow the general HBM4EU sample transfer protocol and SOPs for the shipment of samples when samples need to be sent from one laboratory to another.

The developed SOPs aim to minimize pre-analytical problems that may lead to misrepresentation of the results. Exogenous contamination introduced by environment and bio-sampling procedures, containers, reagents, blood preservatives, or physical and chemical changes in the biomarkers during transport or storage, or changes in the biological matrix (e.g. coagulation of blood or sedimentation of urine samples) are the foremost interfering factors on the quality of results. An overview of the procedures that each participating country is obliged to follow, as far as is it reasonably possible, are reported in the next sections.

3.3. Sample traceability and collection of contextual information

To guarantee sample traceability, a standardized convention of the sample code assigned to each worker will be used as unique identifier for all samples collected. The same coding system is used also for related documents (questionnaires and informed consent). Two questionnaires will be used to collect relevant contextual information (Supplementary material 2). The first being a self-administered questionnaire to be completed by a company representative, prior to the sampling campaign. The second is an interviewer-led post-shift worker questionnaire to be completed while interviewing the worker as close as

Table 2
List of detailed SOPs prepared for the study.

SOP No.	Title	Topic/Sampling matrix
1	Standard operating procedure for selection of participants and recruitment, information to the participants, informed consent	Recruitment and consent
2	Standard operating procedure for completion of company and worker questionnaires	Company and worker questionnaires
3	Standard operating procedure for blood sampling, including sample storage and transfer	Blood
4	Standard operating procedure for the collection of exhaled breath condensate samples	EBC
5	Standard operating procedure for urine sampling, including sample storage and transfer	Urine
6	Standard operating procedure for air sampling of inhalable and respirable dust fraction and (hexavalent) chromium	Air
7	Standard operating procedure for obtaining dermal wipe samples	Dermal
8	Procedure for comparing occupational hygiene measurements with exposure estimates generated using exposure models	Contextual exposure determinant information

possible to the end of work shift.

The company questionnaire aims to collect general information on the company. Some details regarding general training, exposure monitoring, and occupational health and safety practices are included. Details of the operational conditions related to their chrome plating, sanding, spraying or painting tasks and welding operations (as applicable) are also obtained through questions on e.g. the amount of Cr(VI) used, work procedures and frequency of operations leading to metal exposure, size of the worked parts, and number of involved workers.

The interviewer-led post-shift worker questionnaire is more detailed. Different questionnaires have been prepared for workers involved in chrome plating in baths, welders and surface treatment operators. Job descriptions are addressed through questions concerning the characteristics of the specific tasks, including their duration in a work shift, and frequency. Possible background exposures from non-workplace sources are investigated through the questions related to the living environment, i.e. rural vs. urban environment, presence of industrial plants, incinerators or landfill sites, as well as road traffic density. Information on habits that may affect Cr exposure or effect marker levels are requested, e.g. cigarette and/or e-cigarette smoking, tobacco product uses, alcohol consumption, dietary habits and the use of food supplements (including Cr containing weight loss pills), as well as recreational activities or hobbies that may lead in Cr exposure. Presence of dental implants and dental fillings and therapies performed are investigated as possible confounding factors.

Information on the occupational histories of individual workers, and a detailed description of the tasks performed on the day of sampling are collected. Some items are also included to obtain details of the risk management measures (RMM) used during the work activities, e.g. presence of local exhaust ventilation (LEV), availability and use of personal protective equipment (PPE), information and training, hygiene practices, as well as the occurrence of abnormal conditions during work.

3.4. Air sampling and sampling of hand wipes

As workers may be exposed to Cr(VI) via the inhalation and dermal exposure it is important to understand the contribution of each route of exposure in the total exposure seen by biomonitoring. Personal air samples will be collected for the assessment of inhalation exposure and wipe samples to assess skin contamination, which may lead to skin absorption and to gastrointestinal absorption following hand-to-mouth exposure (Gorman Ng et al., 2017).

For electroplaters and surface treatment workers, simultaneous sampling of the inhalable and respirable fractions will be performed (CEN, 1993). Sampling of the inhalable dust fraction is performed at a flow rate of 2 L/min with an IOM-sampler containing an IOM-cassette fitted with a pre-weighed 25 mm PVC-filter (GLA-5000). Sampling of the respirable dust fraction is performed using sampling heads such as the Higgins Dewell type or similar cyclone sampling heads, performed at the required flow rate, with these containing a cyclone cassette fitted with a pre-weighed 25 mm PVC-filter (GLA-5000). For welders, alternatively the SKC Mini-sampler could be used with a pre-weighed 13 mm MCE filter at a flow rate of 0.75 L/min. Samples will be collected for a representative period of the work shift (> 75%).

The air samples are to be analysed firstly gravimetrically for determination of the inhalable/respirable dust fraction. The samples are then analysed for total Cr, Cr(VI) and depending on the activities for other metals using OSHA Method ID-125G (OSHA, 2002) and ISO 16740 Method (ISO, 2005).

The dermal wipe samples will be collected using SKC Ghost sampling wipes (or similar lead wipes) as these have been demonstrated to be suitable for the collection of wipe samples for metals, including Cr (OSHA, 2002; NIOSH, 2003). A standardized wiping technique will be used to collect samples at set periods during the working shift; these being pre-shift, first break period, lunch and post-shift. In each sampling period, using a separate wipe for each hand, five horizontal and five vertical wipes across the surface of the palm of the hand (including the fingers) will be made, followed by a wipe in the clockwise direction. This procedure will then be repeated for the dorsal region of the hand, with each finger then being wiped, taking care to wipe in between the fingers. Field blanks will be collected and comprise of one wipe handled in the same way as the exposure samples, but were not used to wipe a hand. The Ghost wipes are to be analysed for total Cr using OHSA Method ID125G. Average hand areas will be used in subsequent calculations, these being 535 cm^2 per male hand (total 1070 cm^2 for both hands) and 445 cm^2 per female hand (total 890 cm^2 for both hands) (EPA, 2011).

3.5. Blood sampling and exposure markers

The collection of blood requires a clean and private space, the availability of sterile material for blood collection and staff trained in phlebotomy (WHO, 2010). To reduce interferences in Cr analysis appropriate tubes for trace element detection will be used. In addition, all the plastic material used for Cr detection are of trace element quality or soaked in 20% HNO_3 for 24 h prior to use and rinsed three times with deionized distilled water. Regular phlebotomy syringe with a stainless-steel needle can be used but the use of silicone-coated needle or butterfly is recommended.

One blood sample will be collected from each (exposed or non-exposed) worker, preferentially on the 3rd - 5th day of the working week. Four different tubes are collected: two tubes with sodium heparin for micronucleus and Comet assays; one with potassium EDTA or heparin for analysis of Cr in plasma and RBC and PFAS in plasma and one with potassium EDTA for epigenetics, oxidative stress and telomere length analyses (see Fig. 2).

To avoid haemolysis in tube 3 (for Cr analysis), plasma and RBC separation is conducted, preferably within 8 h (and maximum 24 h) from the specimen collection, following the method described by Devoy et al. (2016). Samples are centrifuged (10 min at 1000–2000 × *g* or 5 min at 2700 × *g*) and the supernatant containing the plasma and white blood cells is used for Cr and PFAS analyses. The pellet undergoes three washing steps with 0.9% NaCl solution (with a volume corresponding the initial volume of blood collected), in order to eliminate interfering plasma/Cr residues. Haematocrit (HT) is measured twice, soon after the collection (HT1) and just before centrifuging for the last washing (HT2) and the ratio of HT2:HT1 is calculated for correction of RBC loss along the washing steps. Analyses can be performed by different analytical techniques. For instance, the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) validated the biomonitoring method applied in this study by using GFAAS and reaching a detection limit of 0.5 μg/L (DFG, 1990), while a lower detection limit (0.01 μg/L) and improved accuracy and precision is reached in the case of ICP-MS quantification (Goldoni et al., 2010).

3.6. EBC samplings and exposure biomarkers

EBC is the collection of cooled exhaled breath as a condensate solution, during regular tidal breathing. This exhaled air is mostly water vapour, but also droplets of fluid from the respiratory tract. These droplets of fluid will contain markers and molecules from the mouth, tracheobronchial system and the alveoli regions of the lungs that originate from occupational and environmental exposure (Kharitonov and Barnes, 2001).

In this study, for the harmonized collection of EBC, we will use TurboDECCS (Medivac, Parma Italy), in all settings. This is a portable collection system which consists of a cooling chamber to cool and condense the exhaled breath sample on the surface of a collection tube and a sampling system comprising of a mouthpiece connected to a one-way aspiration valve with a saliva trap.

Two EBC samples will be collected from occupationally exposed

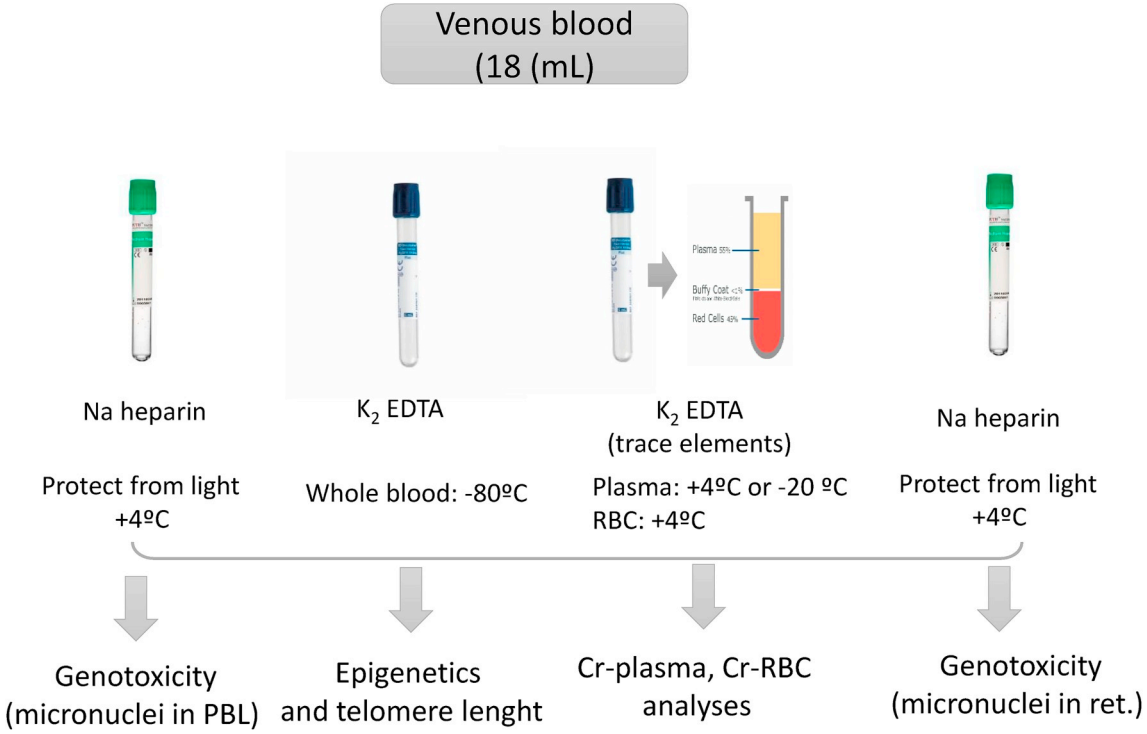

Fig. 2. Blood samples collected.

workers; the first before the start of shift on the first day of the work week and a second at the end of the shift towards the end of the work week. For the control group only one EBC sample will be collected (time not specified).

The collection of EBC is non-invasive and the sampling of EBC does not cause an inflammatory response itself (Hoffmeyer et al., 2007). EBC samples are collected by regular, tidal breathing through the mouth via a disposable mouthpiece for 15 min. For the most part, a complete seal must be maintained around the mouthpiece with periodic swallowing of saliva.

As the stability and integrity of Cr species can be very dependent on pH, to inhibit the degradation or interconversion of Cr(III) and Cr(VI) the EBC sample must be complexed with an EDTA solution and stored refrigerated (not frozen). Immediately after the collection of each EBC sample, an aliquot of EBC is diluted 10-fold with 0.5 mM EDTA (pH adjusted to pH 8 using 10% v/v ammonia solution). In addition, the volume of EBC collected can vary from one individual to the next. This variation in EBC sample volume means the concentration of Cr(VI) will also vary. As there is currently no proposed volume correction marker, the results will be reported in μg/L per volume of EBC collected. Therefore, it is important to register the amount of EBC sample aliquoted to be complexed with the EDTA solution, and to weigh the remainder of the uncomplexed EBC sample upon return to the analysing laboratory.

3.7. Urine sample collections and exposure biomarkers

Two spot urine samples will be collected from the occupationally exposed workers, the first before the start of the shift at the beginning of the work week, and the second one at the end of the shift towards the end of the work week. In order to avoid any contamination of urine samples, participants will be instructed to remove their work clothes and to thoroughly wash their hands before the urine collection.

Urine samples will be collected in previously decontaminated containers (pre-washed with 10% of nitric acid solution) to avoid background contamination. After collection, urine samples will be homogenized and aliquoted in several pre-labeled tubes and stored at − 20 °C in freezers before shipment to the analyzing laboratories. Samples will be analysed for total Cr. Urinary creatinine concentrations will be measured and Cr results normalized to creatinine. In addition, urinary Ni and Mn levels will be analysed in urine samples from welders and Ni in urine samples obtained from workers involved in the use of Ni in metal plating. These are the main hazardous metals which may be present at these workplaces besides Cr(VI). Although it is recognized that urine may not appropriately reflect Mn exposure (Ellingsen et al., 2006), U-Mn is analysed for comparison together with EBC-Mn levels. Urine samples will also be analysed for oxidative stress biomarkers (malondialdehyde, 8-isoprostane, 8-OHdG) and for metabolomic profiles.

3.8. Quality assurance

In order to obtain accurate data, the Cr(VI) study will include a quality assurance (QA) programme in which candidate laboratories for sample analysis will participate in inter-laboratory comparison investigations (ICI). The proficiency tests include Cr in urine, RBCs and plasma and PFAS in plasma. Each laboratory will receive at least two materials, namely biological specimens, containing the parameters at different levels. Each control material is tested for stability and homogeneity by the ICI organizers before distribution. For quantitative evaluation, the results from the participating laboratories will be rated using the classical z-scores indicators, according to ISO 13528 (ISO, 2015). Those laboratories that will be successful in the ICI rounds will be designated qualified to perform analysis of the human samples in agreement to the HBM4EU Quality Assurance programme criteria (www.hbm4eu.eu/online-library).

After approval of the candidate laboratories, QA/QC assurance measures should be maintained concomitant with the analysis of biological samples. To this end, each laboratory selected for the analysis of samples has to perform internal quality control (IQC) measures including at least the purchase or in-house preparation of appropriate IQC materials (at least low and high level). The analysis of at least one sample of each ICQ material in each analytical series and the evaluation of the IQC results using quality Control Charts (CCs) ensure the

comparability of the results over the full study period (Ruggieri et al., 2016). Sustainable continuation of QA/QC measures beyond the ICI runs organized in-house by HBM4EU, will be realised by the use of Certified Reference Materials (CRM) and the participation at public accessible international proficiency tests, like UK Trace Elements EQAS (TEQAS, http://www.surreyeqas.org.uk/trace-elements-teqas/), the Québec multielement EQAS (QMEQAS, https://www.inspq.qc.ca/en/ctq/eqas) and the German External Quality Assessment Scheme (G-EQUAS, http://www.g-equas.de/ (Göen et al., 2012)). Since there is no ICI/EQUAS for Ni and Mn organized under HBM4EU, the laboratories analysing these elements from urine and blood samples need to participate public proficiency tests to ensure the quality of these analyses.

Regarding the analysis of Cr in EBC samples, a suitable ICI/EQUAS scheme does not exist. One of the aims of HBM4EU will be the development and validation of a method for EBC condensate. For this, laboratories with previous experience in this technique and capability for method development will be selected. Once the method is available, a bespoke QA programme will be designed to ensure the comparability of the analyses.

3.9. Power calculations and proposed sample sizes

Power calculations were made for urinary Cr to identify the sample sizes needed to detect meaningful differences between the workers and controls. Calculations were done by using ClinCalc Sample Size Calculator (Kane, 2018), which determines the minimum number of subjects for adequate study power. Study group design was one study group vs. population, primary endpoint was continuous (means), alpha was 0.05, and power was either 80% or 95%. Mean (GM) and standard deviation (SD) of a known population were used in calculations. Urinary total Cr was selected for power calculations because there are background data available for the general population. For other matrixes the data are scarce. A problem with existing urinary Cr data is that in most of the published general population studies no SD data is given. To obtain an estimate on the suitable sample size, we selected general population data from the UK (Morton et al., 2014) and Italy (Aprea et al., 2018) as reference populations. In the UK, the GM of the total urinary Cr is 0.76 μmol/mol creatinine (0.26 μg/L) and GSD 0.55 μmol/mol creatinine (0.24 μg/L). Sample size calculations made for this study with both 80% and 95% power are shown in Table 3. In order to detect an increase of 10% in GM of creatinine adjusted urinary Cr, the required sample size is about 400 (power 80%). For a 20% increase level the respective sample size is about 100 (170 with 95% power). With non-adjusted urinary Cr concentrations, less than 300 samples are needed to detect a 20% increase in GM. The arithmetic mean (AM) and SD of the total urinary Cr data from Italy are 0.317 μg/g creatinine (0.297 μg/L) and 0.334 μg/g creatinine (0.251 μg/L), respectively. In order to detect an increase of 20% in AM of creatinine adjusted urinary Cr, the required sample size is less than 400 (Table 3). With non-adjusted urinary Cr concentrations, fewer samples are needed to detect a 20% increase in AM.

Overall, according to this general population data, 400 samples is a suitable sample size to reliably detect even small differences between workers and controls. It was not possible to perform power calculations for RBC-Cr and EBC-Cr because of the lack of data on background levels in occupationally non-exposed population. In addition, in the case of EBC-Cr(VI), background levels in the occupationally non-exposed population are typically below the detection limits (Leese et al., 2017). Also in the case of RBC-Cr, smaller variation in background levels is expected when compared to the urine total Cr since urine total Cr levels in occupationally non-exposed population are highly affected also by the intake of Cr(III) from different sources.

The final target sample sizes for different matrices are shown in Table 4. Targeted total number of urine samples is 50 pre- and post-shift samples per country from exposed workers and 25 samples from control population per country. In the case of EBC and blood, samples will be collected from 25 exposed workers and 25 controls.

3.10. Data protection, analysis and interpretation

All collected data will be pseudo-anonymized before any treatment, by replacing participant and company names with a code and protecting all electronic and paper records from unauthorized access, in accordance with the EU General Data Protection Regulation (GPDR Regulation (EU) 2016/679 (http://data.europa.eu/eli/reg/2016/679/oj). Any information will be presented in a pseudo-anonymous format so that no external person will be able to identify any person or company who took part in the study.

Data analyses will be done for HBM biomarker variables and some main accompanying variables (e.g. age, sex). Data analysis plans for each specific biomarker include the definition and harmonization of the variables (codebook), the statistical test to be applied, specific exclusion/partitioning criteria for calculation of reference values, uncertainty analysis, data descriptions, and visualizations.

A comparison by industry sectors/types of activities (e.g. welding activities, electroplating and other surface treatment activities) will be done and data will initially be analysed using a (parametric or non-parametric, depending on the distribution) two-way ANOVA, with matching data point. Furthermore, regression models will be developed for comparison by types of activities, size of companies and/or between countries, taking into account specific confounders (like sex, age and tobacco consumption). Multiple linear regression analysis will be

Table 3
Sample size calculations for urinary total chromium.

Reference population						Study population			
Study	Country	Units	N	Mean	SD	Increase (%)	Mean	Required sample size	
								80% power	95% power
Morton et al. (2014)	UK	μmol/mol creat	297	0.76[a]	0.55 [b]	10	0.836	411	681
						20	0.912	103	170
		μg/L	297	0.26[a]	0.24[b]	10	0.286	669	1107
						20	0.312	167	277
Aprea et al. (2018)	Italy	μg/g creat	260	0.317[c]	0.334[d]	10	0.3487	871	1443
						20	0.3804	218	361
		μg/L	260	0.297[c]	0.251[d]	10	0.3267	561	928
						20	0.3564	140	232

[a] Geometric mean.
[b] geometric standard deviation.
[c] Arithmetic mean.
[d] Arithmetic standard deviation.

Table 4
Detailed sample collection plan for the eight participating countries.

Samples	Sampling period			Average number of workers (per country)[a]	Average number of controls (per country)[a]	Overall Number of samples (8 countries)
	Full Shift	Pre-shift/Beginning work week	Post-shift/End work week			
Urine		X	X	50	25[b]	1000
Plasma + RBC		At any time in the work week except the first		25	25	400
Whole blood		work day		25	25	400
EBC		X	X	25	25[b]	600
Air	X			25	–	200
Dermal	4 samples/shift[c]			25	–	800

[a] All matrices will not necessary be collected from all participants.
[b] One urine and EBC sample from each control.
[c] Depends on shift duration and number of breaks. In the case of 8 h shift and one lunch and one rest break, wipe samples will be collected pre-shift, first break period, lunch and post-shift, resulting in four samples per participant.

conducted to examine the associations between inhalation and dermal exposure and biomarkers.

3.11. Use of inhalation and dermal exposure data for exposure model testing

More recently, and probably primarily due to regulatory influence of REACH regulation, the use of predictive exposure models is becoming more frequent as it is not possible for the occupational hygiene community to collect a sufficient number of exposure measurements to generate estimates for all relevant exposure scenarios (Fransman, 2017; Landberg et al., 2018). Several Tier 1 screening models such as ECETOC-TRA, MEASE and others are recommended for use under REACH (ECHA, 2016) and were evaluated under the E-TEAM project of which the results have been reported in several papers (Lamb et al., 2017; Tischer et al., 2017; van Tongeren et al., 2017). Lamb et al. reported a between-user reliability exercise where exposure estimates ranged over several orders of magnitude for the same exposure situation by different users (Lamb et al., 2017). It was also noted that the amount of contextual information provided in the situations could have potentially affected the level of variation between users. To explore this further a standardized proforma will be used to collect contextual information about the work activities observed during the measurement campaign. At a later stage (and without knowledge of the results of the measurement campaigns), several experts with different experience on workplace environments and current activities will be asked to use the ECETOC TRA v 3, MEASE-2 and ART exposure models to estimate the inhalation and dermal exposure (where applicable) at the sites, with these estimates then being compared with the measurement results. The procedure for this parallel study has been described in SOP No. 8.

3.12. Reporting and participants feedback

The results of the study will be reported within the reporting policy of the HBM4EU project, and a general report will be publicly accessible via the project website (https://www.hbm4eu.eu/). It is also anticipated that a number of manuscripts outlining the study findings will be drafted for publication in the peer-reviewed literature.

The participating companies will not receive the results of individual workers. They will receive results on urinary Cr concentrations at a group level and on air samplings in a company-specific occupational hygiene report, which includes any recommendations for the surveillance and monitoring procedures for the workers, and RMMs that should be in place to reduce exposure. Since these recommendations are prepared by the local research teams due consideration can be given to national regulations and guidelines.

Participants will receive their personal lab results of urinary total Cr from the research team or occupational physician, depending on the country specific regulations (unless they have specifically indicated on their consent form not wanting to receive any individual feed-back). In some countries this will be done upon request. This will also show if their result is below or in excess of the national guidance or limit values for Cr. Participants personal results will be accompanied by a fact sheet prepared under HBM4EU on Cr(VI). This will provide information about the possible health effects associated with Cr, the preventative measures their employer must apply and the precautions they should take to protect their health when working with Cr.

It is not intended to provide participants or the participating companies with details of their individual blood, EBC and wipe sample results due to the novelty of the measurement methodology and the difficulty to compare these results with any legal exposure limits. However, the collective results of these sampling matrices will be published in the study report and in various peer-reviewed publications.

4. Expected results and discussion

To our knowledge, this occupational HBM Cr(VI) study is the first that will be performed concurrently in multiple European countries using harmonized protocols for data gathering, sampling and (chemical) analyses. So far, most institutes conducted relatively small surveys, but by combining these national standardized surveys, the power of the study and the strength of the findings are greatly enhanced. This allows also the comparison of several markers of exposure and effect in a variety of exposure scenarios while diluting the influence of the individual workplace visited (which can be an important drawback when studies are only conducted at one or two sites).

The higher number of companies visited and samples collected will enhance the usability of the data in regulatory risk assessment and decision making in EU. The countries involved in the study reflect different parts of Europe; southern (Italy and Portugal), eastern (Poland), mid (France, Netherlands and Belgium) and northern parts (UK and Finland), and the study will also include a range from micro-sized companies and SMEs to large industries. Since a number of different companies (~40 companies) will be engaged in this study and detailed contextual information will be collected by the questionnaire, we will have biomonitoring data linked to different conditions and different RMMs. This way, data can be collected on the used RMMs and it will be possible to identify the best practices. In case of reinvestment or renovation of equipment this information may allow informed decisions to adapt the best available technology.

Hexavalent chromium has been a target for recent legislative actions in EU. These include authorisation of Cr(VI) under REACH and the setting of a BOELV for Cr(VI) under CMD. The main challenge in chromate authorizations has been the paucity of exposure data, particularly in the case of large applications covering more than hundred

individual workplaces. As a self-explanatory example it can be mentioned the Chromium Trioxide Authorisation Consortium (CTAC) application (ECHA, 2019) where 515 companies based in the EU and 36 different worker contributing exposure scenarios were implicated. To grant the authorisation, the Risk Assessment Committee of ECHA requested additional exposure monitoring arrangements that should be based on relevant standard methodologies and be representative of the range of countries and the range of all those tasks undertaken. Therefore, the data generated can be used to support exposure assessment in the scope of the REACH authorisation process, providing background data for regulators on measured levels in several working contributing scenarios and related RMMs in European workplaces. Since the substitution of Cr(VI) in the scope of REACH authorisation is not to be expected in the near future due to the lack of suitable alternatives for many uses, the selection of suitable RMM to reduce exposure is very relevant. Also, very relevant is the comparison of occupational hygiene measurements with exposure estimates generated using exposure models since the use of exposure models is the most common practise in the authorizations processes. This comparison will allow the recognition of how the contextual information and exposure assessor's experience can influence the exposure model outcome. All this can help to harmonize approaches regarding exposure assessment data available in authorisation processes to achieve data representativeness.

In the case of stainless-steel welding, the study will inform on the compliance of companies with the new EU BOELV. This BOELV includes a derogation for welding or plasma-cutting processes or similar work processes that generate fumes: only 5 years after the transposition date the limit will be decreased to 0.005 mg/m^3. It will be important to show whether additional RMMs are needed in European welding workplaces to achieve inhalation exposure levels below 0.005 mg/m^3 or whether they already are able to comply with it.

Both chromate authorisation and CMD oblige employers to conduct exposure assessment at the workplaces. Although air monitoring can provide new and useful information on the airborne levels at the workplace it does not necessarily give much information on the real exposure of the workers if PPE is used and is effective. On the other hand, traditional biomonitoring methods (urinary Cr) may overestimate the exposure since it cannot differentiate exposure to Cr(VI) from the exposure to less hazardous Cr(III). This becomes a problem in work tasks in which co-exposure to different Cr valences occurs (like stainless steel welding) and also at low OELs, i.e. levels close to an OEL of 1 μg/m^3 established for Cr(VI) in France and the Netherlands. At these exposure levels, corresponding biological limit values are close to background urinary Cr levels measured in general population (Fréry et al., 2010; Hoet et al., 2013; Morton et al., 2014; Leng, 2016; Nisse et al., 2017; Aprea et al., 2018) and an individual's Cr background level may have significant impact on total U-Cr level. This means that those workers with higher backgrounds from other (environmental, dietary etc.) sources are likely to exceed limit values more readily than those with lower background levels even though the occupational exposure to Cr(VI) has been similar. Therefore, new, more specific methods (Cr-EBC and Cr-RBC) used in the current study can bring more accurate information on the workers' exposure to Cr and help to improve the occupational exposure and risk assessment. The result will also bring information on the reliability of different biomarkers in different work tasks. It can be hypothesized that U-Cr may be adequate to assess the exposure to Cr(VI) in plating activities, but not necessarily anymore in welding activities.

The measurement of Cr-EBC can provide information specifically on the exposure of the main target organ, lungs, to Cr(VI). Although Cr(VI) has increased also the risk of local gastrointestinal tract cancers in animals when administered orally, in humans, lungs remain the main target organ for its carcinogenic action (ECHA, 2013). Information obtained from EBC analyses can be used to further refine the assessment of lung cancer risk in workers exposed to Cr(VI). Correlations between air Cr(VI), EBC, blood and urinary Cr levels allow further study of the fate and transformation of Cr(VI) to Cr(III) in the human body. When EBC-Cr gives us information on the ratio between Cr(VI) and Cr(III) in mixed exposure scenarios, a simpler U-Cr could be used on a routine basis and the contribution of Cr(VI) calculated.

The current study also analyses effect markers in blood and urine, which might bring additional information on the systemic genotoxicity and epigenetic effects of Cr(VI) at the exposure levels relevant for today's workplaces. This is useful information when considering the potential systemic carcinogenicity of absorbed Cr(VI). In addition, several novel effect markers (reticulocyte MN, epigenetic and oxidative stress markers, telomere length) have been included in the study in order to evaluate which one is the most sensitive to detect subclinical changes upon Cr(VI) exposure.

5. Conclusions

This manuscript describes the design of a multicenter study using HBM in the assessment of Cr(VI) exposure and associated health risks in occupational settings. It describes in detail the methodology and QA procedures in a harmonized multicenter study. This biomonitoring study on occupational exposure to Cr(VI) has a unique set-up including multiple countries collecting biomonitoring and industrial hygiene information on exposure to Cr(VI) using harmonized protocols. In this way, we expect to achieve higher sample numbers than would be feasible in individual studies and create more comprehensive data for EU decision making. This study has also an important role to test new, more sensitive and specific methods for the biomonitoring of exposure to Cr(VI) and their applicability in exposure assessment in occupational health interventions. The reports of the study results are expected to become available in 2020.

Declarations of interests

None.

Disclaimer

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Appendix A. Supplementary data

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