Review article

# Biomarkers of effect as determined in human biomonitoring studies on hexavalent chromium and cadmium in the period 2008–2020

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## ABSTRACT

A number of human biomonitoring (HBM) studies have presented data on exposure to hexavalent chromium [Cr (VI)] and cadmium (Cd), but comparatively few include results on effect biomarkers. The latter are needed to identify associations between exposure and adverse outcomes (AOs) in order to assess public health implications. To support improved derivation of EU regulation and policy making, it is of great importance to identify the most reliable effect biomarkers for these heavy metals that can be used in HBM studies. In the framework of the Human Biomonitoring for Europe (HBM4EU) initiative, our study aim was to identify effect biomarkers linking Cr(VI) and Cd exposure to selected AOs including cancer, immunotoxicity, oxidative stress, and omics/epigenetics. A comprehensive PubMed search identified recent HBM studies, in which effect biomarkers were examined. Validity and applicability of the markers in HBM studies are discussed. The most frequently analysed effect biomarkers regarding Cr(VI) exposure and its association with cancer were those indicating oxidative stress (e.g., 8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdehyde (MDA), glutathione (GSH)) and DNA or chromosomal damage (comet and micronucleus assays). With respect to Cd and to some extent Cr, β-2-microglobulin (B2-MG) and N-acetyl-β-D-glucosaminidase (NAG) are well-established, sensitive, and the most common effect biomarkers to relate Cd or Cr exposure to renal tubular dysfunction. Neutrophil gelatinase-associated lipocalin

*Abbreviations:* 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 8-isoPGF2α, 8-isoprostaglandin-F2α; α1-MG, α1-microglobulin; AKI, Acute Kidney Injury; AO, Adverse Outcome; AOP, Adverse Outcome Pathway; B2-MG, β-2 MicroGlobulin; CAT, catalase; CC-16, Clara Cell protein; CCL5, C-C motif chemokine ligand 5; CKD, Chronic Kidney Disease; Cr(VI), hexavalent chromium; CRP, C-reactive protein; Cys-C, Cystatin C; DPD, deoxypyridinoline; GPx, Glutathione Peroxidase; GR, glucocorticoid receptor; GR, glutathione reductase; GFR, Glomerular Filtration Rate; GSH, glutathione; HBM, Human BioMonitoring; HNE-MA, 4-hydroxy-2-nonenal-mercapturic acid; IARC, International Agency for Research on Cancer; IFN-α, Interferon-alpha; IL, InterLeukin; KE, Key-Event; KDa, kiloDalton; KIM, Kidney Injury Molecule; L-FABP, Liver-type Fatty Acid Protein; LPO, Lipid Peroxidation; MDA, Malondialdehyde; MeSH, Medical Subject Headings; MIE, Molecular Initiating Event; MoA, Mode of Action; MT, Metallothionein; NAG, N-Acetyl-β-D-Glucosaminidase; NGAL, Neutrophil Gelatinase-Associated Lipocalin; OGG1, 8-Oxoguanine DNA Glycosylase; PAH, Polycyclic Aromatic Hydrocarbon; PBA, polyclonal B Cell Activation; PBMC, peripheral blood mononucleate cells; PCR, Polymerase Chain Reaction; PGE2, Prostaglandin E2; ROS, Reactive Oxygen Species; SP-D, surfactant-associated protein D; SOD, Superoxide Dismutase; TCR, T-cell receptor; TNF-α, Tumor Necrosis Factor-alpha; TAC, Total antioxidant capacity.

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(NGAL) and kidney injury molecule (KIM)-1 could serve as sensitive biomarkers of acute kidney injury in response to both metals, but need further investigation in HBM studies. Omics-based biomarkers, i.e., changes in the (epi-)genome, transcriptome, proteome, and metabolome associated with Cr and/or Cd exposure, are promising effect biomarkers, but more HBM data are needed to confirm their significance. The combination of established effect markers and omics biomarkers may represent the strongest approach, especially if based on knowledge of mechanistic principles. To this aim, also mechanistic data were collected to provide guidance on the use of more sensitive and specific effect biomarkers. This also led to the identification of knowledge gaps relevant to the direction of future research.

## 1. Introduction

Human biomonitoring (HBM) assesses human exposure to chemicals and pollutants by analysing these substances, their metabolites or re-action products in blood, urine, saliva, placenta, breast milk, faeces, hair and nails. Regarding metal and metalloid species, blood, urine and hair are the most widely accepted biological matrices to measure their cumulative body burden (CDC, 2005; Gil and Hernández, 2015). HBM allows for taking into account lifestyle factors and individual susceptibilities, which helps to identify subpopulations at risk. Additionally, it enables the identification of spatial and temporal trends in exposure and to detect exposure-health relationships. HBM is also a valuable tool for the detection of emerging pollutants. The evidence gathered through HBM can contribute to human health risk assessment of chemicals (Louro et al., 2019), to priority setting of actions and measures for policy-making, to assessing the effectiveness of implemented environment and health policies in reducing exposure to hazardous substances, and to evaluating more comprehensively the impact of policy measures on human health (Joas et al., 2012; Ganzleben et al., 2017).

The Human Biomonitoring Initiative for Europe (HBM4EU, www. hbm4eu.eu), involving 30 European countries, the European Environment Agency (EEA) and the European Commission, surveys the actual exposure of citizens to chemicals and environmental pollutants, in order to support policy-making and to minimize adverse health outcomes. The heavy metals hexavalent chromium [Cr(VI)] and cadmium (Cd), classified by the International Agency for Research on Cancer (IARC) as group 1 carcinogens (IARC 2012), are included as priority substances within HBM4EU.

Cd is a naturally occurring heavy metal, which is present in small quantities in the environment. Human activities over the last 120 years have significantly increased Cd emissions (WHO 2019; ATSDR 2012). In addition, tobacco and food are the most important sources of exposure of the general population (EFSA 2009; Bocca et al., 2020). Cd is stored in kidney and liver with a biological half-life of 10–30 years (Järup and Åkesson 2009). Cd is primarily toxic to the kidney. Prolonged or high exposure to Cd may result in a reduced glomerular filtration rate (GFR) and ultimately in renal failure. Prolonged urinary levels of >4 μg Cd/g creatinine are associated with renal tubular dysfunction but can also cause bone demineralization in children (Nordberg 2009; Sughis et al., 2011). Cd exposure may also increase the risk of osteoporosis and fracture in pregnant and postmenopausal women and the elderly as well as an increased risk of lung, endometrial, bladder and breast cancer in the general population (EFSA 2009; Nordberg et al., 2015; Grioni et al., 2019). Whether human exposure at doses below 2.5 μg/kg bw/week (equivalent to < 1 μg Cd/g creatinine in urine) affects skeletal, renal, hormonal or reproductive functions is controversial (Åkesson et al., 2014; Nordberg et al. 2015, 2018; Apostoli and Catalani 2015; Bernard 2016; Ronchetti et al., 2016). This lack of clarity, also with regard to occupationally exposed persons, is the main reason for including Cd as a priority substance in HBM4EU.

Cr(VI), the second most stable oxidation state of chromium, is rarely found in nature. Environmental exposure occurs through tobacco smoke, including electronic cigarette smoke (Williams et al., 2017) and inhalation of polluted air or ingestion of contaminated water in citizens living in industrial or contaminated areas (IARC, 2012). Exposure to environmental Cr(VI) through the oral route has been associated to chronic kidney disease (Kulathunga et al., 2019; Tsai et al., 2017) and gastrointestinal disturbances, including stomach ulcers and oral cancer (Zhitkovich, 2011). Even though the meta-analysis conducted by Welling et al. (2015) pointed out that Cr(VI) was a stomach carcinogen for humans, a more recent systematic review and meta-analysis showed that Cr(VI) does not increase the incidence of stomach cancer (Suh et al., 2019).

Nevertheless, anthropogenic activities such as welding, electroplating, surfaces treatment and leather tanning are by far the largest source of Cr(VI). Occupational exposure occurs in workers involved in such industrial activities, the main exposure routes being dermal contact and inhalation of dust and mist or fumes (Elhosary et al., 2014; Lin et al., 2019; Pan et al., 2018; Wang et al. 2011, 2012). Following inhalation, Cr (VI) reaches the respiratory tract and a high percentage of both solubilized Cr(VI) and poorly soluble Cr particles (<5 μm) enter the bloodstream and is distributed to nearly all tissues (ATSDR, 2008). The main adverse health outcomes due to chronic Cr(VI) inhalation are lung impairment, including pneumonia, bronchitis, asthma and lung cancer (Al osman et al., 2019; Saha et al., 2011). Similarly to Cd, total Cr accumulates also in human kidney, besides liver and bone tissues (IARC 2012), increasing the risk of nephrotoxicity, among other undesirable effects. Despite its carcinogenic properties, the use of Cr(VI) compounds (chromates, chromium trioxide and dichromium tris(chromate)) for specific purposes is still authorized under the European regulation (EC, 1907/2006) concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), raising occupational health concerns that have been addressed under the HBM4EU project. Due to potential exposure sources, converging mechanisms of action or target organs, co-exposure to Cd and Cr(VI) has been the subject of research in recent years.

One important objective of the HBM4EU project is to demonstrate the value and applicability of biomarkers of biological effect, further referred to as 'effect biomarkers', to complement biomarkers of exposure in HBM studies (Baken et al., 2019; Mustieles et al., 2020; Steffensen et al., 2020). Effect biomarkers are physiological parameters that mirror an exposure-related response that is associated with an altered biological structure, function, or with a disease phenotype (WHO, 1993; NRC, 2006). These include early biochemical, cellular or molecular effects (Corradi et al., 2015) that are measurable in target or surrogate tissues, e.g., blood cells, and that are associated with the adverse outcome (AO). Thus, mechanistic information about Cr(VI) or Cd Modes of Action (MoA) is of utmost importance to identify the most sensitive as well as robust effect biomarkers, i.e., those that measure alterations recognized as being central to the targeted health effect.

The framework of the Adverse Outcome Pathway (AOP) is based on a series of linked key events (KEs) that begins with a molecular initiating event (MIE) and ends in an AO. The concept allows to evaluate the relevance of an effect biomarker to an expected AO (Leist et al., 2017; OECD 2013, 2018). Concerning the MoAs, Cr(VI) and Cd likely share KEs at the molecular and cellular levels, involving oxidative stress and changes in cell redox status, inhibition of DNA repair mechanisms and altered regulation of gene expression that can induce genetic instability and disturbances of cell homeostasis. In cells, Cr(VI) is reduced to Cr(III), a more thermodynamically stable form of Cr. The intermediate Cr

species, i.e., Cr(IV) and Cr(V) are able to form DNA and DNA-protein adducts (e.g. DNA-amino acid cross-links) that may give rise to DNA single- and double-strand breaks (Stearns et al., 1995). During the intracellular detoxification process, reactive oxygen species (ROS) are also formed, which are considered to mediate Cr(VI)-induced changes in cell signalling and homeostasis leading to cell death by apoptosis (Chen et al., 2019a). ROS accumulation generates oxidative stress and

contributes to chronic inflammation, metabolic reprogramming, and genetic instability, ultimately leading to tumor development (Wang et al., 2016; Chen et al., 2019b). The molecular toxicity of Cd also involves the (indirect) induction of ROS and the reduction of cellular antioxidants. In addition, the inhibition of DNA repair enzymes, the influence on gene transcription and translation, the disturbance of the ubiquitin-proteasome system and the calcium homeostasis are
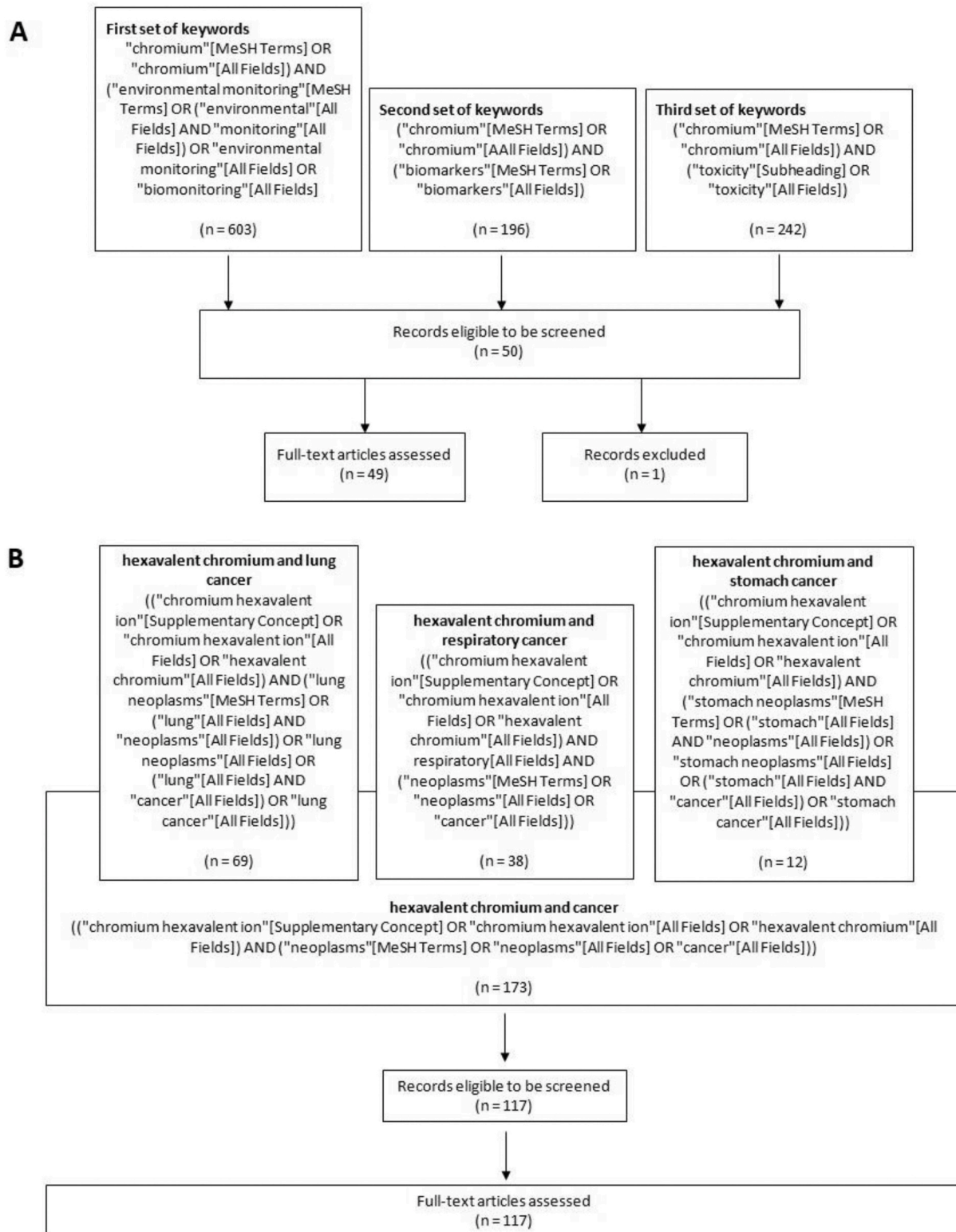


**Fig. 1.** Results of the literature survey on (A) effect biomarkers used in HBM studies related to Cr(VI) exposure or on (B) Cr(VI) exposure and cancer.

recognized to mediate its toxicity. As a result, Cd can interfere with essential cellular processes such as proliferation, differentiation, and apoptosis, which may contribute to its carcinogenicity (Yu et al., 2008; EFSA 2009).

To obtain an overview of the most informative effect biomarkers suitable for use in HBM studies on Cr(VI) and/or Cd exposed populations, a comprehensive literature search was carried out. The most promising biomarkers of effect are presented and discussed considering the mechanism of action and specific AOs for each heavy metal.

## 2. Methods

A literature search in PubMed was conducted to identify studies published between 2008 and 2018 that included effect biomarkers associated with Cr or Cd exposures, supplemented by a targeted search for studies dealing with particular AOs. For Cr(VI), the focus was on cancer, stomach cancer, respiratory cancer and lung cancer (Fig. 1). The search for Cd focused on immunotoxicity, epigenetics/omics, and oxidative stress (Fig. 2). Initially, we did not address on nephrotoxicity, as effect biomarkers for this endpoint have already been widely described (e.g. EFSA 2009). Nevertheless, when searching for Cd and effect markers, we mainly found those effect markers indicating renal damage. As depicted in Figs. 1 and 2, numerous search terms and search term combinations were used, based on MeSH® terms and MeSH supplementary concepts (https://www.nlh.gov/mesh).

The selection of abstracts, after exclusion of inappropriate studies (non-human studies, pure environmental studies, pure exposure studies, publications in non-English language, commentaries, studies for the development of methods and identification technologies), was subjected to a thorough review. With the aim to link the effect biomarkers identified in the search to KEs that ultimately lead to the AOs for Cr(VI) or Cd exposures, articles containing mechanistic information from animal studies or *in vitro* studies were also used (Fig. 1B).

The studies found in the literature search formed the basis for the review. The selected references are listed in Table 1. Where indicated, we have added supplementary information from other reviews and original papers. An additional literature search was conducted in February 2021 for the period 2018–2020. These new articles are summarized in Suppl. Table 1 and their findings discussed when they add relevant information beyond that already found.

## 3. Results

Among the articles retrieved were many studies on established effect biomarkers, which are summarized in Table 1. Their applicability, clinical significance, and mechanistic relevance associated with the respective health outcome are also included in the table. Other effect biomarkers identified for both metals whose utility remains to be confirmed in larger populations and/or other contexts are referred to as candidate biomarkers. The latter are mostly associated with omics approaches and are presented in Table 2 along with some foreseeable advantages and limitations in their use.

The first search for effect biomarkers applied in HBM studies regarding Cr(VI) exposure resulted in 1041 articles, from which 50 were
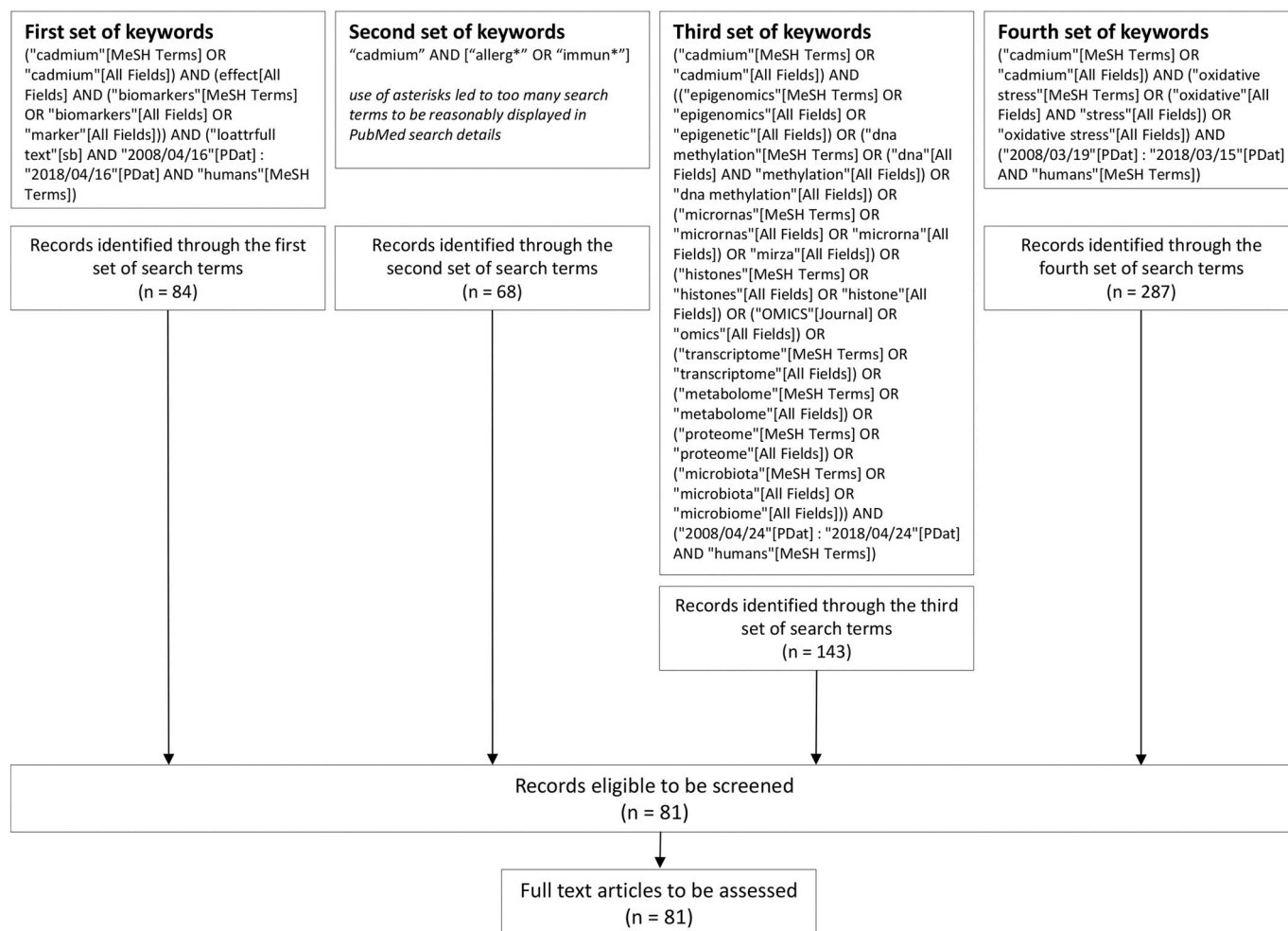


**Fig. 2.** Results of the literature survey on effect biomarkers used in HBM studies related to Cd exposure.

**Table 1**

Effect biomarkers identified for Cr(VI) or Cd-exposed populations according to the respective literature sources (column 'References') and their mechanistic relevance for the selected adverse health effect. The biomarkers are listed separately for Cr(VI) and Cd.

| Biomarker of effect | Mechanistic relevance and link with health outcome | References |
| --- | --- | --- |
| **Cr(VI) Genotoxicity** | | |
| **Chromosome alterations** (Micronucleus or chromosome aberrations in peripheral blood lymphocytes or micronucleus in buccal cells) | Cr(VI) induces DNA damage either mediated by reactive oxygen species (ROS) from its intracellular reduction, by interacting with proteins or with DNA (Wedrychowski et al., 1985; DeLoughery et al., 2015), or through interferences with DNA repair systems causing DNA single- and double-strand breaks (DSBs) (Christie et al., 1984; DeLoughery et al., 2015). Unrepaired or erroneously repaired DBS may give rise to structural chromosome anomalies, which can be experimentally assessed through chromosome aberrations or micronuclei (MN) in human peripheral lymphocytes (Albertini et al., 2000; Bonassi et al., 2007) or in buccal cells (for MN). The frequencies of chromosome aberration or micronucleated cells have been used as effect biomarkers in heavy metals- exposed populations (Annangi et al., 2016) and are known to be associated with cancer risk (Bonassi et al., 2007; Bofetta et al., 2007). | Balachandar et al. (2010) Angelieri et al. (2011) Xiaohua et al. (2012) Sudha et al., (2011) [a] Li et al. (2014) Li et al. (2016) Yang et al. (2016) Wultsch et al., (2017) [b] Franken et al., (2017) [a,c] |
| **DNA damage** (Comet assay in leukocytes or buccal cells; DNA-protein crosslinks) | Different types of DNA damage have been identified following exposure to Cr(VI) and have been implicated in its genotoxicity both *in vivo* and *in vitro*: DNA strand breaks, inter-strand cross-links and DNA adducts. Using UvrABC nuclease and formamidopyrimidine glycosylase (Fpg), and ligation-mediated PCR methods, Arakawa et al. (2012) suggested that inside of the cells, Cr(VI) and Cr(V) interact with adenines and guanines of genomic DNA to cause bulky DNA adducts (e.g., GSH-Cr-DNA) and oxidative DNA damage, which are poorly repaired. Fang et al. (2014) showed the ability of Cr(VI) to intercalate between DNA basepairs (Fang et al., 2014).<br>Unrepaired lesions can lead to mutation formation and, subsequently, to cancer development. The comet assay is one of the most used effect markers in human biomonitoring studies to measure DNA damage; its modification with the Fpg enzyme allows the additional detection of oxidative DNA damage. The information obtained can lead to individual advice on relative risks of genotoxic exposure to environmental or occupational stressors (Dusinska and Collins, 2008). | Grass et al. (2010) [d,e] Balachandar et al. (2010) Sudha et al., (2011) [a] Zhang et al. (2011) Franken et al., (2017) [c] |
| **Mutation frequencies of T-cell receptor (TCR-Mf)** | Cr(VI) exposure induces directly and indirectly (via ROS) DNA lesions that, if unrepaired, may result in increased levels of mutations in somatic cells. The T-Cell Receptor (TCR) mutation assay measures the mutation frequencies of T-cell receptor in somatic cells and has been used in biomonitoring studies to predict the cancer risk following exposure to ionizing radiation (Taooka et al., 2006) or metal(loid)s (Coelho et al., 2013) exposure. | Coelho et al., (2013) [f] |
| **Cr(VI) Oxidative stress** | | |
| **8-hydroxy-2'-deoxyguanosine (8-OHdG)** [r] | One of the major mechanisms that mediate Cr(IV)-induced cellular damage is ROS generation following binding of ROS scavengers (e.g., glutathione and ascorbate) to Cr(VI) and Cr(III) and their reduction to Cr(V) and Cr(IV). Free radicals such as the hydroxyl radicals are able to react with guanine residues and generate DNA adducts. Among these, 8-OHdG has been recognized as a relevant exposure and effect biomarker not only for oxidative damage associated with Cr(VI) exposure (and also Cd exposure; Huang et al., 2009) but with mutagenicity and cancer development (Valko et al., 2004; Chen et al., 2019 a, b). Measurement of urinary 8-OHdG can be carried out using high-performance liquid chromatography with tandem mass spectrometry (Pan et al., 2018) and other methods. | Zhang et al. (2011) Ni et al., (2014) [g] Pizzino et al., (2014) [h] Pesh et al. (2015) Wang et al. (2015) Franken et al., (2017) [c] Savarino et al., (2017) [i] Pan et al. (2018) |
| **Lipid peroxidation (LPO)** [r]<br>**Malondialdehyde (MDA)** [r] | MDA is the major product of polyunsaturated fatty acid peroxidation, a metabolic process of oxidative deterioration of lipids within the cell membrane that is catalysed by iron. This aldehyde is a highly toxic molecule that can react with DNA bases guanine, adenine, and cytosine to form premutagenic adducts (Jomova and Valco, 2011). It has been widely used as a biomarker of lipid peroxidation in epidemiological studies of Cr(VI) exposure. | Khan et al. (2012) Serafim et al., (2012) [j] Ambreen et al. (2014) Zendehdel et al. (2015) Bibi et al. (2016) [k] Gube et al. (2010) Khan et al. (2012) Ambreen et al. (2014) Elhosary et al. (2014) Jamilian et al. (2016) Mozafari et al. (2016) Nascimento et al. (2017) Pan et al. (2018) |
| **Glutathione (GSH)** [r] | In the intracellular moiety Cr(VI) reacts with glutathione, which is a substrate for several enzymes that remove ROS and is also a powerful cellular antioxidant, giving rise to Cr(V). Likewise, Cr(VI) and then Cr(IV) become reduced to the next lower oxidation state by reacting with GSH while the latter become oxidized to glutathione disulfide (GSSG). In lung cells, ascorbate is the most important Cr(VI) reductant, although GSH also plays an important detoxification role. The risk of lung cancer increases when Cr(VI) doses overwhelm the cellular defence mechanisms (Jomova and Valco 2011). Low GSH concentrations and a high GSSG:GSH ratio have been measured in blood of patients with various diseases, including breast and lung cancer. The GSH system is also altered in lung inflammatory conditions and GSH concentrations in | Khan et al. (2012) Ambreen et al. (2014) Bergamo et al., (2016) [l] |

*(continued on next page)*

Table 1 (*continued*)

| Biomarker of effect | Mechanistic relevance and link with health outcome | References |
|---|---|---|
| | the epithelial lining fluid are decreased in idiopathic pulmonary fibrosis, asbestosis and acute respiratory distress syndrome (Comhair et al., 2002). | |
| **Glutathione peroxidase (GPx)**[r] **Superoxide dismutase (SOD)**[r] | Enzymatic antioxidants can intercept, scavenge and neutralize ROS, and can reactivate intermediates generated in excess under physiological conditions. The most important antioxidant enzymes are SOD, catalase, and the glutathione redox system (glutathione peroxidase and glutathione-S-transferase) (Valko et al., 2007). The activity of selected antioxidant enzymes has been measured in blood samples as biomarkers of oxidative stress linked to Cr(VI) exposure. | Bibi et al. (2016)[k] Khan et al. (2012) Ambreen et al. (2014) |
| **Prostaglandin E2 (PGE2) in exhaled breath condensate** **8-iso-prostaglandin-F2α (8-iso-PGF2α) in urine** | 8-iso-PGF2α is an isoprostane (IsoPs) produced by the non-enzymatic peroxidation of arachidonic acid in membrane phospholipids. It has been considered as a reliable biomarker of lipid peroxidation. At present, measurement of F2-IsoPs in plasma or urine is regarded as one of the most reliable approaches for the assessment of oxidative stress status or free-radical–mediated lipid peroxidation *in vivo* and thereby it may be a reliable effect biomarkers for Cr(VI) (Dalle-Donne et al., 2006; Wang et al., 2015). Furthermore, 8-iso-PGF2α is markedly increased, serving as a biomarker, in the bronchoalveolar lavage (BAL) fluid, plasma, urine, or exhaled breath condensate in several pulmonary diseases such as asthma, and interstitial lung disease, among others (Dalle-Donne et al., 2006). | Grass et al., (2010) [d] Hoffmeyer et al., (2012) [m] Wang et al., (2015) [n] |
| **Thiol antioxidants** | Thiols are anti-oxidant molecules in the sulfhydryl group that can be found in plasma (albumin thiols, protein thiols and cysteine, cysteinylglycine, glutathione, homocysteine and γ-glutamylcysteine). Oxidation reaction of thiols with oxidizing molecules causes the formation of reversible disulphide bonds that can again be reduced to thiol groups. Dynamic thiol-disulfide homeostasis plays an important role in anti-oxidant defense, detoxification, and apoptosis, among others (Baba and Bhatnagar 2018). Serum and plasma levels of total thiol (SH groups) were lower in Cr(VI) exposed workers as compared to controls and correlated negatively with oxidative stress. | Pournourmohammadi et al., (2008) [o] Elhosary et al. (2014) Zendehdel et al. (2015) |
| **Total antioxidant capacity (TAC)** | Several assays that determine the ability of a biological sample to reduce a substrate, for instance through hydrogen atom transfer or electron transfer reactions, as measuring the ability of serum to reduce $Fe^{3+}$ to $Fe^{2+}$ or $Cu^{2+}$ to $Cu^+$. Serum levels of TAC were lower in cement industry workers as compared to controls. TAC was also lower in seminal plasma of males living in areas high polluted with toxic waste, but not in their blood. Cr supplements in women with polycystic ovary syndrome resulted in a significant elevation of plasma TAC. | Pournourmohammadi et al., (2008) [o] Zendehdel et al. (2015) Jamilian et al. (2016) |
| **Nitrate and nitrite in exhaled breath condensate** | Production of nitric oxide (NO) is generally increased during inflammation and eventually is oxidized to nitrite ($NO_2$) and nitrate ($NO_3$), both of which are end-products of NO metabolism. Increased in welders. | Gube et al. (2010) Brand et al. (2010) |
| *Cd Oxidative stress* | | |
| **Glutathione peroxidase (GPx)**[r]**, glutathione (GSH)**[r]**, Selenium** | Cadmium is not a Fenton metal and therefore is unable to directly generate free radicals. However, some studies described indirect formation of ROS and RNS involving the superoxide radical, hydroxyl radical and nitric oxide. In rats exposed to Cd through drinking water significantly increased lipoperoxides, MDA and decreased activities of SOD and GPx were found in cardiac tissue (reviewed in Jomova and Valko 2011). | Cabral et al. (2015) |
| **Protein carbonylation of GPx, glutathione reductase (GR), superoxide dismutase (SOD)**[r]**and catalase (CAT)** | | Castillo-Castaneda (2017) |
| **Lipid peroxidation (e.g., MDA)**[r] | | Castillo-Castaneda (2017) Cuypers et al. (2010) |
| *Cd Nephrotoxicity* | | |
| **Urine β2-microglobulin (B2-MG)**[r] | B2-MG is a small protein of about 12 kDa necessary for cell surface expression of major histocompatibility factor (MHC) class I molecules and other (non-classical MHC-1) proteins such as hemochromatosis. It circulates in a soluble form in blood entirely reabsorbed by the nephron. Serum levels rise when glomerular filtration is impaired, while urinary levels increase when tubular reabsorption is harmed. Thus, urinary B2-MG may be a marker for glomerular rather than of tubular pathology (Grangé et al., 2016; Argyropoulos et al., 2017). B2-MG is recommended as marker of chronic kidney disease (CKD)(Foster et al., 2016; Tummalapalli et al., 2016). CKD lasts for at least 3 months and is often an irreversible defect (Kaufman et al., 2019). Cd concentrations in urine, when adjusted for creatinine, age, sex, and smoking are associated with B2-MG in ten studies retrieved by our search (see right column for references). | Nordberg et al. (2009) Trzcinka-Ochocka et al. (2010) Ikeda et al. (2011) Swaddiwudhipong et al., (2012), 2015 Nishijo et al. (2014) Ke et al. (2015) Zhang et al. (2015) Wang et al. (2016) Eom et al. (2017) |
| **N-acetyl-β-D-glucosaminidase (NAG)**[r] | NAG is a large lysosomal brush border enzyme (>130 kDa) of the proximal tubular cells, stably present in urine. Plasma NAG cannot be filtered through the glomerular membrane and its increase in urine is exclusively caused by proximal tubular cell injury. NAG is defined as being more specific and sensitive to renal tubular injury than creatinine especially with its isoenzymes and when combined with other renal biomarkers, for example Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) (Çuhadar and Semerci 2016; Moriguchi et al., 2009; Argyropoulos et al., 2017). NAG is among the best characterized markers and proven to be a sensitive and robust indicator of acute kidney injury (AKI) (Ferguson et al., 2008). AKI is an abrupt increase in serum creatinine - generally over days - and is largely reversible (Kaufman et al., 2019). Cd concentrations in urine, when adjusted for creatinine, age, sex, smoking and other study-specific factors (e.g. concurrent Pb exposure), are associated with NAG in ten studies (see right column for references). | Huang et al. (2009) Trzcinka-Ochocka et al. (2010) Boonprasert et al. (2011) Ikeda et al. (2011) Nordberg et al. (2009) Hambach et al. (2013) Nishijo et al. (2014) Zhang et al. (2015) Wang et al. (2016) Eom et al. (2017) |
| **Retinol binding protein (RBP)** | RBP is a low molecular weight protein belonging to the lipocalin super family. Its main function is to transport retinol (vitamin A). The majority of RBP-retinol circulates in the plasma bound to transthyretin, a complex that prevents its glomerular filtration. 4–5% of serum RBP- | |

**Table 1** (*continued*)

| Biomarker of effect | Mechanistic relevance and link with health outcome | References |
| --- | --- | --- |
| | retinol, however, circulates freely, pass the glomerular barrier and is then reabsorbed and degraded in the proximal tubule, a process mediated by megalin. Due to these properties, urinary RBP is an established biomarker of proximal tubular dysfunction and is used as a diagnostic tool in proximal tubulopathies (Domingos et al., 2016). Cd concentrations in urine, when adjusted for creatinine, age, sex, smoking and other study-specific factors (e.g. concurrent Pb exposure), are associated with RBP in three studies (see right column for references). | Trzcinka-Ochocka et al. (2010) Hambach et al. (2013) Zhang et al. (2015) |
| **Glomerular filtration rate (GFR)** | GFR represents the flow of plasma from the glomerulus into Bowman's space over a specified period. It is the main measure of kidney function calculated from blood creatinine and/or Cystatin-C (Cys-C) in conjunction with age, body size and gender. Combining serum creatinine and Cys-C are superior to equations using either Cys-C or serum creatinine alone (Wasung et al., 2015). Changes in GFR are used to define and diagnose several pathologies. In three studies Cd levels in urine are inversely correlated with GFR (Weaver et al., 2011; Swaddiwudhipong et al., 2012; Eom et al., 2017). | Weaver et al. (2011) Swaddiwudhipong et al. (2012) Eom et al. (2017) Tsai et al. (2017) Wu et al. (2018) |
| **Urine albumin** | Urine albumin serves as a specific and stable biomarker for the early diagnosis of AKI. The combination of creatinine, Cystatin-C and urinary albumin to creatinine ratio enhances risk stratification for kidney disease progression and mortality (Wasung et al., 2015). Much higher levels of urinary albumin are observed with glomerular injury than with tubular injury. In rodents, albuminuria is a marker of cisplatin nephrotoxicity. Cisplatin increases albuminuria, but the clinical relevance is uncertain. The low costs of commercially available tests for the detection of urine albumin is an advantage for routine clinical use. Nonetheless, a panel of biomarkers would be necessary to identify the severity of injury and the type of insult (Bolisettyand Agarwal 2011; Griffin et al., 2019). Two studies found urinary Cd associated with urine albumin. | Trzcinka-Ochocka et al. (2010) Nordberg et al. (2009) |
| **Urine total protein** | In one study, urinary total protein was associated with urine Cd levels (Swaddiwudhipong et al., 2012) | Swaddiwudhipong et al., (2012), 2015 |
| **Urine α₁-microglobulin (A1-MG)** | Urinary A1-MG offers a non-invasive, cost-effective diagnostic alternative for the early detection of tubular diseases caused by e.g. heavy metal intoxication. The 27-kDa glycoprotein, whose exact biological function is unknown, is found in various body fluids. Altered plasma/serum levels are usually due to impaired liver or kidney function, but are also observed in clinical conditions such as HIV (Penders and Delanghe 2004). | Ikeda et al. (2011) |

*Cr(VI) Nephrotoxicity*

| | | |
| --- | --- | --- |
| **Kidney injury molecule-1 (KIM-1)** | KIM-1 is strongly upregulated in the proximal tubule cells after AKI. Its ectodomain is secreted into the lumen and serves as a urinary biomarker. KIM-1 also acts as a blood biomarker specifically reflecting AKI (Prozialeck and Edwards 2012; Sabbisetti et al., 2014). | Cárdenas-González et al. (2016)[p] |
| **Cystatin C (Cys-C)** | Cys-C is a marker of the GFR. It is a low molecular weight protein and a potent cysteine protease inhibitor. The plasma concentration of Cystatin-C is proportional to glomerular filtration, making it ideal for GFR estimation. The protein has the ability to detect early renal failure as it provides a reliable GFR estimate at critical values. For this reason, Cys-C is regarded superior to serum creatinine. Also Cd increases the urinary excretion of Cys-C in rats (Prozialeck et al., 2016). | Wang et al. (2011) |
| **Urine β2-microglobulin (B2-MG)[r]** | See information related to Cd nephrotoxicity | Wang et al. (2011) Li et al. (2015)Cárdenas-González et al. (2016) P |
| **N-acetyl-beta-D-glucosaminidase (NAG)[r]** | See information related to Cd nephrotoxicity | Wang et al. (2011)Cárdenas-González et al. (2016) P |

*Cd Immunotoxicity/inflammatory effect*

| | | |
| --- | --- | --- |
| **Values of blood counts** | Alterations of values of blood counts, i. e. values of neutrophils and lymphocytes, are regarded as a marker of early immunotoxicity. | Ciarrocca et al. (2015) Nygaard et al. (2017) Ohsawa et al. (2009) Ohsawa et al. (2009) |
| **Altered distribution of T cell populations** | Shifts of T-cell subpopulations as innate elements of the immune system are markers of early immunomodulatory effects. Altered distribution of T cell populations at birth could cause immunosuppressive effects observed later in childhood (Nygaard et al., 2017). | |
| **Induction of autoantibodies** | Associated to polyclonal B cell activation, T-cell mediated immunostimulation via cytokines and recognition of MHC-II antigens of cell surface including induction of autoantibodies was found to be the primary immunotoxic effect of Cd and can be linked to autoimmune diseases (Ohsawa et al., 2009). | |

*Cr(VI) Immunotoxicity/inflammatory effect*

| | | |
| --- | --- | --- |
| **High-sensitivity C-reactive protein (hs-CRP)** | CRP is an acute phase protein synthesized by the liver in response to interleukin-6 secretion by macrophages and T cells, being a nonspecific biomarker of inflammation. Serum Hs-CRP was significantly elevated in Cr(VI)-exposed workers. | Jamilian et al. (2016) Wang et al. (2012) |
| **Lymphocyte induced proliferation** | This assay evaluates the proliferative response of lymphocytes, and is usually performed in the presence of a mitogen, a polyclonal activator of lymphocytes that stimulates their proliferation (lymphocyte mitogen-induced proliferation). It is used to evaluate the subject cell-mediated immune responsiveness. The lymphocyte proliferative response to mitogens was higher in shoe, hide, and leather industry workers exposed to Cr(VI). Lymphocytes of Cr allergic contact dermatitis patients were also stimulated by Cr, but in this case the lymphocyte proliferative response had low sensitivity. | Mignini et al. (2009) |
| **Glucocorticoid receptors (GR) on peripheral blood mononucleate cells (PBMC)** | | Mignini et al. (2009) |

**Table 1** (*continued*)

| Biomarker of effect | Mechanistic relevance and link with health outcome | References |
|---|---|---|
| **Cytokines (Interleukins IL-2, IL-5, IL-6, IL-12 and IL-13) and interferon-gamma (IFN-γ)** | GR are ubiquitously expressed nuclear receptors to which cortisol and other glucocorticoids bind. Glucocorticoids are steroid hormones involved in several physiological functions and in controlling inflammation (Scheschowitsch et al., 2017). GR density on PBMC were lower in exposed workers versus controls indicating that Cr(VI) is probably an immunological stressor. Cytokines are small, secreted proteins released by cells having a specific effect on the interactions and communications between cells. There are both anti- and pro-inflammatory cytokines. The latter are produced predominantly by activated macrophages and involved in the up-regulation of inflammatory reactions (Zhang and An, 2007). IL-2, IL-5, IL-6 and IL-12 are pro-inflammatory cytokines that were raised upon Cr exposure, as well as IFN-γ.IFN-γ is primarily secreted by activated T cells and natural killer cells, and can promote macrophage activation, mediate antiviral and antibacterial immunity, enhance antigen presentation, orchestrate activation of the innate immune system, coordinate lymphocyte–endothelium interaction, regulate T helper1/T helper 2 activity of CD4$^+$ T cells balance, and control cellular proliferation and apoptosis (Tau and Rothman 1999). IL-13, a mediator of allergic inflammation, showed higher levels in Cr allergic contact dermatitis patients. | Martins and Reis (2013) |
| **Club (Clara) cell protein (CC16) and surfactant-associated protein D (SP-D) CC16** | CC16, as an immunosuppressive protein, and CC16/SP-D were positively associated with indicators of lung injury and can be used as sensitive serum biomarkers for lung toxicity caused by Cr(VI) exposure. | Li et al. (2015) |

*Cd or Cr(VI) Other biomarkers/effects [q]*

| | | |
|---|---|---|
| **Blood pressure (Cd)** | Altered blood pressure is commonly associated with cardiovascular disease. | Boonprasert et al. (2011) Osorio-Yañez et al. (2016) |
| **Bone demineralization (urinary calcium, deoxypyridinoline (DPD)) (Cd)** | Urinary calcium excretion is a determinant of bone mineral density. Deoxypyridinoline (DPD) represents a specific degradation product of mature collagen found in bones. It is excreted unmetabolized in urine and is a specific marker of bone resorption and osteoclastic activity. Two studies found urinary Cd concentrations associated with B2-MG, U-Ca and DPD levels. | Swaddiwudhipong et al. (2015) Sughis et al. (2011) Eom et al. (2017) |
| **Bone mass density (Cd)** | Decreasing bone mass density is associated with cadmium-induced bone disorders. | Trzcinka-Ochocka et al. (2010) |
| **Increased susceptibility to chronic infections (Cd)** | Commonly linked to reduced immune functions. | Krueger and Wade (2016) |
| **Serum folate level (Cr(VI))** | Long-term Cr (VI) exposure has been associated with decreased serum folate, an essential vitamin, and a cofactor in one-carbon metabolism (Wang et al. 2011, 2012). Epidemiological and experimental evidences have shown that folate deficiency may be implicated in cancer development, e.g., lung and colorectum cancer (Ozkan et al., 2007) and thereby serum folate level is a promising marker of early Cr(VI) effects that needs, however, to be further studied. | Wang et al., (2011), 2012 |

[a] Analysed in buccal exfoliated cells.

[b] Occupational exposure to chromium and cobalt.

[c] Environmental exposure to potentially carcinogenic compounds, including chromium, cadmium, nickel, arsenic, PAHs, benzene, pesticides, etc.

[d] Occupational exposure to chromium, iron, and manganese.

[e] DNA-protein crosslinks measured in blood lymphocytes.

[f] Environmental or occupational co-exposure to metal(loids) including cadmium, chromium, arsenic, manganese, nickel, lead, and selenium.

[g] Occupational co-exposure to cadmium, chromium, lead, and nickel.

[h] Environmental co-exposure to chromium and cadmium.

[i] Chromium and cobalt exposure in patients with metal-on-metal hip resurfacing.

[j] Environmental exposure to chromium, cadmium, lead, copper, nickel, and zinc.

[k] Environmental exposure to chromium, cadmium, lead, copper, nickel, cobalt, manganese, iron, and zinc.

[l] Environmental exposure to toxic waste; biomarkers measured in semen.

[m] Occupational exposure to chromium, iron and nickel.

[n] Occupational exposure to chromium, cadmium, arsenic, lead and nickel.

[o] Occupational exposure to chromium and aluminium.

[p] Environmental exposure to chromium and arsenic.

[q] Biomarkers identified during the literature search that were not associated with the selected health effects. These biomarkers were included because they may provide additional useful information.

[r] Biomarker reported for Cr(VI) or Cd exposure.

eligible (Fig. 1A). One of these articles was excluded afterwards because it consisted of a re-analysis of pre-existent data. The literature searches on Cr(VI)-associated cancer resulted in 173 articles either after using the search terms "Cr(VI)" and "cancer" or after more targeted queries for Cr (VI) and stomach cancer (10), respiratory cancer (29) and lung cancer (51); from these, 117 were considered eligible (Fig. 1B). Out of these, 26 studies were observational, 3 were data modelling or meta-analyses, and 88 were experimental, using either mammalian cell lines (42 articles), animal models (41 articles) or both (5 articles). Those *in vitro* and *in vivo* studies reporting mechanistic information about Cr(VI) were selected for a more detailed analysis (Fig. 1B). With respect to Cd, a total of 582 articles were found, of which 81 publications were eligible (Fig. 2). Relevant information on effect markers for oxidative stress (3 articles), nephrotoxicity (32 articles), and immunotoxicity (3 articles) is summarized in Table 1. Seven more articles provided information on additional markers, which were included in Table 1.

Most biomarkers analysed in the epidemiological studies on Cr(VI) exposure targeted oxidative stress (Table 1). For example, 9 out of the 49 articles selected for a full text analysis reported significantly high levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and eight of malondialdehyde (MDA), three reported depletion of glutathione (GSH) and three showed a depletion of superoxide dismutase (SOD). Additionally, biomarkers of genetic damage in blood cells were also frequently used. Regarding Cd, most of the biomarkers retrieved by this search were related to nephrotoxicity and some to oxidative stress and immunotoxicity (Table 1).

## 4. Discussion

### 4.1. Established effect biomarkers

#### 4.1.1. Cr(VI) exposure, genotoxicity and cancer

Since Cr(VI) is known to induce, either directly or indirectly via oxidative stress, DNA strand-breaks and, consequently, chromosomal alterations, these endpoints have been frequently analysed (9 out of 49 studies) in workers performing several activities, e.g., electroplating, welding, and leather tanning (Table 1). Two non-occupational studies, one in patients undergoing fixed orthodontic therapy (Angeleri et al., 2011) and another one in adolescents (Franken et al., 2017) included the characterization of chromosome damage (micronuclei in exfoliated cells from oral mucosa) or DNA damage (comet assay), respectively. The analysis of micronuclei in cytokinesis-blocked lymphocytes was included in the majority of these studies (Balachandar et al., 2010; Xiaohua et al., 2012; Li et al. 2014, 2016; Sudha et al., 2011), frequently in combination with the assessment of DNA damage (Balachandar et al., 2010; Sudha et al., 2011) or oxidative damage (e.g., 8-OHdG) (Li et al. 2014, 2016). Notably, the frequency of micronuclei in lymphocytes was significantly increased in all studies and, in some of them, correlations were established with exposure biomarkers (Xiaohua et al., 2012; Zhang et al., 2011). Some studies have also reported an increase of DNA strand breaks in lymphocytes of workers exposed to Cr(VI) (Zhang et al., 2011). Franken et al. (2017) reported a positive association between biomarkers of exposure to Cr and the levels of both 8-OHdG in urine and DNA damage in blood cells from the adolescent population of Flanders. Balachandar et al. (2010) combined the analyses of chromosome aberrations, micronuclei and DNA damage (comet assay) in peripheral blood cells and found a higher degree of genetic alterations in 72 Cr (VI)-exposed subjects (36 occupationally exposed in leather tannery industries and 36 environmentally exposed due to their residence in the vicinity of tannery industries) compared to non-exposed individuals. An increase in micronuclei frequency was also detected in cytological smears of tobacco user's tannery workers (Kamil et al., 2019), but it was not observed in epithelial nasal or buccal cells neither in patients undergoing fixed orthodontic therapy (Angeleri et al., 2011) nor in chrome plating workers (Wultsch et al., 2017). The mutation frequency of T-cell receptor (TCR) was included in a study. This is not a widely adopted

effect biomarker and thereby its value has not been proven, although gene mutations are frequent events in malignant cells. Overall, these studies evidenced a clear genotoxic effect associated with Cr(VI) exposure, and confirm the high sensitivity of genotoxicity biomarkers for the biomonitoring of exposed groups in occupational settings. Not surprisingly, test methods for measuring chromosome aberrations, micronuclei, and DNA strand breaks (comet assay) have been adopted as OECD Test Guidelines (OECD TGs 474, 475, 489). It is well-accepted that genetic damage will eventually lead to cancer; hence, safety testing strategies for carcinogenic effects in chemical risk assessment are usually based on proving absence of genotoxic potential (Luijten et al., 2016). Indeed, an increased chromosome breakage or micronuclei frequency in human blood cells (usually lymphocytes) has been associated with increased cancer risk (Bofetta et al., 2007; Bonassi et al., 2011). A similar association has not been demonstrated for the comet assay in human blood cells, yet.

#### 4.1.2. Cd or Cr(VI) exposure and nephrotoxicity

Most of the identified effect biomarkers were related to nephrotoxicity at urinary concentrations >4 µg Cd/g creatinine (Table 1) (Nordberg et al. 2015, 2018). Associations reported at lower urinary Cd levels (<1 µg/g creatinine) are insofar questionable as various confounding factors (renal physiology, diuresis, smoking), may lead to non-causal positive associations. Therefore, blood rather than urine markers were suggested as a better alternative for future studies to assess Cd-associated health risks (Bernard 2016, Stajnko et al., 2017).

A single biomarker is rarely sufficient to clearly define a certain pathological condition of the kidney (Ferguson et al., 2008; Ostermann et al., 2020a). It is recommended to use sets of biomarkers to diagnose acute kidney injury (AKI) that is reversible in many cases, acute kidney disease (AKD), and chronic kidney disease (CKD) (for clinical definition of kidney disease see Ostermann et al. (2020b)). Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule (KIM)-1 are among the most frequently mentioned biomarkers that predict AKI in an early stage (Wasung et al., 2015, Nickolas et al., 2018), also in newborns (Askenazi et al., 2012). NGAL, a 25 kDa glycoprotein produced by epithelial tissues, is excreted via glomerular filtration und undergoes complete reabsorption in healthy tubular cells. NGAL is a marker of injury not of normal function and serves also as a marker in diagnosis of CKD (Ronco et al., 2014). KIM-1 is a type 1 transmembrane protein, whose expression is markedly upregulated in proximal tubule cells from patients with acute tubular necrosis. Since 2002, the soluble form of human KIM-1 in urine has been considered a useful and early biomarker for AKI and renal proximal tubule injury (Han et al., 2002). This was confirmed in 2007 in a study of Cd-induced nephrotoxicity in rats in which KIM-1 appeared in urine 4–5 weeks before the onset of proteinuria and 1–3 weeks before the appearance of metallothionein (MT) and Clara cell protein (CC-16) (Prozialeck et al., 2007). Findings that were later substantiated by a thorough description of the potential underlying pathomechanisms (Prozialeck and Edwards 2012).

Accurate markers for the diagnosis of CKD include NGAL, Cystatin C (marker of GFR), liver-type fatty acid protein (L-FABP; marker of tubular cell damage), B2-MG (marker of GFR), and serum β-trace protein (BTP; marker of GFR) among others (Nickolas et al., 2008; Wasung et al., 2015; Foster et al., 2016; Tummalapalli et al., 2016). It should be noted that several markers such as Cystatin-C, NGAL, L-FABP, or NAG can be analysed in plasma as well as in urine, but the significance may vary. For example, plasma Cystatin-C may be a marker of GFR (i.e. a marker of kidney function), whereas it is detectable in urine only after tubular injury (Ostermann et al., 2012).

Among the established biomarkers, B2-MG, NAG and α1-microglobulin (α1-MG), which is preferred over B2-MG in case of acidic urine (pH < 6), are the most frequently used biomarkers linking Cd exposure to renal tubular dysfunction (Table 1). In a recent study from China, B2-MG was shown to be a more sensitive marker than NAG for renal dysfunction, with 2.2 µg/g cr as threshold for clinical diagnosis (Li et al.,

2020).

While B2-MG is a small protein (12 kDa) necessary for cell surface expression of major histocompatibility factor (MHC) class I molecules and other (non-classical MHC-1) proteins such as the hemochromatosis (HFE) protein, NAG is a large lysosomal brush border enzyme (>130 kDa) of the proximal tubular cells. Plasma NAG cannot be filtered through the glomerular membrane and its increase in urine is exclusively caused by proximal tubular cell injury (Moriguchi et al., 2009; Argyropoulos et al., 2017). B2-MG is recommended as marker of CKD (Foster et al., 2016; Tummalapalli et al., 2016). NAG is proven to be a sensitive and robust indicator of AKI (Ferguson et al., 2008). Other markers of kidney malfunction in Cd-exposed populations included the glomerular filtration rate, creatinine, urinary albumin, urinary total protein, and urinary Retinol Binding Protein (RBP) that were frequently examined together with B2-MG and/or NAG (Nordberg et al., 2009; Trzcinka-Ochocka et al., 2010; Ikeda et al., 2011; Hambach et al., 2013; Swaddiwudhipong et al., 2015; Zhang et al., 2015; Eom et al., 2017).

Despite its widespread use in HBM studies, surprisingly little is known about how Cd damages kidney cells to cause a simultaneous increase in e.g. NAG (regarded as marker of AKI) and B2-MG (marker of CKD) levels in urine. It should be noted that the complex pathophysiology of kidney disease, including AKI, is poorly understood and that many questions regarding biomarkers used in the clinical setting remain unresolved (Ronco et al., 2014; Ostermann et al., 2020a). We see an urgent need to further research on the mechanisms underlying Cd-induced nephrotoxicity in order to better understand the value and importance of effect biomarkers in practice. It is therefore difficult to recommend biomarker sets for the Cd-exposed population. However, since early detection of kidney damage is very advantageous (as it is then often reversible), it could be of great benefit in future HBM studies to investigate sensitive markers for early kidney damage (e.g. NGAL, KIM-1) together with the established markers NAG and B2-MG.

Nephrotoxicity after human environmental exposure to Cr was also observed in three studies (Table 1). A dose-dependent association between urinary KIM-1 across Cr exposure was found in 107 children living in San Luis Potosi, Mexico (mean urine Cr level was 61.7 ppb, higher than the biological exposure index). Other kidney injury/functional biomarkers such as serum creatinine, GFR, albuminuria, and NGAL did not show any association with Cr(VI) exposure, confirming that KIM-1 could be an early and sensitive indicator of proximal tubular damage in children (Cárdenas-González et al., 2016). In another study, the nephrotoxicity of chromate after chronic occupational exposure was evaluated in 115 exposed workers and 60 non-exposed volunteers by determination of Cystatin-C in serum and microalbumin, B2-MG and NAG levels in urine. Either the serum or the urinary biomarkers were significantly increased in the chromate-exposed workers over controls (Wang et al., 2010). The urinary levels of B2-MG were also significantly increased in workers from two chromium-producing factories in China with high Cr exposure (Cr > 20 μg/L in blood) (Li et al., 2015).

A few studies looked into co-exposure to Cr and Cd in relation to nephrotoxicity. Tsai et al. (2017) investigated the effects of co-exposure to Cr and Cd on renal function in 360 Taiwanese adults. They observed an independent association between Cr exposure and decreased renal function, but also evidenced that additional exposure to Cd further reduced the GFR. Such an additive effect on GFR was not found in another study with 934 participants in China (Wu et al., 2018).

### 4.1.3. Cd or Cr(VI) exposure and oxidative stress

Among the effect biomarkers identified in epidemiological studies on human exposure to Cr(VI), the majority targets endpoints related to ROS generation, antioxidant cell defence mechanisms and oxidative stress. The characterization of 8-OHdG, a DNA adduct formed by the reaction of hydroxyl radicals with guanine residues, was one of the most frequently used marker (Table 1) and its association with mutagenicity and cancer development has been recognized (Valko et al., 2004; Chen et al., 2019 a,b). A study that included 201 pregnant women, from which

126 were exposed to e-waste recycling and dismantling activities, showed that the levels of 8-OHdG in the umbilical cord blood (UBC) of neonates were positively correlated to the UBC levels of Cr, suggesting an increase of oxidative DNA damage in neonates (Ni et al., 2014). Several other oxidative stress biomarkers were identified, including lipoperoxidation products (e.g., MDA) and the activity of several antioxidant enzymes in blood (e.g., SOD), as shown in Table 1. In one study, including 22 cement workers exposed to Cr(VI) and 20 tannery workers exposed to Cr(III) the blood Cr level was correlated with the reduction of thiol antioxidants in plasma, and increased MDA and p53 expression (Elhosany et al., 2014). Induction of oxidative stress was observed in chrome electroplating workers by increased lipid peroxidation, decreased plasma antioxidant capacity and plasma total thiol (Zendehdel et al., 2015). Pan et al. (2018) also found an association between Cr exposure and urinary 8-OHdG and MDA in electroplating workers. Serum concentration of MDA, activities of CAT and GSH-Px, as well as urinary concentration of 8-OHdG, were also higher in people living in contaminated rural areas of north-eastern China, and serum SOD activity was significantly lower (Xu et al., 2018).

In contrast, only few studies used biomarkers related to Cd exposure and oxidative stress, including glutathione peroxidase (GPx), selenium and GSH levels in blood and urine samples of Cd-exposed human subjects (Cabral et al., 2015; Goyal et al., 2020; He et al., 2020). Additionally, associations of Cd exposure and lipid peroxidation, e.g., MDA, have been described, as well (Cabral et al., 2015; Cuypers et al., 2010; Krueger and Wade 2016). Castillo-Castaneda et al. (2017) found an association between the levels of protein carbonylation in breast milk, induced by lead (Pb) and Cd oxidation, and the activity of GPx and glutathione reductase (GR), SOD and catalase. Metabolic changes induced by Cd, other heavy metals and PAHs were also found in 252 subjects and linked to the oxidative stress biomarkers 8-OHdG, 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA), 8-isoprostaglandin F2α (8-isoPF2α), and 8-nitroguanine (8-NO$_2$Gua), using two-dimensional gas chromatography time-of-flight (2D-GC-ToF) mass spectrometry. Urine metabolomics identified age-dependent biological pathways (Chen et al., 2017).

Of particular interest is a recent work on Cd-specific induction of metallothionein (MT) isoforms, specifically MT1A and MT1M in Normal Human Urothelial Cells (McNeill et al., 2019), in contrast to a human placental cell line, in which Cd induced mainly the expression of MT2A (whereas hardly of MT1A) (Widhalm et al., 2020).

### 4.1.4. Cd or Cr(VI) exposure and immunotoxicity

Few scientific publications have addressed the effects of Cd or Cr(VI) on the immune system (Table 1). The results of a cross-sectional human health survey (NHANES data) suggested that the immunological effects of Cd and Pb toxicity may result in increased susceptibility to chronic infections (Krueger and Wade 2016) or contribute to auto-immune conditions leading to renal impairment (Ohsawa 2009). Several effect biomarkers, e.g., blood cell counts, and characterization of lymphocyte subpopulations have been proposed (Ciarrocca et al., 2015; Nygaard et al., 2017) but their utility still needs to be confirmed. Concerning the possible Cd effects on the immune system, a study on urban workers showed an association between exposure to airborne Cd and hemogram alterations consisting in a reduction of neutrophils and an increase of lymphocytes counts in blood (Ciarrocca et al., 2015). Also, a relationship between prenatal exposure to arsenic and Cd and specific T cell subpopulations in cord blood was established in a birth cohort study (Nygaard et al., 2017). Moreover, enhanced polyclonal B cell activation (PBA) with autoantibody production was induced by oral exposure to environmental Cd, which may suggest a common effect of metals in nephritis development (Ohsawa 2009).

Cr(VI) immunotoxicity was also observed by a decrease in lymphocytes and an increase in basophils number (Nascimento et al., 2016), as well as lymphocyte mitogen-induced proliferation, a decreased density of glucocorticoid receptors on peripheral blood mononuclear cells

**Table 2**
Candidate omics-based effect biomarkers identified for Cr(VI) or Cd exposure.

| "Omics"-based effect biomarker | Mechanistic relevance and link with health outcome | Comments | References |
|---|---|---|---|
| **Gene expression** (transcriptomics) | Differential expression of genes encoding for proteins involved in critical pathways impacted by Cr(V) or Cd, e.g., DNA repair, may be mechanistically associated with exposure and give insights on health outcomes. Heavy metals, e.g. Cr and Cd induce oxidative DNA damage. The expression pattern of genes involved in detoxification (NQO1, ST13 and MT1A genes) or in DNA repair (OOG1 gene) was associated with environmental exposure to Cr and Cd. | ***Pros:*** Gene expression may be analysed in blood cells (low invasiveness) There is a plausible mechanism of action behind this biomarker It has been implemented in human biomonitoring studies Transcriptomics may be a high throughput method Simple and fast after implementation ***Cons:*** Need of RNA-preserved blood samples Molecular biology infrastructures and bioinformatics software required Discriminating between adaptive and adverse responses is challenging More studies needed for specificity, sensitivity and reproducibility analyses Costs – medium/high – depending on the number of genes analysed; sample preservation add cost to the analysis | Pizzino et al., (2014) [a,b] |
| **Protein expression** (proteomics) | Identification of important deregulated pathways after exposure to xenobiotics, can easily be achieved through proteomics. Hu et al. (2017) showed that 44 significantly differentially expressed serum proteins formed 16 significant signaling pathways and a complex proteins-chemical interaction network, which was associated with the immune system and extracellular matrix organization. C reactive protein (CRP), sonic hedgehog protein (SHH) and calcium were located at critical nodes in proteins-chemical interaction network. CRP and SHH might be potential novel biomarkers of Cr(VI) exposure. | ***Pros:*** The biomarker may be assessed in serum (low invasiveness) There is a plausible mechanism of action behind the biomarker. It has been implemented in human biomonitoring studies ***Cons:*** Requires specialized (expensive) equipment and reagents; bioinformatic tools More studies needed for specificity, sensitivity, and reproducibility analyses Costs – generally high, depending on the number of proteins analysed | Hu et al., (2017) [a, c] |
| **DNA methylation levels** (epigenomics) | DNA methylation patterns can be modified by xenobiotics, including Cr(VI) and Cd. Both genomic and mitochondrial DNA can be methylated and demethylated and consequently gene expression altered. | ***Pros:*** DNA methylation may be analysed in blood cells (low invasiveness) No need for sample special preservation beyond storage at −80 °C There is a plausible mechanism of action behind this biomarker It has been implemented in human biomonitoring studies Several methods can be used, e.g., ELISA-based kits or bisulfite pyrosequencing (gold standard method) ***Cons:*** Molecular biology infrastructures and bioinformatics software may be required More studies needed for specificity, sensitivity and reproducibility analyses Costs – low/high, depending on the method used | Hu et al., (2018) [a] Hossain et al., (2012) [b] Zhang et al., (2013) [b] Virani et al., (2016) [b] |
| **5-methylcytosine (5 mC) levels in leukocyte DNA** (epigenomics) | A methylated cytosine-guanine (CpG) island recovery assay was used to assess over 4.6 million sites spanning 16,421 CpG islands. This study shows distinct patterns of DNA methylation or "footprints" in fetal cord blood and maternal venous blood DNA associated with exposure to Cd | ***Pros:*** The biomarker may be analysed in blood cells (low invasiveness) There is a plausible mechanism of action behind this biomarker It has been implemented in human biomonitoring studies Possesses high throughput capabilities ***Cons:*** Molecular biology infrastructures and bioinformatics software required More studies needed for specificity, sensitivity and reproducibility analyses Laborious Costs – medium/high | Sanders et al., (2014) [b] |
| **miRNA expression** (Epigenomics) | Blood Cr levels were associated with plasma miR-3940-5p level but not with genotoxicity markers. Under high Cr exposure, an over expression of miR-3940-5p-regulated DNA repair genes (XRCC2 and BRCC3) was found. It is hypothesized that miR-3940-5p modulates genetic damage levels at higher Cr(VI) levels due to the regulation of Cr(VI) responsive DNA repair genes expression. | ***Pros:*** The biomarkers may be analysed in blood cells (low invasiveness) There is a plausible mechanism of action behind this biomarker It has been implemented in human biomonitoring studies Possesses high throughput capabilities ***Cons:*** | Li et al., (2014) [a] |

**Table 2** (*continued*)

| "Omics"-based effect biomarker | Mechanistic relevance and link with health outcome | Comments | References |
|---|---|---|---|
| | | Need of RNA-preserved blood samples<br>Molecular biology infrastructures and bioinformatics software required<br>More studies needed for specificity, sensitivity and reproducibility analyses<br>Laborious<br>Costs – medium/high | Kuo et al. 2012[a] |
| **Metabolite changes in urine** (Metabolomics) | Detection of urinary metabolite profiles related to nephrotoxicity, altered levels of citrate, amino acids and cytokines [d] | **Pros:**<br>The biomarker may be assessed in urine (low invasiveness)<br>There is a plausible mechanism of action behind the biomarker.<br>It has been implemented in human biomonitoring studies<br>Possesses high throughput capabilities<br>**Cons:**<br>Requires specialized (expensive) equipment and reagents<br>More studies needed for specificity, sensitivity and reproducibility analyses<br>Costs – generally high, depending on the number of metabolites analysed | Ellis et al., (2012)[b]<br>Dudka et al., (2014)[b]<br>Gao et al., (2014)[b]<br>Lee et al., (2014)[b]<br>Sarma et al., (2018)[b]<br>Xu et al., (2016)[b] |

[a] Related to Cr(VI) exposure.
[b] Related to Cd exposure.
[c] Although the pattern of proteins has been considered by the authors as exposure biomarkers, the results interpretation in terms of functional pathways can give insights into the Cr(VI) effects.
[d] Metabolomics can either be considered as an exposure or as an effect biomarker.

(Mignini et al., 2009), and an increased interleukin-6 (IL-6) plasma levels (Mignini et al., 2009; Li et al., 2015). Moreover, the later study including 84 workers from the shoe, leather, and hide industry, showed that Cr(VI) exposure did not modify natural killer cell-mediated cytotoxicity, the plasmatic levels of inflammatory cytokines and related soluble receptors, and prostaglandin levels, while it tended to increase lymphocyte production of immunomodulant cytokines (IFN-γ, IL-4, and IL-2). The authors concluded that the characterization of mitogen-induced lymphocyte proliferation, glucocorticoid receptors on peripheral blood mononuclear cells, and IL-6 plasma levels may allow to discriminate between Cr(VI)-induced stress and other kinds of stress (Mignini et al., 2009). Lymphocyte proliferative rate and cytokine production (IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17 and CCL5) were also investigated as biomarkers for allergic contact dermatitis in chromium-allergic patients (Table 1), but only IL-13 had an accuracy of about 90% (Martins and Reis, 2013).

### 4.2. Candidate omics-based effect biomarkers

Exposure to Cr(VI) or Cd can influence gene expression and, consequently, protein expression and metabolism. Expression analysis of specific genes involved in the response to xenobiotics, such as detoxifying genes and DNA repair genes, can add valuable information about cellular and molecular pathways that are affected by Cr(VI) and Cd exposures (Table 2). Furthermore, disruption of these pathways may be associated with genotoxicity, carcinogenicity, or other pathological processes, and/or may provide mechanistic insights on potential diseases (Ellinger-Ziegelbauer et al., 2009; Schaap et al. 2012, 2014; Li et al., 2017). From a practical point of view, gene expression analyses can be performed in blood, which is easily collected and allows follow-up sampling through time, although it requires proper samples preservation after collection to avoid RNA degradation, adding some costs., and can be used. High throughput gene expression analyses are current but still expensive while for gene-specific approaches the costs are reasonable, making these biomarkers good candidates for implementation (Pizzino et al., 2014). However, finding a specific gene signature for each mechanism of toxicity from an initial set of thousands of up- or down-regulated genes can be cumbersome, blood may not always reflect what is happening in target tissues, and few studies using gene expression analysis as effect biomarkers for Cr(VI) or Cd exposures were retrieved.

Epigenomics is another recent area of research that has shown promising results. Epigenomic changes include histone modifications, chromatin remodelling, changes in DNA methylation and changes in short non-coding RNAs, including microRNAs (miRNAs). While gene transcription is regulated by DNA methylation and chromatin remodelling, post-transcriptional gene expression is regulated by e.g. miRNAs. These three modes of epigenetic regulation interact in a complex feedback network to ultimately determine gene expression (Chung and Herceg 2020). Modifications in the epigenome can be measured in many types of biological samples, including blood. Moreover, DNA methylation can be often be measured in biobanked samples without special conditions rather than −80 °C. Extracellular circulating miRNAs have shown to be remarkably stable in plasma and serum, and resistant to RNase activity, as well as extreme pH and multiple numbers of freeze-thaw cycles, which favour their potential use as effect biomarkers (Chen et al., 2008; Mitchell et al., 2008). As a current limitation, and depending on the population size, the costs of analysing DNA methylation and/or miRNA expression can be high. Overall, both the whole genome methylation level and the pattern of DNA methylation of a selected set of genes appear as potential effect biomarkers for Cr(VI) or Cd exposure (Table 2) that deserve to be further explored.

Proteomics can be even more robust than transcriptomics, since it detects the effect of post-transcriptional and post-translational modifications that regulate protein quantity and function (for instance, miRNAs and phosphorylation, respectively). Target proteins can be found in

high quantities in biological fluids that are easily collected (though some with invasive techniques), and a large amount of data can be obtained using high-throughput methods, e.g., new mass spectrometry techniques, which allow the detection of low and high molecular weight proteins, depending on the number of samples analysed. Thus, proteomic approaches with the improved accuracy of high-throughput technologies and bioinformatics tools might be an important approach to identify protein functional variants associated to metal exposure that may be linked to cellular pathways dysfunction and consequent health outcomes. Most likely, a signature of deregulated proteins could work as an effect biomarker for Cr(VI) or Cd exposures. As an example, in a study conducted in 25 Cr-exposed workers and 16 controls from a chromate production plant Hu et al. (2017) analysed differentially expressed proteins, proteins-chemical interaction networks as well as critical proteins nodes related to signalling pathways. The results showed regulation of 16 signalling pathways and a complex proteins-chemical interaction network, which was associated with the immune system and extracellular matrix organization.

Regarding metabolomics, several human studies were conducted in relevant epidemiological settings using proton nuclear magnetic resonance (H NMR) or liquid chromatography/mass spectrometry (LC/MS) to detect the specific metabolites, mostly in urine, but also in blood. Accordingly, amino acids profiling may be used as a metabolomics effect marker as shown in some human and animal studies, as well (Ellis et al., 2012; Wang et al., 2012; Gao et al., 2014; Dudka et al., 2014; Lee et al., 2014; Xu et al., 2016; Sarma et al., 2018). As an example, a study reported on the urinary metabolomics profiles of 35 Cr-exposed male welders and 16 male office workers at a Taiwanese shipyard. The results showed higher levels of glycine, taurine, betaine/TMAO (modulators of inflammatory and oxidative tissue injury processes), serine, S-sulfocysteine, hippurate, gluconate, creatinine and acetone, and lower levels of creatine among welders. Further studies are required in order to link metal exposures and classical effect markers with metabolic profiles, as well as to provide more data on mechanistic causalities between AO and molecular markers.

### 4.2.1. Cr(VI) or Cd exposure and genotoxicity

The literature search conducted for omics-based effect biomarkers revealed a highly diverse set of studies (Table 2). In relation to genotoxicity, we identified a transcriptomic study of blood samples from 134 subjects exposed to environmental carcinogens, including Cd. This study revealed an association between low exposures to metals and changes in expression of DNA repair genes. In addition, the authors reported deregulation of carcinogenesis-related pathways using the Comparative Toxicogenomics Database (Espin-Perez et al., 2015). Using epigenetic approaches, Hu et al. (2018) identified associations between Cr(VI) exposure, methylation modification of DNA repair genes and their expression levels, and genetic damage in workers. The results of *in vitro* studies using human bronchial epithelial cells exposed to Cr(VI) have confirmed positive correlations between methylation levels of tumour suppressor genes such as p16 (which encodes a specific inhibitor of cyclins being frequently mutated in cancer, including non-small-cell lung cancer) and genetic damage (Hu et al., 2016). Additionally, an epidemiological study by Y. Li et al. (2014) showed that high blood Cr(VI) levels were significantly associated with the decrease of plasma miR-3940-5p, which modulated Cr(VI)-induced genetic damage by regulating the expression levels of the DNA repair genes XRCC2 and BRCC3 in leukocytes (Li et al., 2014). Furthermore, the same authors reported that this miRNA has an important role in the regulation of double-strand DNA breaks repair in response to lung (16HBE) cells exposure to Cr(VI) by enhancing homologous recombination repair (Li et al., 2016). Using *in vitro* and *in vivo* experiments, Ren et al. (2017) were able to further demonstrate that miR-3940-5p over-expression inhibits proliferation and induces apoptosis in non-small cell lung cancer cells by targeting Cyclin D1 and Ubiquitin Specific Peptidase-28, thus having an important protective role of lung cells transformation. Several

miRNAs have also been shown to be dysregulated in Cd-exposed human hepatocellular carcinoma cells (HepG2) and macrophages (THP-1), which have been associated with apoptosis and autophagy, respectively, and 4 have been proposed as promising biomarkers (Paesano et al., 2020). A potential biomarker of DNA damage based on long non-coding RNA (lncRNA) expression profiling was described in Cr(VI)-treated bronchial epithelial cells (Hu et al., 2019). Overall, these findings suggest that epigenetic biomarkers, either based on DNA methylation of selected genes or on non-coding RNA analysis, may be considered as promising candidate effect biomarkers related to genotoxicity and, possibly, to carcinogenicity.

### 4.2.2. Cr(VI) or Cd exposure and nephrotoxicity

Application of epigenetic approaches to detect nephrotoxic effects in blood samples from 81 residents in Cd-polluted and non-polluted areas revealed that levels of blood and urinary Cd correlated positively with the levels of DNA methylation in the gene of the proteohormone KLO-THO, which inhibits phosphate resorption in the proximal tubules of the kidney, and in the tumour suppressor RAS protein activator like 1 (RASAL1) (Zhang et al., 2013). Hypermethylation of the latter may serve as indicator of the progress for chronic kidney disease. Lemaire et al. (2020) identified several dysregulated miRNAs in renal proximal tubular cell models which may play a role in the pathophysiology of Cd-induced kidney damages and may be promising molecular biomarkers that must be further evaluated. The use of metabolomics may also uncover new biomarkers for Cd- and/or Cr-induced effects. Indeed, the effects from exposure to environmental Cd correlated with six urinary metabolites, of which three (including citrate) were associated with mitochondrial metabolism (Ellis et al., 2012), a pathway also found to be affected in Cd-exposed rodents (Lee et al., 2014). These findings are in concordance with heavy metal accumulation causing mitochondrial dysfunction (see section 5). Other relevant metabolomics studies independently confirmed changes in metabolic pathways (Dudka et al., 2014; Gao et al., 2014; Lee et al., 2014; Sarma et al., 2018). Song et al. (2018), using an AKI model in rats, reported that the urinary level of semaphorin 3A, a protein secreted in several kidney structures, may be a valuable biomarker of early AKI.

### 4.2.3. Cr(VI) or Cd exposure and oxidative stress

For oxidative stress, Pizzino et al. (2014) studied the expression levels of detoxifying genes such as NAD(P)H dehydrogenase (quinone 1) (NQO1), metallothionein-1A (MT1A) and suppression of tumorigenicity 13 (ST13), and the DNA repair gene 8-oxoguanine DNA glycosylase (OGG1) in an adolescent population exposed to Cr and Cd, among other heavy metals, from an industrialized area of Italy. They observed a significant upregulation of the genes OGG1 and MT1A, and a slight, but not statistically significant, upregulation of the NQO1 and ST13 genes in the exposed adolescents compared to non-exposed controls. Likewise, oxidative DNA damage, as assessed by urinary 8-OHdG concentration, was higher in adolescents exposed to Cr and Cd than in controls. Another study reported on the expression levels of inflammatory markers in blood of 35 Cr-exposed male welders and 16 controls. The results showed that TNF-α gene expression was significantly associated with welding fume exposure among all cytokines and inflammatory markers measured (Wang et al., 2012).

## 5. AOPs development and effect biomarkers

Cr(VI) exposure via inhalation increases lung cancer risk as the main AO, whereas Cd exposure via ingestion primarily targets the kidney, leading to nephrotoxicity. It is likely that the MIE for several AOs related to heavy metals exposure, including Cr(IV) and Cd, is the binding (of metal ions) to thiol containing molecules. KEs such as oxidative stress (or increase in ROS), mitochondrial dysfunction, activation of caspases (a family of protease enzymes playing essential roles in programmed cell death) and apoptosis are common effects upon Cr(VI) and Cd exposure.

For Cd and for other heavy metals such as arsenic, lead and mercury, they may lead to the AO of tubular damage in the kidney and nephrotoxicity. KEs as oxidative stress and mitochondrial disruption have also been addressed in an AOP for the neurodevelopmental toxicity of arsenic, Cd, manganese and lead (Stackelberg et al., 2015).

In most studies related to Cr(VI) exposure, oxidative stress and direct or indirect (ROS-induced) DNA damage have been identified as the two major KE underlying genotoxicity. Oxidative stress is probably caused by Cr(VI) interference with mitochondrial respiratory chain complex I activity (the possible MIE) that leads to mitochondrial dysfunction (Wise et al., 2018a; Xiao et al. 2012, 2019). These events are reflected in tissue inflammation (Roy et al., 2016) and epithelial-mesenchymal transition (Ding et al., 2013; Val et al., 2015), chromosome instability (Wise et al., 2018b) and malignant transformation of cells (Medan et al., 2012; Val et al., 2015; Wang et al., 2011), among other possible KEs. Another KE may be the insufficient or incorrect DNA repair, possibly by modulating the expression of DNA repair proteins involved in base excision repair or in double strand break repair by homologous recombination, as observed either in epidemiological studies (Sudha et al., 2011; Long et al., 2019) or in *in vitro* studies using lung cells (Li et al., 2017). Additional KEs may be related to apoptosis resistance (Son et al., 2017) and endoplasmatic reticulum stress (Ganapathy et al., 2017). Indeed, Cr(VI) treatment of human lung epithelial cells or keratinocytes activated the intracellular receptor tyrosine kinase SRC, which further upregulated Ras activity, leading to the increase of ROS and the onset of endoplasmatic reticulum stress. An AOP has been proposed for Cr(VI) and intestinal cancer (Thompson et al., 2013, 2015, 2018). Chronic wounding of intestinal villi, chronic regenerative crypt cell hyperplasia (Thompson et al., 2013, 2015, 2018), and increase of crypt enterocytes were identified as major KEs (Thompson et al., 2015). At the molecular level, the same authors identified several changes related to malignant transformation of several cell types were reported, involving changes in the expression of EGFR, p53, c-Myc, HIF-1 alpha, Bcl-2, PDCD4, FBP1, MM1, VEGF, ERK, NF-kB genes and downstream proteins involved in cell signalling pathways related to cell division, proliferation, and differentiation.

The strong binding affinity of Cd to methyltransferases or other thiol group-containing proteins (Sabolic, 2006; Nordberg et al., 2015) suggest several candidates as first KEs (depending on the AO): these include oxidative stress, disruption of cadherin-mediated cell-cell adhesion, mitochondrial dysfunction, and increase of intracellular calcium (Prozialeck and Edwards 2012; Stackelberg et al., 2015; Bal-Price and Meek, 2017; Rana et al., 2018). After suffering from mitochondrial damage, mitochondrial release of cytochrome C might activate several caspases and, subsequently, these caspases trigger apoptosis of cells. Extensive cell death in the proximal tubule may lead to proximal tubular damage in the kidney.

Overall, several potential KEs of Cr(VI) or Cd exposure at the molecular, cellular and tissue level have been identified. However, our understanding of the mechanistic relationship with effect biomarkers is only partial. Nevertheless, effect biomarkers used in HBM studies are informative and at least some of them, e.g., oxidative stress biomarkers for Cr(VI) exposure, are associated with likely KE in the AOP framework allowing an early identification of biological effects associated with adverse health effects, even at population-based subclinical levels. Greater efforts should still be made to clarify how different effect biomarkers are associated with Cr/Cd exposures. Therefore, the results need to be interpreted jointly with results of exposure biomarkers, particularly the metals internal dose. In general, effect biomarkers are not specific of chemicals, but of physiological/pathophysiological functions. However, the biological plausibility of the effect biomarkers can be improved relying on: i) toxicological knowledge organized following an AOP-like conceptualization; ii) evaluate statistical associations in HBM studies between exposure-effect-outcome designs trying to mimic the exposure-MIE-KEs-AO structure. A recent work suggested that AOPs may be used in the future to derive effect biomarker-based values to address adverse risk levels from chemical exposures (Jeddi et al., 2020). To this regard, the development of a list of suitable effect biomarkers for regulatory use, as well as recommendations on data reporting standardization is needed, in order to ensure the reliability, reproducibility and efficiency of effect biomarkers data, as well as the transparency in its interpretation, ultimately increasing their utility for informing regulatory risk assessment (Jeddi et al., 2020). On the other hand, novel omics effect biomarkers may contribute to identify molecular effect biomarkers that can be mechanistically linked to specific adverse health effects. Within these new effect biomarkers, analyses of gene expression (e.g. of DNA repair and detoxification genes), epigenetics, as well as proteome and metabolome analyses are feasible. Moreover, the combination of a set of classic and omics biomarkers may constitute the strongest approach for assessing the potential health effects of Cr(VI) and Cd.

## 6. Conclusions

To support improved derivation of EU regulation and policy making, it is of great importance to identify the most reliable effect biomarkers for the heavy metals Cr and Cd that can be used in HBM studies, as they could provide evidence of early biological events that can predict (and perhaps prevent) adverse health outcomes. In the present study, we identified effect biomarkers in epidemiological studies published between 2008 and 2020.

For Cr(VI), conventional biomarkers targeting oxidative stress and lipid peroxidation (mainly 8-OHdG and MDA), as well as DNA or chromosome damage, were the most frequently used to associate exposure with cancer development. Despite the high sensitivity of these biomarkers, large-scale HBM studies including complementary effect biomarkers are still required to better understand the relationships between exposure and oxidative stress or DNA/chromosome damage biomarkers. With respect to Cd and to some extent Cr, β-2-microglobulin (B2-MG) and N-acetyl-β-D-glucosaminidase (NAG) are well-established, sensitive, and the most commonly used effect biomarkers to relate exposure to renal tubular dysfunction. Additionally, NGAL and KIM-1 could serve as sensitive biomarkers of acute kidney damage in response to both Cr(VI) and Cd that needs to be further investigated in large HBM studies.

Overall, there is still insufficient information on the potential health effects of chronic low-level Cr(VI) and/or Cd exposure in humans and, in particular, a lack of more specific and sensitive (preclinical) biomarkers for health effects resulting from this exposure scenario.

Further development of omics-based biomarkers may help to fill this gap. The properties of these novel candidate effect biomarkers need to be evaluated for Cr(VI) and Cd exposure and should be validated by comparison with established effect biomarkers to (i) determine their sensitivity, i.e. whether they can reliably detect differences between unexposed and exposed individuals at the low concentrations currently present, ii): investigate their specificity (false positive rate), iii) assess their predictive value for the adverse outcome, iv) investigate their reproducibility, (v) identify possible effects of common confounding factors such as smoking, diet, age, sex, and other metal exposures and assess their inter- and intra-individual variability; and (vi) evaluate their feasibility in terms of speed, cost-effectiveness, and non-invasiveness. Nonetheless, high-throughput approaches enable the analysis of targets at multiple levels (genes, proteins, metabolites) and the identification of potential effect-specific profiles that provide relevant information on gene networks, cell signaling pathways, and/or biological functions, which can advance the (further) elucidation of underlying mechanisms. This mechanistic knowledge should then be used to select an appropriate set of effect biomarkers. If used in HBM studies along with adequate characterization of the environmental or occupational setting and exposure biomarkers, such effect biomarkers would contribute significantly to better protection of human health.

**Declaration of competing interest**

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.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.110998.

**Author's contributions**

Célia Ventura, Critical reviews of the manuscript, edition, and provision of important intellectual content. Bruno Costa Gomes, Work conceptualization and design of search strategies, Articles screening and selection and data extraction. Axel Oberemm, Work conceptualization and design of search strategies, Articles screening and selection and data extraction, Original draft preparation, Critical reviews of the manuscript. Henriqueta Louro, Critical reviews of the manuscript. Pasi Huuskonen, Articles screening and selection and data extraction. Vicente Mustieles, Work conceptualization and design of search strategies, Articles screening and selection and data extraction, Critical reviews of the manuscript. Mariana F. Fernández, Critical reviews of the manuscript. Sophie Ndaw, Articles screening and selection and data extraction. Marcel Mengelers, Critical reviews of the manuscript. Mirjam Luijten, Critical reviews of the manuscript. Claudia Gundacker, Work conceptualization and design of search strategies, Articles screening and selection and data extraction, Original draft preparation, Critical reviews of the manuscript, edition, and provision of important intellectual content. Maria João Silva, Critical reviews of the manuscript, edition, and provision of important intellectual content.

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